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Robust transcriptional indicators of immune cell death revealed by spatio-temporal transcriptome analyses
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Abstract:	Recognition of a pathogen by the plant immune system often triggers a form of regulated cell death traditionally known as the hypersensitive response (HR). This type of cell death occurs precisely at the site of pathogen recognition, and it is restricted to a few cells. Extensive research has shed light into how plant immune receptors are mechanistically activated. However, a central key question remains largely unresolved: how does cell death zonation take place and what are the mechanisms that underpin this phenomenon? Consequently, bona fide transcriptional indicators of HR are lacking, which prevents gaining a deeper insight of its mechanisms before cell death becomes macroscopic and precludes any early or live observation. We addressed this question using the paradigmatic Arabidopsis thaliana – Pseudomonas syringae pathosystem, by performing a spatio-temporally resolved gene expression analysis that compared infected cells that will undergo HR upon pathogen recognition vs by-stander cells that will stay alive and activate immunity. Our data revealed unique and time-dependent differences in the repertoire of differentially expressed genes, expression profiles and biological processes derived from tissue undergoing HR and that of its surroundings. Further, we generated a pipeline based on concatenated pairwise comparisons between time, zone and treatment that enabled us to define 13 robust transcriptional HR markers. Among these genes, the promoter of an uncharacterized AAA-ATPase has been used to obtain a fluorescent reporter transgenic line, which displays a strong spatio-temporally resolved signal specifically in cells that will later undergo pathogen-triggered cell death. In sum, this valuable set of genes can be used to define those cells that are destined to die upon infection with HR-triggering bacteria, opening new avenues for specific and/or high-throughput techniques to study HR processes at a single-cell level.
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Robust transcriptional indicators of immune cell death revealed by spatio-temporal transcriptome analyses

Running title: Plant HR indicators

Short summary: Our work provides a detailed overview of the spatio-temporal transcriptomic landscape of the hypersensitive response (HR), a form of plant-specific immune cell death. Further, a set of robust transcriptional marker genes of the cells that will undergo HR is provided.

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Abstract

Recognition of a pathogen by the plant immune system often triggers a form of regulated cell death traditionally known as the hypersensitive response (HR). This type of cell death occurs precisely at the site of pathogen recognition, and it is restricted to a few cells. Extensive research has shed light into how plant immune receptors are mechanistically activated. However, a central key question remains largely unresolved: how does cell death zonation take place and what are the mechanisms that underpin this phenomenon? Consequently, *bona fide* transcriptional indicators of HR are lacking, which prevents gaining a deeper insight of its mechanisms before cell death becomes macroscopic and precludes any early or live observation. We addressed this question using the paradigmatic *Arabidopsis thaliana–Pseudomonas syringae* pathosystem, by performing a spatio-temporally resolved gene expression analysis that compared infected cells that will undergo HR upon pathogen recognition *vs* by-stander cells that will stay alive and activate immunity. Our data revealed unique and time-dependent differences in the repertoire of differentially expressed genes, expression profiles and biological processes derived from tissue undergoing HR and that of its surroundings. Further, we generated a pipeline based on concatenated pairwise comparisons between time, zone and treatment that enabled us to define 13 robust transcriptional HR markers. Among these genes, the promoter of an uncharacterized *AAA-ATPase* has been used to obtain a fluorescent reporter transgenic line, which displays a strong spatio-temporally resolved signal specifically in cells that will later undergo pathogen-triggered cell death. In sum, this valuable set of genes can be used to define those cells that are destined to die upon infection with HR-triggering bacteria, opening new avenues for specific and/or high-throughput techniques to study HR processes at a single-cell level.

INTRODUCTION

Plants are rich sources of nutrients for pathogens with contrasting lifestyles (Dangl et al., 2013). As opposed to animals, plants do not possess a circulatory system with mobile cells specialized in pathogen defense (Jones and Dangl, 2006). Since their cells are fixed by their cell walls, plants rely on each cell's autonomous immunity and on systemic signals emanating from infection sites to distal cells to prime the plant for future pathogen encounters (Ausubel, 2005). Moreover, instead of a somatic adaptive immune system that produces antigen receptors on demand, plant cells are equipped with extracellular pattern-recognition receptors (PRRs) and intracellular nucleotide-binding leucine-rich repeat immune receptors (NLRs) that recognize microbe-associated microbial patterns (MAMPs) and pathogen effectors required for virulence, respectively (Couto and Zipfel, 2016). PRR activation brings about a broad defense response named pattern-triggered immunity (PTI), while NLR activation triggers a potentiated and prolonged immune response named effector-triggered immunity (ETI) that reinforce defense outputs observed during PTI (Yuan et al., 2021a; Ngou et al., 2021b). ETI often culminates in a macroscopic localized cell death at the attempted pathogen ingress site known as hypersensitive response (HR)-cell death or immune-related cell death (Olvera-Carrillo et al., 2015; Balint-Kurti, 2019; Salguero-Linares and Coll, 2019).

Regulated cell death has a crucial role in both animals and plant immune responses. Extensive research in the animal field supports the notion that the immune system is highly dependent on cell death for a robust and tightly controlled immune response to occur (Lu et al., 2014; Nagata and Tanaka, 2017) . In plants, our knowledge about the biochemical and genetic pathways regulating cell death, particularly in the context of immunity, is still very limited. As an attempt to shed light into how HR is orchestrated in plants, most efforts have been directed towards

26 understanding how NLRs are mechanistically activated, as well as identifying molecular

27 components upstream or downstream of NLRs that are required for HR to occur (Dangl and

28 Jones, 2019; Wang et al., 2019a; Wang et al., 2019b; Ma et al., 2020; Ngou et al., 2021a)

29

30 Plant NLRs can be broadly classified into TNLs and CNLs based on their domain composition:

31 TNLs contain a Toll/Interleukin-1 Receptor (TIR), whereas CNLs harbor a coiled-coiled

32 domain at their N-terminal end (Jones et al., 2016). Groundbreaking research has shown that

33 in plants, pathogen perception leads to NLR oligomerization, which ultimately will result in

34 cell death and immunity (Wang et al., 2019a; Wang et al., 2019b; Ma et al., 2020; Förderer et

35 al., 2022). Oligomerized forms of CNLs can form pores at the plasma membrane that act as

36 Ca^{2+}-permeable channels (Wang et al., 2019a; Wang et al., 2019b; Jacob et al., 2021). Some

37 TNLs, in turn, can oligomerize upon activation to reconstitute a holoenzyme that triggers cell

38 death by a mechanism that is not fully elucidated but that may involve their NAD^+ hydrolase,

39 as well as their 2′,3′-cAMP/cGMP synthetase activities (Ma et al., 2020; Martin et al., 2020;

40 Yu et al., 2021). How oligomerization translates to immune signaling and HR remains to be

41 defined.

42

43 In the context of signaling downstream NLR activation or ETI, large-scale transcriptional

44 studies have highlighted the importance of phytohormone networks for high-amplitude

45 transcriptional reprogramming to mount a fast and efficient response (Mine et al., 2018).

46 Comparisons between host transcriptional responses elicited by PTI and ETI suggest minor

47 qualitative differences in the repertoire of genes differentially expressed (Navarro et al., 2004;

48 Mine et al., 2018). These studies also support the recently evidenced assumption that ETI and

49 PTI share immune signaling components (Pruitt et al., 2021; Yuan et al., 2021a; Ngou et al.,

50 2021b). However, a central key question remains unexplored: which early transcriptional

51 signatures differentiate cells that recognize the pathogen and will undergo HR from by-stander

52 cells that will remain alive and will activate defenses to fight the pathogen? In recent literature,

53 a few studies underscore the importance of zonation during HR (Betsuyaku et al., 2018; Giolai

54 et al., 2019; Lukan et al., 2020). At the hormonal level, it has been shown that salicylic acid

55 (SA) plays a major role at pathogen-inoculated spots that will later undergo HR, while the

56 jasmonic acid (JA) signaling pathway is activated in the cells surrounding the central SA-active

57 cells (Dorey et al., 1997; Betsuyaku et al., 2018). Furthermore, precision transcriptomics during

58 the immune response elicited by the potato Ny-1 gene against potato virus Y (PVY) revealed

59 the importance of SA accumulation and genes involved in the generation of reactive oxygen

60 species (ROS) for efficient confinement of macroscopic cell death lesions caused by PVY

61 (Lukan et al., 2020). The cell wall polymer lignin has also been shown to participate in HR

62 zonation, by forming a physical barrier around the infection site upon pathogen recognition

63 that presumably contributes to confine the invading agents and restricts colonization (Lee et

64 al., 2019). A transcriptional meta-analysis of developmental *vs* HR-cell death in plants could

65 only reveal robust indicators for developmental cell death but not for HR-cell death (Olvera-

66 Carrillo et al., 2015) . We realized that the limitation of previous large-scale transcriptomic

67 analysis lacked the spatial dimension of HR (Lewis et al., 2015; Mine et al., 2018), as dying

68 cells were not compared to by-stander cells, and the focus was not placed on identifying

69 specific cell death markers, but rather bulk-analyzing the ETI response at the inoculated area.

70

71 A systematic gene expression analysis of the zonation of HR overtime would help

72 understanding the process of HR at the molecular level and importantly, would allow defining

73 *bona fide* transcriptional markers of the process. With this purpose, we generated RNA-

74 sequencing (RNA-seq) data to systematically analyze and compare the transcriptional

75 programs taking place at the zone of inoculation/pathogen recognition that will undergo HR vs

76   the surrounding area that will stay alive and activate immunity. We show unique and time-

77   dependent differences in the repertoire of differentially expressed genes (DEGs) and

78   expression profiles derived from tissue undergoing HR and that of its surrounding tissues.

79   Furthermore, we generated a pipeline based on pairwise comparisons between time, zone and

80   treatment that enabled us to define 13 robust transcriptional HR markers and a fluorescent

81   transgenic reporter line. These valuable set of genes can be used to define those cells that are

82   destined to die upon pathogen recognition before the onset of cell death becomes

83   macroscopically visible, opening new horizons to study the processes therein by live, cell-

84   specific and/or high-throughput techniques.

85

86   **RESULTS**

87

88   **Zonally dissected Arabidopsis transcriptomes upon *Pto AvrRpm1* infection reveal unique**

89   **spatio-temporal gene expression.**

90   In our experiments we used the paradigmatic interaction between *Arabidopsis thaliana* Col-0

91   (hereafter Arabidopsis) and the bacterial pathogen *Pseudomonas syringae* pathovar tomato

92   (*Pto*) carrying the effector *AvrRpm1* (hereafter *Pto AvrRpm1)*, which triggers restricted HR at

93   the site of inoculation upon recognition by the CNL RPM1 (RESISTANCE TO

94   PSEUDOMONAS SYRINGAE PV MACULICOLA 1) (Mackey et al., 2002). In order to

95   zonally dissect HR and its surrounding, we syringe-infiltrated a limited area (roughly 3-4 mm)

96   at the side edge of Arabidopsis leaves with either a mock solution or *Pto AvrRpm1*. Collected

97   tissue from this area was designated as the "IN" zone. To ensure proper separation between IN

98   and OUT zones, a buffer zone expanding 1 mm next to the IN area was discarded, and a parallel

99   region expanding 1 to 2 mm towards the vein was designated as "OUT" (**Figure 1A**). We

100   collected tissue at 0, 1-, 2-, 4- and 6-hours post-inoculation (hpi), extracted RNA and assessed

101   transcript abundance by RNA-seq. Under these conditions, macroscopic cell death started

102   appearing at 4 hpi in the *Pto AvrRpm1*-inoculated samples, as visualized by trypan blue staining

103   (**Figure 1B**). As expected, this cell death is concomitant with a dramatic drop in photosynthetic

104   efficiency of photosystem II (Fv/Fm ratio) and electron transport rate (ETR) at the IN area

105   (**Figure 1C**) (Berger et al., 2007).

106

107   To determine whether the obtained RNA-seq data complied with our working hypothesis of

108   spatio-temporal gene expression regulation we performed a Principal Component Analysis

109   (PCA) (**Figure S1A-B**). We observed that at the IN area, *Pto AvrRpm1*-treated samples

110   separated from their mock controls from 2 hpi onwards. At the OUT area, however, only *Pto*

111   *AvrRpm1*-treated samples at 4 and 6 hpi separated from mock controls. Overall, the PCA

112   confirms that the biggest changes in gene expression are produced at IN, particularly at 4 and

113   6 hpi, whereas at OUT there is a subtler modulation that is most pronounced at 4 hpi.

114

115   Next, we identified differentially expressed genes (DEGs) between bacteria and mock-

116   inoculated samples (DEGs; false discovery rate (FDR) < 0.05 and $|log2FC| > 2$), thereby

117   characterizing the transcriptional changes occurring at each tissue area at every time point. We

118   found a total of 5,495 DEGs at the IN zone and 1,785 at the OUT zone (**Figure 2A, Table S1**).

119   Enrichment of Gene Ontology (GO) terms was examined in every group of DEGs at each

120   specific time point (**Figure S2, Table S2**). Upregulated genes at the IN area were enriched in

121   immunity- and phytohormone-associated processes (**Figure S2A**). Immunity-related GO terms

122   associated with PTI and ETI such as "plant-type hypersensitive response" and "pattern

123   recognition receptor signalling pathway" appeared at initial stages of infection (1 and 2 hpi),

124   while at later stages (from 2 hpi onwards) there is enrichment of GO terms associated with

125   more general defense and abiotic stress processes such as "defense response to bacteria" and

126 "response to wounding", respectively (**Figure S2A**). Regarding phytohormone-related

127 processes, we observed an enrichment in SA-related GO terms from 1 hpi onwards, confirming

128 the importance of SA at the HR/IN area (Dorey et al., 1997; Zheng et al., 2015). In contrast,

129 GO terms associated with JA were particularly overrepresented at later time points (4 and 6

130 hpi), in accordance with previous findings demonstrating that SA can activate JA signaling

131 through a non-canonical pathway promoting ETI (Liu et al., 2016). GO terms related to other

132 defense/stress-related phytohormones such as ethylene (ET) and abscisic acid (ABA), were

133 also enriched at 4 and 6 hpi (**Figure S2A**).

134

135 Among downregulated genes at the IN zone, an enrichment in GO terms related to

136 photosynthesis and chloroplast biology occurred at late time points (4 and 6 hpi) (**Figure S2B**).

137 This correlates with the drop in photosynthetic efficiency shown in **Figure 1C**, which is part

138 of the defense/yield trade-off to derive resources to immune responses and shut down

139 production of sugars and nutrients, as they might serve as a source for pathogen survival and

140 multiplication (Lu and Yao, 2018).

141

142 Strikingly, at the OUT area we only observed differential expression at late time points (4 and

143 6 hpi), with an overall reduction in the number of DEGs compared to the IN area (**Figure 2A**).

144 Upregulated genes were enriched in GO terms associated with hormonal regulation,

145 particularly to the JA signaling pathway (**Figure S2C**). Downregulated genes at the OUT area

146 did not show any enriched GO term, possibly due to the low number of genes.

147

148 To identify genes exclusively upregulated (FDR < 0.05 and |log2FC| > 2) at either the IN or

149 OUT areas we first generated Venn diagrams representing the number of genes modulated at

150 each time point upon infection (**Figure S3**). This analysis confirmed that upregulation at both

151   IN and OUT mainly occurs at 4 or 6 hpi (**Figure S3**) and therefore, we selected these two time

152   points to further identify genes that are exclusively upregulated at each tissue area (**Figure 2B**).

153   Specifically, we found a total of 1,840 genes being upregulated exclusively at IN, 1,117 genes

154   upregulated at both IN and OUT and 221 genes being exclusively upregulated at OUT (**Figure**

155   **2B, Table S3**). Among the overrepresented GO terms found in genes exclusive for the IN area

156   were "defense response to bacterium", "response to molecule of bacterial origin" or "response

157   to salicylic acid". We also found various GO terms associated with responses to several other

158   stresses such as salt, oxygen-containing compounds, sulfur compounds, heat and hydrogen

159   peroxide (**Figure 2C, Table S4**), which is not surprising, considering that the tissue is

160   undergoing cell death. In contrast, overrepresented GO terms in genes exclusively upregulated

161   at the OUT area included "regulation of defense response" and, interestingly, "response to

162   wounding" and "response to jasmonic acid" (**Figure 2C, Table S4**). These JA-related genes

163   follow a very distinct expression pattern, with an early peak at 1 hpi both at the IN and OUT

164   areas, and a second peak at 4 hpi of higher intensity in the OUT zone (**Figure S5**, **Table S4**).

165   Although further experimental validation would be required, these data reveal expression

166   patterns of a set of genes that could potentially be used as OUT markers along with previously

167   reported markers such as VSP1 (Chung et al., 2008; Betsuyaku et al., 2018). To better visualize

168   the behavior of the remaining OUT specific genes throughout the course of the infection, we

169   generated heatmaps representing their differential expression at IN and OUT areas (**Figure**

170   **S4**).

171

172   **Clustering of gene expression profiles reveals distinct expression patterns at the IN and**

173   **OUT areas over time**

174   Next, we set out to determine whether genes at the IN and OUT areas followed specific

175   expression patterns and if particular biological processes were associated to those patterns. For

176 this, we first analyzed gene expression profiles using Fuzzy c-means, a soft partitioning

177 algorithm which offers robust clustering with regards to noise by variation of a fuzzification

178 parameter that limits the contribution of ill-behaved profiles to the clustering process (Olsen et

179 al., 2006; Kumar and Futschik, 2007). Based on this, we could define three and five distinct

180 and non-overlapping clusters for *Pto AvrRpm1*-treated samples in the IN and OUT areas,

181 respectively (**Figure 3, Figure S8 and Table S6-S7**). Genes within each cluster were

182 subsequently re-clustered in mock-treated samples, producing two distinct sub-clusters

183 (**Figures 3, Figure S8 and Table S6-S7**). This procedure provided a more detailed overview

184 of the differences and similarities of trajectories between treatments over time and reflected

185 the well-documented wound response that takes place in mock-treated tissue (Mine et al., 2018;

186 Giolai et al., 2019; Vega-Munoz et al., 2020).

187

188 At the IN area of infection, cluster I exhibited a pattern of upregulation from 0 to 2 hpi and

189 mild downregulation from 2 to 6 hpi (**Figure 3A**). Genes near to its centroid (MSV > 0.7; **see**

190 **Materials and Methods**) are mainly associated with immune-related GO terms (**Figure S6A,**

191 **Table S8**). Genes in this cluster followed two distinct trajectories in the mock-treated samples:

192 while mock sub-cluster 1.1 showed a steady increase throughout the experiment, mock sub-

193 cluster 1.2 exhibited a typical wounding immune-related response common with infected

194 samples, peaking at 1 h and rapidly returning to steady-state levels (Savatin et al., 2014).

195

196 Cluster II-IN includes genes with a sharp increase of expression at 4 hpi (**Figure 3A**). Many

197 of the genes following that trajectory are involved in protein degradation processes (autophagy,

198 protein targeting to the vacuole, proteasome mediated degradation) taking place in response to

199 infection (**Figure S6A and Table S8**). Sub-clusters from mock-treated samples predominantly

200 followed a similar steady trajectory throughout the experiment, which points to an infection-

201 specific effect of upregulation on protein turnover due to infection at the IN area (**Figure 3A,**

202 **Figure S7A and Table S10**).

203

204 Cluster III-IN exhibits an expression pattern of steady downregulation from 0 to 4 hpi, followed

205 by a slight recovery of expression from 4 to 6 hpi (**Figure 3A**). This cluster includes mostly

206 genes belonging to GO terms related to photosynthesis (**Figure S6A and Table S8**). In this

207 case, mock-treated samples sub-cluster into two distinct patterns of expression: sub-cluster 3.1

208 follows a similar pattern as infected samples, while sub-cluster 3.2 shows a transient decrease

209 of expression at 1 h followed by a recovery phase from 2 to 6 hpi (**Figure 3A**). Our data show

210 that only certain components of the photosynthetic machinery are specifically affected by the

211 pathogen treatment (**Figure S6A, Figure S7A and Table S10**).

212

213 At the OUT area of infection, cluster I includes genes that display a sharp peak of expression

214 at 4 hpi (**Figure 3B**). From this cluster, genes near the centroid belong to GO terms associated

215 with metabolism, hormonal regulation, and wounding response, among others (**Figure S6B**

216 **and Table S9**). Interestingly, JA- and SA-responsive genes, which are known to act

217 antagonistically and cooperatively during ETI (Liu et al., 2016; Betsuyaku et al., 2018), seem

218 to be highly enriched in the OUT area. Genes comprising the mock-derived sub-clusters follow

219 a similar trend of steady expression throughout the time course of the experiment, suggesting

220 that the peak of high expression is a specific response to the bacterial infection in the

221 surrounding area (**Figure 3B, Figure S7B and Table S11**).

222

223 Cluster II-OUT in *Pto AvrRpm1*-treated samples follows an expression pattern with two sharp

224 upregulation peaks at 1 and 4 hpi (**Figure 3B**). These trajectories are followed by genes

225 associated with JA-related processes and wounding, and is a very specific pattern exclusively

226 found at the OUT zone (**Figure 3, Figure S6B and Table S9**). The early peak at 1 hpi shared

227 between mock and infected samples could account for a wounding response elicited early at

228 the area surrounding the syringe-infiltrated area, whilethe peak at 4 hpi appears as a late

229 response that occurs specifically at the tissues surrounding the pathogen inoculation area

230 (**Figure 3, Figure S6 and Table S11**).

231

232 In cluster III-OUT, the trajectory of genes from *Pto AvrRpm1*-treated samples does not

233 remarkably differ from mock treatment (**Figure 3B**). Genes that comprise this cluster mainly

234 fall into GO terms associated with the photosynthetic machinery (**Figure S6B and Table S9**).

235 These data indicate that photosynthesis at the OUT area of infection does not seem to be altered

236 by pathogen infection as opposed to the IN area (**Figure 3B and Figure S6-S7**) correlating

237 with zonal photosynthesis efficiency values shown in **Figure 1C** and as previously reported

238 (Berger et al., 2007).

239

240 **Novel zonal HR transcriptional indicators can be elucidated from pairwise comparisons**

241 **between time, treatment and area**

242 In order to identify robust HR markers that are exclusively upregulated at the site of cell death

243 (IN area) we conducted a pipeline of differential expression analysis that consisted of

244 concatenated pairwise comparisons considering the three variables in our experimental design:

245 time, treatment and area (**Figure 4A**). Since the highest degree of differential expression

246 between treatments took place at 4 and 6 hpi (**Figure 2A**), we carried out the comparisons at

247 these two time points independently. Firstly, we focused on the time variable and selected

248 genes that were confidently upregulated at the IN area of *Pto AvrRpm1*-infected plants, either

249 at 4 and/or 6 hpi, compared to 0 hpi (1st filter: FDR < 0.05 and log$_2$FC>2). Secondly, we

250 removed genes also upregulated at 4 and/or 6 hpi at the IN area in mock controls (2nd filter:

251 FDR < 0.05 and $\log_2$FC>2). Since we aimed to find genes only upregulated at the IN/cell death

252 area, next, we removed the genes that were upregulated by bacterial inoculation at the OUT

253 area at least to half of the levels than in the IN zone (3rd filter: FDR <0.05 and $\log_2$FC<1).

254 Finally, from the genes that met those three criteria, we kept those that were differentially

255 upregulated at IN compared to the OUT area in *Pto AvrRpm1*-infected plants (4th filter: FDR

256 < 0.05 and $\log_2$FC > 2) (**Figure 4A**).

257

258 A total of 32 genes passed all 4 filters, constituting a set of potential HR indicators (**Figure**

259 **S9**). From these, 24 were extracted from the 4 hpi dataset and 11 from the 6 hpi dataset and 3

260 from both time points (**Figure S9**). Of note, due to the stringency of the filters none of these

261 genes passed all the filters at 1 or 2 hpi, although 7 of them were upregulated after infection at

262 the IN zone at these early time points (M5, M6, M7, M8, M9, M11 and M12). The expression

263 profiles of this putative HR indicators can be visualized as DESeq2 pseudo-counts as a function

264 of time at both areas of infection in **Figure S10**. The expression patterns of these 32 genes at

265 0 and 4/6 hpi were validated by real time quantitative PCR (RT-qPCR) using newly obtained

266 biological samples (**Figure S11**). To ensure that the potential markers were exclusively

267 upregulated as part of the HR response triggered by effector-mediated bacterial recognition

268 and not as part of the defense responses triggered by disease-causing bacteria, we also included

269 samples inoculated with *Pto* DC3000 EV (*Pto* EV), a strain that causes disease but does not

270 trigger HR in Arabidopsis Col-0. Among the 32 genes tested, a total of 13 (10 of them at 4 hpi,

271 4 at 6 hpi, with one at both time points), behaved as *bona fide* HR indicators (**Figure 4B-C**),

272 showing a distinctive upregulation specifically triggered at the IN area by an HR-causing

273 bacterium.

274

The AAA-ATPase *At5g17760* promoter specifically drives expression of GFP to the IN

area of infection, constituting a robust transcriptional live marker of HR

In order to generate much needed tools to extend our understanding of how HR unfolds at the infection site and its surrounding tissue, we generated stable transgenic Arabidopsis plants expressing green fluorescent protein (3xGFP) under the control of the promoters of each of the 13 identified putative HR marker genes. A nuclear localization signal (NLS) was fused to GFP to concentrate the signal in the nucleus and facilitate detection, which enabled us to distinguish promoter-driven fluorescence from the auto-fluorescence derived from HR (Bennett et al., 1996).

We focused our analysis on plants expressing *pAT5G17760:NLS-3xGFP* (corresponding to M13), as they showed high, cell-specific, robust and clear GFP signal in the nuclei of the leaf regions infected with *Pto AvrRpm1* (**Figure 5B, Figure S12**). In several independent transgenic lines, activation of *pAT5G17760* was limited to the syringe-infiltrated area and could not be detected in the surrounding tissues (**Figure S13**). In all *pAT5G17760:NLS-3xGFP* marker lines the GFP signal appeared concomitantly with cell death, as shown by trypan blue staining **(Figure 5B, Figure S12 and S13)**. A clear GFP signal was not detected in all other marker lines tested.

In addition to *Pto AvrRpm1*, we also analyzed the response of *pAT5G17760:NLS-3xGFP* plants to *Pto* expressing *AvrRpt2* (*Pto AvrRpt2),* which induces HR in Col-0 plants via the CNL RESISTANT TO P. SYRINGAE 2 (RPS2) (Mackey et al., 2003) and to *Pto* expressing *AvrRps4 (Pto AvrRps4)*, where HR is mediated by the TNL-pair RPS4/RRS1 and requires helper NLRs (Gassmann et al., 1999; Narusaka et al., 2009). The same pattern was observed after infiltration with *Pto AvrRpt2* or *Pto AvrRps4* (**Figure 5B**), which indicates that

300   *pAT5G17760* robustly responds to pathogen-mediated activation of different classes of NLR

301   receptors. As controls, we included mock, *Pto* EV and a non-pathogenic mutant strain secreting

302   no effectors (*Pto hrcC⁻*) (Alfano et al., 2000). Importantly, infiltration with the mock solution

303   or with non-HR causing bacterial strains did not activate *pAT5G17760*. It is worth noting that

304   for microscopy imaging experiments we used a lower bacterial inoculum ($O.D_{600}$ 0.01) to

305   mimic more natural infection conditions and to delay the onset of HR and tissue collapse

306   (**Figure 5A**), which was necessary for microscopic detection of GFP. At higher inoculum, rapid

307   accumulation of phenolic compounds at the site of infection results in extremely high auto-

308   fluorescence levels that hamper imaging.

309

310   Since pathogens with contrasting lifestyles can trigger HR or HR-like cell death in plants, we

311   tested whether this reporter line can be employed in a broader sense. For this, we infected adult

312   Arabidopsis leaves by drop inoculation with *Botrytis cinerea*, a necrotrophic pathogen that kills

313   plant tissue prior to feeding, using a range of toxic molecules (Muckenschnabel et al., 2002).

314   At 3 dpi, we observed GFP expression in the nuclei of cells at the region inoculated with the

315   pathogen as opposed to mock-inoculated plants (**Figure S14**). Together, our observations

316   indicate that *pAT5G17760* activity is spatially regulated and confined to the area undergoing

317   HR elicited by hemibiotrophic (*P. syringae*) and necrotrophic (*B. cinerea*) pathogens. Thus,

318   the transgenic reporter line *pAT5G17760:NLS-3xGFP* constitutes a very useful tool to monitor

319   this process *in planta*.

320

321   The *AT5G17760* gene encodes a putative AAA ATPase of unknown function. A knock-out

322   mutant of this gene did not show any alteration in HR or pathogen growth restriction compared

323   to wild-type plants (**Figure S14**). The lack of phenotype could be due to functional

324   redundancy/compensation, a very common masking phenomenon in plants.

325

326 HR markers and particularly *At5g1776* are highly upregulated in other RNA-seq data

327 sets from plants undergoing ETI and autoimmunity

328 We looked at the behavior of *At5g17760* and the rest of marker genes in already published

329 RNA-seq data sets from either plants undergoing ETI or autoimmune mutant plants displaying

330 constitutive defense responses and runaway cell death (**Figure S16**) (Mine et al., 2018; Yang

331 et al., 2020; Barragan et al., 2020; Chantarachot et al., 2020). Fold changes from marker genes

332 with significant p values (FDR < 0.05) in these data sets were plotted as heatmaps to reveal

333 their level of upregulation (**Figure S16**). As expected, most gene markers are significantly

334 (FDR < 0.05) upregulated during ETI triggered by *Pto AvrRpm1* and *Pto AvrRpt2* at 4, 6 and

335 9 hpi in Mine et al., (2018) **(Figure S16A)**. Interestingly, *At5g17760* is the highest upregulated

336 gene in *hos15-4* and *rh6812* mutant plants undergoing autoimmunity (Yang et al., 2020;

337 Chantarachot et al., 2020). Likewise, upregulated genes from data sets of incompatible

338 Arabidopsis F1 hybrids (*Cdm-0 x TueScha-9)* exhibiting autoimmunity comprised most HR

339 markers found in this study with *At5g17760* being the highest upregulated gene (Barragan et

340 al., 2020)(**Figure S16B).**

341

342 DISCUSSION

343 Zonation of HR in plants is underscored by distinct gene expression patterns and

344 processes in dying vs by-stander cells

345 In plants, pathogen recognition via intracellular NLR receptors often results in an HR reaction

346 that helps preventing pathogen proliferation (Pitsili et al., 2020). This is a highly zonal response

347 that takes place at the site of infection, whereby dying cells send signals to the surrounding

348 tissues to activate defenses and block pathogen invasion. Traditionally, the plant immune

349 system was considered strictly two-branched, with PTI being elicited by recognition of

350 conserved pathogen patterns via cell surface receptors, and ETI recognizing pathogen effector

351 proteins secreted into the plant cell via intracellular NLR receptors (Jones and Dangl, 2006).

352 Over the last decades, many efforts have been directed towards understanding the

353 transcriptional reprogramming elicited during PTI and ETI (Tao et al., 2003; Lewis et al., 2015;

354 Bozso et al., 2016; Mine et al., 2018; Duan et al., 2020). One of the major conclusions drawn

355 from these studies is that whilst the repertoire of differentially expressed genes in the host is

356 largely similar, ETI leads to a faster and more robust transcriptional response than PTI (Tao et

357 al., 2003; Mine et al., 2018; Yuan et al., 2021a; Yuan et al., 2021b; Ngou et al., 2021b). These

358 findings, together with emerging evidence showing additional levels of synergy and crosstalk

359 between PTI and ETI has somewhat blurred the traditional PTI-ETI dichotomy (Dongus and

360 Parker, 2021; Ngou et al., 2021a; Pruitt et al., 2021). However, despite the large amount of

361 time-resolved transcriptomic data produced (Tao et al., 2003; Lewis et al., 2015; Hillmer et al.,

362 2017; Mine et al., 2018), the spatial consideration of HR upon ETI activation has been partly

363 overlooked, with only few studies pointing to its importance in regulating the process (Dorey

364 et al., 1997; Betsuyaku et al., 2018; Giolai et al., 2019; Lukan et al., 2020). It remains unclear

365 whether and to what extent transcriptional reprogramming takes place at the vicinity of cell

366 death compared to that occurring at the infected area upon bacterial infection.

367

368 Our experimental design (**Figure 1A**) considered the spatio-temporal angle of plant HR to gain

369 a better understanding of how this process is restricted to a few cells upon pathogen recognition

370 and to define robust markers of the dying area over time. This is particularly important since

371 in plants, cell death characterization has largely relied on biochemical and morphological

372 hallmarks most of which are *post-mortem* and which in most cases do not provide unequivocal

373 criteria (van Doorn, 2011; van Doorn et al., 2011). We currently lack a set of genes that can be

374 employed as gene indicators of cell death triggered by pathogens. *In silico* comparisons of

375 transcriptome profiles at different developmental stages and upon environmental stresses

376 leading to cell death, enabled identification of cell death indicators of developmentally

377 regulated programmed cell death that can be used to detect or even isolate cells that are ready

378 to die (Olvera-Carrillo et al., 2015). The same approach did not lead to identification of reliable

379 HR markers, partly because the available datasets were not obtained on zonally resolved

380 samples (Olvera-Carrillo et al., 2015).

381

382 Here, differential expression analysis and clustering of genes based on expression profiles over

383 time enabled us to infer biological processes taking place at each tissue area (IN/OUT) upon

384 bacterial infection, giving us hints on how HR can be spatially restricted. At the IN area, genes

385 involved in a local immune response to ETI-triggering bacteria are greatly induced from 1 hpi

386 onwards (cluster I) (**Figure 2A and Figure S2A, Figure 3A**). Tissue from the IN area, also

387 contains a set of genes that show a peak of upregulation from 2 to 4 hpi (cluster II), involved

388 in diverse biological processes ranging from regulation of immunity, responses to JA and SA

389 or protein turnover (**Figure S6A**). It is now well established that proteasome activity is strongly

390 induced during bacterial infection and that certain subunits of the proteasome are required for

391 efficient fine-tuning of immune responses in plants (Misas-Villamil et al., 2013; Ustun et al.,

392 2016; Ustun et al., 2018). Finally, we identified a strong transcriptional repression of

393 photosynthetic genes at 4 hpi at the IN area (cluster III) (**Figure 2B, Figure S2B, Figure 3A**

394 **and Figure S6A**), in accordance with the previously established notion that infection results

395 in a global downregulation of genes associated with the photosynthetic machinery (Bilgin et

396 al., 2010). This specific decrease in photosynthesis is particularly interesting in light of recent

397 reports of the interplay between bacterial effectors and the chloroplast, whereby certain

398 effectors can suppress chloroplast functions and in turn, chloroplasts can adopt immune

399   functions to fight off pathogens (Kachroo et al., 2021; Littlejohn et al., 2021; Savage et al.,

400   2021).

401

402   Our results also show that transcriptional reprogramming in host cells surrounding the infection

403   area (OUT area) is less extensive with a lower number of differentially expressed genes than

404   at the IN area, and starts later mostly from 4 hpi onwards (**Figure 2A**). Remarkably,

405   photosynthesis is not significantly affected at the OUT area, corroborating our *in vivo*

406   measurements (**Figure 1C**) and previous findings (Bilgin et al., 2010). A relatively functional

407   photosynthetic machinery may be key to maintain effective defense mechanisms and prevent

408   these cells from dying as their neighbors. This finding might have been masked in previous

409   transcriptional studies that have not taken into account the zonal nature of HR, and reveals that

410   the defense-growth trade-off may also have a marked spatial component that needs to be taken

411   into account in future research. Besides photosynthesis, the OUT zone was characterized by a

412   marked upregulation of wound/JA-related genes at 4 hpi (**Figure 2C, Figure 3B and Figure**

413   **S2C**). This response can also be observed at the IN zone but the level of upregulation at the

414   OUT zone is remarkably higher (**Figure S4**), indicating an amplification in JA signaling at the

415   cells surrounding the death zone. In addition, some of the JA-related genes are among those

416   genes exclusively upregulated at OUT at 4/6 hpi, which indicates that they could potentially

417   be used as zonal markers of the surrounding area (**Figure 2B-C and Figure S5**). *In vivo*

418   imaging of marker gene promoter activities of SA and JA signaling during ETI discerned two

419   spatially distinct domains around the infection site, where JA signaling is thought to be

420   important for regulating over-activation of SA signaling (Betsuyaku et al., 2018). Future

421   studies that include mutants deficient in JA could provide mechanistic insights into how JA

422   signaling contributes to the confinement of plant HR. Our analysis also shows that some SA-

423   signaling genes are among the upregulated IN-specific genes at late time points (**Figure 2B-C**

424   **and Table S4**). Although originally considered antagonistic hormones required for immunity

425   against pathogens with contrasting lifestyles (Spoel et al., 2007), the interplay and synergism

426   of these two phytohormones is now well established during ETI (Liu et al., 2016).

427

428   **Zonally resolved transcriptomic analysis of HR in plants allows for the identification of**

429   **robust biomarkers of the process**

430

431   Robust biomarkers are essential to gain mechanistic knowledge of cell- or tissue-specific

432   processes. In mammals, the extensive mechanistic knowledge of molecular constituents

433   underlying regulated cell death has enabled the use of biomarkers for detection of tumor cells

434   or aberrant cell death processes in cancer patients (Abu-Qare and Abou-Donia, 2001; Ward et

435   al., 2008). The field of HR in plants is gaining momentum thanks to recent major discoveries

436   that in one hand are leading to a redefinition of the PTI-ETI relationship and on the other, have

437   provided mechanistic insight into how NLRs become activated and form supramolecular

438   complexes that mediate cell death (Wang et al., 2019a; Wang et al., 2019b; Martin et al., 2020;

439   Bi et al., 2021; Jacob et al., 2021; Pruitt et al., 2021; Tian et al., 2021; Yuan et al., 2021a; Ngou

440   et al., 2021b; Förderer et al., 2022). However, amidst this exciting scenario, the conceptual

441   framework of HR zonation is scarcely defined and will be key to understand its execution,

442   spatial restriction mechanisms and define *bona fide* indicators of the process.

443

444   One of the main goals of our analysis was to define new markers of HR. We made use of the

445   RNA-seq data generated from IN and OUT areas to pinpoint gene indicators of HR that can be

446   used either as transcriptional markers or gene promoter markers for *in planta* detection of cells

447   destined to die using live imaging. Applying stringent filters to our dataset we identified 13

448   genes that can be used as unequivocal transcriptional markers of zonally restricted cells that

449 have activated a death program in response to pathogen perception via NLR activation (**Figure**

450 **4C**).

451

452 This marker set includes genes involved or putatively involved in various processes such as

453 ion transport across the plasma membrane (M1), cell detoxification (M2, M3), lipid

454 metabolism (M5, M6), cell wall remodeling (M7, M8, M9), protein degradation (M10),

455 glycolysis (M11, M12), whereas one of these genes remains largely uncharacterized (M13) and

456 encodes an AAA+ ATPase of unknown function. Interestingly, all these predicted functions

457 are consistent with processes expectedly taking place on cells destined to die or that have

458 started dying, although the function of most of these genes remains to be fully determined. This

459 set of genes provide a glimpse of transcriptional regulation of HR at the site of infection, the

460 tip of the iceberg of the multi-level regulation of the process. For example, the fact that several

461 genes are involved in cell wall remodeling highlights the importance of processes taking place

462 in this extracellular compartment. In line with this, an increase of lignification at the edge of

463 cells undergoing HR was shown in the past and provided a clear picture of the zonal nature of

464 this process (Lee et al., 2019). Interestingly, our transcriptome data clearly shows that many

465 lignin biosynthetic genes are strongly and specifically upregulated at the IN zone at certain

466 time points (**Figure S15**). How this cell wall lignification is regulated upon pathogen

467 perception remains to be clarified and will be an interesting topic of research in the future.

468

469 Our data also reinforces the idea that the proteases involved in degradation of cell components

470 during HR are not particularly regulated at the transcriptional level. We observe specific

471 upregulation of degradative processes at the IN zone such as autophagy, vacuolar degradation,

472 and proteasome-mediated processes and in fact, one of the marker genes is a proteasome

473 subunit (**Figure 3 and Figure 4B**). However, we did not find any protease specifically

474   upregulated at the IN zone, nor did any of them pass the filters that constitute a marker gene in

475   our study.

476

477   In parallel, the changes observed in marker genes involved either in ion transport across the

478   plasma membrane or cell detoxification may be somewhat related with the predicted formation

479   of a pore at the plasma membrane by pathogen-mediated activation of certain NLRs (Bi et al.,

480   2021; Jacob et al., 2021; Förderer et al., 2022). Although crucial pieces of this mechanism have

481   been unveiled, knowledge is still scattered and we lack a more integrated picture that combines

482   NLR activation with downstream processes, including cell death execution. Interestingly, 7 out

483   of the 32 gene markers (*M5*, *M6*, *M7*, *M8*, *M9*, *M11* and *M12*) that pass our filters exhibit an

484   early upregulation at 2 hpi compared to mock controls according to the RNA-seq data (**Figure**

485   **S10**). Although these genes did not pass the stringent 4-tier filtering applied (**Figure 4A**) at 2

486   hpi, the expression profiles of these genes could be compatible with their potential use as earlier

487   markers of HR at the IN area.

488   In sum, our data provides a snapshot of how infected cells respond to pathogen recognition at

489   the transcriptional level, compared to their neighbors, that are not directly exposed to the

490   pathogen but respond to it. Importantly, this analysis has revealed a set of genes that are

491   specifically upregulated at the IN zone and constitute robust markers of HR, opening new paths

492   to deepen our knowledge on the process.

493

494   Importantly, we present an Arabidopsis HR reporter line stably expressing GFP under the

495   control of the AAA+ ATPase *At5g17760* (M13), which shows extremely clear and strong

496   expression exclusively at the inoculated area where pathogen recognition takes place via ETI,

497   before the onset of cell death becomes apparent (**Figure 5A-B and Figure S12**). The other

498   genes (*M1-M12*) constituted very clear qPCR markers but GFP promoter fusions did not result

499   in clear GFP expression. This can be attributed to the limitations from defining an active

500   promoter sequence.

501

502   Interestingly, expression of the marker p*At5g17760:NLS-3xGFP* is similarly regulated by

503   different classes of NLRs (CNLs and TNLs) revealing conservation of the process (**Figure**

504   **5A**). Moreover, the marker is also induced zonally by necrotrophic pathogens such as *B.*

505   *cinerea* that cause an HR-like phenotype (**Figure 5C**). Thus, this transgenic line constitutes a

506   robust *in planta* biomarker of HR triggered by activation of different NLRs upon infection with

507   pathogens with contrasting lifestyles.

508

509   Future in-depth analysis of all HR marker genes identified in this work, including

510   combinatorial genetics, will contribute to a better understanding of HR. This set of genes

511   constitutes an invaluable tool to zonally discriminate cells undergoing pathogen-triggered cell

512   death and mechanistically dissect this process. Of particular interest will be to sort GFP-

513   expressing cells of the p*At5g17760:NLS-3xGFP* transgenic line upon infection and adapt high-

514   throughput cell death monitoring equipment used so far for animal cell death to describe and

515   quantify the features and regulatory networks that define HR in plants at a single-cell level.

516

517   **MATERIALS AND METHODS**

518   **Plant and bacteria materials and growth**

519   The *Arabidopsis thaliana* accession Col-0 was used for all experiments carried out in this study

520   expect for electrolyte leakage. For electrolyte leakage, Col-0, *rpm1-3* (Grant et al., 1995)

521   mutant of the NLR RPM1 and *at5g17760* mutant (GABI-KAT line 592F04_1) which carries

522   T-DNA insertion in exon two, were used. Primers used for identifying the T-DNA mutant and

523   for corroboration null expression by RT-qPCR are listed in **Table S12**.

524

525   Seeds were sown on ½ Murashige and Skoog (MS) media supplemented with 1% sucrose and

526   stratified at 4ºC for two days. Plants were grown in a controlled chamber with a photoperiod

527   of 9 h light and 15 h dark with white fluorescent lamps under 65% relative humidity. Seeds

528   were germinated on plates and grown for 10-7 days, then individually transplanted to Jiffy

529   pellets and grown for 3 additional weeks.

530

531   *Pseudomonas syringae* pathovar tomato *(Pto)* strains *Pto AvrRpm1*, *Pto AvrRpt2*, *Pto AvrRps4*,

532   *Pto hrpC-* and *Pto* empty vector pVSP61 (EV) were grown on selective King's B (KB) medium

533   plates for 48 h at 28 °C. Bacteria was then resuspended in 10 mM $MgCl_2$ and the OD_{600} adjusted

534   to the appropriate inoculum.

535

536   **Bacterial inoculation and RNA-seq data collection.**

537   Bacteria were resuspended and the concentration was adjusted at $5*10^7$ colony-forming units

538   or to an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.05. Fully expanded

539   7th or 8th rosette leaves were used for infiltration with either a mock solution (10 mM $MgCl_2$)

540   or *Pto AvrRpm1*. We syringe-infiltrated an area of roughly 3-4 mm at the side edge of leaves.

541   Upon infiltration, the edge of the infiltrated area was underlined using India ink, and the total

542   area infiltrated designated as "IN". A 1 mm buffer zone next to the IN area was discarded and

543   used as a reference to properly separate between the IN and the OUT zone, that expanded 1-2

544   mm towards the vein. Leaf tissue was separately collected from the IN and OUT area of

545   infiltration at 5 different time points: 0, 1, 2, 4 and 6 hours by making use of a sterile scalpel.

546   Leaf tissue was stored in 2 mL Eppendorf tubes and snapped-frozen in liquid nitrogen until the

547   time of RNA extraction. Each sample collected consisted of tissue from six leaves derived from

548   three different plants. For generation of three biological replicates from each condition (area,

549 treatment and time), three independent experiments were performed. total sum of 60 samples

550 -2 treatments (mock/infected), 5 time points (0, 1, 2, 4 and 6 hpi), 2 areas (IN/OUT) and 3

551 biological replicates- were used for RNA-sequencing.

552

553 For RNA library preparation, 1 µg of RNA from each sample was isolated using the

554 NucleoSpin® RNA isolation kit (Macherey-Nagel, Hoerdt Cedex, France) following the

555 manufacturer's instructions. RNAseq was performed at the GeT-PlaGe core facility, INRA

556 Toulouse. RNA-seq libraries have been prepared according to Illumina's protocols using the

557 Illumina TruSeq Stranded mRNA sample prep kit to analyze mRNA. Briefly, mRNA was

558 selected using poly-T beads. Then, RNA was fragmented to generate double stranded cDNA

559 and adaptors were ligated to be sequenced. 11 cycles of PCR were applied to amplify libraries.

560 Library quality was assessed using a Fragment Analyzer and libraries were quantified by qPCR

561 using the Kapa Library Quantification Kit (Kapa Biosystems, Inc, Wilmington, MA, USA).

562 RNA-seq experiments have been performed on an Illumina HiSeq3000 using a paired-end read

563 length of 2x150 bp with the Illumina HiSeq3000 sequencing kits.

564

565 **Read mapping and differential expression analysis**

566 "FastQC" and "TrimGalore!" software was used for raw Illumina reads quality control analysis

567 and trimming of reads containing adaptor- or vector-derived sequences, respectively

568 (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence

569 Data, 2021). rRNA was detected and removed using "SortMeRNA 2.1b" software (Kopylova

570 et al., 2012). Cleaned reads together with the transcriptome of *Arabidopsis thaliana* (as of 30

571 August 2018), including ncRNA, were used to quantify gene expression at transcript level

572 using the software "Salmon v0.11.3" (Patro et al., 2017). Raw counts aggregated by gene were

573 obtained using "tximport v1.14.2" and the result was used as input to "DESeq2" v1.26.0 (Love

574 et al., 2014; Soneson et al., 2015) to perform differential expression analysis. Then, genes

575 adding up to less than 10 counts across all 60 samples were removed. The pre-filtered DESeq2

576 object contained 32,865 rows that turned to 23,986 after filtering. Counts normalized for

577 sample size and regularized-logarithm transformed were used to produce PCAs.

578

579 Raw counts together with sample size information were used as input for DESeq2's differential

580 expression analysis. Simple pairwise comparisons based on a single factor were performed

581 using DESeq2's "result" function while time course differential expression results were

582 obtained using a likelihood ratio test as previously described (Love et al., 2015). Genes with

583 FDR below 0.05 and |log2FC| higher than 2 were considered as differentially expressed. FDR

584 was calculated according to the Benjamini and Hochberg's (BH) method (Benjamini and

585 Hochberg, 1995).

586

587 **Gene clustering**

588 Gene clustering was performed using Mfuzz v2.46.0 package under the R environment (Kumar

589 and Futschik, 2007; RStudio | Open source & professional software for data science teams,

590 2021) which is based on fuzzy c-means clustering algorithms. IN and OUT samples were

591 independently analyzed. After time course differential expression analysis using DESeq2, only

592 genes with an FDR <0.05 in the likelihood ratio test were selected for clustering.

593

594 The optimal number of non-overlapping clusters with a correlation value below 0.85 was 3 and

595 6 for *Pto AvrRpm1*-treated samples at the IN and OUT areas of infection, respectively.

596 Subsequently, two highly redundant clusters were merged for OUT samples, yielding 5 final

597 clusters. Genes that integrated each cluster derived from *Pto AvrRpm1*-treated samples were

598 re-clustered for mock-treated samples in order to inspect the differences and similarities of

599   trajectories between treatments over time. Between two and four mock-based sub-clusters were

600   obtained for every infected-cluster. To avoid overlap, we reduced the number of sub-clusters

601   to two in mock-treated samples. Each gene belonging to a cluster returned an associated

602   membership score value (MSV) that ranged from 0 to 1 depending on how well it fitted the

603   expression profile dictated by the overall genes comprising the cluster. Genes that integrate

604   each cluster in Figure 3 area found in Tables S6-S7.

605

606   **Enriched Gene Ontology analysis.**

607   The set of genes that belonged to expression profile clusters or that exhibited differential

608   expression were input into TAIR for Gene Ontology enrichment analysis for biological

609   processes, which uses the PANTHER Classification system that contains up to date GO

610   annotation data for Arabidopsis (Berardini et al., 2004). The most specific term belonging to a

611   particular family of GO terms was always selected for plotting. Only those GO terms exhibiting

612   an FDR < 0.05 after Bonferroni Correction for multiple testing and a fold enrichment above 2

613   were selected for representation in dot plots.

614

615   **Identification of HR indicators**

616   For identification of HR indicators, we concatenated four pairwise comparisons using DESeq2,

617   in which we set different thresholds of log2FC, while keeping a stringent cut-off of FDR <0.05

618   throughout all comparisons. Briefly, we firstly selected genes that were upregulated (log2FC

619   > 2) after *Pto AvrRpm1* infection at 4 or 6 hpi *vs* 0 hpi. From the genes that complied with this

620   first filter, we selected those that were specifically upregulated in *Pto AvrRpm1*-infected *vs*

621   mock-inoculated samples at 4 or 6 hpi (log2FC >2). From the genes that passed these two filters

622   we kept those with a log2FC <1 at the OUT area in *Pto AvrRpm1*-infected vs mock-inoculated

623   samples at 4 or 6 hpi. Since genes with log2FC near 0 do not usually have a low FDR, we kept

624　our stringent FDR threshold while setting the log2FC threshold below 1 in order to capture

625　with statistical confidence downregulated and only mildly upregulated genes at this tissue area.

626　Finally, from the genes that met those three criteria, we kept those that were differentially

627　upregulated at the IN area compared to the OUT area in *Pto AvrRpm1*-infected plants.

628

629　**Validation of gene expression by real time quantitative PCR.**

630　The same experimental setup used for RNA-seq data generation was followed for experimental

631　validation by RT-qPCR including infections with *Pto AvrRpt2*, *Pto AvrRps4*, *Pto hrpC*- and

632　*Pto* EV. Briefly, tissue was snap frozen and RNA isolated with the Maxwell® RSC Plant RNA

633　kit (Promega). 1 µg of RNA was reverse transcribed into cDNA with the High-Capacity cDNA

634　Reverse Transcription Kit with RNase inhibitor (Applied Biosystems™). RT-qPCRs were

635　performed with LightCycler® SYBRgreen I master (Roche) in a LightCycler® 480 System

636　(Roche). Data was analyzed using the *ΔΔCT* method and represented as fold enrichment of the

637　time point tested (4 or 6 hpi) relative to 0 hpi. Primers for RT-qPCR used in this study are listed

638　in **Table S12** along with primer concentrations. RT-qPCR results in numeric format along with

639　Cp values of Targets and Cp values of reference housekeeping gene are listed in **Table S13**.

640

641　**Cell death analysis**

642　Trypan blue staining of Arabidopsis leaves was performed by collecting whole leaves in 50 ml

643　tubes (each leaf in a separate tube) at the specified time-points after treatment and covered with

644　a 1:3 dilution of the stain. Tubes were incubated in previously boiled water for 15 min, and

645　then cleared overnight with chloral hydrate on an orbital shaker. After removal of staining

646　solution, leaves were covered in a 50% glycerol solution and photographed using a Leica DM6

647　microscope.

648

649 **Electrolyte leakage**

650 Whole leaves from four to five-weeks-old *Arabidopsis* Col-0, *rpm1-3* or *at5g17760* (GABI-

651 KAT: 592F04) grown in short-day with a photoperiod of 9h light and 15h dark, were infiltrated

652 with *Pto AvrRpm1* at a wavelength of 600 nm (OD_{600}) of 0.05 using a 1-ml needleless syringe.

653 Leaf discs were dried and subsequently collected with a 0.8-cm-diameter cork borer from

654 infiltrated leaves. Discs were washed in deionized water for 1 h before being floated on 2 ml

655 deionized water. Electrolyte leakage was measured as water conductivity with a pocket water

656 quality meter (LAQUAtwin-EC-11; Horiba, Kioto, Japan) at the indicated time points.

657 **Bacterial growth assay**

658 Whole leaves from four to five-weeks-old *Arabidopsis* Col-0, *rpm1-3* or *at5g17760* (GABI-

659 KAT: 592F04) grown in short-day conditions (9h light and 15h dark) were infiltrated with *Pto*

660 *AvrRpm1* at a wavelength of 600 nm (OD_{600}) of 0.001 using a 1-ml needleless syringe. Two

661 leaf discs from two different leaves were collected using a 6 mm-diameter cork borer (disc area

662 0.282 cm^2). Samples at day 0 and day 3 post-infection were grounded in 10 mM $MgCl_2$ and

663 serially diluted 5, 50, 500, 5,000 and 50,000 times on a 96-well plate. Subsequently, dilutions

664 were spotted (10 μl per spot) on KB medium with antibiotics. The number of colony forming

665 units (CFUs) per drop was calculated and bacterial growth represented as log_{10} CFU per cm^2

666 of tissue.

667

668 **Chlorophyll fluorescence imaging**

669 An IMAGING-PAM (Pulse-Amplitude-Modulated) M-Series Chlorophyll Fluorometer

670 system (Heinz Walz, Effeltrich, Germany) was used to investigate spatio-temporal changes in

671 photosynthetic parameters at the IN and OUT areas of infection (Schreiber, 2004). Plants were

672 kept in the dark for 30 minutes before measurement. Plants were exposed to 2 Hz frequency

673 measuring light pulses for Fo (minimum fluorescence in the dark-adapted state) determination.

674 Saturating pulses (800 ms) of white light (2400 mmol photons.m-2 s-1) were applied for Fm

675 (maximum fluorescence in the dark-adapted state) determination. The photosynthetic

676 efficiency or maximum quantum yield of PSII photochemistry (Fv/Fm) was determined as

677 (Fm-Fo)/Fm. The relative PSII electron transport rate (ETR) was calculated by performing a

678 kinetic analysis for 10 minutes with 60 second pulses (Schreiber et al., 2012). Areas of interest

679 (AOI) included IN and OUT to evaluate spatial heterogeneity. The measurements were taken

680 after 0, 1, 2, 4 and 6 hpi. Results are shown from 6 different AOI.

681

682 **Generation of transgenic promoter reporter lines**

683 Regions of approximately -2.5 kb upstream of the transcription starting site of *AT1G79710*,

684 *AT4G18050*, *AT1G78380*, *AT4G24160*, *AT5G18480*, *AT4G30390*, *AT5G54650*, *AT5G16910*,

685 *AT5G20000*, *AT2G36580*, *AT5G56350* and *AT5G17760* were amplified from Arabidopsis Col-

686 0 genomic DNA by PCR and cloned into the pGGA (plasmid Green Gate A) entry vector to

687 generate pGGA-pMarkerGene. A region of approximately -1.5 kb upstream of the transcription

688 starting site of *AT1G30270* was synthetized by GENEWIZ, Inc. (South Plainfield, NJ) and

689 subsequently cloned into the pGGA entry vector as well. (Lampropoulos et al., 2013). Each

690 entry vector was then recombined with the following plasmids: pGGB-SV40-NLS, pGGC-

691 3xGFP, pGGD-RBCSt (D-F), pGGF-AlliYFP (seed coat selection cassette for transgenic seed

692 selection) and pGGZ-empty destination vector. Primers used for cloning and sequencing the

693 final constructs are listed in **Table S12**. All plasmids were transfected by electroporation into

694 *Agrobacterium tumefaciens* GV3101 strain containing the plasmid pSoup and then transformed

695 into Arabidopsis Col-0 by the floral dipping method (Clough and Bent, 1998). Transgenic

696 seeds from transformed plants were identified as those displaying a clear fluorescence signal

697 under the stereo microscope Olympus SZX18.

698

699 **Pathogen inoculation and microscopy of reporter lines**

700 For microscopy of the reporter line p*AT5G17760*:NLS-3xGFP, plants were grown as

701 previously described. Leaves of Col-0 p*AT5G17760*:NLS-3xGFP were infiltrated in the IN

702 area with either a mock solution (10 mM MgCl$_2$) or different *Pto* strains. *Pto* strains expressing

703 the following effectors were used: *AvrRpm1*, *AvrRpt2* and *AvrRps4*. As controls, the *Pto* EV

704 and *Pto hrcC-* strains were also used. All *Pto* strains were infiltrated at a wavelength of 600

705 nm (OD$_{600}$) of 0.01 for microscopy imaging. For *B. cinerea* infection, B05.10 strain

706 was grown for 14 days in Potato Dextrose Agar (PDA) at 22°C under dark conditions. Spores

707 collected, washed in 5 mL of PDA and filtered through two layers of Miracloth (Merck

708 Millipore). Subsequently, number of spores per cm^2 were counted under the microscope and

709 diluted to 1 x 10^5 spores per mL. For inoculation a 6 µl droplet was placed on the upper surface

710 of the 7th or 8th leaf of an adult Arabidopsis plant grown in short day conditions. A dome

711 covering the plants was placed throughout the course of *B. cinerea* infection.

712

713 Leaves were imaged at 16 hpi with *Pto* stains and 3 dpi with *B. cinerea*. Whole leaves were

714 photographed using a Leica DM6 microscope (Leica Microsystems) equipped with DFC365

715 FX 1.4 MP monochrome digital camera. Bright field and GFP filter pictures were taken of each

716 leaf. Confocal images were obtained using a FV1000 Olympus confocal microscope with the

717 following excitation/emission wavelengths for GFP: 488 nm/500 to 540 nm. Confocal

718 microscopy images were taken of the epidermal layer (20 Z-stacks with stack size of 1 µm) and

719 fluorescent nuclei were counted using ImageJ software.

720

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976   **Zheng, X., Zhou, M., Yoo, H., Pruneda-Paz, J. L., Spivey, N. W., Kay, S. A., and Dong,**

977   **X.** (2015). Spatial and temporal regulation of biosynthesis of the plant immune signal

978   salicylic acid. *Proc Natl Acad Sci U S A* **112**:9166–9173.

979

980   **Author Contribution Statement**

981   JS-L designed and performed experiments, analyzed and interpreted data and wrote the

982   manuscript

983   IS designed and performed experiments and analyzed and interpreted data and helped writing

984   the manuscript

985   NR-S designed and performed experiments, analyzed and interpreted data and helped writing

986   the manuscript

987   MS performed experiments

988   UP performed experiments

989   VMG performed analysis and interpreted data

990   MB-F performed analysis and interpreted data

991   MV interpreted data and helped writing the manuscript

992   DR performed experiments, analyzed and interpreted data, and helped writing the manuscript.

993 NSC conceptualized the research, designed the experiments, interpreted data and wrote the
994 manuscript.

995

1008

1009 **Conflict of Interest Statement**

1010 The authors declare no conflict of interest.

1011

1012 **Ethics Statement**

1013 The present study did not require ethical approval.

1014

1029

1030   **Data Availability Statement**

1031   RNA-seq raw and processed data generated in this study can be found in GEO (GSE198022).

1032   All code used for analysis can be found at

1033   https://doi.org/10.34810/data174

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1043 **Figure legends**

Figure 1. HR in plants can be spatio-temporally dissected. (A) Experimental design of the study. A limited area (3-4 mm) at the side edge of four-week-old Arabidopsis thaliana Col-0 leaves was syringe-infiltrated with either *Pto AvrRpm1* at $2.5*10^7$ cfu/ml (INFECTED) or a 10 mM $MgCl_2$ solution (MOCK) and samples were collected at 5 different time points after infection: 0, 1, 2, 4 and 6 hpi. Upon infiltration, the edge of the infiltrated area was marked, and the total area infiltrated designated as "IN". A 1 mm buffer zone right next to the IN zone ensured proper separation between the IN and "OUT" area, which was the parallel region that expanded from the edge of the buffer zone to 1-2 mm towards the vein. Three biological replicates per area, treatment and time point were collected and subjected for RNA-seq analysis. **(B)** Analysis of macroscopic cell death upon infection with either *Pto AvrRpm1* or 10 mM $MgCl_2$ solution. Leaves were infected as described in (a) and subsequently stained with trypan blue. Scale bar 3 mm **(C)** Representative images of mock or *Pto AvrRm1*-treated plants subjected to pulse-amplitude modulated (PAM) chlorophyll fluorescence measurement to monitor photosynthesis. Scale bar 3 mm. Photosynthetic efficiency (Fv/Fm ratio) and electron transport rate (ETR) were measured in the infiltrated area (IN) and the neighboring tissue (OUT). Measurements were taken at 0, 1, 2, 4 and 6 hpi. Results are representative of 6 different measurements of each tissue area from 6 different plants. Letters indicate statistically significant differences in either Fv/Fm ratio or ETR values following a two-way ANOVA with Tukey´s HSD test ($\alpha = 0.05$). Exact p values are provided in **Table S5**.

Figure 2. Spatio-temporal dynamics of the transcriptome reveal time and zone-dependent gene expression signatures upon infection. (A) Differentially expressed genes (FDR < 0.05 and $|\log_2 FC| > 2$) in *Pto AvrRpm1*-infected plants compared to mock-treated plants at each time point at the IN (left) and OUT (right) areas. Red color denotes upregulated genes whereas blue color indicates downregulated genes. Yellow color shows genes with FDR < 0,05 but $|\log_2 FC| < 2$, whereas grey indicates genes not complying with neither FDR nor $\log_2 FC$ criteria. **(B-C)** Genes exclusively upregulated (FDR < 0.05 and $\log_2 FC > 2$) at either IN or OUT areas of infection at 4 and 6 hpi. **(B)** Venn diagrams shows sizes of gene sets that are upregulated (FDR < 0.05 and $\log_2 FC > 2$) upon bacterial infection at 4 and/or 6 hpi at either IN, OUT or both areas. **(C)** GO terms representing enriched biological processes derived from genes exclusively upregulated at either IN or OUT areas at 4 and/or 6 hpi. The most specific term from each family term provided by PANTHER was plotted along with their corresponding gene number, fold enrichment (FE) and FDR (Bonferroni Correction for multiple testing) represented as $\log_{10}$. Only GO Terms with a FE above 2 and FDR below 0.05 were plotted.

Figure 3. Gene expression profile clustering reveals three distinctive expression patterns at the IN and OUT areas of infection. Non-overlapping clusters derived from *Pto AvrRpm1*- and mock-treated plants for IN **(A)** and OUT **(B)** areas. Standardized expression to Z-scores (Y-axis) is calculated by subtracting the mean and normalizing to standard deviation. The trajectory that defines the overall expression profile of each cluster through the course of the infection is shown in red for *Pto AvrRpm1*-treated plants. Genes derived from *Pto AvrRpm1*-treated samples were re-clustered for mock-treated samples and their trajectories are

represented in grey. Since the expression profile of these genes in mock-treated samples was very distinct among the overall number of genes, they were divided into two sub-clusters represented either in dotted or dashed grey lines. The number of genes that constitute each cluster is indicated below each cluster. Genes comprising each cluster along with their MSV can be found in **Table S6-S7**.

Figure 4. Identification of HR markers specific for the IN area of infection. (A) Schematic representation of the sequence of filters applied to identify indicators. Four filters were concatenated considering the three variables of our experimental design: time, treatment and tissue area. Briefly, in the first filter, we selected genes differentially upregulated from 0 to 4/6 hpi (FDR < 0.05 and $\log_2$FC > 2) at the IN area (colored in red) upon bacterial infection. From the genes that passed this first filter, we selected those that were exclusively upregulated (FDR < 0.05 and $\log_2$FC > 2) due to bacterial infection at the IN area at 4/6 hpi. Subsequently, from the genes that made it into the third filter, we selected those that were not highly upregulated in the OUT area (colored in blue) upon bacterial infection at 4/6 hpi (FDR < 0.05 and $\log_2$FC < 1). Finally, we applied a fourth filter to discard genes that could potentially be basally upregulated at the OUT area upon pathogen treatment at 4/6 hpi (FDR < 0.05 and $\log_2$FC > 2). The starting number of genes and the genes passing the different filtering criteria are indicated. **(B)** RT-qPCR and RNA-seq expression profiles of marker genes that behave as *bona fide* HR indicators. Relative expression levels to the housekeeping gene *EIF4a* were represented as fold enrichment between 4/6 and 0 hpi. Error bars represent standard error of the mean from three independent experiments. Letters indicate statistically significant differences between treatments following one-way ANOVA with Tukey´s HSD test ($\alpha = 0.05$) performed independently at IN and OUT. NS (non-significant after one-way ANOVA). Exact p values

are provided in **Table S5. (C)** List of HR indicators along with their gene ID, gene name and description.

igure 5. *AT5G17760* **encodes an AAA-ATPase and is a reliable HR indicator specifically induced at the IN area by activation of different classes of NLR receptors. (A)** Representative images of trypan blue-stained leaves, epifluorescence microscope and confocal microscope from *pAT5G17760:NLS-3xGFP* Arabidopsis transgenics. A small region of 4-week-old *pAT5G17760::NLS-3xGFP* leaves was syringe-infiltrated with *Pto* expressing the effectors *AvrRpm1*, *AvrRpt2* or *AvrRps4* at $1*10^7$ colony-forming units (CFU)/ml (O.D$_{600}$ = 0.01). Besides mock treatment, the non-cell death-causing bacterial strains *Pto* DC3000 EV and *Pto* DC3000 *hrcC-* were included as negative controls. Images were taken 16 hpi. Scale bar 3 mm. Images were taken 16 hpi on a Leica DM6 microscope and a confocal microscope prior to trypan blue staining. Scale bar 3 mm. Expression of p*AT5G17760* is detected as green dots corresponding to nuclei with positive GFP signal. Scale bar 100 μm. **A** representative close-up magnified image of a *Pto AvrRpm1*-infected leaf expressing *pAT5G17760:NLS-3xGFP* at 16 hpi is shown. Scale bar 3 mm. **(B)** Quantification of fluorescent nuclei from confocal microscopy pictures in **(A)**. Nuclei count was performed using ImageJ software. Data is representative of three independent experiments each one of them containing 4 leaves. Letters indicate statistically significant differences in number of nuclei following one-way ANOVA with Tukey´s HSD test (α = 0.05). Exact p values are provided in **Table S5. (C)** Activation of *pAT5G17760* occurred upon drop inoculation infection with the necrotrophic pathogen *B. cinerea* or a mock solution. Four- to 5-week-old leaves from *pAT5G17760::*3xGFP transgenics were drop inoculated with *B. cinerea* at concentration of $1x10^5$ spores per mL. Images in left panels represent trypan blue stained leaves and right panels represent leaves imaged under the epifluoresecent microscope at 3 dpi. Scale bars 3 mm.

Figure 1

Click here to access/download;Figure;Figure 1.TIF ⬇

Figure 1
A

Figure 2

Figure 2
A

B

C

Figure 3

Figure 3

A IN

Cluster I

Cluster II

Cluster III

Standardized expression

Time (hpi)

Pto AvrRpm1 (2,937 genes)
Mock 1.1 (638 genes)
Mock 1.2 (2,299 genes)

Pto AvrRpm1 (4,183 genes)
Mock 2.1 (2,570 genes)
Mock 2.2 (1,613 genes)

Pto AvrRpm1 (6,428 genes)
Mock 3.1 (3,172 genes)
Mock 3.2 (3,256 genes)

B OUT

Cluster I

Cluster II

Cluster III

Standardized expression

Time (hpi)

Pto AvrRpm1 (1,552 genes)
Mock 1.1 (850 genes)
Mock 1.2 (702 genes)

Pto AvrRpm1 (1,100 genes)
Mock 2.1 (453 genes)
Mock 2.2 (647 genes)

Pto AvrRpm1 (925 genes)
Mock 3.1 (612 genes)
Mock 3.2 (313 genes)

Figure 4

Click here to access/download;Figure;Figure 4.TIF ⤓

Figure 4

Marker ID	Gene ID	Gene	Description	Time point	Process
M1	AT1G30270	CIPK23	CBL-interacting serine/threonine-protein kinase 23	4 hpi	Ion transport PM
M2	AT1G79710		Probable folate-biopterin transporter 3	4 hpi	
M3	AT4G18050	PGP9	P-glycoprotein 9	4 hpi	Cell detoxification
M4	AT1G78380	GSTU19	Glutathione S-transferase U19	6 hpi	
M5	AT4G24160		1-acylglycerol-3-phosphate O-acyltransferase	4 hpi	Lipid metabolism
M6	AT5G18480	IPUT1	Inositol phosphorylceramide glucuronosyltransferase 1	4 hpi	
M7	AT4G30390		UDP-arabinopyranose mutase	4 hpi	
M8	AT5G54650	FH5	Formin-like protein 5	4 hpi	Cell wall remodeling
M9	AT5G16910	CSLD2	Cellulose synthase-like protein D2	6 hpi	
M10	AT5G20000	RPT6B	26S proteosome regulatory subunit 8 homolog B	4 hpi	Protein degradation
M11	AT2G36580		Pyruvate kinase	4 hpi	
M12	AT5G56350		Pyruvate kinase	4/6 hpi	Uncharacterized
M13	AT5G17760		AAA-ATPase	6 hpi	

Figure 5
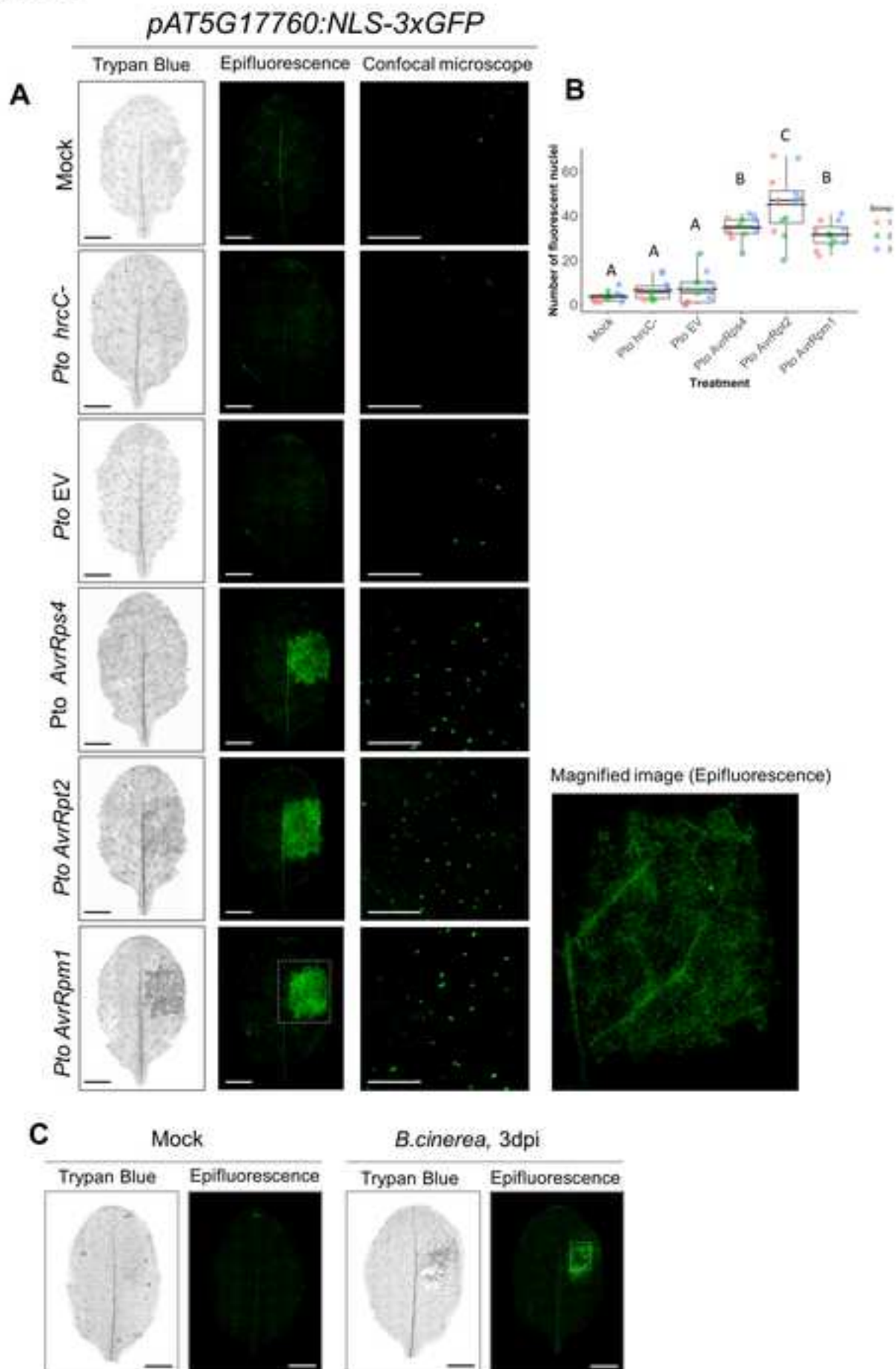
Click here to access/download;Figure;Figure 5.TIF ±

Figure 5

Robust transcriptional indicators of immune cell death revealed by spatio-temporal transcriptome analyses

Jose Salguero-Linares[a,#], Irene Serrano[b, ,#,†], Nerea Ruiz-Solani[a], Marta Salas-Gómez[a], Ujjal Jyoti Phukan[a], Victor Manuel González[a], Martí Bernardo-Faura[a], Marc Valls[a,b], David Rengel[b,c,¥,§,*], Nuria S. Coll[a,d,§,*]

Supplemental Information

Supplementary figures and supplementary tables

Figure S1:

A

B
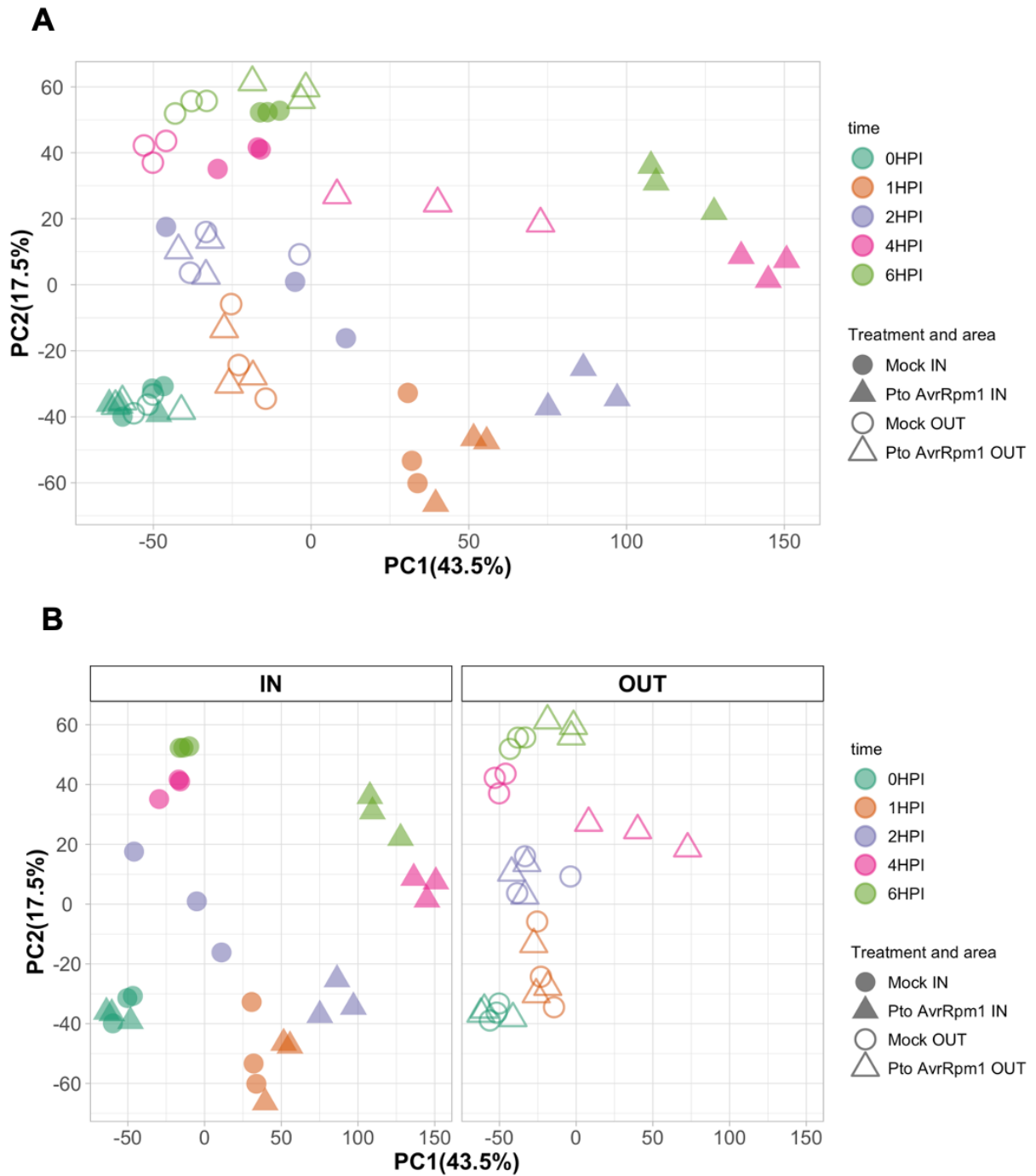
Figure S1. Principal component analysis (PCA) from the RNA seq-data. Circles represent mock-treated plants and triangles represent *Pto AvrRpm1*-infected plants. Different colors are assigned for each time point. **(A)** PCA comprising all data sets in our study (IN and OUT samples together). **(B)** PCA with IN and OUT data sets separated in order to ease visualization of the data.

Figure S2:
A

IN area UP-REGULATED GO terms: Biological process
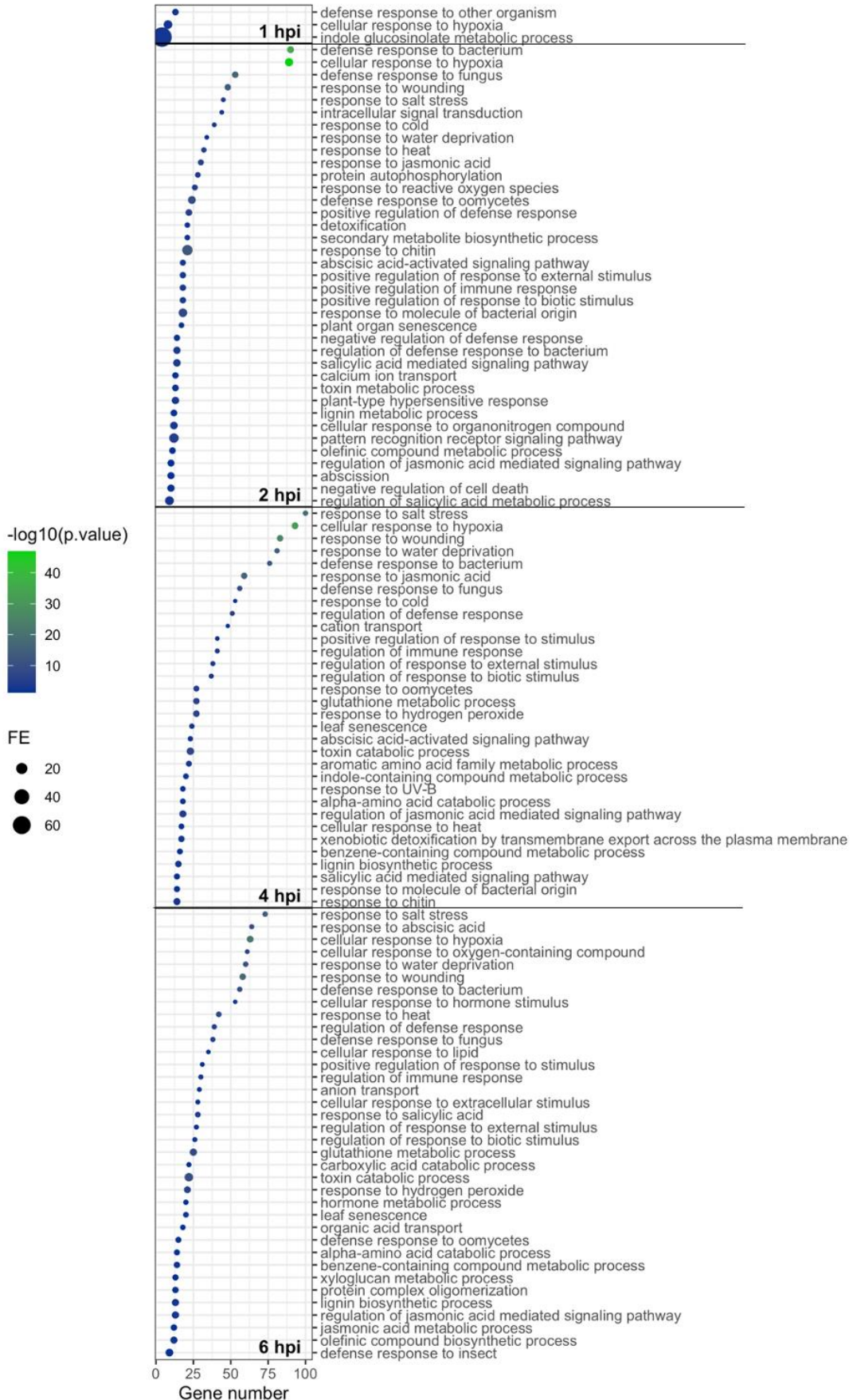
B

IN area DOWN-REGULATED GO terms: Biological process
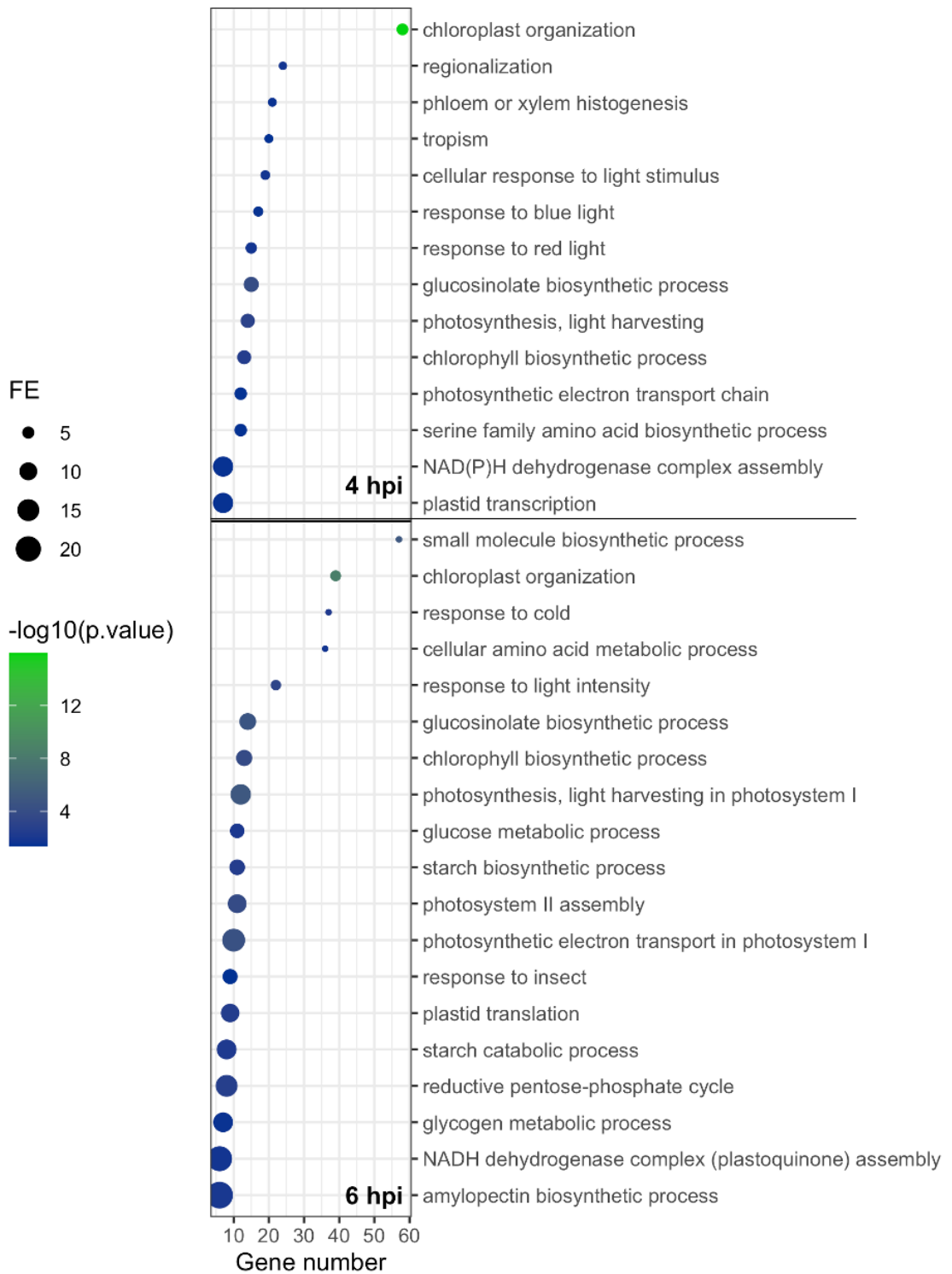
C

OUT area UP-REGULATED GO terms: Biological process
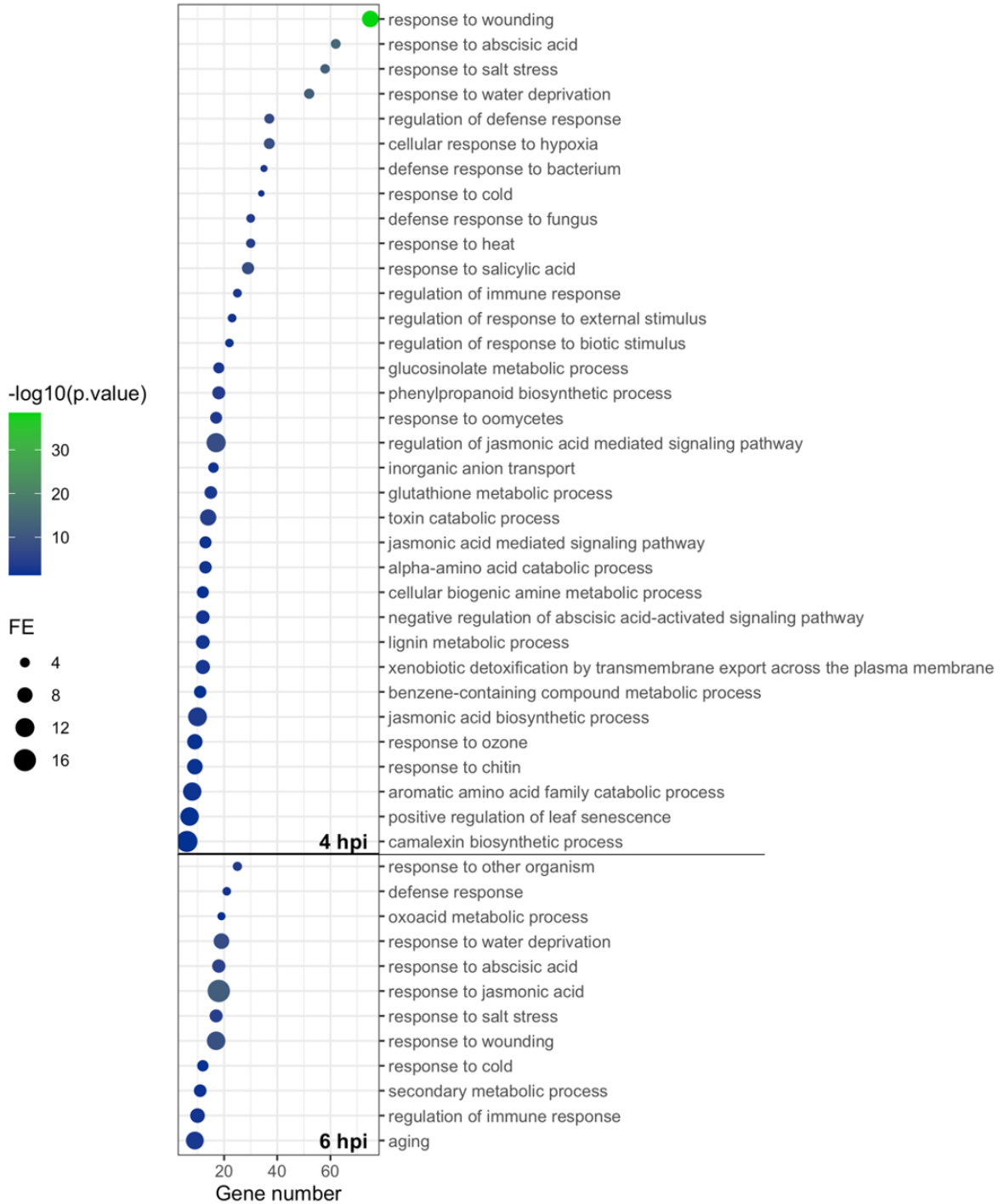
Figure S2. GO term enrichment analysis of upregulated and downregulated genes at each time after infection at the IN **(A-B)** and OUT **(C)** areas. The most specific term from each family term provided by PANTHER was plotted along with their corresponding gene number, fold enrichment and adj p value (Bonferroni Correction for multiple testing) represented as $\log_{10}$. Only GO terms with a fold enrichment above 2 and adj p value below 0.05 were plotted.
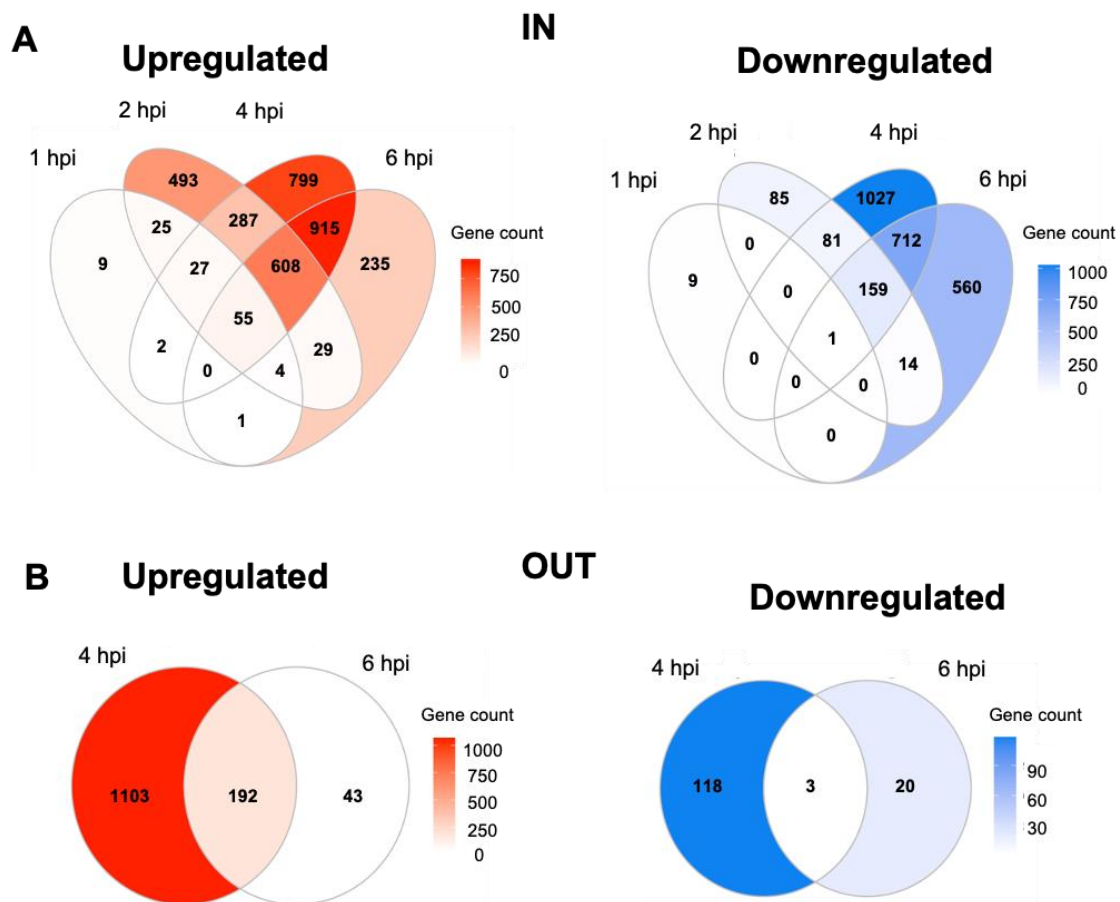
Figure S3. The majority of differentially expressed genes at both IN and OUT are specific to 4 and 6 hpi. Venn diagrams showing sizes of gene sets that are differentially expressed (red: upregulated and blue: downregulated) at IN **(A)** or OUT **(B)** at each time point.

Figure S4
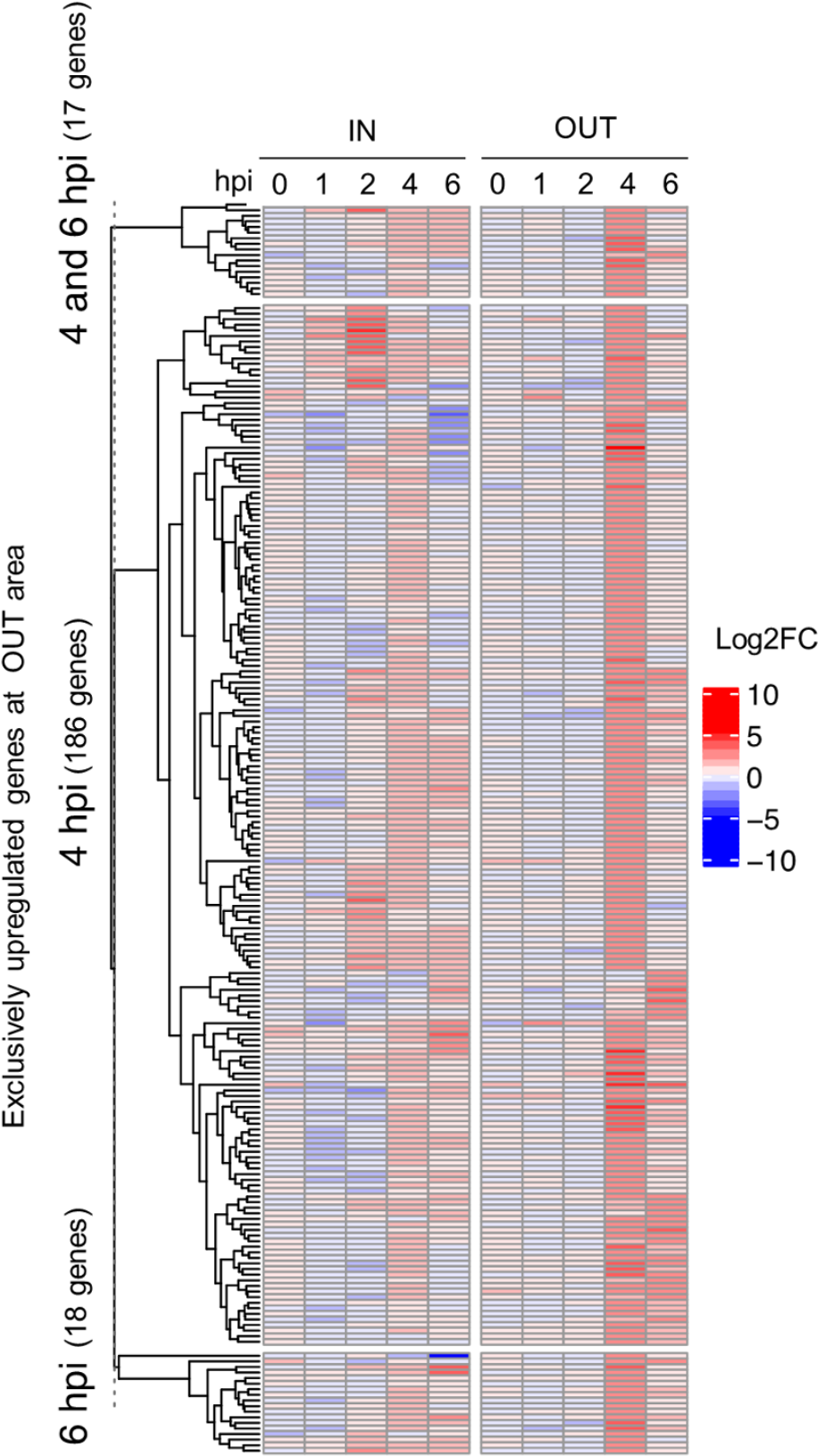
Figure S4. Heatmap representing differential expression of genes exclusively upregulated at 4 and/or 6 hpi at the OUT area (log2FC > 2 and BTH <0.05) throughout the course of the infection (0,1,2,4 and 6 hpi) at IN and OUT areas.

Figure S5
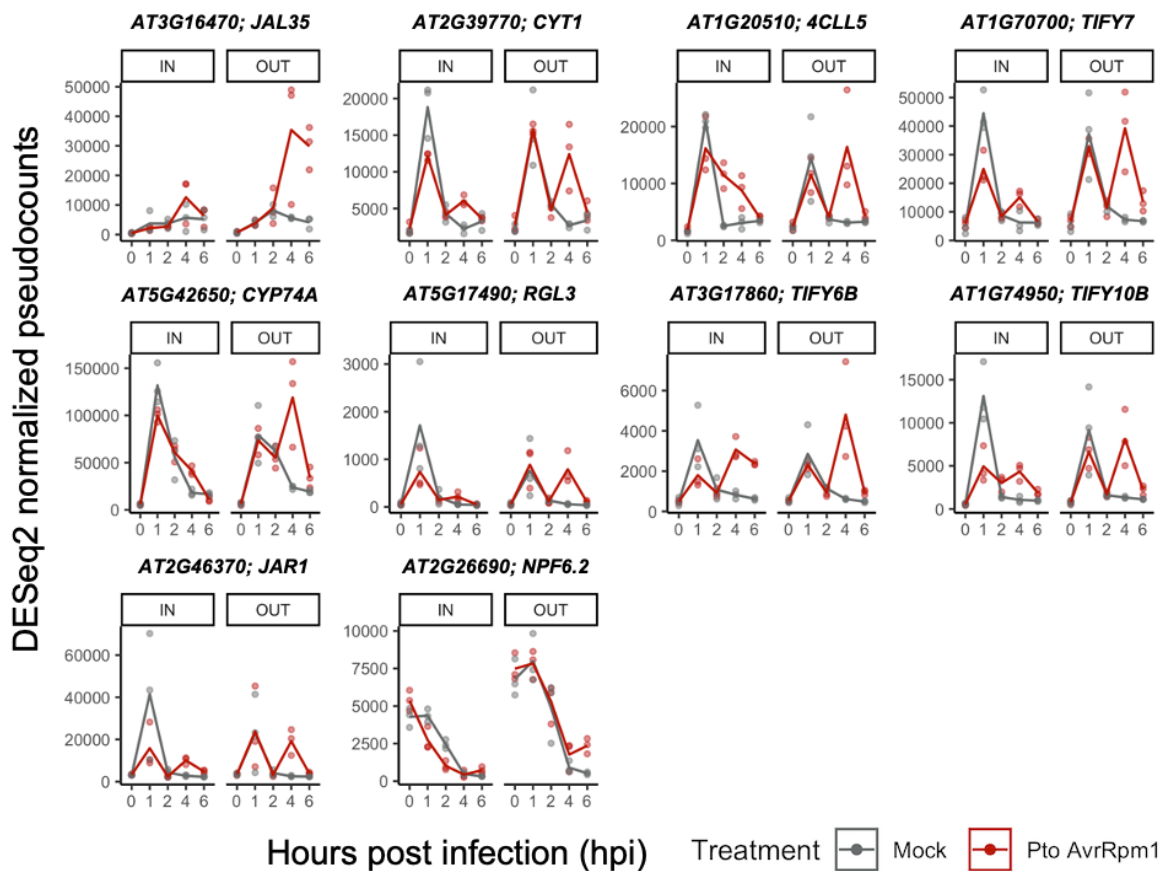
Figure S5. RNA-seq expression profiles of JA responsive genes exclusively upregulated at the OUT area upon *Pto AvrRpm1* infection. Gene expression of genes from *Pto-AvrRpm1* or mock-infected plants is represented as DESeq2 pseudocounts.

JAL35, Jacalin-related lectin 35; *CYT1,* Mannose-1-phosphate guanylyltransferase 1; *4CLL5,* 4-coumarate--CoA ligase-like 5; *TIFY7,* Protein TIFY 7; *CYP74A,* Allene oxide synthase, chloroplastic; *RGL3,,* DELLA protein RGL3; *TIFY6B,* Protein TIFY 6B; *TIFY10B,* Protein

TIFY 10B; *JAR1*, Jasmonoyl--L-amino acid synthetase JAR1; *NPF6.2*, Protein NRT1/ PTR

FAMILY 6.2

Figure S6.

A

Pto AvrRpm1-derived clusters GO terms: Biological process
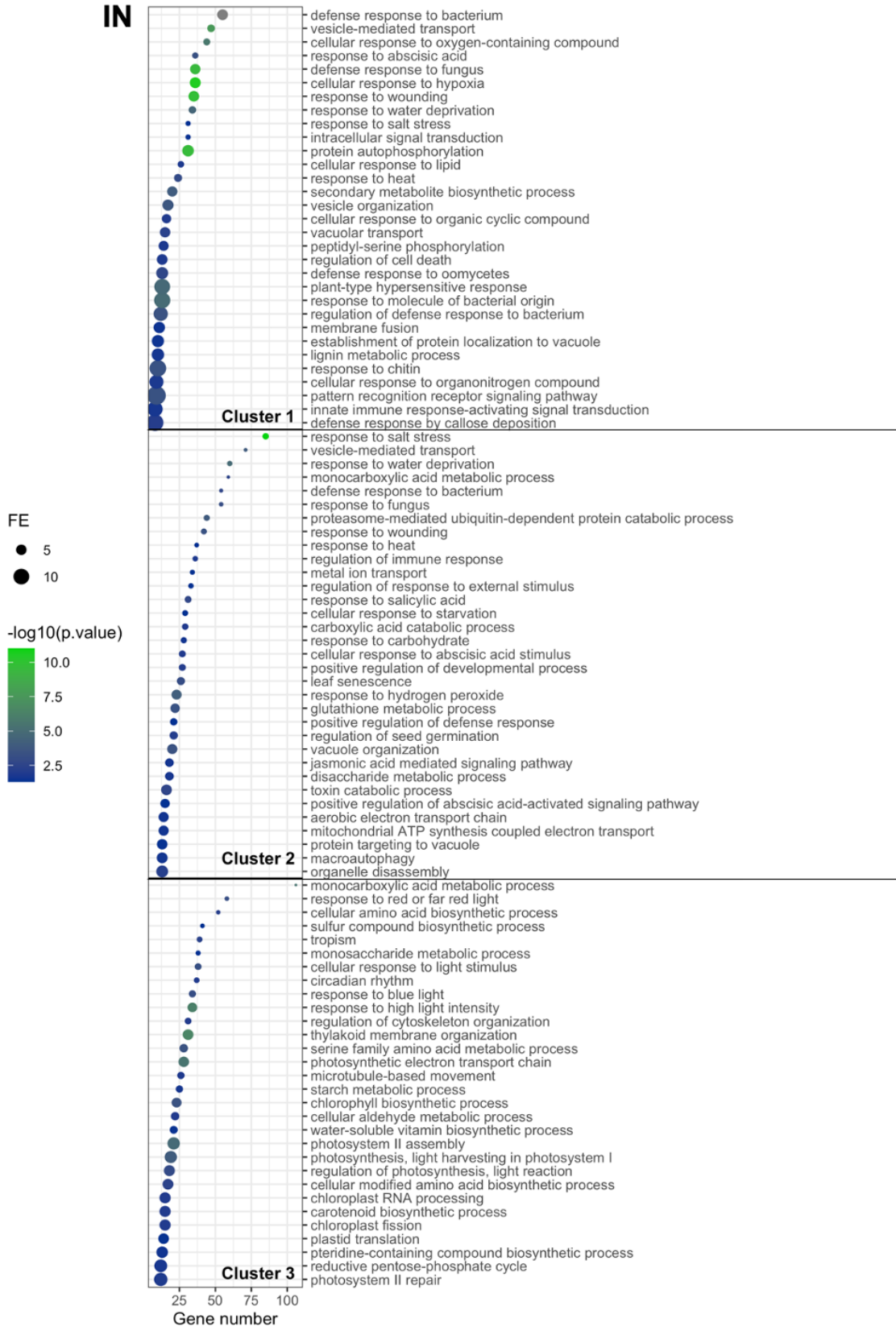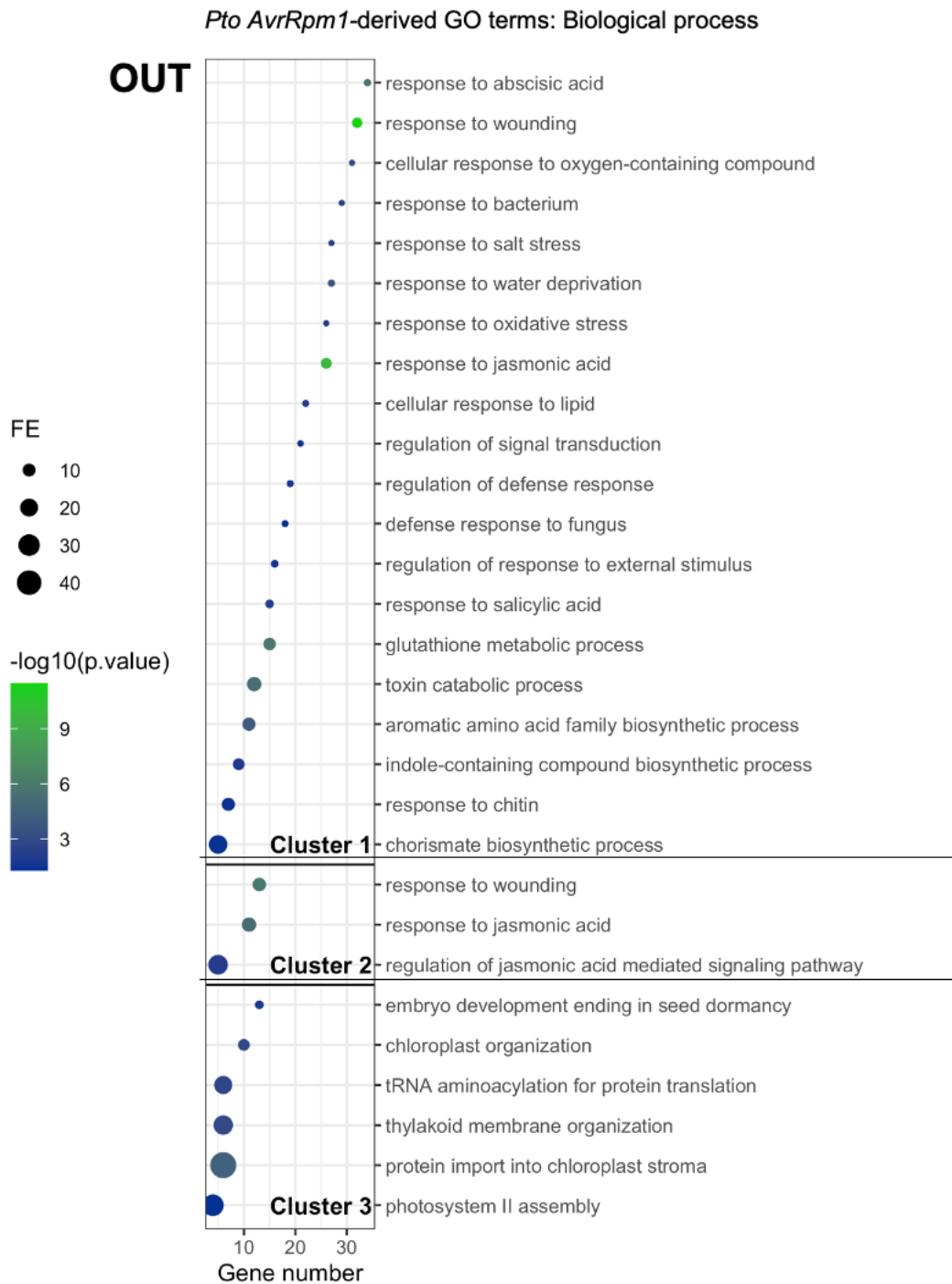
Figure S6. GO terms representing enriched biological processes derived from each cluster in *Pto AvrRpm1*-treated plants. GO term enrichment analysis was performed on those genes that had a membership score value (MSV) above or equal to 0.7 (see Materials and Methods). The most specific term from each family provided by PANTHER was plotted along with their corresponding gene number, fold enrichment (FE) and adj p value (Bonferroni Correction for

multiple testing) represented as $\log_{10}$. Only GO Terms with a FE above 2 and adj p value below 0.05 were plotted. Enriched GO terms from cluster I (2,937 genes; MSV > 0.7 → 1069 genes), cluster II (4,183 genes; MSV > 0.7 → 2613 genes) and cluster III (6,428 genes; MSV > 0.7 → 4885 genes) at the IN area **(A)** in *Pto AvrRpm1*-treated plants were predominantly linked to processes related to immunity, protein turnover and photosynthesis, respectively. At the OUT area **(B)**, enriched GO terms from cluster I (1,552 genes; MS > 0.7 → 747 genes) and II (1,100 genes; MS > 0.7 → 184) suggest the importance of processes related to hormonal regulation in by-stander cells, whereas genes comprising cluster III (925 genes; MS > 0.7 → 181 genes) infer that photosynthesis and rearrangements in the chloroplast occur similarly compared to mock-treated samples at the OUT area

Figure S7

A

Mock-derived clusters GO terms: Biological process

IN
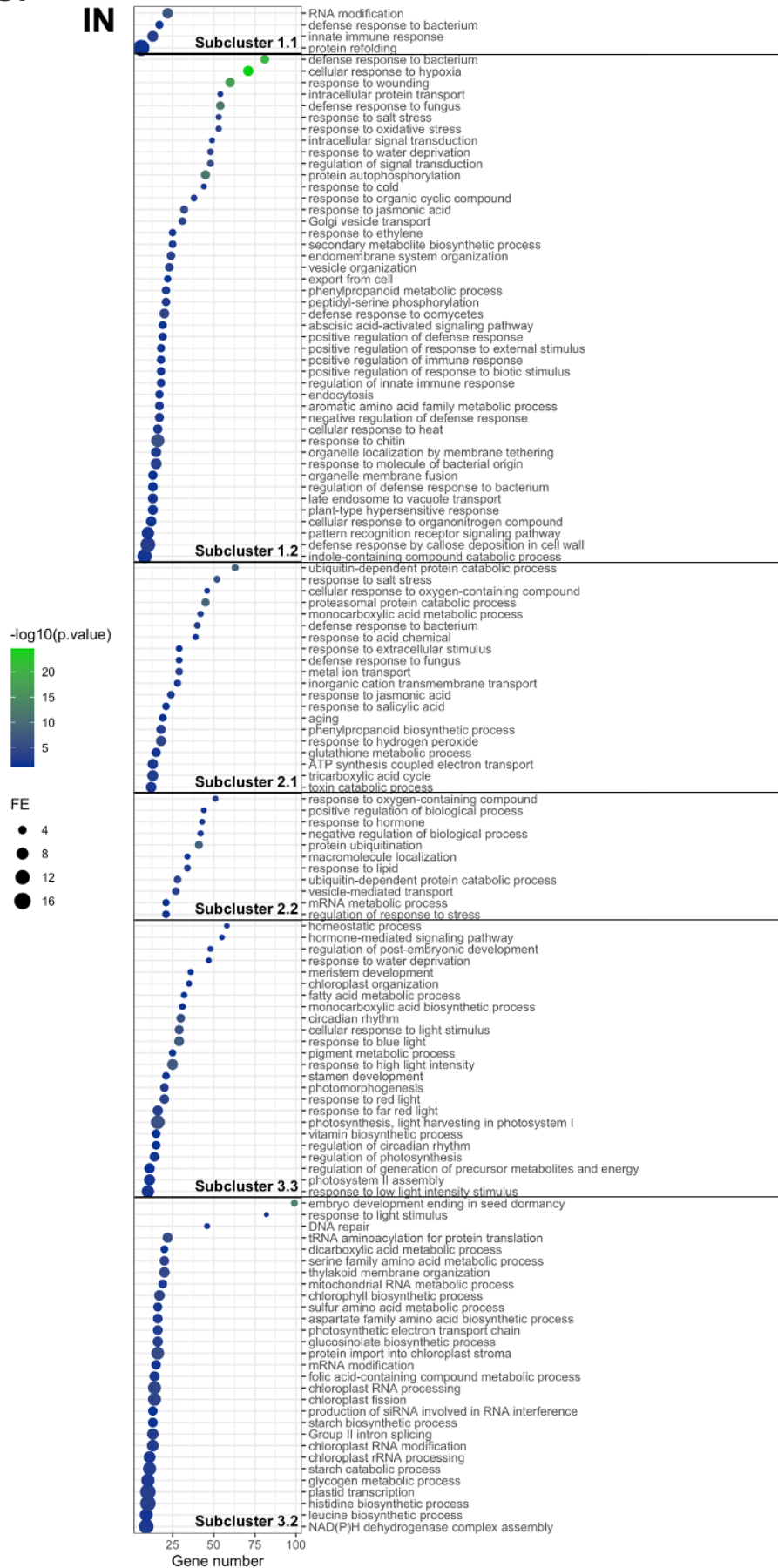
B Mock-derived clusters GO terms: Biological process
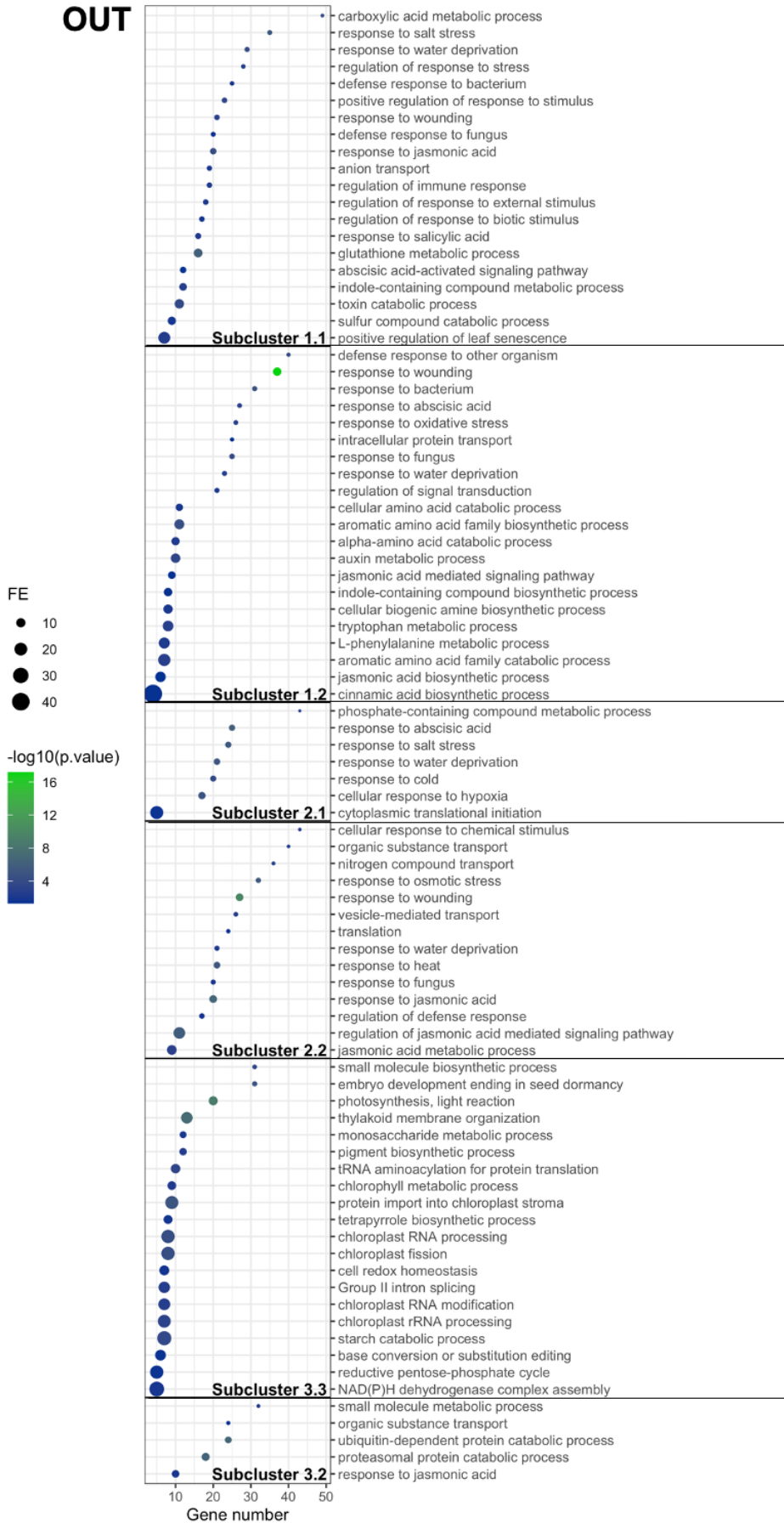
Figure S7. GO terms representing enriched biological processes derived from each sub-cluster in mock-treated plants at the IN and OUT areas. From each cluster belonging to mock-treated samples, GO term enrichment analysis was performed on those genes that had a membership score value (MSV) above or equal to 0.7 at the IN (a) and OUT areas (b). The most specific term from each family term provided by PANTHER was plotted along with their corresponding gene number, fold enrichment and adj p value (Bonferroni Correction for multiple testing) represented as $\log_{10}$. Only GO Terms with a fold enrichment above 2 and adj p value below 0.05 were plotted. **(A)** Sub-cluster 1.1 (638 genes; MSV >= 0.7 → 467 genes), sub-cluster 1.2 (2299 genes; MSV >= 0.7 → 1942 genes), sub-cluster 2.1 (2570 genes; MSV >= 0.7 → 1573 genes), sub-cluster 2.2 (1613 genes; MSV >= 0.7 → 649 genes), sub-cluster 3.1 (3172 genes; MSV >= 0.7 → 2391 genes), sub-cluster 3.2 (3256 genes; MSV >= 0.7 → 2557 genes). **(B)** Sub-cluster 1.1 (850 genes; MSV >= 0.7 → 319 genes), sub-cluster 1.2 (702 genes; MSV >= 0.7 → 183 genes), sub-cluster 2.1 (453 genes; MSV >= 0.7 → 286 genes), sub-cluster 2.2 (647 genes; MSV >= 0.7 → 389 genes), sub-cluster 3.1 (612 genes; MSV >= 0.7 → 555 genes), sub-cluster 3.2 (313 genes; MSV >= 0.7 → 257 genes).

Figure S8
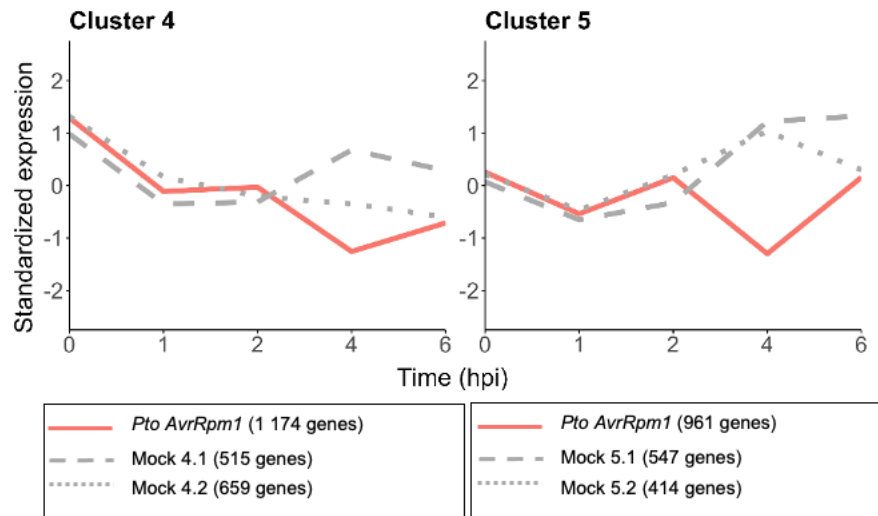
Figure S8. Clusters 4 (1,174 genes; MSV >= 0.7 → 57 genes) and 5 (961 genes; MSV >= 0.7 → 314 genes) from *Pto AvrRpm1*-treated plants at the OUT area share similar expression profiles and do not contain any relevant enriched GO terms associated with biological processes, possibly due to low gene number.

Figure S9

A
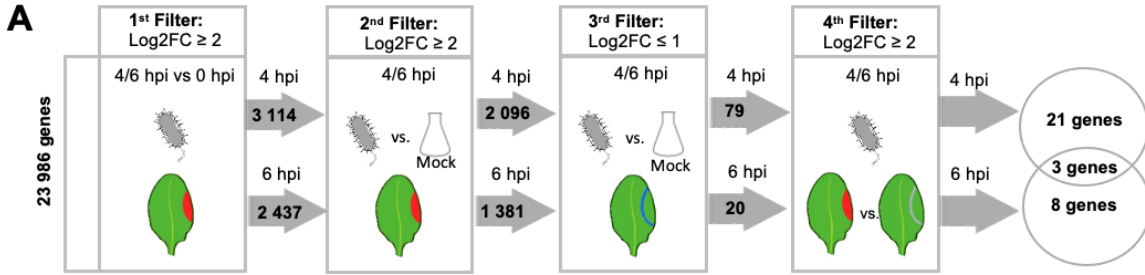
B

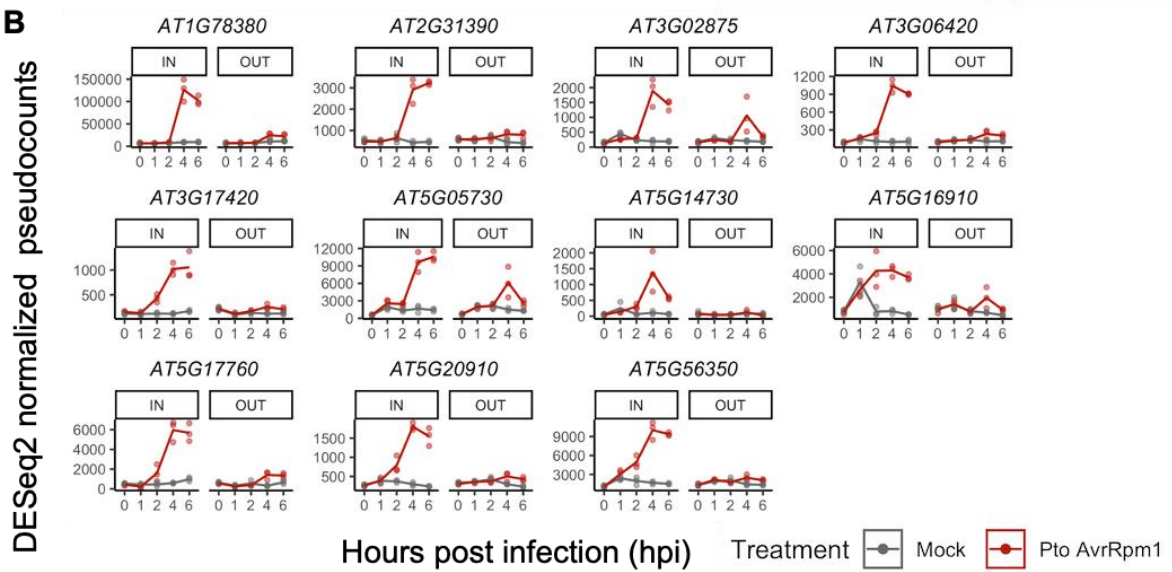|	Gene ID	1st Filter	2nd Filter	3rd Filter	4th Filter	Gene description
4 hpi	AT1G24095	4.03	2.49	0.77	2.36	Putative thiol-disulfide oxidoreductase DCC
	AT1G27720	3.51	3.01	0.94	2.55	Transcription initiation factor TFIID subunit 4 (TAF4)
	AT1G30270	2.59	3.07	0.67	2.54	CBL-interacting serine/threonine-protein kinase 23 (CIPK23)
	AT1G31880	3.00	3.27	0.84	2.56	DZC domain containing protein (NLM9)
	AT1G74810	3.70	3.09	0.90	2.51	Putative boron transporter 5 (BOR5)
	AT1G79710	3.50	2.94	0.85	2.11	Probable folate-biopterin transporter 3
	AT2G33120	2.74	2.71	0.86	2.13	Vesicle-associated membrane protein 722 (SAR1/VAMP722)
	AT2G39400	3.46	2.38	-1.08	3.61	Alpha/beta-Hydrolases superfamily protein
	AT3G04120	2.24	2.29	0.50	2.01	Glyceraldehyde-3-phosphate dehydrogenase (GAPC1)
	AT3G13782	3.38	2.95	0.83	2.85	Nucleosome assembly protein 1;4 (NAP 1;4)
	AT3G28850	2.72	2.16	0.79	2.14	Glutaredoxin family protein
	AT3G60680	2.03	2.87	0.70	2.05	DUF641 family protein
	AT4G18050	4.47	4.57	0.79	3.56	P-glycoprotein 9 (PGP9)
	AT4G24160	2.66	3.43	0.99	2.68	1-acylglycerol-3-phosphate O-acyltransferase
	AT4G30390	3.33	3.42	0.98	2.54	UDP-arabinopyranose mutase
	AT5G10820	3.55	2.01	0.72	2.05	Probable folate-biopterin transporter 6
	AT5G18480	2.86	2.54	0.94	2.04	Inositol phosphorylceramide glucuronosyltransferase1(IPUT1)
	AT5G20000	3.67	2.50	0.79	2.25	26S proteasome regulatory subunit 8 homolog B (RPT6B)
	AT2G36580	2.51	2.58	0.77	2.36	Pyruvate kinase
	AT5G37710	2.14	2.29	0.56	2.02	alpha/beta-Hydrolases superfamily protein
	AT5G54650	4.05	3.21	0.99	2.21	Formin-like protein 5 (FH5)
6 hpi	AT1G78380	3.88	3.45	0.96	3.88	Glutathione S-transferase U19 (GSTU19)
	AT3G02875	3.35	2.92	0.93	3.35	IAA-amino acid hydrolase (ILR1)
	AT3G06420	3.41	3.14	0.83	3.41	Autophagy-related protein 8h (ATG8H)
	AT3G17420	2.75	2.63	0.72	2.75	Probable receptor-like protein kinase (GPK1)
	AT5G05730	4.10	2.90	0.96	4.10	Anthranilate synthase alpha subunit 1 (ASA1)
	AT5G14730	3.47	3.17	-1.41	3.47	Unknown protein
	AT5G16910	2.26	2.83	0.96	2.26	Cellulose synthase-like protein D2 (CSLD2)
	AT5G17760	3.64	2.54	0.98	3.64	AAA-ATPase
4/6 hpi	AT5G20910	2.78	2.59	0.74	2.06	E3 ubiquitin-protein ligase (AIP2)
	AT2G31390	2.55	2.73	0.84	2.04	Probable fructokinase-1
	AT5G56350	3.20	2.55	0.79	2.22	Pyruvate kinase

Log2 FC
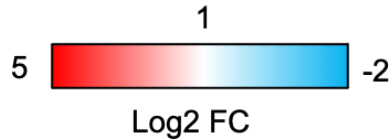
Figure S9. List of *in silico* HR indicators obtained after filtering at 4 and 6 hpi. **(A)** Briefly, we firstly selected genes that were upregulated (log2FC > 2) after *Pto AvrRpm1* infection at 4 or 6 hpi vs 0 hpi. From the genes that complied with this first filter, we selected those that were specifically upregulated in *Pto AvrRpm1*-infected vs mock-inoculated samples at 4 or 6 hpi (log2FC >2). From the genes that complied these criteria, we kept those with a log2FC <1 at the OUT area in *Pto AvrRpm1*-infected vs mock-inoculated samples at 4 or 6 hpi. Finally, from the genes that met those three criteria, we kept those that were differentially upregulated at the IN area compared to the OUT area in *Pto AvrRpm1*-infected plants. **(B)** Log$_2$FCs resulting from pairwise comparisons in the 1st, 2nd, 3rd and 4th filters applied are indicated for each gene marker along with its corresponding gene description.

Figure S10

A

Hours post infection (hpi)   Treatment —●— Mock   —●— Pto AvrRpm1

B

Hours post infection (hpi)   Treatment —●— Mock   —●— Pto AvrRpm1

DESeq2 normalized pseudocounts
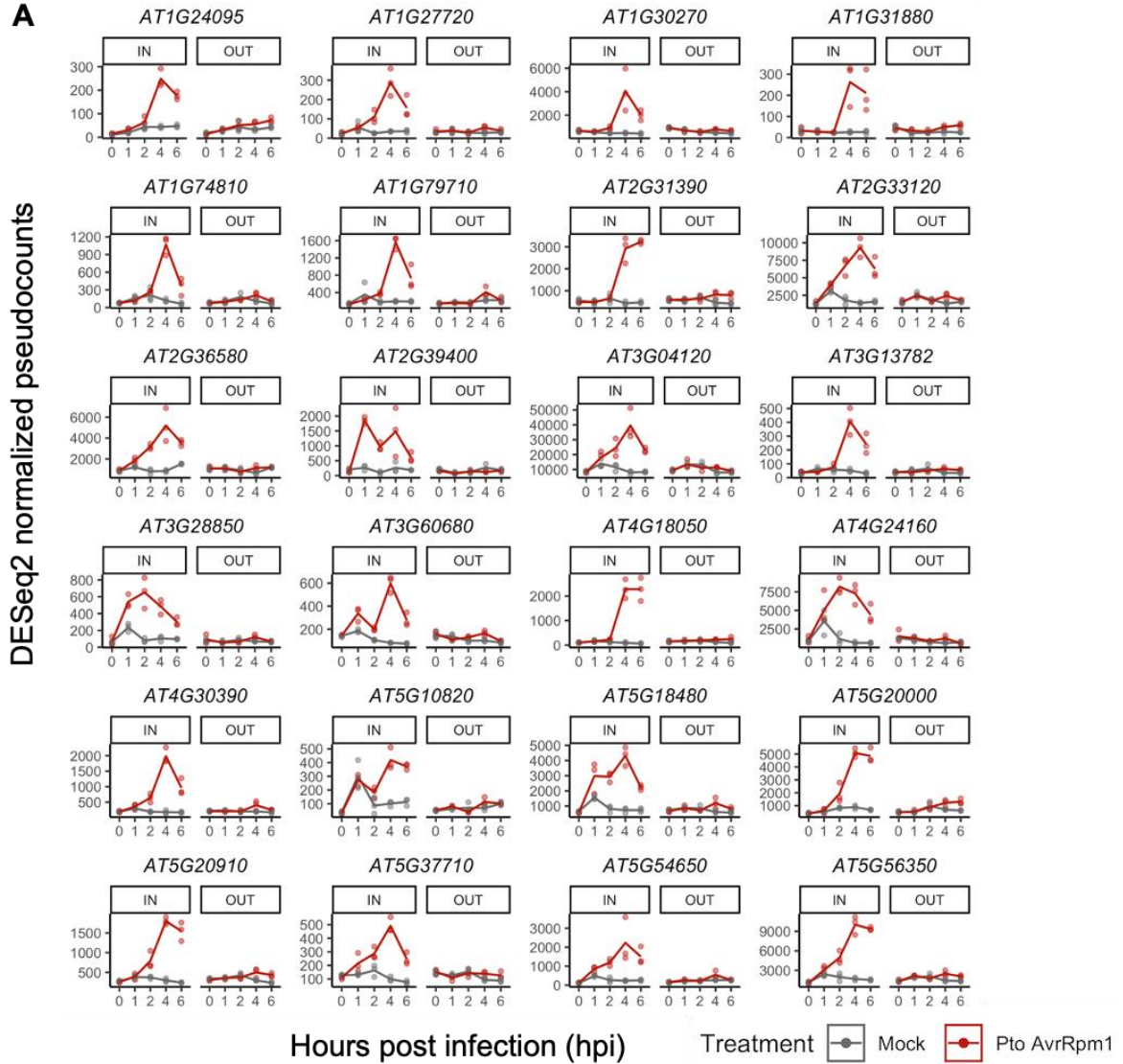
Figure S10. RNA-seq expression profiles of 4 **(A)** and 6 **(B)** hour candidate HR indicators at the IN and OUT areas of infection. Gene expression of genes from *Pto-AvrRpm1* or mock-infected plants is represented as DESeq2 pseudocounts.
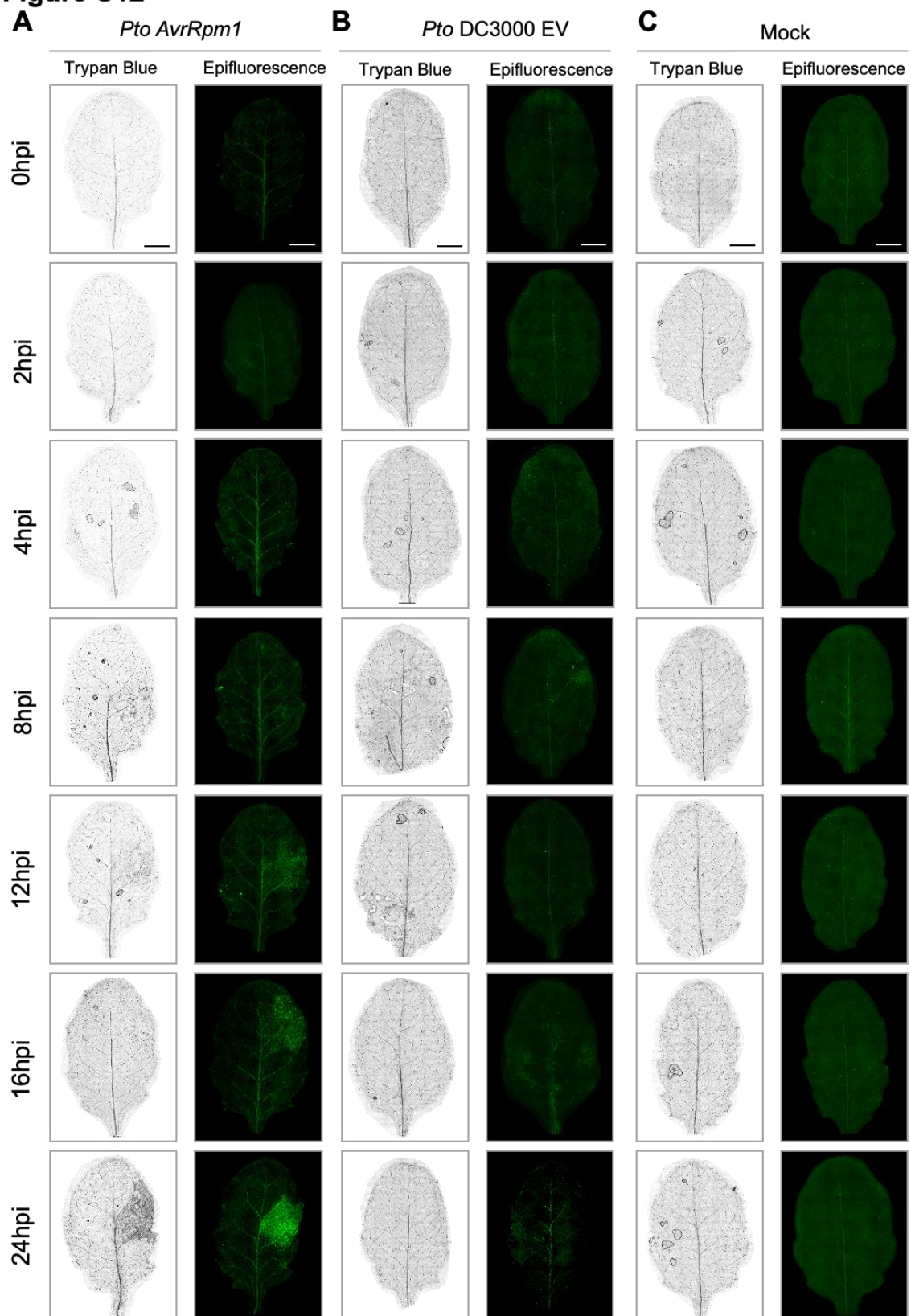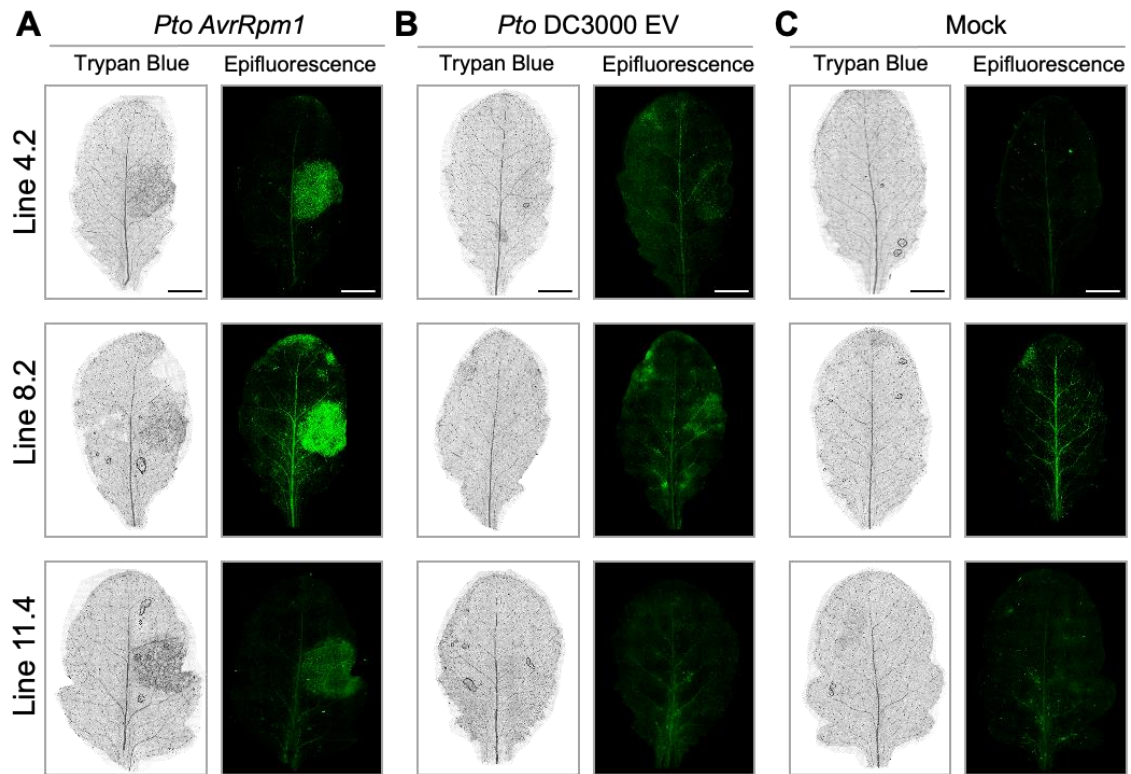
Figure S11

Treatment ⊟ Mock ⊟ Pto AvrRpm1 ⊟ Pto EV
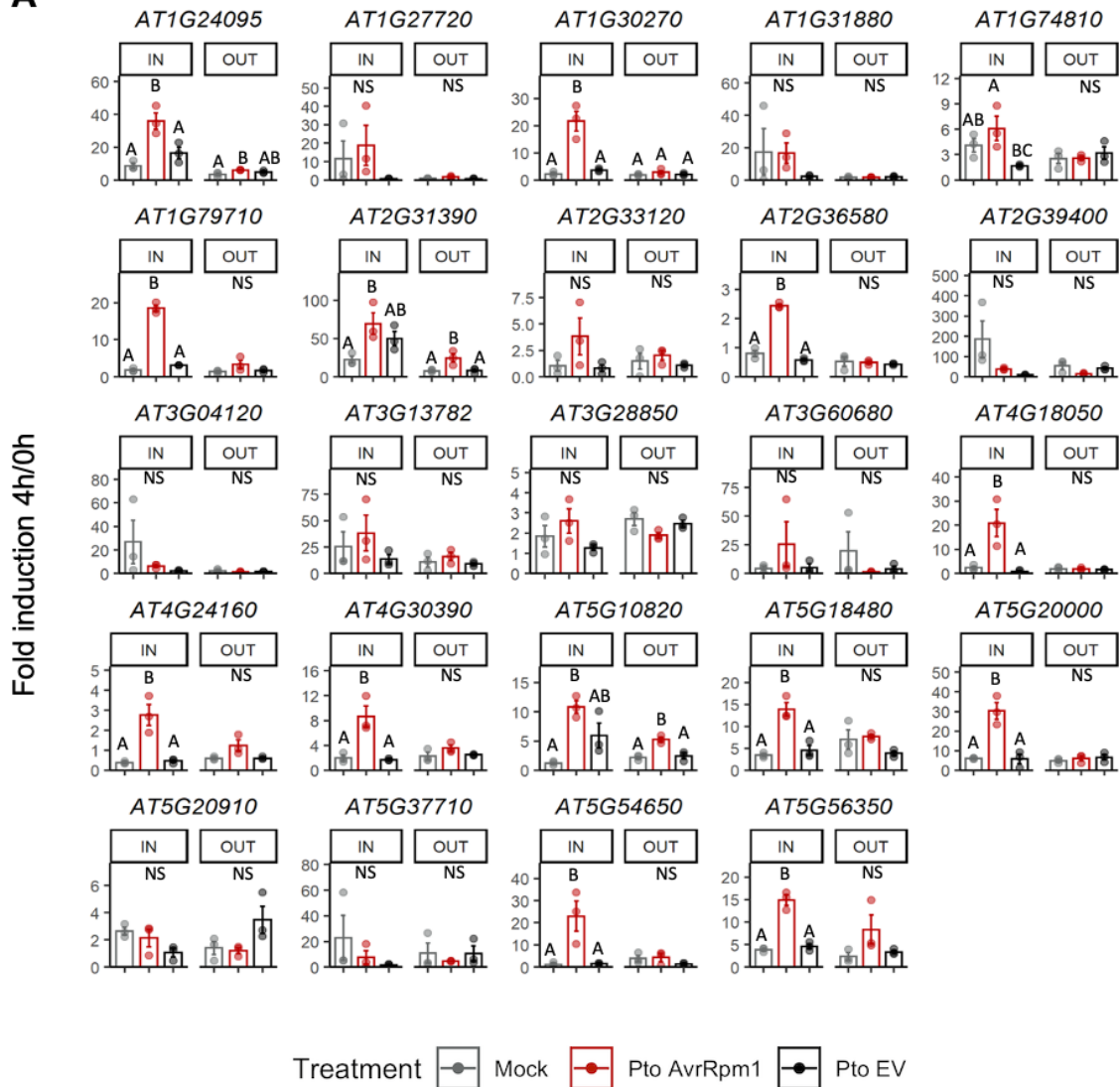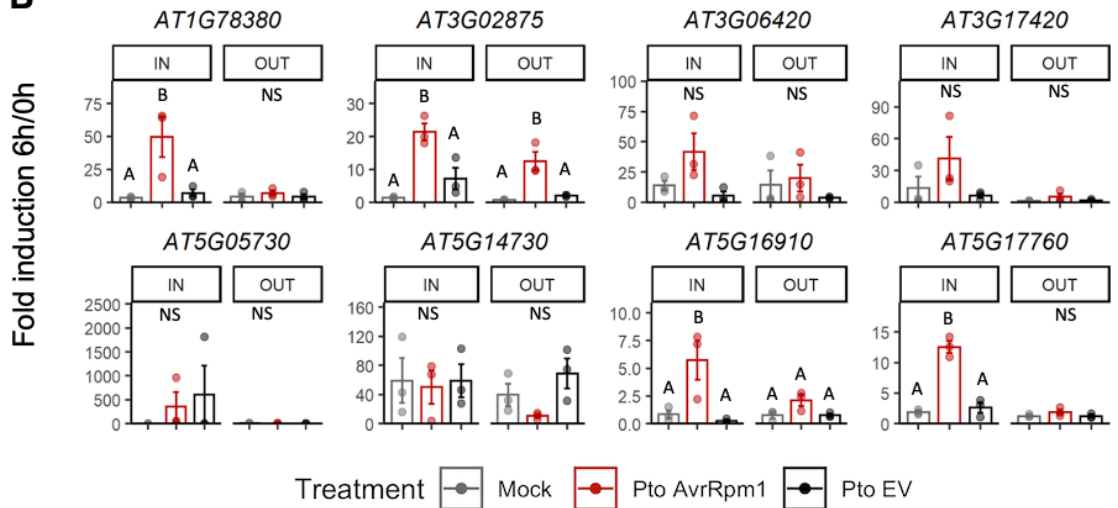
Figure S11. RT-qPCR of 4- and 6-hour transcriptional HR indicators at IN and OUT areas upon treatment with either mock, *Pto AvrRpm1* or *Pto* DC3000 EV. Relative expression levels to the housekeeping gene *EIF4a* were represented as fold induction between 4 **(A)** or 6 **(B)** and 0 hpi. Error bars represent standard error of the mean from three independent experiments. Letters indicate statistically significant differences between treatments following one-way ANOVA with Tukey´s HSD test ($\alpha = 0.05$) performed independently at IN and OUT. NS (non-significant after one-way ANOVA). Exact p values are provided in **Table S5**.

Figure S12

A *Pto AvrRpm1*

Trypan Blue   Epifluorescence

B *Pto* DC3000 EV

Trypan Blue   Epifluorescence

C Mock

Trypan Blue   Epifluorescence

0hpi

2hpi

4hpi

8hpi

12hpi

16hpi

24hpi

Figure S12. Time course imaging of *pAT5G17760:NLS-3xGFP* Arabidopsis transgenic leaves infected with *Pto AvrRpm1* **(A)**, *Pto DC3000 EV* **(B)** or mock solution (10 mM MgCl$_2$) **(C)**. A small region of 4-week-old *pAT5G17760::NLS-3xGFP* leaves was syringe-infiltrated with *Pto* strains at $1*10^7$ colony-forming units (CFU)/ml (O.D$_{600}$ = 0.01). Fluorescent microscopy images were taken at 0, 2, 4, 8, 12, 16 and 24 hpi (right panels). Afterwards, leaves were subjected to trypan blue staining (left panels). Scale bar 3 mm.

Figure S13

Figure S13. Activation of *pAT5G17760* in the syringe-infiltrated area occurred in several independent *pAT5G17760::*3xGFP transgenic lines. Leaves of Arabidopsis transgenics in the T2 generation were syringe infiltrated with *Pto AvrRpm1* (**A**), *Pto DC3000* EV (**B**) at $1*10^7$ colony-forming units (CFU)/ml (O.D$_{600}$ = 0.01) and imaged at 16 hpi. Mock solution was used as a control (**C**). Images in left panels are leaves stained with trypan blue whereas images in right panels are leaves under the epifluorescence microscope. Scale bars 3 mm.

Figure S14
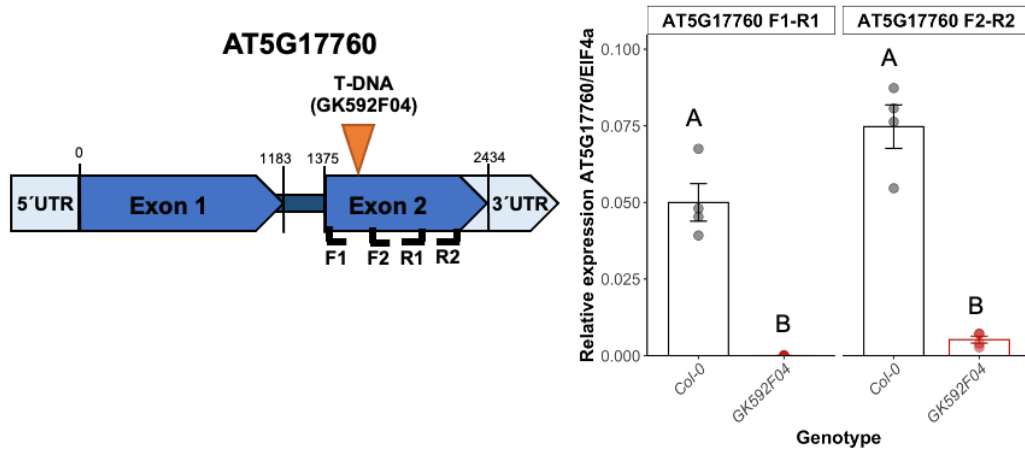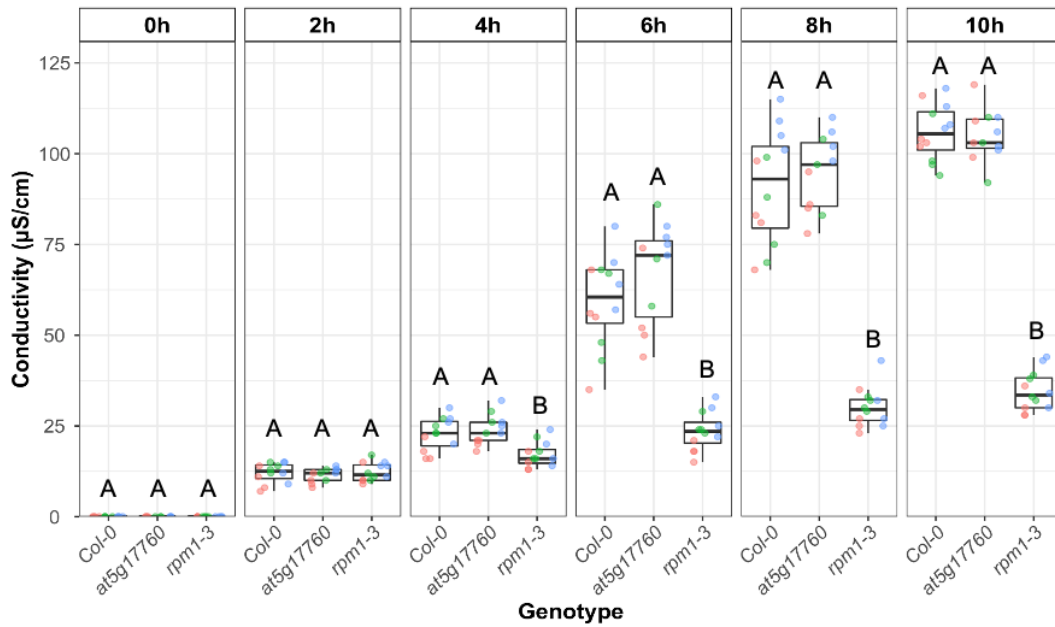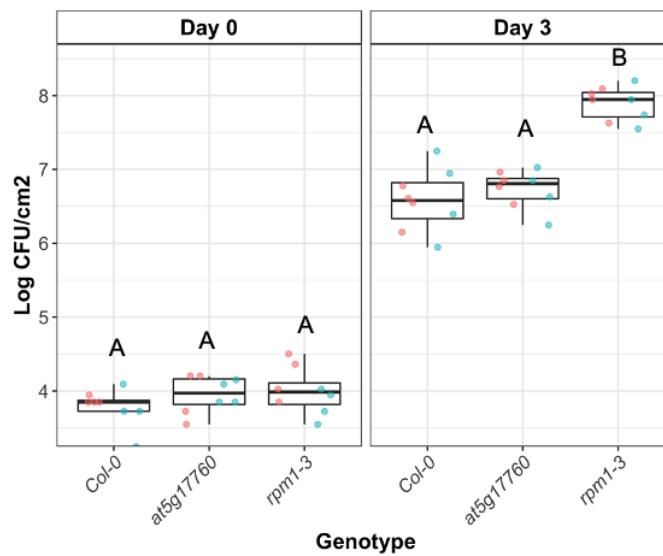
Figure S14. Disease resistance and cell death triggered by avirulent Pto *AvrRpm1* strain is not compromised in Arabidopsis mutant lacking AT5G17760. (A) Scheme of AT517760 gene indicating the position of the T-DNA insertion in GK-59F04 mutant line (left panel) and RT-qPCR of two regions (F1-R1 and F2-R2) of exon 2 in Col-0 and GK-59F04 plants. RT-qPCR data is represented as relative expression levels of *AT5G17760* to the housekeeping gene *EIF4a* (right panel). Error bars represent standard error of the mean from four biological replicates. Letters indicate statistically significant differences between treatments following a Welch Two Sample t-test. Exact p values are provided in **Table S5**. **(B-C)** Four to 5 week-old Col-0, *at5g17760* and *rpm1-3* plants were syringe-infiltrated with *Pto* DC3000 *AvrRpm1* at O.D$_{600}$=0.05 for electrolyte leakage (b) and O.D$_{600}$=0.001 for bacterial growth assays **(C)**, *rpm1-3* mutant is used as a negative control since it is defective in the cognate NLR that recognizes the effector AvrRpm1. **(B)** Conductivity measurements of electrolyte leakage from dying cells were recorded at 0, 4, 6, 8 and 10 hpi. Dots represent data from 3 biological replicates (represented in different colors) consisting of 4 technical replicates each with 2 leaf discs measured per replicate. Letters indicate statistically significant differences between genotypes following one-way ANOVA with Tukey´s HSD test performed at each time point. Exact p values are provided in **Table S5**. **(C)** Bacterial growth at 0 and 3 days post-infection (dpi) was measured in Col-0, *at5g17760* and *rpm1-3*. Dots represent bacterial CFU (colony-forming units) per cm^2 from 2 biological replicates (represented in different colors) consisting of 4 technical replicates each with 2 leaf discs measured per replicate. Letters indicate statistically significant differences between genotypes following one-way ANOVA with Tukey´s HSD test performed at 0 and 3 days post infection. Exact p values are provided in **Table S5.**
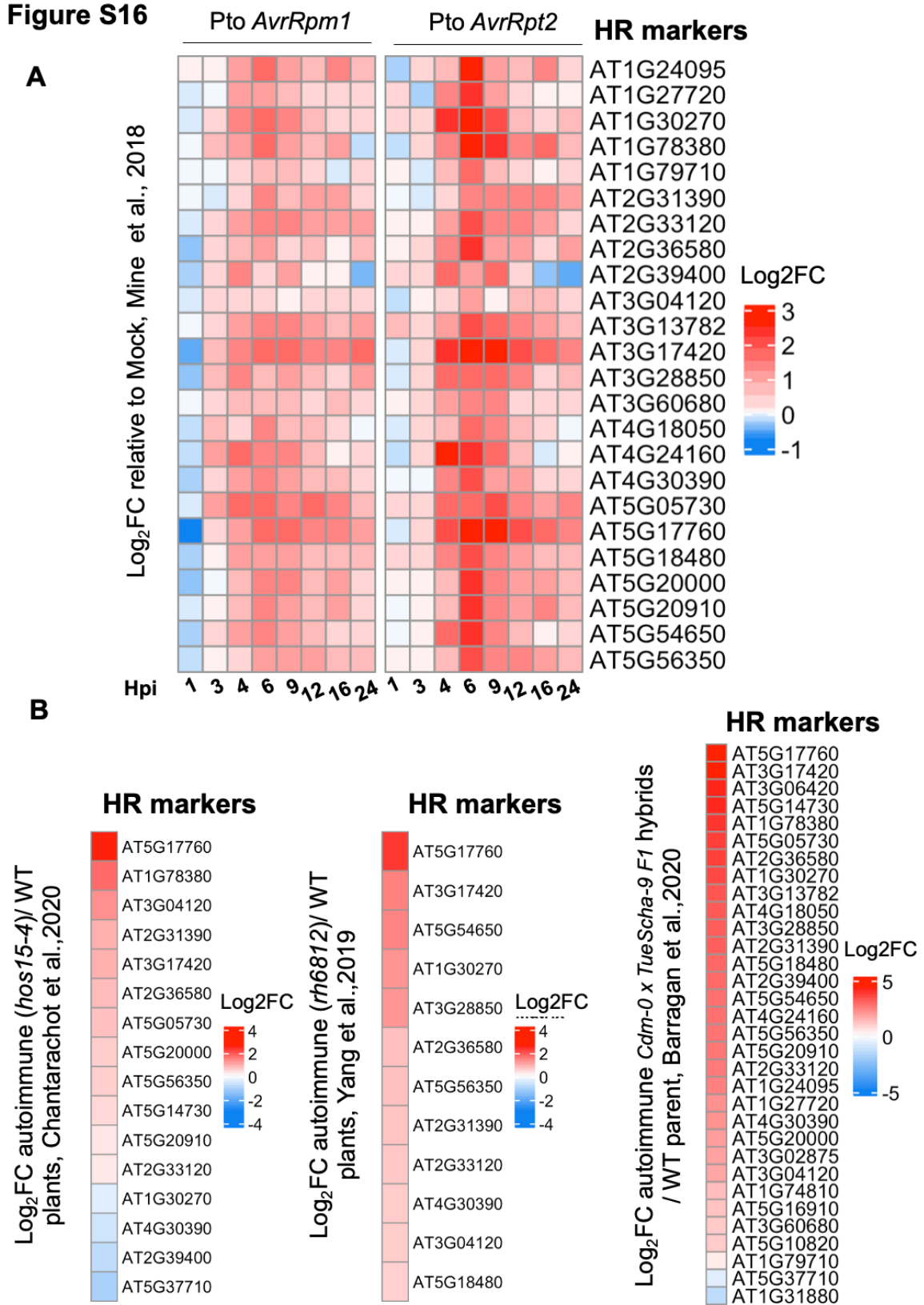
Figure S15

A

DESeq2 normalized pseudocounts

Hours post infection (hpi)

Treatment — Mock — Pto AvrRpm1
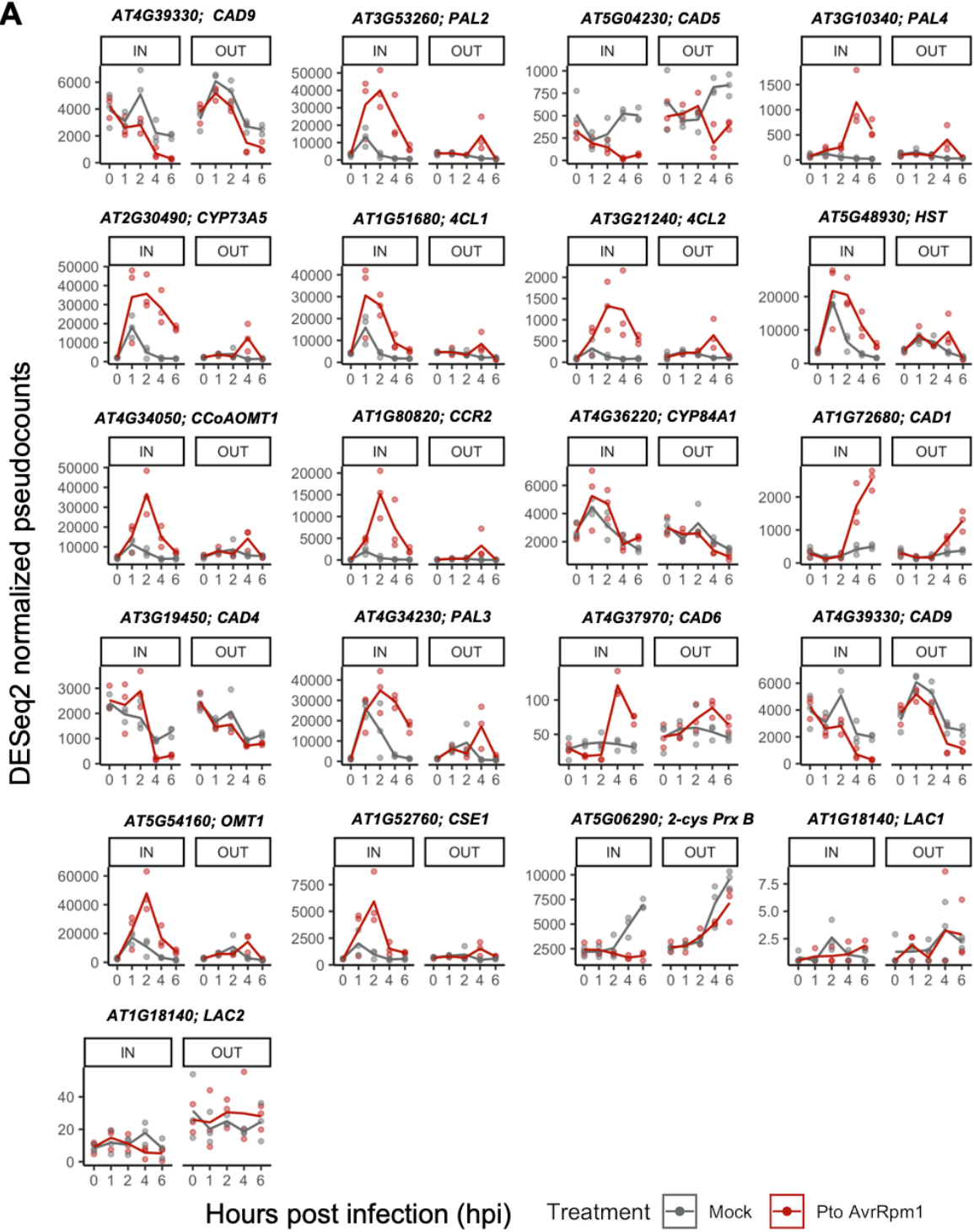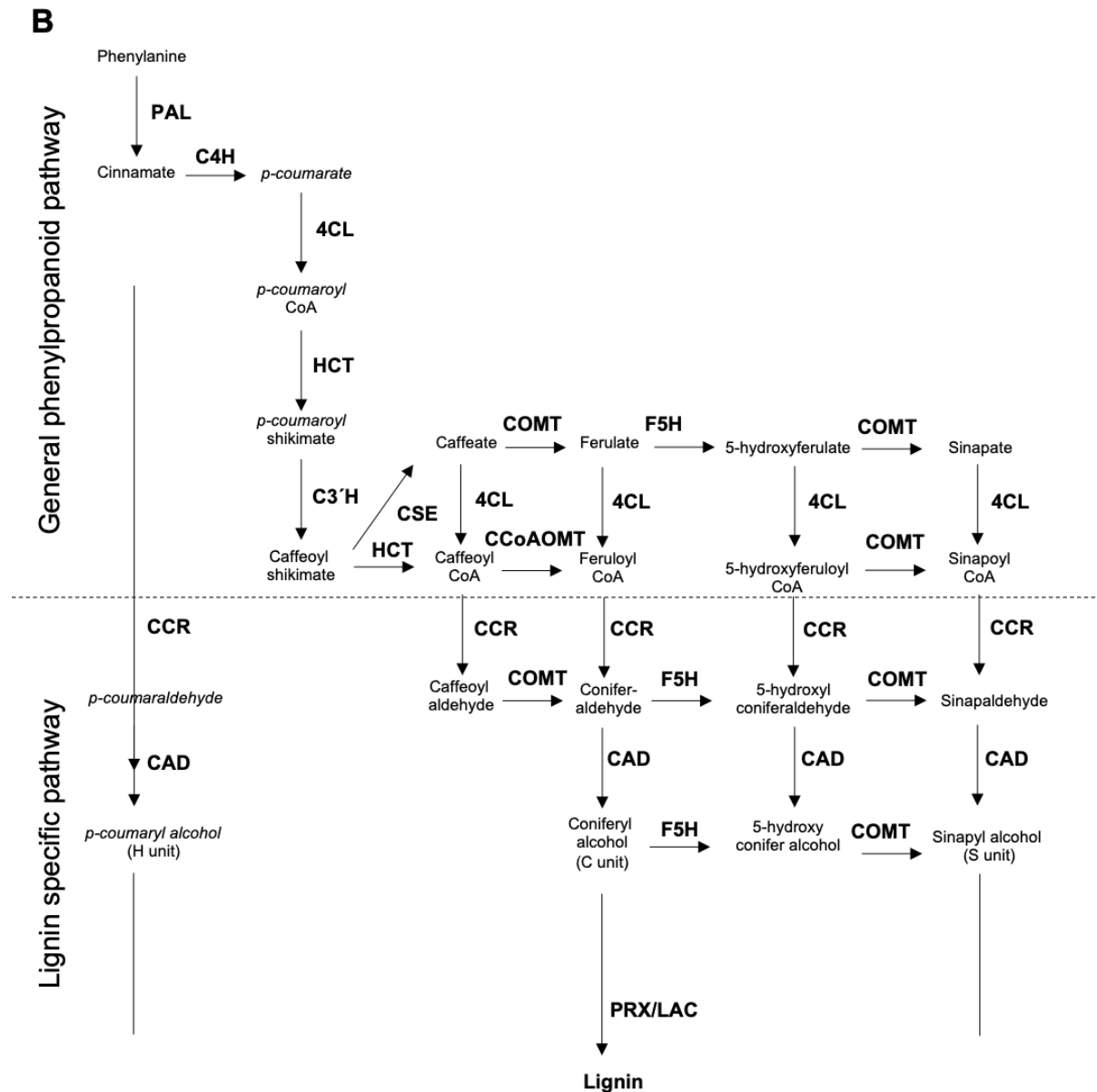
Figure S15. **RNA-seq expression profiles of genes involved in lignin biosynthesis.**

(**A**) Gene expression of genes from *Pto-AvrRpm1* or mock-infected plants is represented as DESeq2 pseudocounts. (**B**) Scheme of lignin biosynthesis in plants. Black arrow indicates the canonical lignin biosynthesis in plants. Bold font indicates enzymes involved in the different steps of the pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT, quinateshikimate *p*-hydroxycinnamoyltransferase;

C3′H, *p*-coumaroylshikimate 3′-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoAreductase; F5H, ferulate 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid *O*-methyltransferase; CSE, caffeoyl shikimate esterase; PRX, peroxidase; LAC, laccase (Adapted from Meng Chie et al., 2018)

Figure S16

Figure S16. **Transcriptional regulation of HR markers found in this study compared to RNA-seq data sets from plants undergoing ETI and autoimmunity.** (A) HR markers found in this study were searched in Mine et al., 2018. In pairwise comparisons between infection with ETI-causing bacteria (Pto *AvrRpm1* and Pto *AvrRpt2*) and mock, only genes with high statistical confidence (q value ≤ 0,01) in at least one time point were plotted on a heatmap indicating Log$_2$FC for the different times tested in their study. (B) HR markers found in this study were searched in RNA-seq data sets of Arabidopsis *hos15-4* (Yang et al.,2019), *rh6812* autoimmune plants (Chantarachot et al., 2020) and *Cdm-0 x TueScha-9 F1* hybrids (Barragan et al., 2020). Genes with high statistical confidance in their data sets (FDR <0.05) were plotted on a heatmap indicating Log2FC between expression of WT (Col-0) and autoimmune plants.

Supplementary tables/dataset legends.

Table S1 (Associated to Figure 2A). List of differentially expressed genes upon *Pto AvrRpm1* infection at each time point and tissue area.

Table S2 (Associated to Figure S2). List of genes constituting each GO term in Figure S2. GO term enrichment analysis of upregulated and downregulated genes at either IN our OUT areas. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni Correction for multiple testing and a fold enrichment above 2 are shown.

Table S3 (Associated to Figure 2B). List of genes that are upregulated upon *Pto AvrRpm1* at 4 and 6 hpi exclusively at IN, both at IN and OUT or exclusively at OUT.

Table S4 (Associated to Figure 2C). List of genes constituting each GO term in Figure 2C.GO term enrichment analysis of genes that are exclusively upregulated at either the IN or OUT area

upon *Pto AvrRpm1* infection. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni Correction for multiple testing and a fold enrichment above 2 are shown.

Table S5 (Associated to Figure 1C, Figure 4, Figure 5, Figure S11 and Figure S14). Tukey HSD p-values and Welch two sample t-test p-values obtained from statistical tests applied in the study.

Table S6 (Associated to Figure 3A). List of genes comprising each cluster derived from *Pto AvrRpm1* and mock-treated plants along with their corresponding MSV at IN.

Table S7 (Associated to Figure 3B). List of genes comprising each cluster derived from *Pto AvrRpm1* and mock-treated plants along with their corresponding MSV at OUT.

Table S8 (Associated to Figure S6A). List of genes constituting each GO term in Figure S6A. GO term enrichment analysis of genes from clusters of *Pto AvrRpm1*-inoculated plants at the IN area with a MSV of 0.7 or above. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni Correction for multiple testing and a fold enrichment above 2 are shown.

Table S9 (Associated to Figure S6B). List of genes constituting each GO term in Figure S6B. GO term enrichment analysis of genes from clusters of *Pto AvrRpm1*-inoculated plants at the OUT area with a MSV of 0.7 or above. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni Correction for multiple testing and a fold enrichment above 2 are shown.

Table S10 (Associated to Figure S7A). List of genes constituting each GO term in Figure S5. GO term enrichment analysis of genes from clusters of mock-inoculated plants at the IN area

with a MSV of 0.7 or above. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni

Correction for multiple testing and a fold enrichment above 2 are shown.

Table S11 (Associated to Figure S7B). List of genes constituting each GO term in Figure S6.

GO term enrichment analysis of genes from clusters of mock-inoculated plants at the OUT area

with a MSV of 0.7 or above. Only those GO terms exhibiting an FDR < 0.05 after Bonferroni

Correction for multiple testing and a fold enrichment above 2 are shown.

Table S12. Primers used in this study and primer concentration for RT-qPCRs.

Table S13. RT-qPCR results in numeric format along with Cp values of Targets and Cp value

of Reference housekeeping gene.
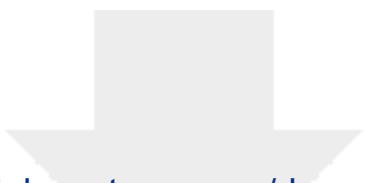
Dataset 1

Click here to access/download
Dataset
Table S1_Primers.xlsx

Dataset 2

Click here to access/download
Dataset
Table S2_Statistics.xlsx

Dataset 3

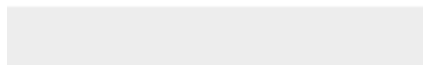Click here to access/download
Dataset
Table S3_DEGs.xlsx
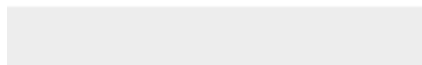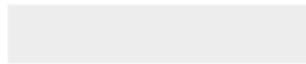
Dataset 4

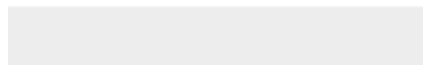Click here to access/download
Dataset
Table S4_DEGsINvsOUT.xlsx

Dataset 5

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Dataset
Table S5_GOsINvsOUT.xlsx

Dataset 6

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Dataset
Table S6_GeneListClusters_IN.xlsx

Dataset 7

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Dataset
Table S7_GOsClusters_IN_PtoAvrRpm1.xlsx

Dataset 8

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Dataset
Table S8_GOsClusters_OUT_PtoAvrRpm1.xlsx

Dataset 9

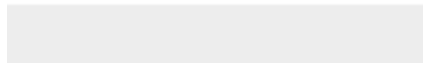Click here to access/download
Dataset
Table S9_GOsDEGs.xlsx

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Dataset
Table S10_GOsCluster_IN_Mock.xlsx

Dataset 11

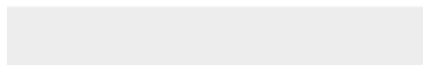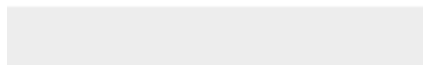Click here to access/download
Dataset
Table S11_GOsCluster_OUT_Mock.xlsx

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Dataset
Table S12_Primers.xlsx

Dataset 13

Click here to access/download
Dataset
Table S13_qPCR_RawData.xlsx