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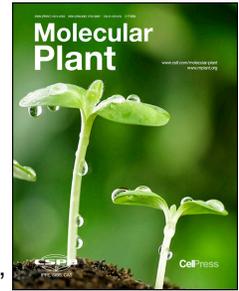
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1 **Cell-specific pathways recruited for symbiotic nodulation in the *Medicago truncatula* legume**  
2 Sergio Alan Cervantes-Pérez<sup>1#</sup>, Sandra Thibivilliers<sup>1,2#</sup>, Carole Laffont<sup>3</sup>, Andrew D. Farmer<sup>4</sup>,  
3 Florian Frugier<sup>3</sup>, Marc Libault<sup>1,2,\*</sup>

4  
5 <sup>1</sup> Department of Agronomy and Horticulture, Center for Plant Science Innovation, University of  
6 Nebraska-Lincoln, Lincoln, NE, 68503, USA

7 <sup>2</sup> Single Cell Genomics Core Facility; Center for Biotechnology, University of Nebraska-Lincoln,  
8 NE 68588, USA.

9 <sup>3</sup> Institute of Plant Sciences Paris-Saclay (IPS2), Université Paris-Saclay, CNRS, INRAE,  
10 Université Paris-Cité, Université d'Evry, Gif-sur-Yvette, 91190, France

11 <sup>4</sup> National Center for Genome Resources, Santa Fe, NM, 87505, USA

12 # Contributed equally

13  
14 Contact: [marc.libault@unl.edu](mailto:marc.libault@unl.edu)

15  
16 **Running Title:** Medicago single-cell RNA-seq response to rhizobia

17  
18 **Short Summary:** Legume nodulation is the result of the symbiotic interaction between legume  
19 plants and soil bacteria collectively named rhizobia. In this study, upon applying single-nucleus  
20 RNA-seq technology, we generated a single-cell resolution transcriptomic map of the Medicago  
21 root. Using this map, we conducted a comprehensive transcriptomic analysis of the early root  
22 symbiotic responses at a cell-type-specific level.

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**Abstract**

*Medicago truncatula* is a model legume species that has been studied for decades to understand the symbiotic relationship between legumes and soil bacteria collectively named rhizobia. This symbiosis called nodulation is initiated in roots with the infection of root hair cells by the bacteria as well as the initiation of nodule primordia from root cortical, endodermal, and pericycle cells, leading to the development of a new root organ, the nodule, where bacteria fix and assimilate the atmospheric dinitrogen for the benefit of the plant. Here, we report the isolation and use of nuclei from mock and rhizobia-inoculated roots to conduct single nuclei RNA-seq (sNucRNA-seq) experiments to gain a deeper understanding of early responses to rhizobial infection in *Medicago* roots. A gene expression map of the *Medicago* root was generated, comprising 25 clusters, which were annotated as specific cell-types using 119 *Medicago* marker genes and orthologs to *Arabidopsis* cell-type marker genes. A focus on root hair, cortex, endodermis, and pericycle cell-types, showing the strongest differential regulations in response to a short-term (48 hours) rhizobium inoculation, revealed both known genes and functional pathways, validating the sNucRNA-seq approach, but also numerous novel genes and pathways, allowing a comprehensive analysis of early root symbiotic responses at a cell-type-specific level.

**Keywords:** *Medicago* root, single-cell transcriptomic, rhizobium, nodule initiation, root hair cells, cortical cells

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## 63 Introduction

64 Legumes symbiotically interact with nitrogen-fixing soil bacteria collectively named  
65 rhizobia. The molecular, physiological, and cellular responses of this symbiosis named nodulation  
66 have been extensively studied over the past decades (Roy et al., 2019). Legume nodulation is a  
67 complex biological process that requires the activation of temporally and spatially coordinated  
68 programs in a limited number of root cells. Briefly, legume nodulation is initiated by the perception  
69 of the rhizobial lipochitooligosaccharide Nod factors (NFs) and the subsequent infection of plant  
70 root hair cells by rhizobia. Concomitantly, a nodule primordium emerges. In *Medicago truncatula*,  
71 a legume species generating indeterminate nodules, these primordia are initiated from cell  
72 divisions within the root inner cortex, endodermis, and pericycle layers, and an apical meristem is  
73 then established and maintained during the entire life of the nodule. Rhizobia infect the developing  
74 root nodule primordia, differentiate into bacteroids, and fix and assimilate the atmospheric  
75 dinitrogen allowing a steady supply of nitrogen for the plant.

76 Functional genomic studies revealed the role of many legume genes controlling the early  
77 stages of nodulation, notably in root hair cells (trichoblasts) where the initial microsymbiont  
78 perception and the rhizobial infection take place, but also to a lower extent in pericycle and cortex  
79 inner root cell layers where nodule organogenesis initiates (Roy *et al.*, 2019). Several *M.*  
80 *truncatula* genes have been functionally characterized to control the infection of the root  
81 epidermis. Among them, *MtNPL* [Nodule Pectate Lyase; (Xie et al., 2012)], a gene encoding a cell  
82 wall degrading enzyme required for the initiation of infection threads in curled root hairs, *MtLIN*  
83 [Lumpy Infections; (Kiss et al., 2009; Liu et al., 2019a)], which encodes a putative E3 ligase,  
84 *MtRPG* [Rhizobium Directed Polar Growth; (Arrighi et al., 2008)], a gene encoding a protein with  
85 a coiled-coiled domain, *MtFLOT4* (Haney and Long, 2010), *MtVPPY* (Murray et al., 2011),  
86 NADPH oxidase/respiratory burst oxidase homologs [e.g., Rboh; (Montiel et al., 2016)], and  
87 *MtCBS1*, a gene encoding a Cystathionine- $\beta$ -Synthase-like Domain-Containing Protein (Sinharoy  
88 et al., 2016), are all upregulated in response to rhizobium inoculation. In addition, other rhizobium-  
89 upregulated genes participate in the NF signaling pathway such as LYsM receptors that perceive  
90 NF bacterial signals. These genes belong to the LysM receptor kinase and LYK-related gene  
91 families [*MtLYK* and *MtLYR*, respectively; e.g. *MtNFP* (NF Perception; (Gough et al., 2018))],  
92 *MtDMII*, 2 and 3 (Does not Make Infections) genes (Ané et al., 2004; Endre et al., 2002; Gleason  
93 et al., 2006), *MtPUB1* and 2 (Plant U-box protein 1 and 2) involved in protein degradation (Liu et

94 al., 2018; Mbengue et al., 2010), *MtNFHI* [NF Hydrolase 1; (Cai et al., 2018)] that controls NF  
95 degradation, *MtIPD3* [Interacting with DMI3; (Messinese et al., 2007)], and various transcription  
96 factor (TF) genes [e.g., *MtNSPI/2* (Nodulation Signaling Pathway 1/2; (Kaló et al., 2005; Smit et  
97 al., 2005)), *MtNF-YA1* (Nuclear factor-YA1; (Combier et al., 2006)), *MtERN1* (ERF (Ethylene  
98 Response Factor) Required for Nodulation; (Andriankaja et al., 2007; Middleton et al., 2007)), and  
99 *MtNIN* (Nodule Inception; (Schauser et al., 1999))].

100 To date, a limited number of studies have highlighted the regulatory mechanisms  
101 controlling the response of inner root cell layers to rhizobial inoculation (i.e., pericycle,  
102 endodermis, and cortex). Among them, cytokinin signaling is necessary and sufficient to promote  
103 the initiation of nodule primordia and inhibit the response of epidermal root cells to rhizobia and  
104 NFs (Boivin et al., 2016; Gamas et al., 2017; Gonzalez-Rizzo et al., 2006; Jardinaud et al., 2016;  
105 Lin et al., 2021; Murray et al., 2007; Plet et al., 2011). Genes controlling root development were  
106 also shown to be recruited for nodule development, such as *MtPLT* [Plethora; (Franssen et al.,  
107 2015)], *MtKNOX* [Knotted homeobox; (Di Giacomo et al., 2017)], *MtLBD16* [LOB Binding  
108 Domain 16; (Schiessl et al., 2019; Soyano et al., 2019)], *MtSHR* [ShortRoot; (Dong et al., 2021)],  
109 *MtSCR* [Scarecrow; (Dong et al., 2021)], and *MtNOOT1* (Shen et al., 2019). On some occasions,  
110 genes were shown to have a dual function to promote root epidermal infection and nodule initiation  
111 at the level of the pericycle and cortical cells, such as *MtNIN* (Liu et al., 2019b).

112 To gain a more accurate picture of the symbiotic transcriptional programs controlling the  
113 root hair signal perception and infection, transcriptomic studies were conducted on populations of  
114 isolated legume root hair cells using root hair shaving or laser dissection (Breakspear et al., 2014;  
115 Libault et al., 2009). Similarly, -omics analyses targeting the zone of emerging nodules revealed  
116 the transcriptomic programs controlling the initiation and development of nodule primordia  
117 (Larrainzar et al., 2015; Lohar et al., 2005; Schiessl et al., 2019; van Zeijl et al., 2015). While  
118 valuable, these approaches suffer from the cellular heterogeneity of the isolated root hair  
119 populations [i.e., a mixture of unresponsive, responsive but uninfected, and infected root hair cells  
120 (Bhuvanewari et al., 1981)], and from the cellular complexity of the root. For instance, as a  
121 reflection of the cellular heterogeneity of the root hair population, it has been estimated that only  
122 1-5 % of the root hair cells are infected by rhizobia (Nutman, 1959).

123 While these approaches increased the resolution of plant transcriptomic analyses, they have  
124 been recently superseded by the emergence of single-cell (scRNA-seq) and single-nucleus

125 (sNucRNA-seq) transcriptomic technologies. Here, we report the use of the sNucRNA-seq  
126 technology on *M. truncatula* roots mock-inoculated or inoculated with its symbiont, *Ensifer*  
127 (*Sinorhizobium*) *meliloti*, to precisely capture the transcriptomic programs induced during the early  
128 stages of the nodulation process in each cell-type composing the *M. truncatula* root. The  
129 establishment of a single-cell resolution transcriptomic map of the *M. truncatula* root allowed  
130 characterizing the transcriptomic response of the *M. truncatula* root hair, cortical, endodermal, and  
131 pericycle cells at an early stage (48 hours) after *E. meliloti* inoculation. The transcriptomic analysis  
132 of these different cell-types revealed the dynamic regulation of *M. truncatula* genes in response to  
133 rhizobial infection, including many novel genes and functional pathways, as well as the differential  
134 recruitment of previously known nodulation-related and hormonal genes depending on cell-types.

135

## 136 **Results**

### 137 **Establishment of a transcriptional map of the rhizobium-inoculated *M. truncatula* root at a** 138 **single-cell level resolution**

139 Isolated plant protoplasts and nuclei have been successfully used to establish single-cell  
140 resolution transcriptomes notably from Arabidopsis root cells (Denyer et al., 2019; Farmer et al.,  
141 2021; Jean-Baptiste et al., 2019; Ryu et al., 2019; Shulse et al., 2019; Zhang et al., 2019). The high  
142 correlation between cellular, nuclear, and whole root transcriptomes (Farmer *et al.*, 2021) supports  
143 the biological relevance of both scRNA-seq and sNucRNA-seq approaches. However, the nuclear-  
144 based transcriptomic technology has unique advantages compared to the protoplast-based  
145 transcriptomic technology, including the ease of nuclei isolation from various plant species and  
146 organs, and the limited induction of stress-related genes [i.e., in contrast, protoplastization leads  
147 to the induction of hundreds of stress-responsive genes; (Birnbaum et al., 2003; Denyer *et al.*,  
148 2019)]. Therefore, we conducted sNucRNA-seq experiments to characterize the transcriptomic  
149 profiles of the different cell-types composing the Medicago root apex and their early response to  
150 rhizobial inoculation.

151 Shortly, Medicago seedlings were inoculated 4 days post-germination with water or a  
152 bacterial suspension of *E. meliloti* (OD<sub>600nm</sub>=0.1). Forty-eight hours post-rhizobium inoculation,  
153 root tips including fully elongated root hair cells were collected and committed to nuclei isolation  
154 (see “Methods” for details). Three independent *E. meliloti*-inoculated and three independent mock-  
155 inoculated sNucRNA-seq libraries were generated using the 10x Genomics Chromium platform.

156 To establish transcriptomic profiles of Medicago root cells, we applied a “pre-mRNA” strategy  
157 using Cell Ranger (10x Genomics) to map sequencing reads against transcripts and introns of the  
158 v1.8 annotation of the MtrunA17r5.0-ANR genome (Pecrix et al., 2018). This first step was  
159 followed by the removal of the ambient transcriptomic noise and the detection and removal of  
160 doublets (i.e., two nuclei encapsulated into the same reaction volume) (see Methods). To support  
161 the quality of the libraries, we analyzed the distribution of the number of expressed genes per  
162 nucleus and observed an expected normal distribution (Supplemental Figure 1). To remove outliers  
163 and low-quality nuclei, we applied a 95% confidence interval to the normal distribution of the six  
164 integrated libraries (Supplemental Figure 1). Taken together, the transcriptomes of 15,854 *E.*  
165 *meliloti*-inoculated and 12,521 mock-inoculated *M. truncatula* root nuclei were further analyzed  
166 (Supplemental Table 1), with a median value of 1,053 expressed genes per nucleus and a total of  
167 31,307 expressed protein-coding genes detected [70.2% of the 44,615 Medicago protein-coding  
168 genes (Pecrix *et al.*, 2018)]. Considering that the number of Arabidopsis and Medicago expressed  
169 genes per nucleus are similar (i.e., 1,124 expressed genes per Arabidopsis nucleus), the percentage  
170 of Medicago protein-coding genes expressed is less compared to Arabidopsis [i.e., 89.4% of  
171 27,420 protein-coding genes (Farmer *et al.*, 2021)]. Such a limited percentage might be a  
172 consequence of the neo- or sub-functionalization of Medicago genes following the whole-genome  
173 duplication that occurred 58 million years ago, or the result of an over-estimation of the number  
174 of protein-coding genes in Medicago compared to the reference Arabidopsis genome.

175 Using the Seurat package, we normalized and integrated 28,375 nuclei transcriptomes  
176 before applying the uniform manifold approximation and projection (UMAP) technique to cluster  
177 the nuclei according to their transcriptomic profiles. Twenty-five clusters were identified [Figure  
178 1A; the dataset can be interrogated using the  
179 [https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1\\_6.expr.Cervantes-  
180 Perez\\_Thibivilliers\\_2022/](https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1_6.expr.Cervantes-Perez_Thibivilliers_2022/) web interface (Ouyang et al., 2021)], for which the percentage of nuclei  
181 per cluster was not statistically different between inoculated and mock-inoculated conditions  
182 (Student t-test > 0.05; Figure 1B; Supplemental Table 1). While *E. meliloti* inoculation did not  
183 lead to changes in the number of clusters or the overall topography of the UMAP projection, we  
184 repetitively noticed a local modification in the distribution of a subpopulation of cluster #2 nuclei  
185 (Figure 1A, red circle). These results highlight that the *E. meliloti* inoculation induces significant  
186 changes in the transcriptome of these Medicago root cells.

187

**188 Functional annotation of Medicago root nuclei clusters**

189 The functional annotation of root cells/nuclei according to their transcriptomic profile was  
190 previously successfully achieved in the model species *A. thaliana* by exploring the transcriptional  
191 pattern of a large number of functionally characterized cell-type-specific marker genes (Denyer *et al.*,  
192 2019; Farmer *et al.*, 2021; Jean-Baptiste *et al.*, 2019; Ryu *et al.*, 2019; Shulse *et al.*, 2019;  
193 Zhang *et al.*, 2019). To annotate the 25 nuclei clusters of the UMAP, we first analyzed the  
194 transcriptional pattern of the few available functionally characterized Medicago root cell-type-  
195 specific markers (Supplemental Table 2). The *MtPLT1-4* genes are specifically expressed in the  
196 quiescent center of the root and nodule primordia (Franssen *et al.*, 2015). Looking at their  
197 expression pattern in the Medicago UMAP, they are all preferentially expressed in the central star-  
198 shaped cluster #9, supporting its annotation as the “stem cell niche” cluster (Figures 2A and B).  
199 To annotate Medicago epidermal cells, the expression of the phosphate transporter *MtPT1* gene,  
200 which is specifically expressed in Medicago root hairs and epidermal cells (Chiou *et al.*, 2001),  
201 was analyzed. *MtPT1* was mostly expressed in clusters #1 and 2, and to a lesser extent, in cluster  
202 #5 (Figure 2B). The root hair-specific *MtRbohF* gene (Marino *et al.*, 2011) was mostly expressed  
203 in cluster #3 (Figure 2B), suggesting its annotation as “root hair cells”. To support the annotation  
204 of the root epidermal cells, we also analyzed the expression of 45 genes previously identified as  
205 specifically expressed in the root hair (Breakspear *et al.*, 2014) and 25 Medicago genes  
206 orthologous to the 168 root-hair-specific Arabidopsis genes (Cvrčková *et al.*, 2010). Taken  
207 together, most of these genes are preferentially or specifically expressed in the Medicago root  
208 epidermal cells (Supplemental Figure 2, red rectangles), especially in cluster #3. Based on the  
209 expression pattern of these different Medicago markers, clusters #1 and 2 could be thus confidently  
210 annotated as “root epidermal cell” clusters, and cluster #3 as a “root hair cell” cluster (Figure 2A).  
211 To identify the Medicago cortical cells on the UMAP projection, we analyzed the transcriptional  
212 activity of the cortical cell-specific genes *MtIFS1*, *MtIFS3*, and *MtPAL5* (Biala *et al.*, 2017).  
213 *MtIFS1* was almost specifically expressed, and *MtIFS3* and *MtPAL5* were preferentially expressed  
214 in cluster #14 (Figure 2B). Besides, *MtIFS3* and *MtPAL5* are also detected in clusters #7 and 10,  
215 and in clusters #18 and 19, respectively. Taken together, these results support the annotation of  
216 cluster #14 as a “cortical cell” cluster. *MtSCR* is mostly expressed in the endodermis as well as in  
217 cortical and epidermal cells (Dong *et al.*, 2021) and was thus used for annotating the endodermis

218 (Figure 2A). *MtSCR* was mostly detected in clusters #17, 18, and 19 that are co-localized on the  
 219 UMAP projection (Figure 2B), which were thus annotated as “endodermal cell” clusters.  
 220 *MtPHO1.1*, *MtPHO1.3*, and to a lesser extent, *MtPHO1.2*, are preferentially expressed in the root  
 221 stele and more specifically in pericycle cells (Nguyen et al., 2020). *MtPHO1.1* and *MtPHO1.3*  
 222 were most expressed in cluster #20, where *MtPHO1.2* is also highly expressed, as well as in cluster  
 223 #5. The transcriptional activity of these three *PHO1* genes supports that cluster #20 is associated  
 224 with the root pericycle and/or stele (Figure 2A). Other genes expressed in the root stele, such as  
 225 *MtHext1/STP13*, *MtSHR1*, *MtSHR2*, and *MtPAL* (Biala et al., 2017; Dong et al., 2021; Gaude et  
 226 al., 2012), were co-expressed in clusters #19 to 24 (Figure 2B). Considering that *MtSCR*, an  
 227 endodermal cell marker gene, is highly expressed in cluster #19, this suggests that clusters #20 to  
 228 24 correspond to root stele cell-types (Figure 2A). Besides, *MtYUC8* and *MtABCG20* genes that  
 229 are active in the vascular bundle of the Medicago root (Pawela et al., 2019; Schiessl et al., 2019),  
 230 were mostly expressed in cluster #25 (Figure 2A). Taken together, this supports the annotation of  
 231 cluster #20 as the Medicago pericycle cell cluster, and of clusters #21 to 25 as Medicago root  
 232 vasculature cell-type clusters. Finally, the expression pattern of *MtSUNN*, a receptor-like kinase  
 233 acting in the Autoregulation of Nodulation (AON) pathway specifically expressed in the phloem  
 234 (Schnabel et al., 2012), precisely maps to cluster #25, thus refining its annotation as the phloem  
 235 cells cluster, and the *MtRDNI* AON-related enzyme modifying CLE signaling peptides (Kassaw  
 236 et al., 2017) to cluster #24, thus refining its annotation as a xylem cells cluster (Figure 2A).

237

### 238 **Use of Medicago orthologs of Arabidopsis root cell-type markers for a more exhaustive** 239 **functional annotation of clusters**

240 To further support the functional annotation of these Medicago root clusters, we  
 241 additionally analyzed the transcriptional activity of Medicago genes orthologous to 1,086  
 242 Arabidopsis root cell-type marker genes (Supplemental Table 3), assuming the conservation of  
 243 their cell-type-specific/-enriched transcriptional patterns. Among these genes, 101 were previously  
 244 validated markers (Böhme et al., 2004; Denyer et al., 2019; Fendrych et al., 2014; Jean-Baptiste  
 245 et al., 2019; Olvera-Carrillo et al., 2015; Ryu et al., 2019; Shulse et al., 2019; Turco et al., 2019;  
 246 Zhang et al., 2019) [see (Farmer et al., 2021) for an exhaustive list], 324 were identified in at least  
 247 two independent Arabidopsis root single-cell RNA-seq studies (Denyer et al., 2019; Jean-Baptiste  
 248 et al., 2019; Ryu et al., 2019; Shulse et al., 2019; Zhang et al., 2019), and 868 genes were identified

249 as the most specifically expressed genes within the 21 sNucRNA-seq clusters of the Arabidopsis  
250 root (Farmer *et al.*, 2021). We found that 196 Medicago orthologs share microsyntenic  
251 relationships with the 1,086 Arabidopsis root cell-type-specific marker genes thanks to the  
252 Comparative Genomic database [CoGe; <https://genomeevolution.org/coge/>; (Lyons and Freeling,  
253 2008; Lyons *et al.*, 2008)]. Among these 196 genes, 38 (19.4%) and 58 (29.6%) genes were very  
254 low/not expressed, or ubiquitously expressed across all Medicago root clusters, respectively. In  
255 the end, we considered 100 Medicago genes orthologous to root cell-type-specific Arabidopsis  
256 marker genes to annotate Medicago root clusters (Supplemental Table 4).

257         Among these 100 genes, 10, 2, and 4 are orthologous to Arabidopsis trichoblast-,  
258 atrichoblast-, and root cap-specific marker genes, respectively, including the *MtPT1* gene (Chiou  
259 *et al.*, 2001) (Supplemental Table 2). Nine trichoblast-specific genes are mostly expressed in  
260 cluster #3, whereas *MtPT1* and the remaining six atrichoblast markers are most expressed in  
261 clusters #1, 2, 4, and 5 (Figure 2B). This conclusion is further supported by the transcriptional  
262 activity in cluster #3 of the *MtLAT52/POE\_9* and *MtERN3* genes orthologous to the soybean root-  
263 hair-specific *Glyma.18G025200* and *Glyma.05G157400/Glyma.08G115000* genes (Qiao *et al.*,  
264 2017), respectively (Figure 2B). Using the same approach, we found that the expression pattern of  
265 other Medicago genes orthologous to Arabidopsis cell-type marker genes similarly supported the  
266 annotation of the different Medicago root cell-type clusters. The “stem cell niche” annotation was  
267 confirmed for cluster #9 based on the transcriptional activity of nine Medicago genes orthologous  
268 to Arabidopsis root meristematic genes (Franssen *et al.*, 2015) (Figures 2B, dark grey). Confirming  
269 the expression of the endodermis-specific *MtSCR* gene in clusters #17 to 19 (Figure 2B, pink), we  
270 additionally identified 23 Medicago genes orthologous to Arabidopsis endodermal cell-specific  
271 genes strongly expressed in clusters #15 to 19 (Figure 2B, pink). The annotation of clusters #20 to  
272 25 as stele cells was also confirmed based on the transcriptional activity of 49 orthologous genes  
273 in these clusters. Among them, xylem (i.e., clusters #22, 23, and 24) and phloem cells (i.e., cluster  
274 #25) were identified based on the expression of 23 and 6 Medicago genes orthologous to  
275 Arabidopsis xylem- and phloem-specific genes, respectively (Figure 2B, brown and light brown).  
276 As a note, three genes orthologous to Arabidopsis xylem marker genes (i.e.,  
277 *MtrunA17Chr2g0282871*, *MtrunA17Chr2g0324131*, and *MtrunA17Chr3g0127561*) were also  
278 expressed in clusters #4 and 5. We assume that their activity in these two root epidermal cell  
279 clusters could reflect the induction of the cell-death program, which was previously reported to be

280 shared between xylem and root cap cells (Farmer *et al.*, 2021; Heo *et al.*, 2017; Kumpf and  
281 Nowack, 2015), refining the annotation of clusters #4 and 5 as containing root cap cell-types.  
282 Finally, the transcriptional activity of three Medicago genes orthologous to Arabidopsis cortical  
283 cell-specific genes in cluster #7 (Figure 2B, grey) suggests that it is composed of developing  
284 cortical cells. This result is further supported by the activity of the cortical cell-specific *MtIFS3*  
285 and *MtPAL5* genes in cluster #7 (Figure 2B, purple).

286 Taken together, the combined use of previously characterized Medicago root cell-type  
287 marker genes with the analysis of the transcriptional activity of Medicago genes orthologous to  
288 Arabidopsis root cell-type marker genes led to the functional annotation of 19 out of the 25  
289 Medicago root cell clusters of the UMAP (Figure 2A). To annotate the remaining six clusters (#6,  
290 8, 10, 11, 12, and 13), all located at the center of the UMAP, we conducted a correlation analysis  
291 between the different Medicago root cell clusters, hypothesizing that two clusters sharing highly  
292 correlated transcriptomic profiles would relate to the same cell-type. As expected, this analysis  
293 revealed several high correlation scores such as between the root epidermal clusters #1 and 2, and  
294 for stele/pericycle clusters #20, 21, and 23 (Supplemental Figure 3, highlighted in orange and red  
295 squares, respectively). As a note, the remaining non-annotated cluster #8 did not share a correlation  
296 with any other cluster, suggesting that cells composing this cluster have a very different  
297 transcriptomic profile compared to all other clusters. However, we were able to associate cluster  
298 #8 with an intense activity of mitochondrial and ribosomal genes (Supplemental Table 5;  
299 Supplemental Figure 4). Previous studies linked the high expression of ribosomal genes with plant  
300 developmental processes in maize, Arabidopsis, and tobacco plants (Makabe *et al.*, 2017; Ponnala  
301 *et al.*, 2014). These results thus suggest that cluster #8 is composed of cells with high biological  
302 activity. Interestingly, we found high correlation scores between clusters #6, 7, 10, 11, 12, 13, and  
303 14 (Supplemental Figure 3, yellow squares). As the cell-type marker-based analyses identified  
304 clusters #7 and 14 as cortical cells, we assume that clusters #6, 10, 11, 12, and 13 are also composed  
305 of cortical cells (Figure 2A), potentially at different stages in their differentiation process and/or  
306 corresponding to different layers of the cortex, knowing that there are 4 to 5 layers of cortical cells  
307 in *M. truncatula* roots.

308

309 **Conservation of expression patterns between orthologous Arabidopsis and Medicago genes**  
310 **at the single-cell level**

311 The previous Arabidopsis-Medicago comparative genomic and transcriptomic analysis  
312 used to annotate Medicago root clusters (Figure 2) suggested that transcriptional patterns of  
313 orthologous genes could be largely conserved upon speciation of the two plants 108 million years  
314 ago (Zeng et al., 2017). To further explore the extent of this conservation at the single-cell level, a  
315 correlation analysis was conducted on 3,921 pairs of orthologous genes sharing microsyntenic  
316 relationships based on the CoGe database (Lyons and Freeling, 2008; Lyons *et al.*, 2008)  
317 (Supplemental Table 6). To maximize the biological significance of the analysis, we processed the  
318 previously generated Arabidopsis sNucRNA-seq datasets (Farmer *et al.*, 2021) similarly to the  
319 Medicago sNucRNA-seq datasets (see Methods). This updated analysis led to the identification  
320 and re-annotation of 16 Arabidopsis root clusters (Farmer *et al.*, 2021) (Figure 3A). We observed  
321 a similar topology between the Medicago and Arabidopsis sNucRNA-seq UMAPs (Figures 2A  
322 and 3A), with the stem cell niche (#9) located in a star-shaped cluster at the center of the UMAP  
323 projection whereas the most differentiated cell-types [e.g. epidermal (#1, 2, and 3), phloem (#25),  
324 xylem cells (#22, 23, and 24)] were retrieved in the periphery. To evaluate the conservation of the  
325 transcriptional profiles between Arabidopsis and Medicago orthologs, the average gene  
326 transcriptional activity of one-to-one orthologs was similarly calculated for each of the 25 mock-  
327 inoculated Medicago (Figures 1A) and of the 16 Arabidopsis root clusters (Figure 3A), and a  
328 correlation analysis was then conducted (Figure 3B). Stem cell niche (#9; Figure 3B, black square)  
329 and stele cells (#20 to 25; Figure 3B, peach-orange square) shared the highest correlation between  
330 the two species, suggesting that the transcriptional activities of orthologous genes were most  
331 conserved across these root cell-types. To a lesser extent, the transcriptomic profiles of orthologous  
332 genes were also conserved between the two species in trichoblasts/epidermal root hair cells (#1 to  
333 3; Figure 3B, blue square). Similar conservation was recently reported for root hair, xylem, and  
334 phloem cells between the more distantly related rice and Arabidopsis roots (Liu et al., 2021; Zhang  
335 et al., 2021). We hypothesize that the unique functions of vascular tissues (xylem and phloem) and  
336 trichoblast cells for plant nutrition, as well as the role of the stem cell niche in root development,  
337 favored the evolutionary conservation of a core transcriptome between these plant species. In  
338 contrast, the transcriptomes of endodermal (#15 to 19) and cortical cell-types (#6, 7, and 10 to 14)  
339 were most divergent between Arabidopsis and Medicago (Figure 3B, purple and violet squares),  
340 suggesting either difference in nutrient provision when growing the Arabidopsis and Medicago  
341 plants, a lower pressure to maintain the transcriptomic signature of these cell-types between the

342 two species, and/or diverging biological functions of these cell-types between Fabaceae (legumes)  
343 and Brassicaceae, such as their differential capacity to interact with soil beneficial microbes  
344 through endosymbiosis.

345

### 346 **A differential transcriptional regulation in response to *E. meliloti* of nodulation and** 347 **hormonal genes depending on cell-types**

348 To date, transcriptomic responses of legume roots to rhizobial inoculation were  
349 investigated mainly at the whole root level (Mergaert et al., 2019) or on isolated populations of  
350 root hair and epidermal cells (Breakspear *et al.*, 2014; Libault *et al.*, 2009). These bulk analyses  
351 however lacked resolution, considering that only a subset of plant cells respond to and are infected  
352 by rhizobia and that the transcriptional response of cell-types located deeper within roots cannot  
353 be easily assessed. Here, we had a unique opportunity to explore the differential regulation of gene  
354 expression across the different cell-types in response to *E. meliloti*.

355 To estimate the percentage of individual Medicago root epidermal cells responding to *E.*  
356 *meliloti*, we quantified the number of epidermal root cells (i.e., clusters # 1, 2, and 3, excluding  
357 the epidermal/root cap clusters #4 and 5) expressing typical early rhizobial infection marker genes,  
358 namely *MtRPG*, *MtFLOT4*, and *MtVPY* (Roy *et al.*, 2019). In *E. meliloti*-inoculated roots, 19.5%  
359 (433/2225 epidermal nuclei) of epidermal nuclei expressed at least one of these symbiotic marker  
360 genes, whereas only 2.3% (39/1694 epidermal nuclei) were detected in mock-inoculated roots. The  
361 increase in the number of rhizobia-responsive epidermal cells upon *E. meliloti* inoculation  
362 identified is thus significantly larger than the previously estimated size of the epidermal cells  
363 population infected by rhizobia, which was about 1-5 % of root hair cells (Nutman, 1959). These  
364 results suggest that only a subset of the transcriptionally-responsive root epidermal cells from these  
365 three clusters is effectively infected by rhizobia.

366 To identify the set of Differentially Expressed Genes (DEGs) in response to rhizobium  
367 inoculation from each Medicago root cell cluster, we used DEsingle, a bioinformatics package  
368 designed to identify DEGs from single-cell RNA-seq datasets (Miao et al., 2018). Using a p-value  
369 threshold  $< 0.05$  and a  $|\text{Fold Change (FC)}| > 1.5$ , we identified a total of 8,513 DEGs (Supplemental  
370 Table 7). Focusing on the most transcriptionally responsive clusters (i.e.,  $> 500$  DEGs; Figure 4A;  
371 Supplemental Table 7, bold characters), the root hair cells cluster #2 was retrieved, as well as the  
372 cortical clusters #7 and 11, the endodermal clusters # 15, 16, and 18, and the pericycle cluster #20.

373 This result nicely fits with knowledge previously gained using microscopy during early nodule  
 374 ontogeny (Xiao et al., 2014), which showed that cellular symbiotic processes mainly affected  
 375 epidermal and cortical cells, and to a lesser extent, endodermis and pericycle cells. We thus further  
 376 focused our analysis on these clusters, as well as on genes previously known to be acting in  
 377 nodulation and/or previously annotated as related to hormonal pathways regulating early  
 378 nodulation stages (Roy *et al.*, 2019).

379 In the trichoblast cluster #2, several known infection-related genes were retrieved as up-  
 380 regulated, namely *MtCBS1*, *MtRbohG* and *MtRbohH* (Montiel et al., 2018), *MtRPG*, *MtVPY*,  
 381 *MtAnn1* (de Carvalho Niebel et al., 1998), and *MtNMNI* [i.e., an ortholog of *GmNMNI* (Libault  
 382 et al., 2011)]. Other genes that belong to the NF signaling pathway were also up-regulated in root  
 383 hair and epidermal cells in response to *E. meliloti*, namely *MtNFH1*, *MtLYK10* (Larrainzar *et al.*,  
 384 2015), *MtPUB1* (noting that *MtPUB2* is repressed in this same cluster), *MtIPD3*, *MtDMI1*,  
 385 *MtDMI2*, *MtDMI3*, *MtNSP1*, *MtNSP2*, *MtERN1*, *MtERN2* (Cerri et al., 2016), and *MtNIN*  
 386 (Supplemental Tables 8 and 9, Figure 5). Unexpectedly, *MtKNOX3* and *MtKNOX5* genes,  
 387 previously proposed to control nodule development (Di Giacomo *et al.*, 2017; Dolgikh et al.,  
 388 2020), and *MtDNF2* [*Does Not fix Nitrogen 2* (Bourcy et al., 2013)] and *MtNCR112* [*Nodule*  
 389 *Cysteine Rich peptide 112* (Alunni et al., 2007)] genes, regulating later stages of the nodulation  
 390 process, were also upregulated in cluster #2 48 hours after rhizobium inoculation. In addition, we  
 391 noticed that *MtNF-YA2* and *MtNF-YA6* genes, phylogenetically related to the early nodulation  
 392 *MtNF-YA1* gene (Baudin et al., 2015), were also upregulated in cluster #2. Genes belonging to  
 393 hormonal regulatory pathways were also induced in this cluster in response to rhizobial  
 394 inoculation. These include notably cytokinin [e.g., *MtIPT1*, *CYP735A1-like*, *MtHPT1*, *MtRRB6*,  
 395 *MtRRA2*, and *MtRRA5* (Azarakshsh et al., 2018; Tan et al., 2019)] and gibberellin [*MtCPS1*,  
 396 *MtGA2ox10*, *MtGA3ox1*, and *MtDELLA2*; (Fonouni-Farde et al., 2016; Kim et al., 2019)]  
 397 biosynthesis and signaling genes. In addition, the expression of genes related to the jasmonic acid  
 398 [*MtLOX6* (Gao et al., 2007), and *MtJAZ3* (Ge et al., 2016)], auxin [*MtARF10*; (Shen et al., 2015)],  
 399 abscisic acid [*MtABI5*; (Verdier et al., 2013)], strigolactone [*MtD27*, *MtMAX1a*; (Liu et al., 2011;  
 400 Müller et al., 2019)], ethylene [*MtETR4* (Tan *et al.*, 2019)], and brassinosteroid [*MtBAK1*  
 401 (Tavormina et al., 2015)] pathways were also upregulated upon rhizobium inoculation in cluster  
 402 #2. Conversely, the expression of several genes encoding signaling peptides was repressed by  
 403 rhizobium in cluster #2, such as *MtPIP1*, *MtIDA20*, *MtIDA31*, and *MtIDA35* (Inflorescence

404 Deficient in Abscission) (de Bang et al., 2017) (Supplemental Tables 8 and 9, Figure 5). To further  
405 estimate the relevance of the trichoblast DEGs identified, we conducted a comparative analysis  
406 with the list of 267 DEGs previously reported in the Medicago root hair cells in response to  
407 rhizobium inoculation (Breakspear *et al.*, 2014). We found that 99 of these genes (37%) were  
408 differentially expressed in the sNucRNA-seq dataset and, among them, 68 genes (69%) were  
409 significantly differentially expressed in at least one of the epidermal clusters #1, 2, or 3  
410 (Supplemental Figure 5). Considering the difference in sensitivity between the technologies used,  
411 these results overall support the identification of genes differentially expressed in Medicago  
412 trichoblasts in response to rhizobia inoculation.

413 The cortical cell cluster #7 DEGs are all repressed upon rhizobium inoculation, including  
414 one gene, *MtCASTOR*, homologous to an *L. japonicus* nodulation gene (Charpentier et al., 2008),  
415 several cytokinin-related genes (two *LOG-like* genes and two *RRA* signaling genes, *MtRRA4* and  
416 *MtRRA9*), the gibberellin signaling gene *MtDELLA1*, and the abscisic acid signaling gene *MtABI5*  
417 (Supplemental Tables 8 and 9, Figure 5). Conversely, the cortical cell cluster #11 DEGs are all  
418 upregulated and include several genes controlling the rhizobia-infection process (*MtRbohA*  
419 (Marino *et al.*, 2011), *MtRbohB*, *MtLIN*), NF signaling (*MtPUB2*, *MtDMI1*, *MtDMI3*, *MtERN2*),  
420 and nodule development [*MtSHR1*, *MtKNOX4* and *MtKNOX9* (Di Giacomo *et al.*, 2017),  
421 *MtNOOT1*, *MtCCS52a* (Cebolla et al., 1999)] and function [*MtNAC969* (de Zélicourt et al., 2012)]  
422 (Supplemental Tables 8 and 9, Figure 5). In addition, hormonal genes related to cytokinin  
423 [*MtCHK1/MtCRE1* (Gonzalez-Rizzo *et al.*, 2006), *MtHPT3*, *MtRRB5*, *MtRRB8*, *MtRRA5*], auxin  
424 (*MtARF10*, *MtARF13*, *MtARF24*), gibberellin (*MtDELLA2*), ethylene (*MtETR1*, *MtEIN3-like*),  
425 jasmonic acid (*MtLOX3*, *MtLOX6*), and strigolactone (*MtMAX2b*), were also upregulated upon  
426 rhizobium inoculation in cluster #11. Finally, two nitrate signaling-related TFs were upregulated  
427 [*MtNLP1* and *MtNLP4* (Luo et al., 2021)] as well as a specific signaling peptide (*MtRTF/DVL11*),  
428 in agreement with the symbiotic function previously reported for *MtDVL1* (Combier et al., 2008)  
429 (Supplemental Tables 8 and 9, Figure 5). Of note, despite the opposite transcriptional responses  
430 between clusters #7 and 11 to rhizobial inoculation, 232 genes out of the 736 DEGs in cluster #7  
431 (31.5%) were shared as significantly differentially expressed with cluster #11 (Figure 4B). This  
432 indicates that an unexpectedly high number of genes show opposite transcriptional regulation in  
433 response to rhizobium within different cortical cell clusters. This exemplifies that reaching a cell-  
434 type specific level allows identifying that strong up- or down-regulations can occur simultaneously

435 in different clusters at the same 48 hours post-rhizobium inoculation time-point, which would be  
436 thus likely missed if whole roots would be used. In addition, the differential transcriptional  
437 responses observed between cortical cell clusters for several hormonal pathways and root/nodule  
438 developmental genes upon rhizobial inoculation might mark cells that are activated for nodule  
439 organogenesis from those that are not, and may relate to the different layers of cortex (i.e. inner  
440 *versus* outer), or correspond to cortical cells opposite to proto-phloem *vs* proto-xylem poles where  
441 nodule organogenesis is differentially initiated (Heidstra et al., 1997).

442 The endodermal clusters #15, 16, and 18, showing more than 500 DEGs, once more  
443 comprise either only downregulated genes (#15 and 18), or upregulated genes for cluster #16  
444 (Figure 4A; Supplemental Table 7). In contrast to the cortical cell clusters, these endodermal  
445 clusters shared a limited number of DEGs (Figure 4C). Surprisingly, among clusters #15 and 18  
446 downregulated genes, many early nodulation genes were observed, including for cluster #15 the  
447 infection-related genes *MtLIN*, *MtPUB2*, *MtENODL13*, the NF signaling-related gene *MtDMI2*,  
448 the cytokinin signaling genes *MtCHK1/MtCRE1* and *MtRRA2*, as well as the late nodulation genes  
449 *MtZPT2-1* (Frugier et al., 2000) and *MtDNF2*; and for cluster #18, the NF signaling gene *MtDMI3*,  
450 the cytokinin signaling gene *MtRRB24*, the late nodulation genes *MtZPT2-1* and *MtZPT2-2*, and a  
451 signaling peptide, *MtPIPI* (Supplemental Tables 8 and 9, Figure 5). Concerning endodermis  
452 cluster #16 upregulated genes, the symbiotic-related gene *MtCASTOR* was retrieved, as well as  
453 several hormone-related genes including *MtARF2* and *MtARF8* (auxin), *MtDELLA1* and  
454 *MtDELLA2* (gibberellin), *MtRRB9* (cytokinin), *MtEIN3* (ethylene), and the co-receptor *MtBAK1*.  
455 In addition, the expression of the nitrate signaling-related *MtNLP1* gene was induced  
456 (Supplemental Tables 8 and 9, Figure 5).

457 Finally, in the pericycle (cluster #20), whereas the rhizobium-induced genes do not include  
458 any previously studied early nodulation genes, the *MtNRLK1* receptor-like kinase gene (Laffont et  
459 al., 2018) was retrieved as slightly induced in response to rhizobia. The *MtRbohG*, *MtENOD40*,  
460 *MtZPT2-1*, and *MtZPT2-2* nodulation genes were unexpectedly repressed by rhizobium, as well as  
461 the *MtKNOX9* developmental gene. Regarding hormonal-related genes, the cytokinin *MtRRB5*, the  
462 auxin *MtYUC8*, and the ethylene *MtEBF1* gene, as well as the *MtIDA33* signaling peptide and the  
463 *MtSERK* co-receptor, were also repressed (Supplemental Tables 8 and 9, Figure 5).

464 Taken together, our unique dataset allowed a refined expression pattern analysis which  
465 revealed unexpected cell-type specificity/enrichment for some of the already well-known early

466 nodulation genes (e.g., a differential expression across cortical cell clusters and repression in  
 467 endodermal cell clusters) or for the *MtCASTOR* gene which currently has no symbiotic function  
 468 reported in *M. truncatula* unlike its homolog from *L. japonicus* (Venkateshwaran et al., 2012). In  
 469 addition, some nodulation genes that were previously linked to later symbiotic stages (e.g.,  
 470 *MtNCR112*, *MtZPT2-1*, *MtZPT2-2*, *MtDNF2*, *MtNAC969*) also showed a differential expression  
 471 in response to rhizobium in specific root cell-type clusters. Surprisingly, a few anticipated early  
 472 nodulation genes were missing from the DEG dataset (e.g. *MtLIN*, *MtNF-YA1*, *MtNPL*), but a  
 473 manual inspection of their expression profiles revealed differential regulations by rhizobia that  
 474 were below the statistical threshold used. This indicates that our statistical analysis is conservative,  
 475 allowing providing a robust dataset of DEGs, but also likely missing other genes of interest. More  
 476 refined statistical analyses could be however performed in the future on this dataset, focussing  
 477 only on a subset of specific clusters to extract such additional information that is currently lost due  
 478 to the high variance between all nuclei analyzed.

479

#### 480 **Cell-type-specific expression of nodulation and cytokinin-signaling related genes**

481 As many nodulation-related genes can be already expressed in cells prior to bacterial  
 482 inoculation, we additionally conducted a comprehensive analysis of their expression patterns  
 483 independently of their response to rhizobial inoculation, focusing notably on genes showing cell-  
 484 type-enriched or -specific patterns that were not previously identified as DEGs (see above).  
 485 Epidermal cells (#1 to 5) most specifically expressed the *MtROP5* (Riely et al., 2011), *MtLIN*, and  
 486 *MtPT5* (Wang et al., 2022) genes associated with rhizobial infections (clusters #2 and 3) (Damiani  
 487 et al., 2016); *MtNFP*, *MtLYK3* (Smit et al., 2007), *MtLYK6*, *MtCNGC15c* (Charpentier et al.,  
 488 2016), and *MtNF-YA1* genes, related to NF signaling (clusters #2 and 3); *MtCHIT5a* that is linked  
 489 to NF degradation [clusters #4 and 5; (Tian et al., 2013)]; as well as genes related to late nodulation  
 490 stages, namely *MtRab7A1* (Limpens et al., 2009), *MtSYP132* (Pan et al., 2016), *MtVPE*, *MtSPK1*  
 491 (Andrio et al., 2013), *MtZIP6* (Abreu et al., 2017) (clusters #2 and 3), *MtDNF2* (cluster #4),  
 492 *MtDGD1* (Si et al., 2019) (clusters #4 and 5), and *MtNAC969* (cluster #5); and *MtNLP1*, a gene  
 493 linked to nitrate signaling (clusters #2 and 3) (Figure 6A).

494 In contrast, only a small number of previously characterized nodulation-related genes were  
 495 specifically expressed/enriched in the non-annotated cluster #8, in the stem cell niche cluster #9,  
 496 and in the cortical clusters #6, 7, and 10 to 14 [i.e., *MtCHK1/CRE1*, a cytokinin receptor required

497 for nodule organogenesis in cluster #7; *MtLATD/NIP*, an abscisic acid transporter linked to early  
 498 nodulation in cluster #8 (Bagchi et al., 2012); *MtBR11*, a brassinosteroid receptor linked to  
 499 nodulation (Cheng et al., 2017), and *MtPIN2*, an auxin efflux carrier linked to nodule  
 500 organogenesis in cluster #9 (Huo et al., 2006); and *MtGlb1-1*, a gene involved in later nodulation  
 501 stages in cluster #11 (Berger et al., 2020)] (Figure 6B).

502 In the endodermis (#15 to 19), we identified several nodulation-related genes specifically  
 503 expressed in one or several clusters [i.e., *MtKNOX5* (cluster #16), *MtRbohG*, *MtZPT2-1*, and  
 504 *MtCDPK1* (cluster #18, as well as in the cortical cell cluster #14, (Ivashuta et al., 2005)),  
 505 *MtCDPK3* (clusters #18 and 19), *MtKNOX3* (clusters #16 and 19), *MtANN1* (clusters #15 and 19),  
 506 *MtDMI3* (cluster #19 in addition to the epidermal cell clusters #1, 2, and 3), *MtSYT3* (Gavrin et  
 507 al., 2017), *MtTOP6A* (an ortholog to *LjSUNERGOS1* (Yoon et al., 2014)), and *MtMAPK6* (cluster  
 508 #19; (Chen et al., 2017))] (Figure 6C).

509 In the stele (#20 to 25), a *LjTRICOT-like* gene (Suzaki et al., 2013), *MtMATE69* (Wang et  
 510 al., 2017), and *MtTML2* (Gautrat et al., 2019) were specifically expressed in cluster #20 (pericycle  
 511 cells) while *MtDNF1* (Van de Velde et al., 2010; Wang et al., 2010), *MtVAMP721a* (Sinharoy et  
 512 al., 2013), *MtPIN3* (Huo et al., 2006), *MtRab7a2* (Limpens et al., 2009), *MtSUC1* (Hohnjec et al.,  
 513 2003), *MtARP3* (Gavrin et al., 2015), *MtSYT1* (Gavrin et al., 2017), TOR-like, *MtCDC16*  
 514 (Kuppusamy et al., 2009), *MtKNOX9*, *MtPIN4* (Huo et al., 2006), *MtEFD* (Vernié et al., 2008),  
 515 *MtLAX2* (Roy et al., 2017), *MtTPS2*, *MtVAMP721d* (Ivanov et al., 2012), *MtNF-YC2* (Baudin et  
 516 al., 2015), *MtCCS52a*, and *MtRDN1* (Schnabel et al., 2011) were enriched in at least one of the  
 517 xylem clusters (i.e., #22 to 24). Finally, in cluster #25 (phloem), we identified several genes  
 518 associated with the systemic regulation of nodulation (Gautrat et al., 2021), namely *MtCRA2*  
 519 (Huault et al., 2014; Mohd-Radzman et al., 2016), *MtSUNN*, *MtTML1* (Gautrat et al., 2019),  
 520 *MtIPT3*, and *MtMPKK5* (Figure 6D). These observations are well supported by previous studies  
 521 reporting the cell-type specificity of these genes.

522 Considering the cytokinin signaling pathway which is key for early nodulation (Gamas et  
 523 al., 2017), we found *MtRRA5* most expressed in epidermal clusters #1 to 3, as well as in clusters  
 524 #7 (cortex) and 19 (endodermis), *MtRRA2* in clusters #2 and 3 (epidermis/root hairs), *MtRRA11* in  
 525 clusters #3 (epidermis) and 7 (cortex), *MtCHK1/CRE1*, *MtCHK4*, *MtHPT8*, and *MtRRA4* in cluster  
 526 #7 (cortex), *MtCHK2* and *MtHPT3* in clusters #17 and 18 (endodermis), *MtRRB5* in cluster #18  
 527 (endodermis), *MtRRB1*, *MtRRA3*, *MtRRA4*, *MtRRA6* and *MtRRA8* in cluster #20 (pericycle),

528 *MtHPT8* and *MtRRA9* in clusters #22, 23, and 24 (xylem), and *MtCHK3*, *MtHPT1*, *MtHPT4*,  
529 *MtRRB4* and *MtRRB20* in cluster #25 (phloem) (Figure 6E).

530         These results overall support that many early nodulation genes, including those involved  
531 in cytokinin signaling, have a cell-type specific/enriched expression pattern, indicating a  
532 coordinated activity between cell-types to successfully promote nodule initiation. Interestingly,  
533 the co-regulation in a specific cluster of different genes belonging to a large family, or even to the  
534 same functional pathway, combined with phylogenetic analyses, now provides critical information  
535 to develop more efficient functional analyses to overcome functional redundancy. As an example,  
536 the *RRB* family related to cytokinin signaling contains 12 genes for which expression was detected  
537 in at least one cluster of the UMAP. Noteworthy, *MtRRB4* and *MtRRB20* have overlapping  
538 expression patterns in the phloem (cluster #25), suggesting a likely functional redundancy, also  
539 knowing their close phylogenetic relationship in the same clade (Tan *et al.*, 2019). When  
540 combining this information with the expression pattern of genes from other cytokinin signaling  
541 families, a specific cytokinin signaling pathway preferentially acting in the phloem cells can now  
542 be identified, involving the *MtCHK3* receptor, the *MtHPT1* and *MtHPT4* phosphotransfer  
543 proteins, and the *MtRRB4* and *MtRRB20* TFs. Similarly, when considering systemic pathways  
544 regulating nodulation (Gautrat *et al.*, 2021), our analysis strikingly demonstrates that most known  
545 genes (i.e. *MtCRA2*, *MtSUNN*, *MtIPT3*, *MtTML1*) are specifically expressed, and even induced by  
546 rhizobium in phloem cells (cluster #25). Importantly, getting access to such very detailed and  
547 clearcut spatial expression information allows generating innovative working hypotheses to be  
548 further tested functionally.

549

### 550 **Root cell-type specific vs shared functional pathways enriched in response to a short-term** 551 **rhizobium inoculation**

552         To reveal new biological functions potentially controlling the response of *Medicago* root  
553 cell-types to *E. meliloti*, we performed a gene ontology analysis using the Mapman software  
554 (Schwacke *et al.*, 2019; Tellström *et al.*, 2007) on the DEGs from clusters #2, 7, 11, 15, 16, 18, 20  
555 containing more than 500 DEGs (Figure 4A). The full list of enriched functional pathways is  
556 shown in Supplemental Table 10 and Supplemental Figure 6.

557         In cluster #2, functional pathways corresponding to changes in cellular organization,  
558 including modification of the cell wall (pectin esterases) and vesicle transport were enriched for

559 genes upregulated in response to bacterial inoculation (Wilcoxon rank-sum test,  $p < 0.05$ ). This  
560 likely reflects the curling of root hairs associated with rhizobial infections. Among metabolic  
561 pathways, the flavonoid metabolism was enriched, notably the biosynthesis of dihydroflavonols,  
562 as well as the cytokinin and gibberellin biosynthesis pathways. This result strikingly fits with  
563 knowledge gained during the last decades where these three plant signaling pathways were shown  
564 as crucial for the regulation of early stages of rhizobial infections in the *Medicago* root epidermis  
565 (Fonouni-Farde et al., 2017; Fonouni-Farde *et al.*, 2016; Gonzalez-Rizzo *et al.*, 2006; Plet *et al.*,  
566 2011; Roy *et al.*, 2019). In addition, both lysine motif and leucine-rich repeats receptor kinases  
567 were enriched in this trichoblast cluster #2 in response to rhizobium inoculation, which includes  
568 already known receptors required for rhizobial recognition and infection, as well as the  
569 phosphoinositide metabolism and PHD finger or GRAS TFs, the latter family including also already  
570 known early nodulation genes. Concerning genes downregulated by rhizobia, the brassinosteroid  
571 and ethylene pathways were enriched, the latter hormone being previously extensively  
572 characterized as an inhibitor of rhizobial infections, notably in *Medicago* (Penmetsa et al., 2008).

573 Cortical cell clusters #7 downregulated DEGs showed enrichment for cell wall  
574 modifications and lipid metabolism, several hormonal pathways namely abscisic acid metabolism,  
575 ethylene signaling, as well as AP2/EREBP and Trihelix TF families; and cluster #11 upregulated  
576 DEGs for biotic stress responses, lipid, terpenoid, phenylpropanoid and glucosinolate metabolic  
577 functions, as well as C2H2 zinc finger, CCAAT box binding factors, G2-like (GARP), and MYB-  
578 related TF families, the protein targeting secretory and protein degradation pathways (subtilases  
579 and autophagy), and cytoskeleton reorganization.

580 Endodermal cells clusters #15 and 18 (downregulated genes) were enriched for cell wall  
581 degradation and lipid metabolism functions, as well as ethylene and jasmonate metabolism, GRAS  
582 TF families, leucine-rich repeat receptor kinases, and protein degradation via ubiquitination.  
583 Regarding cluster #16 (upregulated genes), the biotic stress response and glucosinolate  
584 degradation functions were enriched, as well as the jasmonate hormone and the ARR TF family  
585 related to cytokinins, cell division and cell cycle, and sugar transport.

586 Finally, in pericycle cells (cluster #20), rhizobium downregulated genes were once more  
587 enriched for ethylene signaling pathway genes and AP2/EREBP TFs, as previously observed for  
588 the root hair cluster #2, the cortex cluster #7, and the endodermal clusters #15 and 18, highlighting  
589 one of the few shared biological responses across different cell-types.

590 Overall, as previously noticed for the analysis of known nodulation-related genes, the novel  
591 cell-type specific information gained allows for generating new hypotheses, such as the existence  
592 of a tight interaction in inner root tissues of symbiotic responses with defense pathways through  
593 the modulation of specific specialized metabolite production. It also highlights specific hormonal  
594 pathways and TF families for which functional studies remain still limited, or even lacking, and  
595 that could thus be targeted in the future in relation to cell-type specific phenotypes (e.g. rhizobial  
596 infections or nodule organogenesis).

597

## 598 **Conclusions**

599 Plant root development requires tightly coordinated regulation of transcriptomic programs.  
600 We and others revealed root transcriptomic profiles at a single-cell level notably in the model plant  
601 *A. thaliana* (Denyer *et al.*, 2019; Farmer *et al.*, 2021; Jean-Baptiste *et al.*, 2019; Ryu *et al.*, 2019;  
602 Shulse *et al.*, 2019; Zhang *et al.*, 2019). In this study, we provide a comprehensive annotation of  
603 the Medicago root cell-types according to their transcriptomic profiles, as well as an analysis of  
604 the transcriptomic response of Medicago root cells to rhizobial infection. Our study largely  
605 confirmed knowledge gained during the last decades in legume nodulation (i.e., the regulation of  
606 the expression of nodulation-related and hormonal genes known to regulate rhizobial infection  
607 and/or nodule organogenesis), but also nicely illustrates the gain of knowledge obtained using such  
608 single nuclei transcriptomic approaches to better understand the cell-type specifically restricted  
609 responses of plants to microbial infection. The robust and high-quality dataset generated is also a  
610 resource to enable the discovery of new genes of interest not previously highlighted by bulk  
611 transcriptomic analyses. In particular, accessing cell-type information allows for generating more  
612 precise hypotheses regarding the symbiotic processes potentially affected by these novel candidate  
613 DEGs, and thus facilitates planning more appropriately experimental designs, notably by using  
614 tissue-specific promoters for which single-cell datasets are a key resource, and for performing  
615 refined focused phenotyping of rhizobial infections *versus* nodule organogenesis. We foresee that  
616 the application of single-cell -omics technologies to other symbiotic and pathogenic plant-microbe  
617 interactions will lead to a better understanding of the intimate complexity of the relationships  
618 between plants and microbes.

619

## 620 **Methods**

621 Plant materials, root nucleus isolation, library preparation, and sequencing

622         Medicago seedlings were sterilized as described in Pingault et al., 2018. Eight seeds were  
623 then placed on agar B&D medium (Broughton and Dilworth, 1971) without nitrogen, and placed  
624 in a growth chamber in the dark for four days (26 °C for 16h and 20 °C for 8h). On the fourth day,  
625 four mL of an *E. meliloti* suspension (OD<sub>600nm</sub>=0.1), or water for the mock-inoculated samples,  
626 were applied to the seedlings' roots. The plates were placed back into the growth chamber for 48  
627 hours in the dark. On the sixth day, a subset of the *E. meliloti*-inoculated plants was transferred in  
628 vermiculite:perlite (3:1) and grown in the growth chamber (16 hours daylight) for three weeks to  
629 confirm rhizobial infection and the formation of nodules. The remaining roots were used to collect  
630 the nuclei as described in Thibivilliers et al., 2020. The root samples used for these experiments  
631 were around 3 to 4 cm long, starting from the tip and ending in the zone where root hairs are fully  
632 differentiated, thus including the zone susceptible to rhizobial infection. Briefly, roots were then  
633 chopped and passed through a 30µm cell strainer. The filtered nuclei were purified by cell sorting  
634 using FACS Aria II™ cell sorter (BD Biosciences). An average of 80-100,000 nuclei were  
635 collected for each sample, centrifuged, and re-suspended in phosphate buffered saline-bovine  
636 serum albumin 0.5%-RNA-inhibitor solution. The six sNucRNA-seq libraries (i.e., three *E.*  
637 *meliloti*- and three mock-inoculated root libraries) were constructed following the Chromium™  
638 Single Cell 3' Library & Gel Bead Kit v3.1 protocol (10x Genomics). The sequencing of single-  
639 indexed paired-end libraries was performed on an Illumina™ NovaSeq 6000 platform according  
640 to the 10x Genomics recommendations.

641

642 Pre-processing of raw data, integration, clustering, and annotation. The six Medicago sNucRNA-  
643 seq libraries were preprocessed individually using the 10x Genomics Cell Ranger software  
644 v6.1.1.0, and then aligned against the latest version of the *Medicago truncatula* reference genome  
645 and genome annotation (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>) (Pecrix *et al.*,  
646 2018). Upon removal of background contamination using the SoupX software (Young and Behjati,  
647 2020), filtration of doublets using the DoubletDetection prediction method (Adam Gayoso, 2022),  
648 and applying a statistical threshold on the data distribution (i.e., an interval of confidence of 95%  
649 to remove outliers; see Supplemental Figure 1 to access the parameters for each sNucRNA-seq  
650 library), the normalization of individual sNucRNA-seq datasets and their respective integration to  
651 generate UMAPs was performed using Seurat V4 (Hao et al., 2021), selecting “top 2000 variable

652 genes” for feature selection. Integration anchors were defined for the combined set of six  
653 sNucRNA-seq datasets based on the first 20 dimensions of the canonical correlation analysis  
654 method. After integration, the dimensionality reduction was performed with the first 40 principal  
655 components to generate the UMAP projection. Besides, the clustering was generated with the  
656 method FindClusters from Seurat with a resolution of 0.6. For downstream analyses, the  
657 expression values of each gene were calculated for each cluster using the AverageExpression  
658 function from Seurat.

659 For the annotation of cell-types, the cluster-specific genes were identified with the  
660 FindAllMarkers function in Seurat. In addition, the expression patterns of known cell-type specific  
661 gene markers from *M. truncatula* (Supplemental Table 2 and Medicago genes orthologous to *A.*  
662 *thaliana* root cell-type marker genes were further analyzed (Farmer *et al.*, 2021) (Supplemental  
663 Table 4).

664

665 UMAP visualization. For visualization purposes, all sNucRNA-seq libraries were combined  
666 using the Cell Ranger aggr function from 10x Genomics to combine all counts in a single cloupe  
667 file, and to show the UMAP coordinates projections and cell cluster assignments obtained from  
668 the Seurat analysis. We use ShinyCell (available at <https://github.com/SGDDNB/ShinyCell>), a  
669 web application allowing the visualization of single-cell data, to allow direct inquiries of the  
670 Medicago root single-cell transcriptome atlas (available from the  
671 [https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1\\_6.expr.Cervantes-](https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1_6.expr.Cervantes-Perez_Thibivilliers_2022/)  
672 [Perez\\_Thibivilliers\\_2022/](https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1_6.expr.Cervantes-Perez_Thibivilliers_2022/) web interface).

673

674 Differential Gene expression analysis. To identify DEGs, raw read counts were extracted to  
675 calculate a normalized average expression for each gene, in each cluster, and for each condition  
676 (Supplemental Table 9) before applying the DEsingle package (Miao *et al.*, 2018) using p-  
677 value<0.05 and  $|FC| > 1.5$  thresholds. This package allows the identification of DEGs between  
678 *E. meliloti*- and mock-inoculated nuclei in a raw read count matrix employing the Zero-Inflated  
679 negative binomial model (Wang *et al.*, 2019).

680

681 Correlation analysis between plant root single nuclei transcriptomes. To support the functional  
682 annotation in the *M. truncatula* root clusters, we compared the transcriptomes of the different

683 Medicago root clusters upon extracting the pseudo-bulk expression of each gene among all clusters  
684 and then conducted Pearson's correlation analyses to reveal the most similar transcriptomes among  
685 all nuclei clusters.

686 To compare the transcriptome of Medicago and Arabidopsis root clusters, *A. thaliana*  
687 sNucRNA-seq datasets were obtained from previously published data (Farmer *et al.*, 2021), based  
688 on the following SRA files: GSM4698755, GSM4698756, GSM4698757, GSM4698758,  
689 GSM4698759. The five replicates were processed individually using the 10x Genomics Cell  
690 Ranger v6.1.1.0 pipeline, and then mapped against a reference genome constructed with  
691 TAIR10.26 genome and Araport11 annotations. The same parameters were then used for  
692 preprocessing the Arabidopsis datasets as previously used for the Medicago sNucRNA-seq  
693 datasets (see above). To correlate the *A. thaliana* and *M. truncatula* root sNuc-transcriptomes, we  
694 extracted pseudo-bulk information for the one-to-one orthologs between the two plant species  
695 [CoGe; <https://genomevolution.org/coge/>; (Lyons and Freeling, 2008; Lyons *et al.*, 2008)] and  
696 conducted a Pearson's correlation analysis between the expression of these orthologous genes for  
697 each cell cluster.

698  
699 *Genes of interest and functional classification analyses.* The Legoo knowledge base ([https://lipm-](https://lipm-browsers.toulouse.inra.fr/k/legoo/)  
700 [browsers.toulouse.inra.fr/k/legoo/](https://lipm-browsers.toulouse.inra.fr/k/legoo/)) was used to identify genes of interest (Carrère *et al.*, 2019), as  
701 well as the Mapman software (<https://mapman.gabipd.org/>) for the analysis of gene functions  
702 (Thimm *et al.*, 2004).

703

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710

711 **Author contributions**

712 S.T. performed experiments. S.A.C.P., S.T., C. L., A.D.F., F.F., and M.L. carried out data  
713 analysis. M.L. coordinated the study. S.A.C.P., S.T., F.F., and M.L. drafted the manuscript. All  
714 authors contributed to the preparation of the manuscript.

715

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723

724 **Accession numbers**

725 Expression data are available at the Gene Expression Omnibus (GEO: GSE210881). The  
726 Medicago root single-cell transcriptome atlas can be accessed through  
727 [https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1\\_6.expr.Cervantes-](https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1_6.expr.Cervantes-Perez_Thibivilliers_2022/)  
728 [Perez\\_Thibivilliers\\_2022/](https://shinycell.legumeinfo.org/medtr.A17.gnm5.ann1_6.expr.Cervantes-Perez_Thibivilliers_2022/).

729

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1261

## 1262 **Figure legends**

1263 **Figure 1. Single-nuclei RNA-seq of the *M. truncatula* roots reveals 25 different root**  
 1264 **clusters. A.** UMAP clustering of *M. truncatula* / *E. meliloti*- and mock-inoculated root nuclei  
 1265 according to their transcriptomic profiles. While the overall topography of these two UMAPs is  
 1266 well conserved, subtle differences are observed (e.g., highlighted in red for cluster #2). **B.**  
 1267 Percentage of *E. meliloti* (dash bars) and mock-inoculated (solid bars) nuclei allocated in the 25  
 1268 clusters composing the *M. truncatula* root UMAP.

1269

1270 **Figure 2. Functional annotation of the 25 *M. truncatula* root clusters. A.** UMAP clustering  
 1271 and functional annotation of the Medicago root cell-types clusters based on the expression of  
 1272 Medicago marker genes and of genes orthologous to Arabidopsis root marker genes. **B.**  
 1273 Normalized expression levels of cell-type marker genes functionally characterized in Medicago  
 1274 (detailed in Supplemental Table 2), or orthologous to Arabidopsis root cell-type-specific marker  
 1275 genes (detailed in Supplemental Table 4) across the 25 Medicago root clusters, shown on the y-  
 1276 axis. The percentage of nuclei expressing the gene of interest (circle size), and the mean  
 1277 expression (circle color) of genes, are shown for each sub-panel.

1278

1279 **Figure 3. Comparative transcriptomic analysis of the *M. truncatula* and *A. thaliana* root cell**  
 1280 **clusters. A.** Functional annotation of Arabidopsis root nuclei clusters based on the expression  
 1281 profile of cell-type marker genes defined from (Farmer *et al.*, 2021). **B.** Pairwise correlations of  
 1282 Arabidopsis (*x*-axis) and Medicago (*y*-axis) root cell clusters. Only correlation numbers greater  
 1283 than 0.4 (black numbers) or 0.5 (white numbers) are shown in the heatmap. EC: Epidermal cells;  
 1284 CC: Cortical cells; SCN: Stem cell niche; EC: Endodermal cells; SC: Stele cells.

1285

1286 **Figure 4. Differential expression of the *M. truncatula* genes in response to *E. meliloti***  
 1287 **inoculation across the 25 root cell clusters. A.** The transcriptional response of Medicago root  
 1288 cells to *E. meliloti* inoculation differs between cell-type clusters. The number of up- and down-  
 1289 regulated genes are highlighted in green and red bars, respectively. The dashed bar reflects the  
 1290 500 DEGs thresholds. **B** and **C.** Comparison of the number of DEGs between the cortical cell  
 1291 clusters #7 and 11 (**B**) and between the endodermal cell clusters #15, 16, and 18 (**C**). EC:  
 1292 Epidermal cells; CC: Cortical cells; SCN: Stem cell niche; EC: Endodermal cells; SC: Stele  
 1293 cells.

1294

1295 **Figure 5. Summary of the *M. truncatula* root cell-type specific transcriptional response to**  
 1296 **rhizobial inoculation.** Selected genes previously known as related to nodulation and hormonal  
 1297 pathways and identified as differentially expressed in the clusters showing more than 500 DEGs  
 1298 are listed. Besides the expected induced expression pattern of numerous nodulation-related genes  
 1299 such as in the root hair cells cluster #2, more unexpected expression profiles were also highlighted  
 1300 notably for some late nodulation genes, and for repressed early nodulation and hormone-related  
 1301 genes in cortical and endodermal clusters #7 and 15-18. Genes are listed in the following  
 1302 categories: rhizobial infection-related, Nod factor (NF) signaling, other nodulation stages,  
 1303 hormones, signaling peptides. Upward arrows indicate gene inductions by rhizobia, and downward  
 1304 arrows, repressions.

1305

1306 **Figure 6. Cell-type enrichment of known *M. truncatula* nodulation and cytokinin signaling**  
 1307 **genes. A-E.** Normalized expression levels of Medicago nodulation-related genes specifically  
 1308 expressed/enriched in the epidermal (**A**), cortical (**B**), endodermal (**C**), and steles cells (**D**), as well  
 1309 as of Medicago cytokinin-signaling-related genes (**E**). The 25 Medicago root clusters identified  
 1310 are shown on the *x*-axis. The percentage of nuclei expressing the gene of interest (circle size), and  
 1311 the mean expression (circle color) of genes, are shown for each sub-panel. M = mock-inoculated  
 1312 condition; I: rhizobia-inoculated condition.

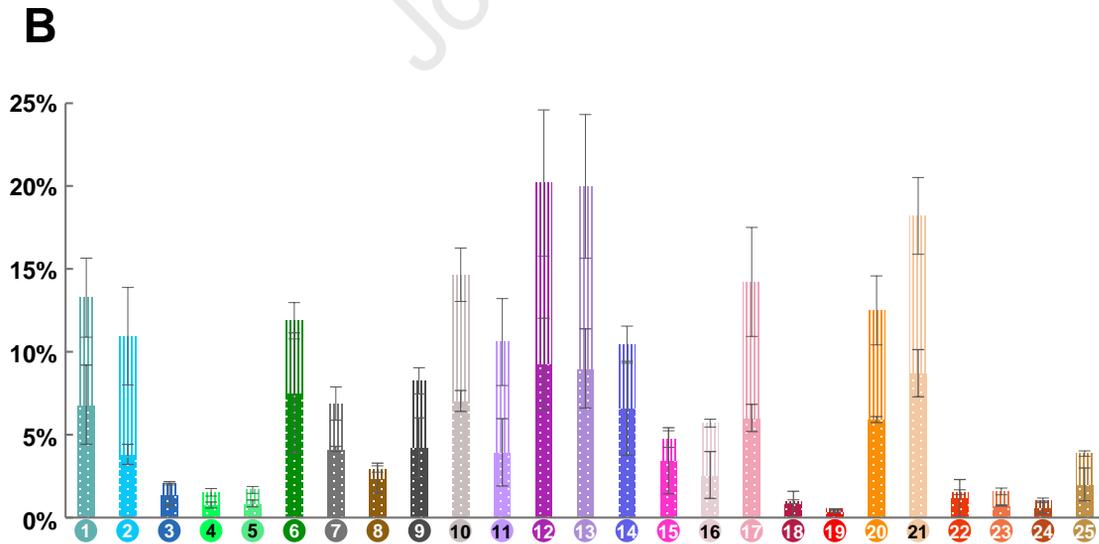
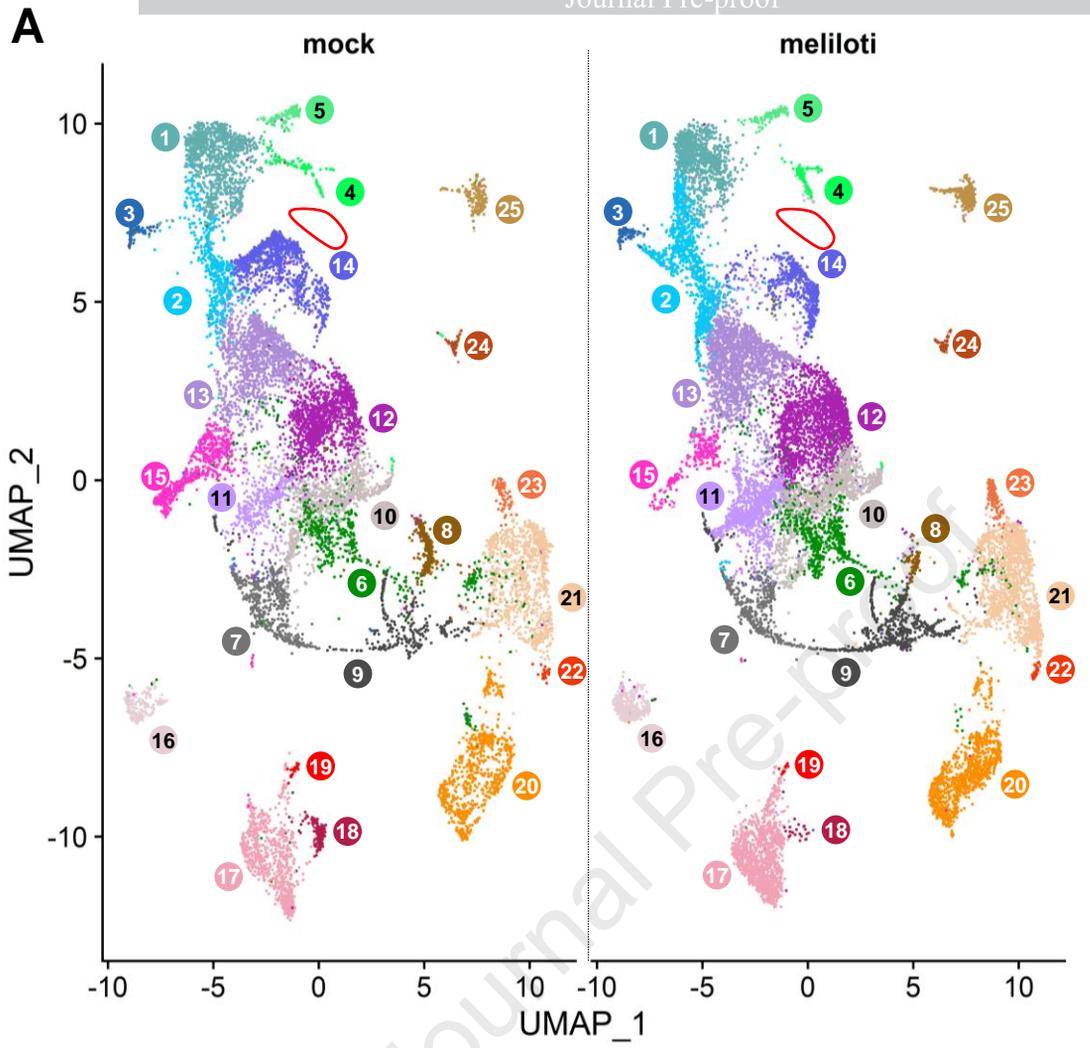


Figure 1.

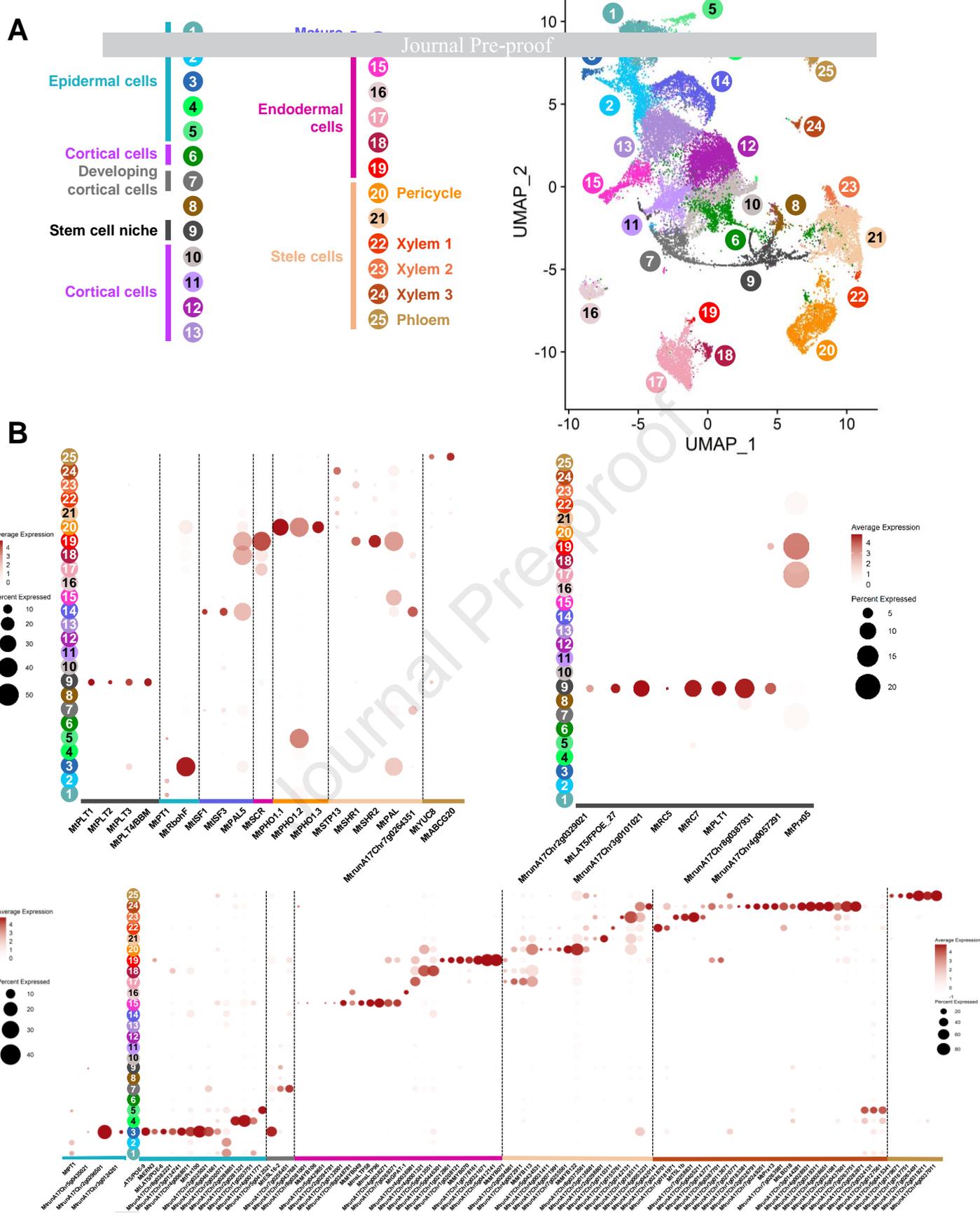
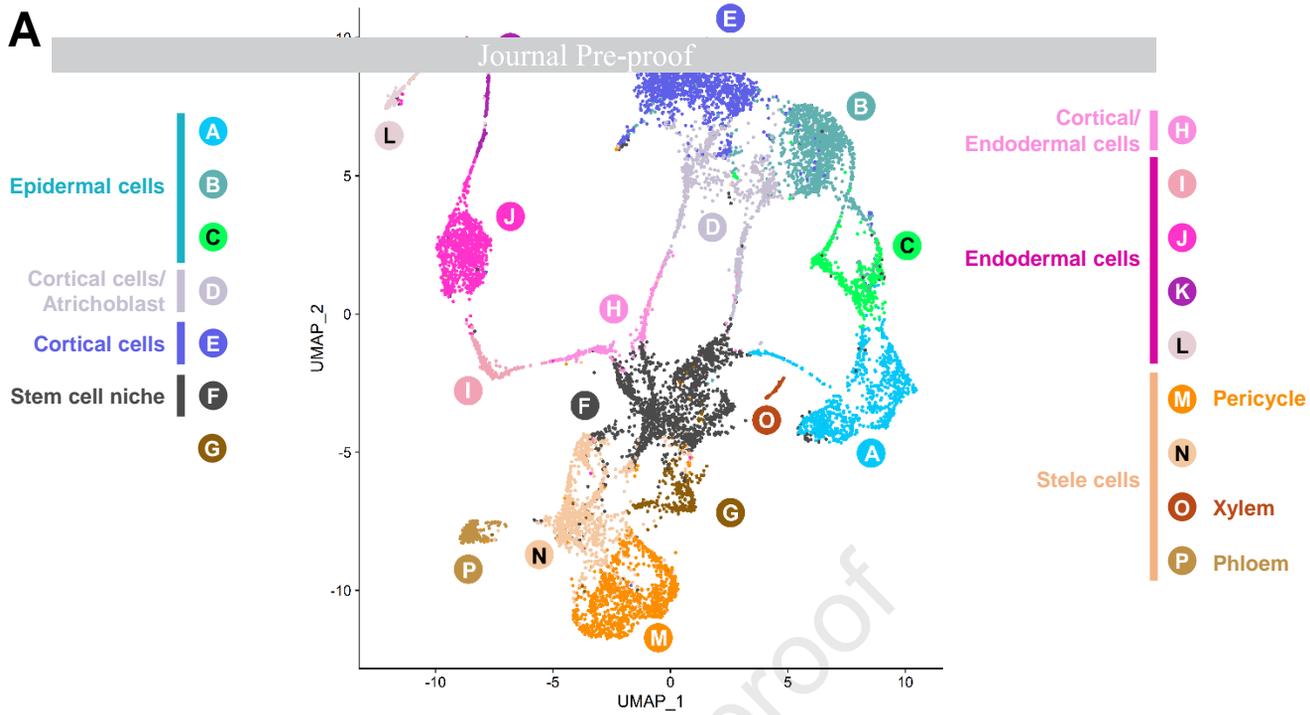


Figure 2

**A**



**B**

*Arabidopsis thaliana* root

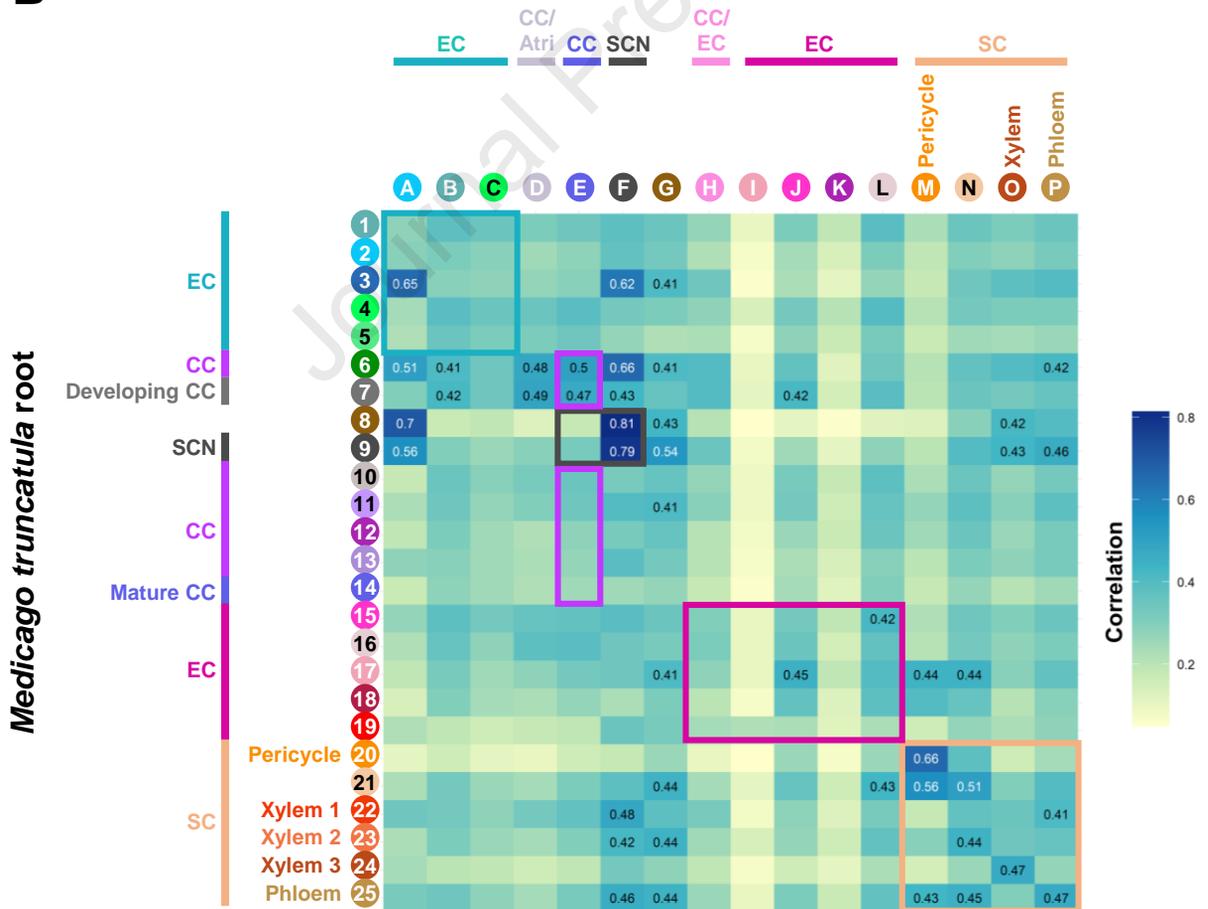


Figure 3.

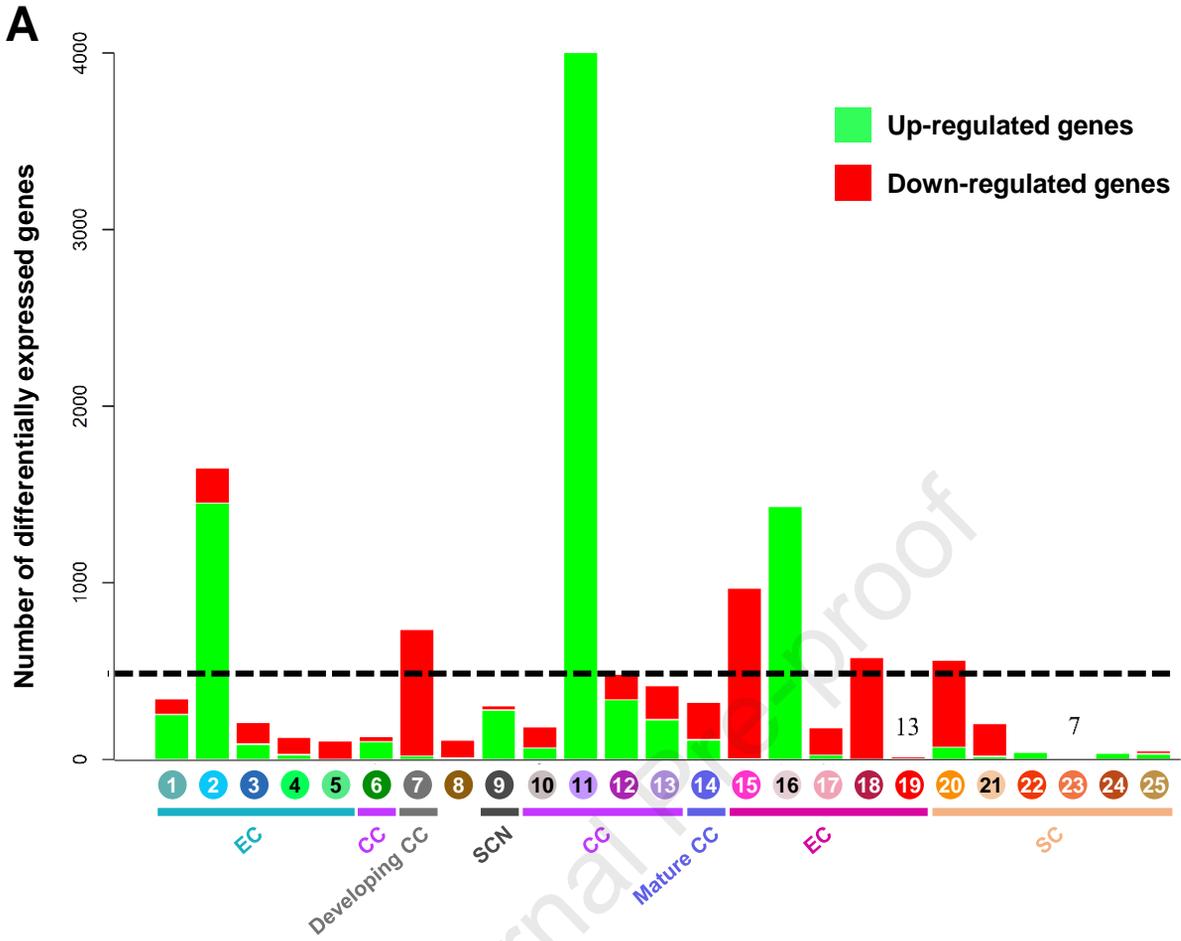
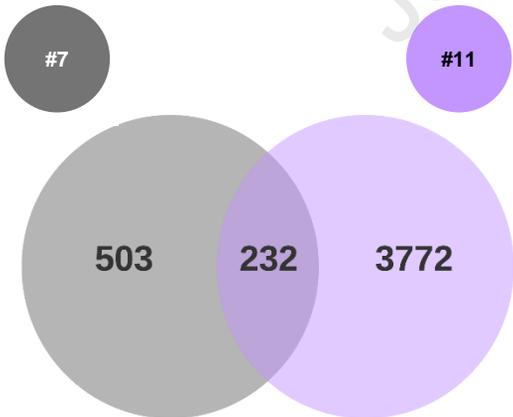
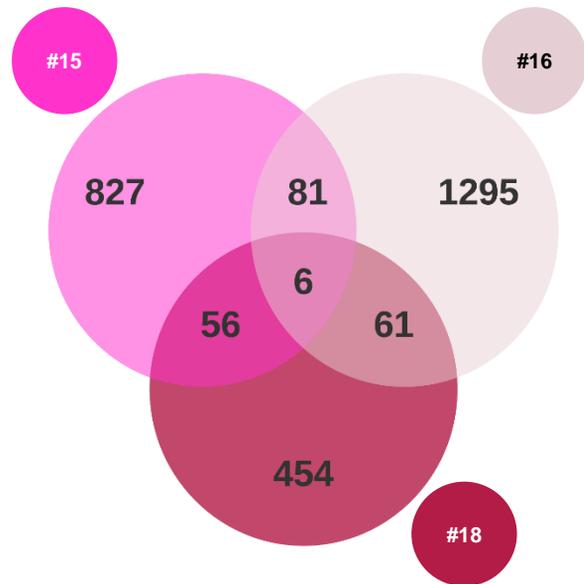
**B****C**

Figure 4.

**CLUSTER #4**

↑ **Infection-related:** *MtRbohA*, *MtRbohB*, *MtLIN*  
 ↑ **NF signaling:** *MtPUB2*, *MtDMI1*, *MtDMI3*, *MtERN2*  
 ↑ **Other stages:** *MtSHR1*, *MtKNOX4*, *MtKNOX9*, *MtNOOT1*, *MtCCS52a*, *MtNAC969*  
 ↑ **Hormones:** cytokinin (*MtCHK1/MtCRE1*, *MtHPT3*, *MtRRB5*, *MtRRB8*, *MtRRA5*); auxin (*MtARF10*, *MtARF13*, *MtARF24*); gibberellin (*MtDELLA2*); ethylene (*MtETR1*, *MtEIN3*); jasmonic acid (*MtLOX3*, *MtLOX6*); strigolactone (*MtMAX2b*)  
 ↑ **Signaling peptides:** *MtRTF/DVL11*

*MtVVPY*, *MtAnn1*, *MtNMN1*  
 ↑ **NF signaling:** *MtNHF1*, *MtLYK10*, *MtPUB1*, *MtIPD3*, *MtDMI1*, *MtDMI2*, *MtDMI3*, *MtNSP1*, *MtNSP2*, *MtERN1*, *MtERN2*, *MtNIN*.  
 ↑ **Other stages:** *MtKNOX3*, *MtKNOX5*, *MtDNF2*, *MtNCR112*  
 ↑ **Hormones:** cytokinin (*MtIPT1*, *CYP735A1-like*, *MtHPT1*, *MtRRB6*, *MtRRA2*, *MtRRA5*); gibberellin (*MtGA2ox10*, *MtGA3ox1*, *MtDELLA2*); jasmonic acid (*MtLOX6*, *MtJAZ3*); auxin (*MtARF10*); abscisic acid (*MtABI5*); strigolactone (*MtD27*, *MtMAX1a*); ethylene (*MtETR4*); brassinosteroid (*MtBAK1*)  
 ↓ **NF signaling/infection:** *MtPUB2*  
 ↓ **Signaling peptides:** *MtPIP1*, *MtIDA20*, *MtIDA31*, *MtIDA35*

**CLUSTER #7**

↓ **NF signaling/infection:** *MtCASTOR*  
 ↓ **Hormones:** cytokinin (*MtRRA4*, *MtRRA9*), gibberellin (*MtDELLA1*), abscisic acid (*MtABI5*)

**CLUSTER #16**

↑ **NF signaling/infection:** *MtCASTOR*  
 ↑ **Hormones:** cytokinin (*MtRRB9*); gibberellin (*MtDELLA1*, *MtDELLA2*); auxin (*MtARF2*, *MtARF8*); ethylene (*MtEIN3*); brassinosteroid (*MtBAK1*)

**CLUSTER #18**

↓ **NF signaling:** *MtDMI3*  
 ↓ **Other stages:** *MtZPT2-1*, *MtZPT2-2*.  
 ↓ **Hormones:** cytokinin (*MtRRB24*)  
 ↓ **Signaling peptides:** *MtPIP1*

**CLUSTER #20**

↑ **Other stages:** *MtNRLK1*  
 ↓ **Other stages:** *MtRbohG*, *MtENOD40*, *MtZPT2-1*, *MtZPT2-2*, *MtKNOX9*  
 ↓ **Hormones:** cytokinin (*MtRRB5*); auxin (*MtYUC8*, *MtSERK*); ethylene (*MtEBF1*)  
 ↓ **Signaling peptides:** *MtIDA33*

**CLUSTER #15**

↓ **Infection-related:** *MtLIN*, *MtPUB2*, *MtENODL13*  
 ↓ **NF signaling:** *MtDMI2*  
 ↓ **Other stages:** *MtZPT2-1*, *MtDNF2*  
 ↓ **Hormones:** cytokinin (*MtCHK1/MtCRE1*, *MtRRA2*)

Figure 5.

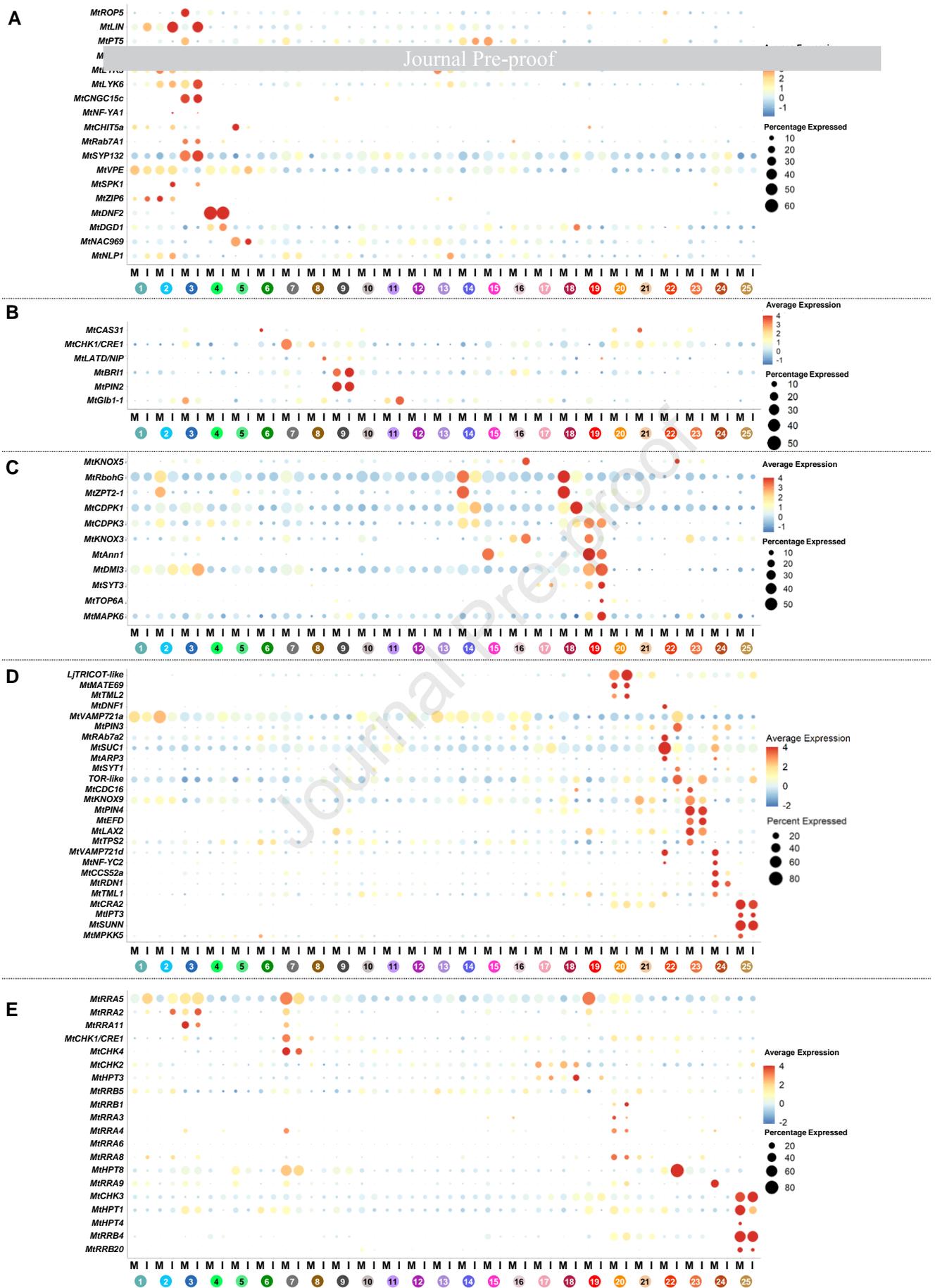


Figure 6.