BABA-induced pathogen resistance: a multi-omics analysis of the tomato response reveals a hyper-receptive status involving ethylene

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Running title: The multi-omics analysis of BABA-treated tomato

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1. ABSTRACT

Prior exposure to microbial-associated molecular patterns or specific chemical compounds can promote plants into a primed state with stronger defence responses. β-aminobutyric acid (BABA) is an endogenous stress metabolite that induces resistance protecting various plants towards diverse stresses. In this study, by integrating BABA-induced changes in selected metabolites with transcriptome and proteome data, we generated a global map of the molecular processes operating in BABA-induced resistance (BABA-IR) in tomato. BABA significantly restricts the growth of the pathogens *Oidium neolycopersici* and *Phytophthora parasitica* but not *Botrytis cinerea*. A cluster analysis of the upregulated processes showed that BABA acts mainly as a stress factor in tomato. The main factor distinguishing BABA-IR from other stress conditions was the extensive induction of signaling and perception machinery playing a key role in effective resistance against pathogens. Interestingly, the signalling processes and immune response activated during BABA-IR in tomato differed from those in *Arabidopsis* with substantial enrichment of genes associated with jasmonic acid (JA) and ethylene (ET) signalling and no change in Asp levels. Our results revealed key differences between the effect of BABA on tomato and other model plants studied until now. Surprisingly, salicylic acid (SA) is not involved in BABA downstream signalization whereas ET and JA play a crucial role.

2. INTRODUCTION

Throughout their lives, plants are constantly exposed to many stressful situations caused by changing environmental conditions or attacks by various pests and pathogenic microorganisms. Therefore, they have evolved structural barriers, microbicidal secondary metabolites, and inducible defence mechanisms to repel potential attackers. Unfortunately, the basal immune responses of plants are usually only sufficient to slow their colonisation by pathogens. As a result, a significant portion of the world's plant production is destroyed each year by fungi, oomycetes, bacteria, insects, and nematodes [1]. Although plants do not have the adaptive immune system of vertebrates, it has long been known that components of the innate immune system of plants can learn from the past [2]. When exposed to microbe-associated molecular patterns (MAMPs) or specific chemical compounds, plants can enter a state of enhanced defence characterised by more rapid and robust responses to stressful stimuli. Although the term "defence priming" was proposed in the 1930s, the molecular mechanisms underlying this phenomenon have only recently been partially elucidated, particularly in the model plant *Arabidopsis thaliana* [3,4]. Defence priming causes increased expression of genes related to stress and defence [5], including many transcription factors that regulate defence [6], and is now considered an essential component of several types of systemic

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62   plant immunity, including acquired systemic acquired resistance (SAR), induced systemic resistance

63   (ISR) [3,7], wound-induced resistance [8], and resistance induced by chemical compounds. Unlike

64   strategies based on single resistance genes, defence priming activates multigenic defence

65   mechanisms, conferring relatively durable resistance [3]. One of the most effective priming agents is

66   the non-protein amino acid β-aminobutyric acid (BABA), which protects various plant species against

67   a wide range of stresses [9]. Importantly, this resistance is long-lasting and can be transferred to

68   vegetative progeny [10,11]. BABA was previously considered a xenobiotic, but it has recently been

69   shown to accumulate in stress-exposed plants [12], suggesting that it is an endogenous stress

70   metabolite [13]. BABA induces resistance via the action of several hormones, including salicylic acid

71   (SA) [14,15], jasmonic acid (JA) [16], abscisic acid (ABA) [17], and ethylene (ET) [15]; the signalling

72   pathway that is activated appears to depend strongly on the particular plant-pathogen combination

73   [6]. Recently, it was discovered that the aspartyl-tRNA synthetase (AspRS) IBI1 in *Arabidopsis*

74   *thaliana* serves as an enantiomer-specific BABA receptor that interacts with the transcription factors

75   VOZ1 and VOZ2 [17,18]. In BABA -primed cells, this interaction represses the expression of ABA

76   genes, resulting in increased expression of PTI genes and callose-associated defence [17]. The

77   previous study of our laboratory showed that, as in potato, effective BABA-IR is also associated with

78   the formation of HR -like lesions in tomato [19]. However, while BABA-IR appears to activate SA

79   signalling pathways in *Arabidopsis* and potato plants, our results suggest that ET signalling pathways

80   play a key role in BABA-IR in tomato plants [19].

81   Here, we present a study in which a combination of nontargeted approaches was used to

82   elucidate the molecular basis of BABA-IR in tomato (*Solanum lycopersicum* cv. *Marmande*), an

83   important crop [20]. BABA was applied by spraying onto leaves, as this application strategy is easy to

84   implement in practical agriculture. Global transcriptomic and proteomic analysis of tomato plants

85   allowed us to identify the molecular processes and signalling pathways that occur in tomato at BABA-

86   IR.

87   **3.   RESULTS**

88   *3.1   Growth of pathogens having different lifestyles after BABA treatment*

89   To determine the protective effect of BABA treatment towards pathogens with different

90   lifestyles (biotrophic *Oidium neolycopersici*, hemibiotrophic *Phytophthora parasitica*, and

91   necrotrophic *Botrytis cinerea)* in *S. lycopersicum* cv. *Marmande* plants, we treated plants with 10 mM

92   BABA. This dose was selected based on a previous study [21] showing maximal (95%) protection

93   against *Phytophthora infestans* with no effect on the general growth of the *S. lycopersicum* cv. F1

94   hybrid cv Baby plants. Indeed, BABA was unable to trigger effective resistance, in our experimental

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95 conditions, below concentration of 5mM, as shown previously [21]. More than two days after
96 treatment we observed not reproducibly the appearance of HR-like microlesions on some BABA
97 spayed leaves (S1 Fig) not connected to plant age or leaf position, as described previously [21]. BABA
98 treatment significantly reduced the sporulation of *O. neolycopersici* and the spreading of *P. parasitica*
99 but had no effect on *B. cinerea* disease (Fig 1A). These findings are in agreement with previous
100 studies showing that BABA-IR to biotrophic and hemibiotrophic pathogens in tomato and potato
101 plants but gave inconsistent results against the necrotrophic pathogen *B. cinerea*, possibly due to the
102 use of different methods to evaluate resistance [22–24].
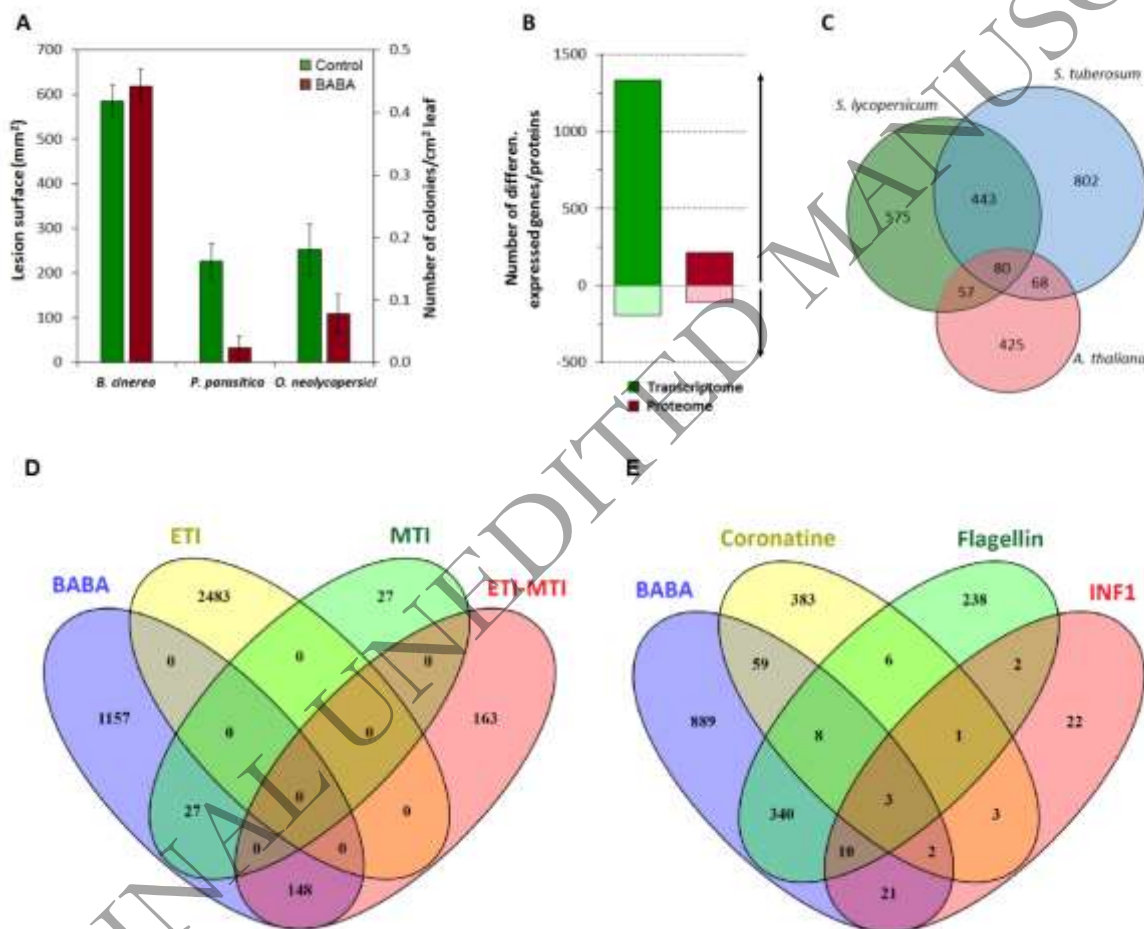
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104 **Figure 1. Resistance, Transcriptomic, and Proteomic changes associated with BABA-IR, overlap**
105 **between BABA-induced genes in different plant species and overlap between genes induced by**
106 **BABA and selected elicitors.** (A) Lesion surface area in 6-8 weeks-old tomato plants after spraying
107 with 10 mM followed by challenge with the phytopathogens *Botrytis cinerea*, *Phythophtora*
108 *parasitica*, and *O. neolycopersici*. (B) Total numbers of differentially expressed genes and proteins
109 during BABA-IR (calculated as the ratio of the expression in 10 mM BABA-sprayed and water-sprayed
110 leaves) 24 h after treatment. Cut-offs of a ≥2-fold difference in expression and P ≤ 0.01 (for genes) or
111 P ≤ 0.05 (for proteins) were applied. The number of genes or proteins in each category is shown. (C)
112 Degrees of overlap between orthologous groups identified in this study and previous transcriptomic

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113 studies on BABA-treated potato (Bengtsson et al., 2014) and *Arabidopsis* plants (Zimmerli et al.,
114 2008). (D) Overlap between genes induced in BABA-treated tomato Marmande plants and in tomato
115 Rio Grande (RG)-PtoR resistant plants during MTI, ETI, or both (Pombo et al., 2014). (E) Overlap
116 between genes induced in BABA-treated tomato Marmande plants and in tomato plants treated with
117 the virulence factors coronatine (COR) from *Pst* DC3000 (Geng et al., 2014), flagellin from *P. syringae*
118 (Rosli et al., 2013), and elicitin INF1 secreted by *P. infestans* (Kawamura, 2009). Induced genes were
119 identified by applying cut-offs of a ≥2-fold difference in expression and P <0.05.

120 *3.2   Transcriptome and proteome modifications during BABA-IR in tomato*

121   Three leaf biological replicates were sampled 24 h and 48 h after spraying with 10 mM BABA.
122 Six samples collected at 24 hrs were used for RNA sequencing and six samples collected at 48 hrs
123 were used for label-free LC-MS/ analysis of the proteome excluding microsomal fraction (thereafter
124 proteome). RNA sequencing generated 334,275,668, high-quality reads with an average of
125 55,712,611 reads per sample (S1 Table). The reads for each sample were mapped to the *S.*
126 *lycopersicum* reference genome sequence, with 74% of reads being mapped successfully. Only genes
127 with a median count above 20% in at least one treatment were considered in the subsequent
128 analysis. The cut-offs used in the comparison were P < 0.01, and ≥ 2-fold expression change. Using
129 these criteria, we identified 24,562 genes from 34,725 annotated genes (ITAG 2.4), with 1,523 genes
130 being differentially expressed (Fig 1B, S2 Table). In the proteome analysis, protein identification was
131 only performed for peptides of at least six amino acids with a statistically significant peptide score (q
132 < 0.01, FDR 1 %; FDR based on decoy search against the reverse database). The cut-offs used for the
133 comparison were q < 0.05 and ≥ 2-fold expression change. Using these criteria, we identified 1808
134 protein groups (S3 Table) and 319 differentially expressed proteins (Fig 1B, S2 Table). As with the
135 transcriptome, we found that far more proteins were upregulated (67%) than downregulated (33%)
136 after BABA treatment (Fig 1B, S2 Table), however the correlation between proteins and transcripts
137 changes was only about 10% as discussed previously [25]. These results confirm that BABA treatment
138 causes extensive reprogramming of cellular processes in tomato plants, as previously observed in
139 *Arabidopsis* and potato [22,26] . However, a comparison of orthologous groups between our
140 transcriptomic study and previous studies on *Arabidopsis* [26] and potato [22] plants exposed to
141 BABA revealed significantly greater overlap between the potato and tomato datasets than between
142 *Arabidopsis* and tomato (Fig 1C, S4 Table).

143 *3.3   BABA exhibits common features with MAMPs-triggered immunity in tomato*

144   Sets of genes whose transcript abundance was specifically increased at 6 hours post-infection
145 (hpi) during MAMPs-triggered immunity (MTI) and effector-triggered immunity (ETI) were recently
146 identified using RNA-Seq technology in tomato Rio Grande (RG)-PtoR resistant plants [27].

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147   Interestingly, almost 50% of the BABA-upregulated genes were also differentially regulated during

148   MTI, but there was no overlap between ETI-upregulated genes and BABA treatment (Fig 1D, S5

149   Table). Following this result, the response of tomato plants to BABA was far more similar to that

150   induced by exposure to a well-characterized MAMP flagellin flgII-28 from the bacterium

151   *Pseudomonas syringae* [28] or elicitin INF1 secreted by *Phytophthora infestans* [29] than to the

152   response induced by the virulence factor coronatine (COR) secreted by *Pst* DC3000 [30] (Fig 1E, S5

153   Table). Specifically, almost 48% and 57% of BABA-upregulated genes were also upregulated

154   following exposure to flgII-28 analysed using RNA-Seq technology at 6 hpi [28] and INF1 analysed

155   using a GeneChip tomato genomic array 12 hpi [31], respectively. Almost 85 % of COR-upregulated

156   genes analysed using a TOM1 cDNA microarray 24 hpi of tomato var "Glamour" seedlings [32] were

157   unaffected by BABA treatment (Fig 1E, S5 Table). The only genes upregulated by both BABA and COR

158   were associated with ET (ACC synthase and ACC oxidase) and JA signalling pathways.

159   *3.4   BABA functions as a stress factor*

160   A gene ontology (GO) term enrichment analysis was performed to identify critical processes

161   upregulated by BABA treatment. The sets of terms obtained using the transcriptomic and proteomic

162   data were similar (S2 Fig, S6 Table), with many common terms in the GO categories "Process" and

163   "Function".

164   The protein-protein interaction network based on RNA-Seq data was generated by directly

165   mapping upregulated genes to proteins in the String database [33] (Fig 2A, S7 Table). The network is

166   highly aggregated with clustered sub-networks comprising proteins associated with defence

167   responses (PR proteins), JA and ET signalling and synthesis, regulation of transcription related to

168   mitogen-activated protein kinase MPK3, and processes related to reactive oxygen species (ROS)

169   production. This is also consistent with maps produced after the ReviGO [34] analysis showing

170   significant enrichment of stress-associated clusters (S2 Fig, S6 Table). The protein-protein interaction

171   network based on proteins exhibiting significant changes in abundance 48 hours after BABA

172   treatment (Fig 2B, S7 Table) featured notable clusters relating to photosynthesis, secondary

173   metabolite biosynthesis, and translation. The photosynthesis cluster shows that BABA induces

174   complex changes in the regulation of photosynthesis-related energetic processes and carbohydrate

175   metabolism. The cluster related to secondary metabolites includes the enzymes prephenate

176   aminotransferase (PAT), arginase (ARG2), and lactate dehydrogenase. PAT plays a role in the

177   biosynthesis of aromatic amino acids, while lactate dehydrogenase plays an important role in

178   detoxifying D-lactate, a product of the glyoxalase pathway for detoxifying methylglyoxal, which

179   accumulates under stress conditions [35]. The arginase in tomato leaves was suggested to be

180   involved in ROS homeostasis when its expression was induced by JA signalling following wounding

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181 and exposure to *Pseudomonas syringae* pv. *tomato* strain DC3000 [36]. The upregulation of the

182 proteins in the cluster related to translation is probably related to translational switching from

183 growth to defence [37] and supports the reported role of non-canonical functions of aminoacyl-tRNA

184 synthetase in BABA responses [18].
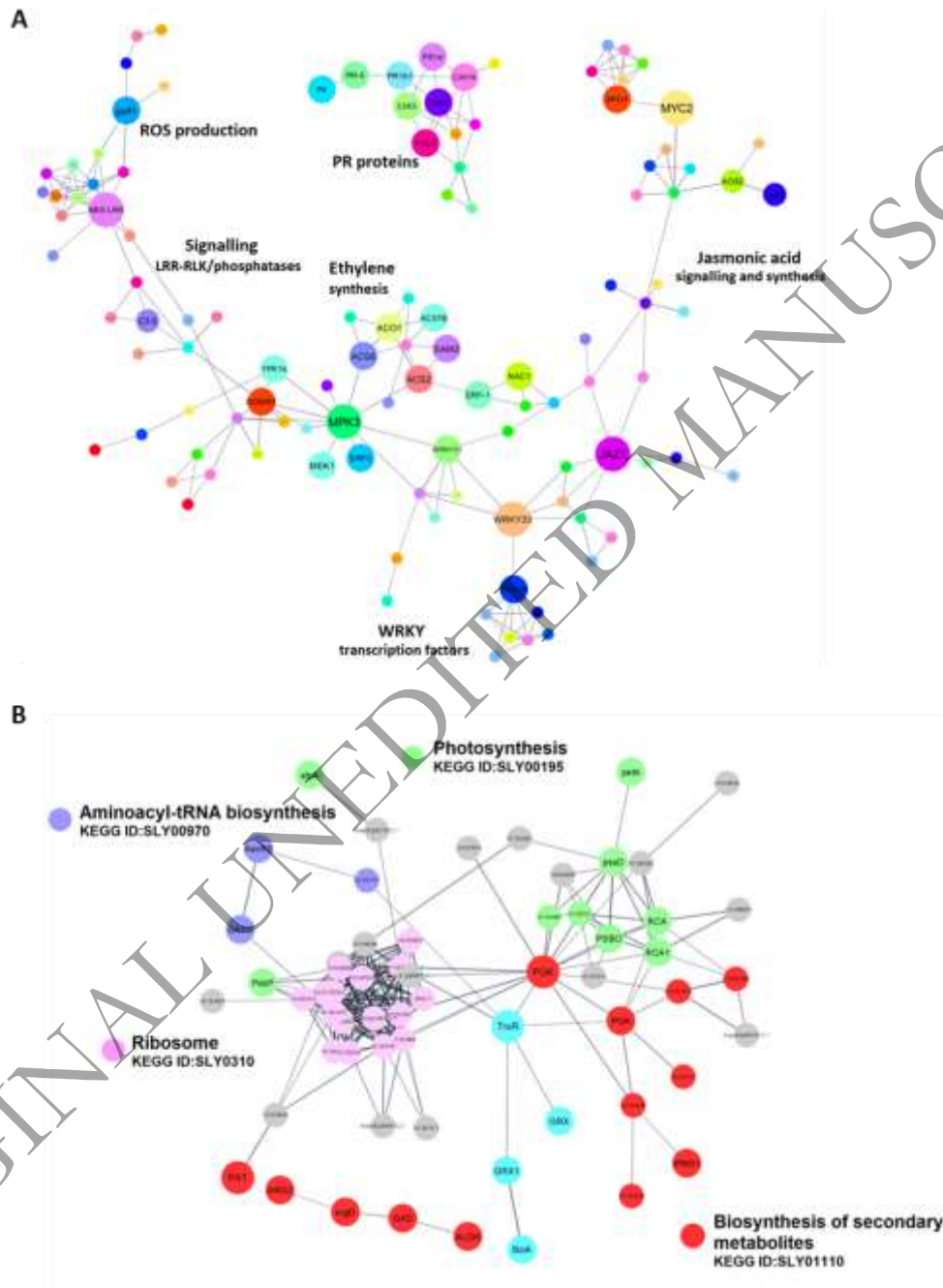
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186 **Figure 2. Transcriptomic and proteomic comparisons of plants treated with BABA revealed the**
187 **existence of sets of genes and proteins that are specifically induced during BABA-IR.** Protein-
188 protein association networks were generated for significantly induced (A) genes and (B) proteins,
189 applying cut-offs of a ≥2-fold difference in expression and $P \leq 0.01$ (for genes) or $P \leq 0.05$ (for

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190 proteins). Interaction networks were constructed in STRING using a minimum required interaction
191 score of 0.7 [33] and visualized with Cytoscape [96].

192   To distinguish between stress-related genes upregulated following BABA treatment and
193 defence-related genes involved in BABA-IR, we compared the BABA-upregulated genes to the sets of
194 genes upregulated in previous transcriptomic studies on tomato plants subjected to temperature
195 [38] and salinity [39] stress as well as those upregulated in tomato plants infected by the fungus
196 *Stemphylium lycopersici* [40] and the FIRE (flagellin-induced repressed by effectors) genes, which
197 represent a pathogen-defined core set of immune-related genes [28]. We found that 50% of the
198 BABA-upregulated genes overlapped only with the sets of genes upregulated by abiotic stress,
199 confirming the hypothesis that BABA acts on tomato plants primarily as a stress factor. However, 30
200 % of the BABA-upregulated genes were also FIRE genes or genes upregulated following *S. lycopersici*
201 infection, suggesting that these genes are associated with BABA-augmented defence expression
202 against *P. parasitica* and *O. neolycopersici*. Moreover, about 20% of genes unique to the BABA
203 treatment were significantly enriched in KEGG pathways related to plant-pathogen interaction,
204 MAPK signalling, and phenylpropanoid synthesis, indicating that these genes are also involved in
205 BABA-IR (Fig 3B, S5 Table).
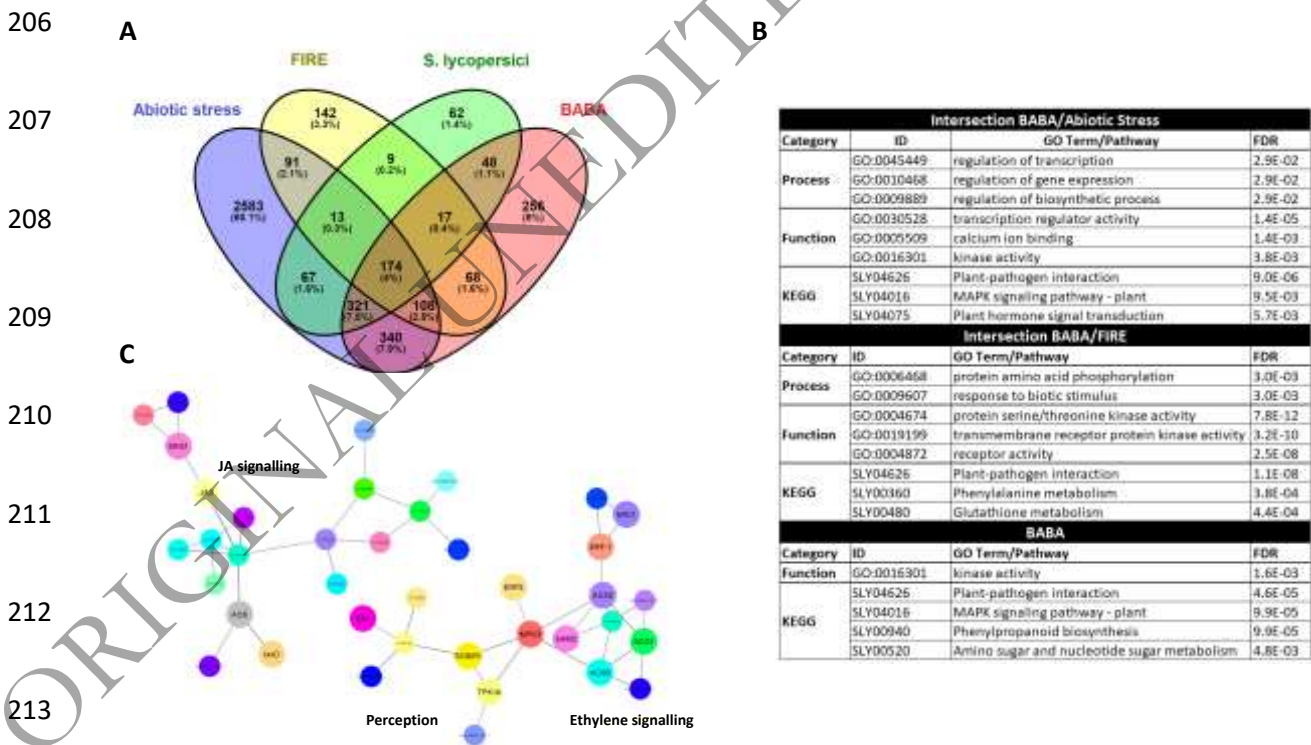
214 **Figure 3. Overlap between genes induced by BABA and genes induced in tomato plants subject to**
215 **various abiotic and biotic stresses.** (A) Overlap between genes induced in BABA-treated tomato
216 Marmande plants and genes upregulated in tomato plants subject to abiotic heat stress [38], genes
217 upregulated in abiotic salinity stress [39], genes upregulated following infection by the fungus

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218 *Stemphylium lycopersici* (biotic stress; [40]), and FIRE (flagellin-induced repressed by effectors)
219 genes, representing a pathogen-defined core set of immune-related genes [28]. Cut-offs of a ≥2-fold
220 difference in expression and q <0.05 were applied. (B) KEGG and Gene Ontology (GO) term analysis
221 were performed for genes in each category. The most-enriched GO terms in the process and function
222 categories are shown. (C) Protein-protein association network for genes significantly induced by both
223 BABA treatment and abiotic stress. The interaction network was constructed in STRING using a
224 minimum required interaction score of 0.7 [33] and visualized with Cytoscape [96].

3.5   The BABA stress response is orchestrated via ethylene and jasmonic acid signalling

226   The preceding analysis revealed a clear enrichment of genes associated with JA and ET
227 signalling pathways. Moreover, both ET and JA accumulated in tomato plants during the first few
228 hours after BABA treatment (Fig 4A, B). Accordingly, BABA induced upregulation of several isoforms
229 of ACC synthase (*ACS*) and ACC oxidases (*ACOs*) (Fig 4C, S8 Table), essential enzymes for ET
230 biosynthesis [41,42]. The high upregulation of the *ACS2* and *ACS6* isoforms is also consistent with
231 their reported activation during defence reactions in *Arabidopsis* [43] and with the observed
232 upregulation of *WRKY33*, which activates *ACS2* and *ACS6* expression downstream of the *MPK3/MPK6*
233 cascade [44]. Our data also support the findings of an earlier study on *Arabidopsis* [45] showing that
234 the conversion of methylthioadenosine (MTA) to Met via the Yang (or Met salvage) cycle is generally
235 not controlled by ET signalling because BABA treatment had no detectable effect on the regulation of
236 the Yang cycle genes *MTN*, *MTK*, *MTI*, and *ARD* (Fig 4C, S8 Table).

237   Among the BABA-upregulated genes were the patatin-like proteins PLA1 and PLA3 (Fig 4D, S8
238 Table), which have been implicated in wound responses and resistance to necrotrophic pathogens
239 via JA signalling [46]. In addition, there was significant upregulation of genes encoding enzymes
240 involved in the synthesis of endogenous JA (*TomloxD*, *AOS2* and *OPR3*). The upregulation of these
241 genes was accompanied by the accumulation of JA in BABA-treated leaves (Fig 4D). Also upregulated
242 were 6 of the 12 Jasmonate ZIM Domain (*JAZ*) genes, key regulators of JA signalling that govern host
243 and non-host pathogen-induced cell death in tomato. The most highly upregulated *JAZ* genes (*SlJAZ1*,
244 *SlJAZ2* and *SlJAZ9-11*) were also induced by treatment with COR [47].

245   Surprisingly, unlike in previous studies on the effects of BABA in *Arabidopsis* [14] and potato
246 plants [22], there was no significant enrichment of genes associated with the phytohormone SA. In
247 keeping with this result, the levels of total SA declined slightly in leaves treated with BABA (Fig 4B). A
248 recent study showed that ABA signalling is suppressed during BABA-IR in *Arabidopsis* plants [17]. In
249 accordance with this result and our previous study [48], we observed no enrichment of genes
250 associated with ABA signalling following BABA treatment in tomato and several genes involved in

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251 ABA catabolism were upregulated including *CYP707A* (*Solyc08g005610.2.1*) and UDP-
252 glucosyltransferase (*UGT*; *Solyc09g098080.2.1*) (Fig 4E, S8 Table).
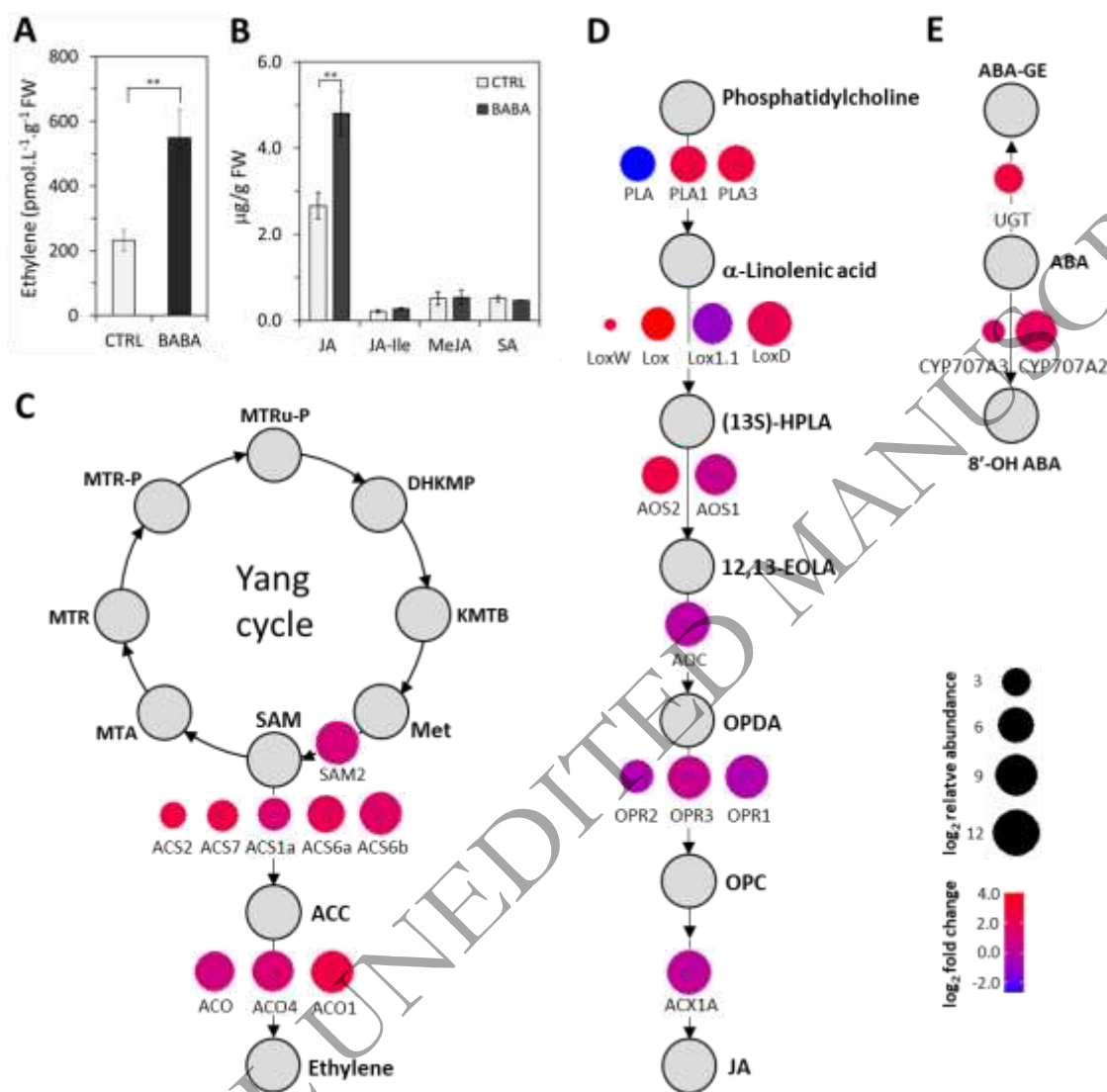
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254 **Figure 4. Involvement of signalling pathways in BABA-IR.** (A) ET accumulation was measured at 24
255 hrs after BABA treatment (10 mM) of leaflets (n = 8) by gas chromatography. (B) Levels of JA,
256 jasmonic acid-isoleucine (JA-Ile), methyl-jasmonate (MeJA) and SA were measured by LC-MS 24 h
257 after BABA treatment (10 mM) leaves (n = 6). The control tissue (CTRL) was a water-treated control
258 sample. Each bar represents the mean ± SE. Asterisks denote mean values that differ significantly
259 from that for the control group based on Student's t-test at P ≤ 0.01 (**). (C-E) The BABA-induced
260 induction of genes involved in the synthesis of ET (C) and JA (D) and the degradation of ABA (E) is
261 shown.

262 SAM – S-Adenosyl-L-methionine, MTA – 5′-Methylthioadenosin, MTR – 5′-Methylthioribose, MTR-P – 5′-
263 Methylthioribose-1-phosphate, MTRu-P – 5′-Methylthioribulose-1-phosphate, DHKMP – 1,2-Dihydroxy-3-keto-
264 5-methylthiopentene, KMTB – 2-keto-4-methyl-thiobutyrate, SAM2 – S-Adenosyl-L-methionine synthase, ACS
265 – 1-aminocyclopropane-1-carboxylic acid synthase, ACC – 1-aminocyclopropane-1-carboxylic acid, ACO – 1-
266 aminocyclopropane-1-carboxylic acid oxidase, PLA – Phospholipase A, Lox – Lipoxygenase, (13S)-HPLA – 13-

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267 hydroperoxylinolenic acid, AOS – Allene oxide synthase, 12,13-EOLA – 12,13-epoxyoctadecatrienoic acid, AOC –
268 Allene oxide cyclase, OPDA – (9S,13S)-12-oxo-phytodienoic acid, OPR – 12-oxo-phytodienoic acid 10,11
269 reductase, OPC – 3-oxo-2-(2´-pentenyl)-cyclopentane-1-octanoic acid, ACX1A – acyl-CoA oxidase, JA – Jasmonic
270 acid, ABA – Abscisic acid, UGT – Abscisic acid glucosyl-transferase, ABA-GE – Abscisic acid glucosyl ester, 8´-OH
271 ABA – 8´-hydroxy abscisic acid   N.C. – transcript not changed, N.D. – transcript not detected.

272 *3.6   BABA upregulates perception and signalling machinery related to abiotic stress*

273   Protein kinases comprised 142 of the BABA-upregulated genes (S3 Fig, S9 Table), accounting
274 for 15% of the total set of expressed kinases in our analysis. Similarly, 44 protein kinases were
275 identified in the proteomic analysis, of which 14 were upregulated and only 3 were downregulated
276 (S3 Fig). These results suggest that protein kinases play an important role in the BABA stress
277 response (S3 Fig, S9 Table). An enrichment analysis of the BABA-responsive families using the chi-
278 squared test revealed overrepresentation of the large Receptor-Like Kinase/Pelle (RLK-Pelle) family,
279 which is crucial for plant-specific adaptation [49] (S3 Fig) at both the transcriptomic and proteomic
280 levels. This finding agrees well with the upregulation of 27 Receptor-Like Proteins (RLP), representing
281 23% of the total expressed RLPs in our analysis. It is also notable that all previously reported PRRs in
282 tomato plants were upregulated including those for flagellin (*FLS2* and *FLS3*), the fungal elicitor *EIX*
283 (*LeEix1* and *LeEix2*), *Ave1* from *V. dahlia* (*Ve1* and *Ve2*) [50], and *Avr* factors (*Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*)
284 [51]. This increased expression of perceptual proteins is consistent with the observed protein-protein
285 interaction between *WRKY33* and the *MPK3* kinase from the CMGC family (Fig 2B, S3 Fig). The
286 *MPK3/MPK6* cascade causes the phosphorylation of *WRKY33* and the closely related *WRKY25*,
287 leading to ET production due to activation of the enzyme *ACS* as described above. Two other RLKs
288 interacting with RLPs involved in MAMPs perception, *SOBIR1* [52] and *TARK1* [53], were also
289 upregulated.

290   In parallel with the induction of pathogen perception machinery, we also observed massive
291 upregulation of genes encoding transcription factors (TFs): BABA treatment induced the upregulation
292 of 130 TF genes (S4 Fig). The largest numbers of induced genes were found in the ERF, WRKY, MYB
293 and NAC families (S4 Fig), which play essential roles in regulating stress responses in plants, mainly
294 through ET and JA signalling pathways. The strong induction of ERFs following BABA treatment was
295 particularly notable: of the 137 identified ERF genes in tomato [54], 113 were expressed, 33 were
296 upregulated, and 5 were downregulated. The BABA response has many features in common with the
297 responses to cold, salt, and mechanical stress observed in previous studies on tomato [38,55,56].
298 However, the BABA-upregulated genes *SlERF*5 (*ERF*5), *SlERF*43 (*RAV2*), *SlERF*55 (*TSRF*1), *SlERF*60
299 (*Pti*5) or *SlERF*69 (*ERF*1) were previously linked to the activation of defence responses against diverse
300 pathogens (S9 Table). Unlike in previous studies on tomato, no WRKY genes were downregulated
301 after BABA treatment; their expression patterns resembled those induced by salt stress, the tomato

11

302 spotted wilt virus (TSWV), and the fungal elicitor *EIX* [57]. The largest class of plant MYB factors are

303 the 2R proteins, which regulate primary and secondary metabolism, hormone signal transduction,

304 development, and responses to biotic and abiotic stresses [58]. Of the 91 expressed 2RMYB genes,

305 11 were upregulated following BABA treatment and 6 were downregulated. Their expression profile

306 most closely resembled that seen in tomato plants treated with MeJA [59] or stressed by infection

307 with the bacterial pathogens *Pst* DC3000 or *P. putida* (S9 Table).

308   Finally, a comparison of the BABA-upregulated RLPs/WRKY, ERF, and MYB genes to recent

309 RNA-Seq results for tomato plants under biotic [27] and abiotic stress [38] confirmed that the pattern

310 of upregulation induced by BABA is significantly more similar to that for abiotic stress than that for

311 biotic stress (S9 Table).

312 *3.7   Amino acid metabolism in BABA-treated plants*

313   Transcriptomic and metabolomic studies have consistently shown that amino acid (AA)

314 homeostasis plays a role in stress responses [60,61]. Moreover, BABA induces the stress-induced

315 morphogenic response (SIMR). In *Arabidopsis*, Asp levels were increased 3-fold by treatment with the

316 active (R)-BABA enantiomer but were unaffected by the (L) enantiomer, suggesting that BABA

317 obstructs canonical AspRS activity [18]. Surprisingly, we observed no change in Asp levels in tomato

318 plants after BABA treatment. However, there was a significant increase in the levels of the enzymes

319 Asparagine-tRNA synthetase (GlnRS) and plastid Glutamyl-tRNA(Gln) amidotransferase (GATC) as

320 well as the Glu, GABA, Pro, Phe, and Tyr, together with a reduction in Ala levels (Fig 2B, 5A). The

321 glutamate family pathway is strongly activated under stress conditions, leading to the accumulation

322 of GABA and proline. GABA is synthesized from Glu by a decarboxylation reaction in response to

323 abiotic stress, viral infection, and herbivore attack [60]; its increased concentration in BABA-treated

324 tomato plants is almost certainly connected to the strong transcriptomic and proteomic upregulation

325 of its key biosynthetic enzyme glutamate decarboxylase (*Solyc04g025530.2.1*) (Fig 5C). Proline, which

326 plays a pivotal role in responses to abiotic stresses, osmotic, salinity, and low temperature stresses, is

327 synthesized predominantly from glutamate by two successive reductions catalysed by P5C

328 synthetase (*P5CS*) and P5C reductase (*P5CR*), with *P5CS* being the rate-limiting enzyme for proline

329 synthesis [62]. However, although BABA treatment increased the abundance of *P5CS*, it did not

330 upregulate *P5CS* transcription. This suggests that the increase is due to a change in post-

331 transcriptional regulation, as observed previously [63]. Conversely, there was significant

332 transcriptional upregulation of proline dehydrogenases (*ProDH*) (*Solyc02g089630.2.1*,

333 *Solyc02g089620.2.1*), which catalyse the catabolic conversion of Pro into the toxic intermediate P5C

334 (Fig 5C, S10 Table). Spraying *Arabidopsis* leaves with Pro or P5C causes the formation of HR-like

335 lesions resembling those induced by BABA [64]. We therefore suggest that the lesion formation

12

336 observed in tomato after BABA foliar spraying [19,22] spraying can be attributed to the generation of
337 very high local BABA concentrations on leaf surfaces (white deposits) and subsequent P5C
338 accumulation.

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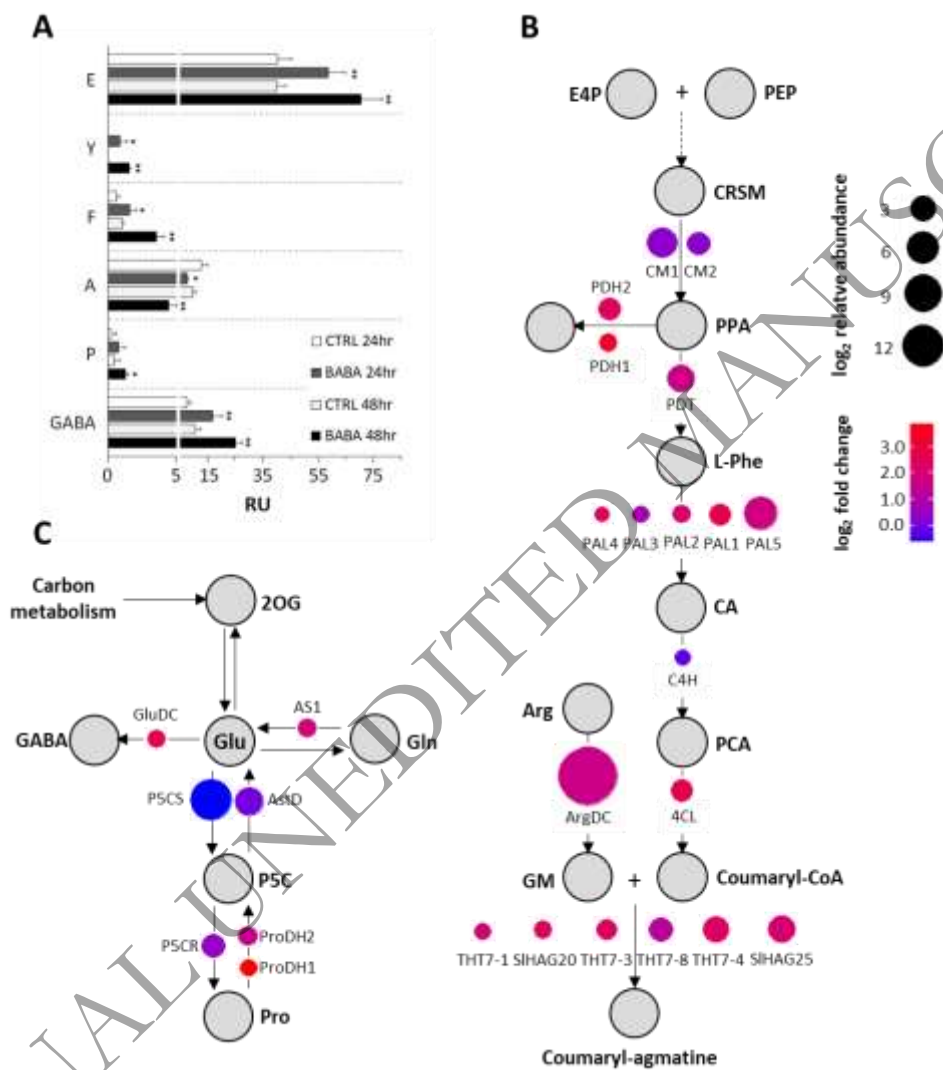
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342 **Figure 5. BABA-induced changes in amino acid levels and their metabolic pathways.** (A) Amino
343 acids whose abundance in BABA-treated Marmande tomato plants differs significantly from that in
344 water-treated controls. Data are means from three replicates; the errors are standard errors of
345 means. Statistically significant differences recorded for each amino acid as determined by the t-test
346 are indicated with different numbers of asterisks (*P < 0.05, **P < 0.01). (B, C) Induction of genes
347 belonging to the phenylpropanoid pathway (B) and glutamate metabolic pathway (C).

348 E4P – erythrose-4-phosphate, PEP – phosphoenolpyruvate, CRSM – chorismic acid, CM – chorismic acid
349 mutase, PPA – prephenate, PDH – prephenate dehydrogenasa, PDT – prephenate dehydratase, PAL –
350 Phenylalanine ammonia-lyase, CA – cinnamic acid, C4H – Cytochrome P450, PCA – p-coumaric acid, 4CL – 4-
351 coumarate CoA ligase, THT – Tyramine N-(hydroxycinnamoyl) transferase, GM – Agmatine, ArgDC – Arginine

13

352 decarboxylase, 2OG – 2-ketoglutarate, GluDC – Glutamate decarboxylase, GABA – gamma-Aminobutyric acid,
353 AS1 – Asparagine synthase, P5CS – Gamma-glutamyl phosphate reductase, AstD – N-succinylglutamate 5-
354 semialdehyde dehydrogenase, P5C – 1-pyrroline-5-carboxylate, P5CR – Pyrroline-5-carboxylate reductase,
355 ProDH – Proline dehydrogenase, N.C. – transcript not changed, N.D. – transcript not detected.

356

357 BABA treatment also upregulated transcription of branched-chain amino acid
358 aminotransferase (*Solyc03g043880.2.1*) and cysteine desulfurase (*Solyc11g005840.1.1*) (Fig 5C). Both
359 of these enzymes are important in the degradation of branched AAs, whose complete oxidation in
360 the mitochondria allows large amounts of ATP to be generated under stress conditions that impair
361 photosynthesis [60]. BABA treatment also caused upregulation of the glutamine-dependent
362 asparagine synthetase *AS1* (*Solyc06g007180.2.1*) and LHTs, which function as high-affinity
363 transporters of uncharged/acidic AAs in the mesophyll plasma membrane (*Solyc11g066800.1.1*,
364 *Solyc01g111980.2.1*, *Solyc10g055740.1.1*). These changes indicate an effect on overall nitrogen
365 metabolism in the plant (Fig 5C, S10 Table). The upregulation of all these proteins was previously
366 observed during chlorosis caused by proteolytic activity and amino acid deamination during *P.*
367 *syringae* infections [65]. BABA also upregulated four glutamate receptor genes (*SlGLR1.2*, *SlGLR2.1*,
368 *SlGLR2.2* and *SlGLR2.5*) implicated in various processes including the response to aluminium [66] and
369 enhanced drought tolerance in plants [67].

370 Two prephenate dehydrogenases (*Solyc09g011870.1.1*, *Solyc06g050630.2.1*) and one
371 prephenate dehydratase (*Solyc06g074530.1.1*) were strongly upregulated at the transcript level
372 following BABA treatment, and prephenate aminotransferase was upregulated at the protein level.
373 These changes are clearly connected to the BABA-induced upregulation of the phenylpropanoid
374 pathway and especially its lignin/lignan branch. Important upregulated enzymes of this branch
375 include 4-coumarate CoA ligase (4-CL) and several caffeoyl-CoA O-methyltransferase (COMT),
376 laccases (LAC), and peroxidases (PER), which catalyse the synthesis of several secondary metabolites
377 (Fig 5B). BABA treatment also caused transcript-level upregulation of arginine decarboxylase
378 (*Solyc10g054440.1.1*), which converts arginine into agmatine, a precursor of polyamines including
379 putrescine, spermidine, and spermine (Fig 5B, S10 Table). Interestingly, however, we observed no
380 increase in polyamine levels, suggesting that agmatine serves some other metabolic purpose in
381 BABA-treated tomato plants [68]. It could possibly be converted into *p*-coumaroylagmatine; this
382 hypothesis is supported by the upregulation of *4-CL* (*Solyc06g035960.2.1*) and *PAL* together with
383 several hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl) transferases (*THT1-3*, *THT1-4*, *THT7-*
384 *1*, *THT7-8*), which are also upregulated during incompatible interactions of *Pst* with tomato [69] (Fig
385 5B, S10 Table).

386 **4. DISCUSSION**

14

387   BABA has long been described as an agent inducing highly effective resistance against a wide

388   spectrum of biotic and abiotic stresses in multiple plant species [9,14]. Although much has been

389   learned about the perception and molecular action of BABA in *Arabidopsis* [17,18], many questions

390   remain regarding its mechanisms of action. Since recent studies have shown that during plant

391   immune responses translation is tightly regulated and poorly correlated with transcription [25], as

392   observed here, we characterized BABA-induced changes in both the transcriptome and the

393   intracellular proteome. In our experimental conditions only high BABA concentration after foliar

394   spraying of the crop tomato cultivar Marmande (which would be a viable agricultural application

395   strategy) behaves as a strong stress inducer that does not only triggers highly effective resistance and

396   that deeply remodels transcriptome and subsequent proteome of tomato. Whether BABA primed

397   tomato defences at lower concentrations without any protective effect was not our aim since it

398   cannot be useful for crop protection. Transcriptomic and proteomic analyses showed a similar trend

399   when most of differentially expressed genes and proteins were upregulated and have been linked to

400   general stress responses and defence. Besides, many proteins exhibiting decreased amounts can be

401   linked to the general decrease in the rate of photosynthesis and carbon metabolism under stress

402   conditions as previously described [61]. It is this stress-induced reduction in plant growth induced by

403   BABA that is the major factor limiting its commercial exploitation [18].

404   Analysis of the genes and proteins upregulated following BABA treatment revealed

405   enrichment of cellular processes related to primary metabolism and responses to stimuli when BABA

406   increased the amount of enzymes involved in carbohydrate metabolism. Previous transcriptomic and

407   genetic analyses have demonstrated the induction of genes involved in carbohydrate metabolism

408   upon challenge by pathogens or PAMPs, and the expression of these genes was shown to affect

409   downstream defence responses including ROS production and PR gene expression [61]. Induction of

410   these genes was also observed in genome-wide studies in *Arabidopsis* plants infected with the

411   avirulent pathogen *P. syringae* pv. Tomato [70] and in rice leaf sheaths infected by *Rhizoctonia solani*

412   [71]. Thus, the genes upregulated in our study and publicly available datasets of potato [22] and

413   *Arabidopsis* [26] (Fig 1C) can be seen as common defence response genes that contribute to BABA-IR.

414   Previous studies have also shown that BABA-IR is driven by different signalling molecules in

415   different plant species [15,16,72,73]. In *Arabidopsis*, BABA treatment induces accumulation of SA and

416   SA glucoside (SAG) and causes significant changes in the abundance of isochorismate synthase (ICS),

417   which is directly involved in SA biosynthesis which, in turn, is associated with the expression of acidic

418   PR proteins [15,26]. Although we paid a particular attention to the role of SA in BABA-induced

419   resistance, none of these responses were observed in tomato as in our recent study in which we

420   determined decreased level of SA after BABA treatment of tomato leaflets via petiole aspiration [48].

421   In addition, unpublished results from our lab, clearly show that SA treatment on tomato leaves failed

15

422   to induce both a resistance to mildews and the classical defence marker genes outlined in the

423   literature and in this paper (mainly PR-proteins coding genes). It seems that SA is probably not so

424   important in tomato defence to biotic stresses since attempts to protect tomato with SA and SA

425   mimics always failed in available literature with the exception of a protective effect observed

426   towards tomato canker (*Clavibacter michiganensis*) with acibenzolar-S-methyl. Indeed, some plants,

427   like rice or wintergreen, exhibit a high constitutive level of SA or SA methyl ester without being

428   protected from diseases [74] and we have previously demonstrated that SA sensitizes carnation to

429   disease by inhibiting N-benzoate-based phytoalexins biosynthesis [75]. Thus, what was demonstrated

430   in Arabidopsis and even in tobacco should not signify that all the plant kingdom, especially among

431   crops diversity, would follow the same defence regulation scheme. However, treatment of tomato

432   with BABA did affect genes involved in the biosynthesis of JA and ET as well as basic isoforms of PR

433   proteins, which were also observed in an earlier study on BABA-treated potato and tomato plants

434   [22,48]. This finding is in accordance with impaired BABA-IR towards *P. infestans* in the tomato *def*

435   mutant, which is defective in JA accumulation [73] and pivotal importance of ET in chitosan- and

436   Flg22-induced local and systemic defence responses of tomato plants previously proved in Never-ripe

437   (*Nr*) tomato mutants exhibiting insensitivity to ET in all vegetative tissues due to mutation in SlETR3

438   receptor [76,77]. All these findings clearly emphasize the need to study plant biology with a

439   necessary hindsight when comparisons to model plants fail to confirm established mechanisms.

440   A recent screening of *Arabidopsis* mutations affecting BABA-IR revealed defects associated

441   with the gene *IBI1*, which encodes aspartyl-tRNA synthetase (AspRS). The specific binding of R-BABA

442   to the L-Asp-binding domain of *IBI1* primes the protein for non-canonical defence activity [18]. In

443   accordance with these findings, we found that BABA had no effect on the regulation of the *IBI1*

444   orthologue in tomato plants. However, we found no evidence of any change in aspartate levels

445   driven by the accumulation of uncharged tRNA^{Asp} leading to inhibition of translation activity via

446   phosphorylation of the initiation factor eIF2α [18]. Instead we observed elevated glutamate levels

447   and increased expression of the enzymes GlnRS and GATC, which are involved in tRNA^{Gln} synthesis

448   [78]. Following these findings, BABA treatment of tomato plants upregulated transcription of HSF24

449   (*Solyc02g090820.2.1*), a HSF-type homologue of TBF1. The heat shock factor(HSF)-like transcription

450   factor TBF1 was proven to play a crucial role in the growth-to-defence switch that activates multiple

451   defence mechanisms and inhibits primary growth and development upon pathogen challenge [79].

452   Interestingly, one suggested activation mechanism for TBF1 is related to the GCN2-dependent

453   phosphorylation of eIF2α, which is regulated via the accumulation of uncharged tRNAs. This is

454   consistent with the reported inhibitory activity of R-BABA towards the cellular AspRS activity of *IBI1*

455   in *Arabidopsis* [18]. Moreover, TBF1 controls distinct output genes in SAR and MTI, which could be

456   connected to our observation that BABA-IR in tomato is mediated by genes involved in MTI.

16

457 Together, these observations convincingly explain the finding that BABA has dual activities in tomato,

458 simultaneously activating defence mechanisms and downregulating protein synthesis and carbon

459 metabolism. The latter activity is similar to the stress responses [61] associated with SIMR in

460 *Arabidopsis*. It has been suggested that BABA primes defence responses simply via this stress

461 imprinting process because L-glutamine treatment reduced both BABA-induced SIMR and BABA-IR

462 [80]. In keeping with this hypothesis, 70% of the BABA-induced transcripts overlapped with the set of

463 transcripts upregulated by abiotic salt [39].

464 Two important processes in pathogen recognition and the subsequent activation of plant

465 defence mechanisms are the secretion and spotting of diverse pattern-recognition receptors (PRRs)

466 to the plasma membrane and the activation of protein kinases involved in signal transduction

467 cascades. Augmented perception of stress signals by plant cells seems to be essential in BABA-IR, as

468 demonstrated by the significant enrichment of GO terms and pathways related to receptor activity

469 after BABA treatment. Moreover, extensive induction of signalling and perception machinery was

470 one of the main factors distinguishing BABA-treatment from the other stress conditions and put

471 forward in our data set. BABA induced a significant number of receptor and receptor-like kinases

472 involved in abiotic stress responses (L-type lectin receptor kinases) [81], MAMPs perception,

473 *Phytophthora* resistance (*LysM*, *Bti9*, *SOBIR1*) [52,82], and responses to pathogen infection,

474 mechanical wounding, and oxidative stress (*TPK1b*) [83]. This is the first demonstration that BABA-IR

475 in tomato is connected to a hyper-receptive status. In that way, BABA acts as a real priming agent,

476 preparing the plant to rapidly recognize pathogens and to set-up strong defences.

477 However, as stated above, BABA also induce a major plant stress. Whether these two aspects

478 can be disconnected is a pending question to only keep the hyper-receptive side unless this status

479 could be a consequence of the major stress. Plant defence responses are also modulated by AA

480 homeostasis and treatment with high concentrations of AAs. For example, the *Arabidopsis thaliana*

481 *lht1* mutant, which has reduced levels of Gln, Ala, and Pro, exhibits SA dependent resistance to a

482 wide range of diseases [84]. In addition, treatment of rice roots with the AAs Glu, Asn, Met, and Asp

483 induced systemic disease resistance against rice blast that was partially dependent on SA signalling

484 and did not cause any change in the content of free AAs in leaves [85]. In our experiments, treatment

485 with BABA increased levels of Pro and the expression of the Glu biosynthetic enzyme ProDH, as well

486 as the levels of free Glu in the leaves (Fig 5A, C). The ProDH is an enzyme that plays a crucial role in

487 plant metabolism. Recent studies have shown that ProDH activity is upregulated in response to

488 pathogen infection and contribute to HR and disease resistance, which apparently potentiates the

489 accumulation of ROS. In addition, ProDH may also regulate the balance between proline and P5C,

490 which has been shown to affect the accumulation of defence-related metabolites and the expression

491 of defence genes [86–88]. Decreases in Glu and Pro levels are also associated with microbial

17

492 community breakdown and disease incidence , supporting the idea that they play an important role

493 in the plant's defence response [89]. Free Glu may be recognized by glutamate receptor-like proteins

494 (GLRs), which have been implicated in enhanced resistance to *Hyaloperonospora arabidopsidis*, and

495 *Pseudomonas syringae* in *Arabidopsis* [90]. The initial cellular events in BABA-IR in tomato may thus

496 involve GLRs as suggested previously in a study on AA-ISR to rice blast in leaves [85]. Interestingly,

497 despite significant differences, the upregulated gene clusters in BABA-treated tomato overlap

498 extensively with the sets of orthologous upregulated genes identified by microarray analysis of *A.*

499 *thaliana lht1* plants [84] and the genes upregulated in Glu-treated rice plants [85] (Fig 6A). Notably,

500 the orthologous genes common to all three sets exhibited functional enrichment in the plant-

501 pathogen interaction pathway (KEGG) and in protein domains related to signal transduction

502 (INTERPRO), suggesting that, in all three cases, induced resistance is driven by similar molecular

503 mechanisms based on sensitization to stress responsiveness (Fig 6B), which may be characteristic of

504 priming phenomena [3].
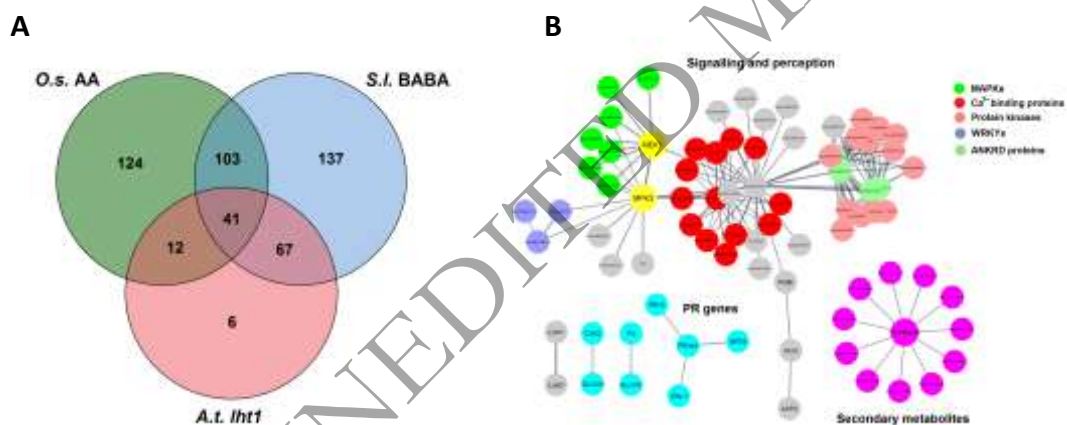
505

506 **Figure 6. Overlap between genes induced by BABA and genes induced in selected plants with**
507 **altered amino acid homeostasis.** (A) Degrees of overlap between orthologous groups identified in
508 this study and previous transcriptomic studies on A. thaliana lht1 plants [84], and Glu-treated rice
509 plants [85]. (B) Functional enrichment of the plant-pathogen interaction pathway (KEGG) and protein
510 domains related to signal transduction (INTERPRO) among the orthologous genes common to all
511 three sets.

512   Collectively, here we demonstrate that a strong BABA-IR towards *Phytophthora parasitica*

513 and *Oidium neolycopersici* in tomato cv Marmande resemble in many aspects responses to general

514 stress. This resistance was largely explained by the activation of the ET and JA pathways resulting in

515 a strong defence set-up involving PR-proteins as well as phenyl propanoid pathway, lipid

516 peroxidation but in the same time revealed a complete remodelling of plant functions including

517 decrease in primary metabolism and in photosynthesis together with an enhanced ability to perceive

518 (P)(M)(D)AMPs and to set-up downstream signalling. In conclusion, much more attention should be

18

paid to comparative studies between plants of agronomic interest submitted to R-BABA treatment. We clearly evidenced that multiomics as well as targeted approaches can bring original insight on who to who, even though many black boxes still remain closed.

5. MATERIALS AND METHODS

5.1 Plant material and growing conditions

Tomato plants (*Solanum lycopersicum* cv. *Marmande*) were grown at 75% humidity and 14 hours of light (day 24°C, night 22°C). After 6 or 7 weeks of growth, whole tomato plants were sprayed with 10 mM DL-BABA or water. Leaflets were then removed from plants 24 or 48 hours after spraying for transcriptome and proteome analysis and processed immediately or stored at -80°C until use. Plants assigned to each treatment were randomly selected, labelled, and then returned to the growth chamber. Three biological replicates were selected for each treatment.

5.2 *Botrytis cinerea* and *Phytophthora parasitica* inoculation and measurement

Two days after spraying with 10 mM BABA or water, leaflets were removed from 7-8 week-old tomato plants and placed in clear Styrofoam boxes with moist absorbent paper to maintain high relative humidity. The centre of each leaflet was inoculated with a mycelial plug (5 mm in diameter) from the growth margin of a 3-day-old culture of the BC21 strain of *B. cinerea*. Alternatively, leaflets were pricked with a needle at a marked spot and 20 μl of a *P. parasitica* zoospore suspension (40 000 zoospores/ml) was applied to the wounded spot. Five replicates in styrofoam boxes with 3 leaflets each were used. After inoculation, the detached leaflets were incubated in a growth chamber under conditions conducive to disease development (21°C, 14-h photoperiod, 114 μmol.s-1.m-2). Symptoms were recorded after 3 days of incubation for *P. parasitica* or 4 days for *B. cinerea*. Photographs were analysed using the ImageJ image analysis programme to quantify the surface area of necrotic lesions (in mm²). Analysis of variance was used to evaluate whether differences between controls and treatments were significant for each of the three independent experiments.

5.3 *Oidium neolycopersici* inoculation and measurement

Spraying with 10 mM BABA or water was done on whole 5-6 week old tomato plants. Two days later, each plant was inoculated with approximately 10 ml of a spore suspension of *O. neolycopersici* at a concentration of 10^4 sp/ml. The inoculated plants were then incubated in a growth chamber under conditions conducive to disease development (21°C, RH > 80%, 14 hours of light). The number of powdery mildew colonies was counted 14 days after inoculation on 2 leaves per plant with 5 plants per test. Three independent tests were performed.

5.4 Identification and quantification of proteins by LC-MS/MS

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551   Three biological replicates in the form of separate plants were subjected to analysis. For each

552   replicate, 4 g of leaflets from 3 different plants were harvested on ice and homogenised at 4°C in 20

553   ml of extraction buffer (50 mM Tris-Mes (pH=8.0), 20 mM EDTA, 500 mM sucrose, 10 mM DTT, 100

554   mM PMSF, cOmplete Mini Protease Inhibitor Cocktail tablets) using an Ultraturrax homogenizer (IKA,

555   DE) at 15 000 rpm. Samples were filtered through Miracloth and centrifuged at 20 000 x g and 4°C.

556   The supernatant was collected and centrifuged in a Beckman Optima (Beckman-Coulter)

557   ultracentrifuge at 35 000 rpm and 4°C with a Ti45 rotor. The supernatant was collected and

558   concentrated using Vivaspin® 3 kDa (GE Healthcare) sample concentrators. The concentrated

559   samples were dialyzed overnight to 10 mM ammonium acetate and finally concentrated to 0.5 ml

560   using 4 ml Amicon® Ultra 4 3 kDa (Merck Millipore Ltd.) sample concentrators. Each sample was then

561   fractionated into 5 fractions by HPLC using an IEX PolyWAX LP mixed bed column (200 x 4.6 mm, 5

562   µm particles, PolyLC Inc., Columbia, USA) and a gradient of ammonium acetate. The collected

563   fractions were dried under vacuum and subjected to LC-MS /MS analysis (S3 Table). The dried

564   protein fractions were processed using a philtre filter-aided sample preparation (FASP) method [83].

565   LC-MS /MS analyses of the peptide mixtures were performed using the RSLCnano system connected

566   to the Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific). For more details, see the

567   supplemental material (S1 Appendix).

568   *5.5   RNA sequencing analysis*

569   Total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and

570   checked for integrity on a Bioanalyzer 2200 (RIN ≥ 7.40). Libraries for sequencing were prepared

571   according to a standard protocol for the SOLiD 5500 system (Life Technology). Sequencing was

572   performed using the SOLiD 5500W platform. Raw reads of 75 bp in length were mapped to the

573   Solanum lycopersicum build 2.40 reference using ITAG2.4 as the gene model in colour space with the

574   Maxmapper algorithm implemented in Lifescope software (Life Technologies, Ltd). RNA content was

575   assessed using a whole-transcriptome workflow with the quality threshold set to 10, resulting in an

576   assignment probability of greater than 90. The raw sequencing data with corresponding metadata

577   are available in the NCBI Gene Expression Omnibus (GEO) repository under accession number

578   GSE108421. Analytical comparison between BABA and the control treatments was performed using

579   the DESeq package [91].

580   *5.6   Orthology and Gene Ontology enrichment analysis*

581   Orthologous gene clusters were compared and annotated using the OrthoVenn web platform [92],

582   and the results obtained were visualised using the eulerr R package [93]. Significantly differentially

583   expressed genes and proteins were analysed by Singular Enrichment Analysis (SEA) for GO term

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584 enrichment using agriGO [94] based on GO terms retrieved from the PLAZA 3.0 database [95].

585 Summarisation and visualization of SEA were done using REViGO [34] and Cytoscape [96] with DyNet

586 [97].   Protein-protein association networks for significantly differentially expressed genes and

587 proteins were generated using the STRING database with an interaction score of 0.9 [33] and

588 visualized using Cytoscape [96].

589 *5.7   HPLC analysis of amino acids*

590 Twenty-four hours after treatment with BABA, tomato leaves were collected, frozen in liquid

591 nitrogen, and ground to a fine powder. A portion of 250 mg of this powder was then extracted with 1

592 ml of extraction buffer (0.1 M HCl and 4.6 µg/ml L-2-aminoadipic acid as an internal standard), mixed

593 thoroughly, incubated on ice for 5 minutes, and centrifuged. A 500 µl aliquot of the resulting

594 supernatant was then diluted with 100 µl methanol and loaded onto an SPE C18 column to adsorb

595 interfering secondary metabolites, which had previously been wetted with 1 ml MeOH and

596 equilibrated with 20% MeOH in 0.1M HCl. The sample was loaded and the column was washed with

597 400 µl of 20% MeOH in 0.1 M HCl. Amino acids were recovered in both the flow-through and wash

598 fractions and derivatized and analyzed as previously described [98]. See supplemental material (S1

599 Appendix) for further details.

600 *5.8   Quantitative analysis of salicylic acid, jasmonic acid, and jasmonic acid-isoleucine*

601 The tomato leaflest (100 mg) was frozen immediately after the harvest using liquid nitrogen,

602 and the frozen materials ground under liquid nitrogen and extracted with 750 µl of MeOH-H_2O-HOAc

603 (90:9:1, v/v/v) containing 100 ng of o-anisic acid as an internal standard. The mixture was centrifuged

604 at 10,000 x g for 1 min, the supernatant was collected, and the pellet was repeatadly extracted. The

605 pooled supernatants were dried under nitrogen, resuspended in 200 µl of 0.1% HOAc in H_2O-MeOH

606 (90:10, v/v), and a portion of the mixture (2–5 µl) was subjected to LC-MS analyzes using a TOF mass

607 spectrometer (Agilent Technologies) as previously described [99]. Further details can be found in the

608 supplemental material (S1 Appendix).

609 *5.9   Measurement of ethylene production*

610 Single tomato leaflets 24 hours after treatment with BABA or water were placed in 20 ml test

611 tube when the cut end of the petiole was in sterile water and sealed with a air-tight rubber syringe

612 cap. ETwas accumulated for 4 hours before a 1-ml sample was withdrawn for analysis. ET production

613 was measured using gas chromatography with a flame ionization detector quantified by using a gas

614 chromatograph flame ionization detector (Agilent GC 6890, Agilent Technologies) as previously

615 described [19].

616

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Contributions

Conceptualization: JL, MP, CR and ZZ; Data curation: MZ, CR, BI, MB, KLB, PN, VM, AS, PB, and DP; Data analysis: MZ, CR, DP, AS and BI; Funding acquisition: JL, MP and ZZ; Methodology: MZ, CR, JL, MP and ZZ; Project administration: JL and MP; Resources: JL, MP and ZZ; Validation: MZ, CR, JL, MP and ZZ; Writing, review, and editing: MZ, JL, and MP; Supervision: JL and MP.

Data availability statement

Raw sequencing data with appropriate metadata are available in the NCBI Gene Expression Omnibus (GEO) repository under accession number GSE108421.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD038074

Competing interests

We declare that none of the authors have any competing interests.

7. SUPPLEMENTARY INFORMATION

S1 Appendix - Supplementary methods

S1 Figure. HR-like microlesions on BABA spayed leaves.

S2 Figure. ReviGO analysis of enriched GO terms.

S3 Figure. BABA perception affects gene expression of certain protein kinase families.

S4 Figure. BABA perception affects gene expression of certain transcription factor families.

S1 Table. Workflow of sequence analysis.

S2 Table. Significant differentially expressed proteins and transcripts.

646   **S3 Table.** Identified protein groups.

647   **S4 Table.** Orthologous across our and previous transcriptomic studies of BABA treated potato and
648   *Arabidopsis* plants.

649   **S5 Table.** Overlap between genes induced by BABA and different elicitors.

650   **S6 Table**. GO analysis of up-regulated proteins and transcripts in tomato leaves after treatment with
651   10 mM BABA.

652   **S7 Table**. Protein-protein association network for significantly induced genes and proteins.

653   **S8 Table.** Involvement of signalling pathways in BABA-IR.

654   **S9 Table**. Transcripts of protein kinases, receptor like proteins and transcription factors found in our
655   study and their comparison with other studies.

656   **S10 Table.** Induction of genes in glutamate metabolic pathway and phenylpropanoid pathway.

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