A novel cross talk of AtRAV1, an ethylene responsive transcription factor with MAP kinases imparts broad spectrum disease resistance in plants

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Short title: RAV1 promotes disease resistance in plants

34 **Abstract**

35 **Plant diseases pose a serious threat to sustainable agriculture as controlling them in**
36 **eco-friendly manner remains a challenge. In this study, we establish RAV1 as a master**
37 **transcriptional regulator of defense genes in model plant Arabidopsis. The**
38 **overexpression of *AtRAV1* provided disease resistance against necrotrophic fungal**
39 **pathogen (*Rhizoctonia solani*) infection in *A. thaliana*. The transgenic lines exhibited**
40 **enhanced expression of several defense genes including mitogen associated protein**
41 **kinases (MAPKs) and the amplitude of their expression was further enhanced upon**
42 **pathogen infection. Conversely, the *atrav1* mutant plants were unable to induce the**
43 **expression of these defense genes and were highly susceptible to infection. Our data**
44 **suggests that upon pathogen attack, AtRAV1 transcriptionally upregulate the**
45 **expression of *MAPKs* (*AtMPK3, AtMPK4* and *AtMPK6*) and AtMPK3 and AtMPK6 are**
46 **essential for AtRAV1 mediated disease resistance. Further, we demonstrate that**
47 **AtRAV1 is a phosphorylation target of AtMPK3 (but not AtMPK6) and the phospho-**
48 **defective variants of AtRAV1 are unable to induce disease resistance in *A. thaliana*.**
49 **Considering the presence of AtRAV1 orthologs in diverse plant species, we propose**
50 **that they can be gainfully deployed to control economically important diseases. In deed**
51 **we observe that overexpression of tomato ortholog of *AtRAV1* (*SlRAV1*) provides**
52 **broad spectrum disease resistance against bacterial (*Ralstonia solanacearum*), fungal**
53 **(*R. solani*) and viral (Tomato leaf curl virus) infections in tomato.**

54 **Key words**: Bacterial wilt disease, disease resistance, MAP kinase, plant defense response,
55 post translational modification, protein phosphorylation, signalling cascades, transcription
56 factor, transcriptional regulator, reactive oxygen species

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58 Plants have evolved specific receptors to perceive pathogen attack. The PRRs (Pattern
59 recognition receptors) is deployed to perceive pathogen associated molecular cues while
60 leucine rich repeat (LRR) receptors recognize effector proteins of the pathogens (1–3). In
61 this process, plant mount strong defense response to ward off most of the pathogens (1, 4–
62 6). Increase in production of reactive oxygen species (oxidative burst), alkalization of
63 cytoplasm, production of phenolics, phytoalexins, deposition of lignin and callose,
64 hypersensitive response associated programmed cell death, etc are part of plant defense
65 strategies (7). The phytohormones such as jasmonic acid, salicylic acid and ethylene also

66 play a critical role in elaborating the plant defense response (8–12). Moreover, an extensive

67 crosstalk (both synergistic and antagonistic) between various phytohormones modulate the

68 defense response (13, 14).

69 On the other hand, for successful colonization phytopathogens have evolved diverse

70 strategies to suppress the induction of plant defense response. With extensive

71 polymorphisms in various isolates/strains, some phytopathogens are able to cause disease on

72 diverse host species. *Ralstonia solanacearum* is one of the notable examples which causes

73 devastating bacterial wilt disease in tomato, potato and over two hundred other plant species

74 (15–17). Similarly *Rhizoctonia solani* a necrotrophic fungal pathogen infects diverse plants

75 including rice, potato, tomato etc. and imparts huge economic losses (18, 19). Notably *R.*

76 *solani* and *R. solanacearum* share many common hosts, including agriculturally important

77 crops such as tomato, potato, etc. Moreover viruses also pose a serious threat for crop

78 production (20). Thus, strategy to simultaneously control bacterial, fungal as well as viral

79 diseases in an eco-friendly and sustainable fashion will be important for ensuring food

80 security.

81 Manipulation of some of the PRR receptors, LRR receptors and host defense related genes

82 had been shown to provide broad spectrum disease resistance in plants (21, 22). The

83 overexpression of *AtNPR1* (Non expressor of *PR* genes; encoding a positive regulator of

84 SAR) provided broad spectrum disease resistance in various crop plants (23, 24). Similarly,

85 the overexpression of an *AtEFR* (a PRR receptor) gene could enhance tolerance against

86 bacterial pathogen (*R. solanacearum, Xanthomonas perforans*) infection in tomato (25).

87 Further, overexpression of an anti-apoptotic vaculoviral p35 protein also imparted broad

88 spectrum disease resistance in tomato against bacterial (*Pseudomonas syringae* pv. tomato)

89 and fungal (*Alternaria alternata* and *Colletotrichum coccodes*) infections (26). However,

90 the deployment of transgenes in disease management has to face strong biosafety

91 regulations (27). In this regard, utilization of endogenous gene(s) with broad spectrum

92 disease resistance will be helpful in preventing yield loss due to pathogen attack.

93 In this study, we endeavoured to identify master regulator of plant defense genes in model

94 plant *A. thaliana* and explore the potential of identified gene to impart broad spectrum

95 disease resistance in economically important crops such as tomato. Based upon network

96 centrality parameters, we identified 16 proteins to be topologically central to Arabidopsis

97 defense proteins interaction network. The RAV1 transcription factor binding motifs was

98 present in the promoter region of each of the genes encoding them. It is worth mentioning

3

99   that RAV1 is an ethylene responsive transcription factor which contains AP2 domain (which

100   participates in activation of ethylene mediated signalling pathway) at its N-terminal region

101   and B3 domain (involved in abscisic acid mediated signalling) at its C-terminus (28, 29).

102   RAV1 has been shown to be a positive regulator of leaf senescence (30) and upon

103   overexpression it provides ABA insensitive phenotype in *A. thaliana* (29).

104   In this study we identified AtRAV1 as a master regulator of defense gene expression in *A.*

105   *thaliana* including mitogen activated protein kinases (MAPKs; AtMPK3, AtMPK4 and

106   AtMPK6) and when overexpressed it provides disease resistance against *R. solani*.

107   Similarly, overexpression of *SlRAV1* (ortholog of *AtRAV1*) confers broad spectrum disease

108   resistance in tomato against fungal (*R. solani)*, bacterial (*R. solanacearum*) and viral

109   (tomato leaf curl Joydebpur virus; *ToLCJoV*) infections. The data presented in this study

110   highlights a novel cross talk between RAV1 with MAPKs in imparting disease resistance.

111   The RAV1 transcriptionally induces the expression of *MAPKs* (*AtMPK3, AtMPK4* and

112   *AtMPK6*) and the AtMPK3/AtMPK6 is essential for RAV1 mediated disease resistance.

113   Further the AtMPK3 (but not AtMPK6) phosphorylates AtRAV1 and potentially stabilize it

114   to facilitate sustained activation of defense response.

Results

Identification of *AtRAV1* as a key transcriptional regulator of plant defense genes. *In-silico* analysis of protein-protein interactions between Arabidopsis defense proteins (31); identified 16 proteins to be important for the topology and dynamics of the network (**Fig. S1)**. Here onwards, we refer these proteins as key defense proteins of Arabidopsis. Some of the previously reported plant defense proteins such as SKP1 (32), MAPKs (33, 34), heat shock proteins (35, 36) and cyclophilins (37, 38) were noteworthy in this list. Interestingly, the RAV1 binding sites were present in the promoter region of each of these genes (**Fig. S2**). The phylogenetic analysis revealed RAV1 to be conserved in different monocot as well as dicot plants (**Fig. S3**). Presence of RAV1 binding motifs in the *AtRAV1* promoter (**Fig. S4**) suggested it to be under auto-regulation.

Overexpression of *AtRAV1* induces the expression of key defence genes. We reasoned that overexpression of *AtRAV1* would simultaneously induce the key defense genes and promote disease resistance in *A. thaliana*. To test this, transgenic *A. thaliana* (Col-0) lines that constitutively overexpress *AtRAV1* (At1G13260) under CaMV 35S promoter were generated. Two independent overexpression lines (OE1 and OE2) having relatively higher

131 fold expression of *AtRAV1* along with an EV line were propagated to T$_4$ generation for

132 further analysis (**Fig. S5**). Compared to EV plants, the OE lines (OE1 and OE2)

133 demonstrated enhanced expression of key defense genes (**Fig. 1A**).

134 To validate that AtRAV1 can bind to the promoter and induces expression, we randomly

135 selected few of key defense genes namely *MPK4, MPK6, ROC1, WD40, BRL2, SKP1* and

136 *HSP70* and performed yeast one hybrid (Y1H) as well as GUS reporter assays. For Y1H

137 assay potential AtRAV1 binding motifs in the promoter region of these genes (**Table S1**)

138 were individually cloned in a bait vector (pAbAi) while the full length *AtRAV1* was cloned

139 in a prey vector (pGADT7-AD). The Y1H Gold bait reporter yeast strain expressing both

140 the plasmids grew on Aureobasidin A (AbA) containing double drop out (SD-URA-LEU)

141 plates while the strain co-expressing empty vectors (pAbAi and pGADT17) failed to grow

142 on such plates (**Fig. 1B**). GUS reporter assay was performed in *N. benthamiana* plants to

143 validate that co-expression of AtRAV1 modulates GUS expression through the promoters of

144 selected   key   defense   genes.   Limited   GUS   expression   was   observed   in

145 pBI101:promoter:*GUS* infiltrated leaves, while significantly enhanced GUS expression was

146 observed when promoter:*GUS* and *AtRAV1* constructs were co-infiltrated (**Fig. 1C**). The

147 qRT-PCR further reinforced that expression of *AtRAV1* enhances *GUS* expression (**Fig. 1D**).

148 Taken together, our result suggests that AtRAV1 modules the expression of various key

149 defense genes including AtMPK4 and AtMPK6. As AtMPK3 is an important player in plant

150 defense (39), we performed Y1H and promoter:*GUS* reporter assays to test whether

151 AtRAV1 can modulate *AtMPK3* gene expression. As shown in **Fig. S6**, the AtRAV1 did

152 bind to the promoter of *AtMPK3* and induced its expression.

153 **Overexpression of *AtRAV1* confers disease resistance in *A. thaliana.*** We further analysed

154 whether the overexpression of *AtRAV1* enhances disease resistance against *Rhizoctonia*

155 *solani*, a notorious necrotrophic fungal pathogen infection. Both OE1 and OE2 lines

156 demonstrated only mild necrotic symptoms when infected with *R. solani*, however severe

157 necrosis was observed in the infected *atrav1* mutant (a previously reported mutant line

158 *Salk_021865*; obtained from Arabidopsis Biological Resource Center, ABRC; **Fig. S7**), EV

159 as well as WT plants (**Fig. 2A**). Compared to others the extent of host cell death (**Fig. 2B**)

160 and ROS accumulation was relatively less in the infected OE lines (**Fig. 2C**). Moreover, the

161 disease severity index (**Fig. 2D**) and abundance of fungal (estimated through monitoring the

162 abundance of *R. solani* 18S ribosomal gene through qRT-PCR) biomass (**Fig. 2E**) was

163 significantly less in infected OEs plants, compared to the infected *atrav1* mutant, WT and

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164 EV plants. Also the chlorophyll content was relatively higher in infected OE lines compared

165 to that of WT, EV and atrav1 mutant plants (**Fig. 2F**).The confocal microscopic analysis

166 revealed limited growth of *R. solani* and absence of infection cushion in OE lines (**Fig. 2G**).

167 Taken together, these results reinforced that overexpression of *AtRAV1* imparts enhanced

168 resistance against *R. solani* infection.

169 **Expression of key defense genes gets enhanced upon pathogen infection in *AtRAV1***

170 **overexpressing lines.** In comparison to WT and EV plants, the expression of *AtRAV1* was

171 up-regulated upon pathogen *(R. solani)* infection in OE lines but not in the *atrav1* mutant

172 lines (**Fig. S8**). Similarly, the expression of most of the selected key defense genes (*BRL2,*

173 *ROC1, SKP1, WD40* and *HSP70*) as well as previously reported Salicylic acid (SA),

174 Jasmonic acid (JA) and Ethylene (ET) mediated defense marker genes (**Table S2**) were

175 significantly enhanced upon *R. solani* infection in the OE lines but not in the *atrav1* mutant

176 plants (**Fig. S9A and B**). Also the enhanced expression of *AtMPK3*, *AtMPK4* and *AtMPK6*

177 was observed in *R. solani* infected OE lines (**Fig. 3A**). Western blot analysis further

178 revealed enhanced accumulation of AtMPK3, AtMPK4 and AtMPK6 proteins in the

179 infected OE lines but not in the atrav1 mutant plants (**Fig. 3B**). Here it is worth mentioning

180 that compared to AtMPK4, the extent of up-regulation of AtMPK3 and AtMPK6 was

181 significantly high (**Fig. 3**).

182 **AtMPK3 and AtMPK6 are required for AtRAV1 mediated disease resistance in *A.***

183 **thaliana.** We obtained *AtMPK3* (*atmpk3*, SALK_100651), *AtMPK4* (*atmpk4-2*,

184 SALK_056245) and *AtMPK6* (*atmpk6-2*, SALK_073907) mutants from ABRC stock centre

185 and subjected them to *R. solani* infection. The *mpk3* and *mpk6-2* mutants were hyper

186 susceptible to *R. solani* infection while the *mpk4-2* mutant was moderately susceptible (**Fig.**

187 **4A**). We crossed each of MAP kinase mutants individually with the *AtRAV1* OE1 plants to

188 obtain the AtRAV1[OE1]/mpk3, AtRAV1[OE1]/mpk4-2 and AtRAV1[OE1]/mpk6-2 lines; wherein

189 the respective MAP kinase protein has been knocked out (**Fig. S10**). Interestingly, the

190 AtRAV1[OE1]/ mpk3 as well as AtRAV1[OE1]/ mpk6-2 lines demonstrated hyper susceptibility

191 to *R. solani* infection (**Fig. 4A**). On the other hand the AtRAV1[OE1]/mpk4-2 showed

192 moderate disease tolerance; however the amplitude of tolerance was significantly less

193 compared to that observed in OE1 lines (**Fig. 4A-C**). Also ROS accumulation, extent of host

194 cell death and pathogen load (*R. solani* biomass estimated through qRT-PCR) were

195 significantly high in AtRAV1[OE1]/mpk3 and AtRAV1[OE1]/mpk6-2 lines, compared to the

196 AtRAV1[OE1]/mpk4-2 or OE1 lines (**Fig. 4A and 4B**). The total chlorophyll content of

6

197 infected AtRAV1^{OE1}/mpk3 and AtRAV1^{OE1}/ mpk6-2 lines was significantly less compared

198 to that of AtRAV1^{OE1}/mpk4-2 or OE1 line (**Fig. 4C**). Taken together these results

199 highlighted that AtMPK3/AtMPK6 is predominantly required for AtRAV1 mediated

200 enhanced disease resistance in *A. thaliana*.

201 **AtRAV1 is phosphorylated by AtMPK3 under in-vitro condition.** Bioinformatics

202 analysis revealed that AtRAV1 protein contains three TP and one SP amino acid residues as

203 putative MAP kinase phosphorylation sites (**Fig. S11**). We ectopically overexpressed and

204 purified AtRAV1 protein as well as its different variants wherein potential phosphorylation

205 residues had been mutated (SDM1: Ser310Ala; SDM2: Thr19Ala; SDM3: Thr23Ala;

206 SDM4: Thr193Ala; SDM5: having all four potential phosphorylation sites mutated) from *E.*

207 *coli* cells to analyse their phosphorylation by AtMPK3 and AtMPK6 under in-vitro

208 condition. The assay revealed that AtMPK3 but not AtMPK6 phosphorylate the AtRAV1

209 (**Fig. 5A and 5B**). Compared to others, the SDM2 demonstrated weak phosphorylation

210 signal by AtMPK3 whereas the phosphorylation was completely abolished in case of

211 SDM5.

212 **Overexpression of phospho-defective variants of AtRAV1 is unable to induce disease**

213 **resistance in *A. thaliana***

214 In order to test whether phosphorylation of AtRAV1 is required for inducing disease

215 resistance, we overexpressed phospho-defective variants of AtRAV1 (SDM2 and SDM5) in

216 the WT and *atrav1* mutant *A. thaliana* plants. Interestingly in both the cases (WT^{SDM2}/^{SDM5}

217 and atrav1^{SDM2}/^{SDM5}) the plants were highly susceptible to *R. solani* infection (**Fig. 6A**). The

218 severity of disease symptoms, extent of host cell death and pathogen load were also

219 significantly higher in WT^{SDM2}/^{SDM5} and atrav1^{SDM2}/^{SDM5} plants, compared to the *AtRAV1*

220 OE1 plants (**Fig. 6A and 6B**). In contrary to the OE1 line, the WT^{SDM2}/^{SDM5} and

221 atrav1^{SDM2}/^{SDM5} lines were unable to induce the expression of key defense genes including

222 MAP kinases (*AtMPK3*, *AtMPK4* and *AtMPK6*) (**Fig. 6C**) and defense marker genes (**Fig.**

223 **6D**) upon pathogen infection. Overall, this suggested that phosphorylation of AtRAV1 is

224 required for eliciting defense response and imparting disease resistance in *A. thaliana*.

225 **Overexpression of *SlRAV1* imparts broad spectrum disease resistance in tomato.** To

226 further substantiate the role of RAV1 in plant defense, we analysed whether the *AtRAV1*

227 ortholog of tomato (*SlRAV1;* EU164416) can impart disease resistance in tomato

228 (*Lycopersicon esculentum* mill). Two independent *SlRAV1* OE (OE:L1 and OE:L2) lines

229 were generated in tomato cultivar Pusa Ruby (**Fig. S12**). The qRT-PCR revealed both the

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230 OE lines to have higher fold expression of *SlRAV1* (**Fig. S12E**) and the expression were
231 significantly enhanced upon *R. solani* infection in OE lines (**Fig. S13A**). The western blot
232 analysis also suggested enhanced accumulation of SlRAV1 in pathogen infected OE lines
233 (**Fig. S13B**). The disease symptoms (**Fig. 7A**), disease severity index (**Fig. 7B**) and
234 chlorophyll content of the infected leaves (**Fig. 7C**) suggested enhanced disease tolerance in
235 OE lines, compared to EV and WT tomato plants. Also, the expression of most of the
236 selected Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) mediated defense
237 marker genes were significantly enhanced upon *R. solani* infection in the OE lines (**Fig.**
238 **7D**).
239 We further tested the susceptibility of OE lines against a deadly pathogen (*Ralstonia*
240 *solanacearum*) which causes bacterial wilt disease in tomato. As shown in **Fig. 8A**, the
241 wilting symptoms was remarkably less in infected OE lines (OE:L1 and OE:L2), compared
242 to WT plants. Notably, the pathogen (*R. solanacearum*) load and disease severity index
243 were also significantly less in the infected OE:L1 and OE:L2 lines (**Fig. 8B** and 8**C**). The
244 enhanced accumulation of SlRAV1 protein in *R. solanacearum* (**Fig. 8D**) infected tomato
245 OE lines, supported its pathogen inducible nature. Also gene expression (**Fig S14**) as well as
246 enzymatic activity of some of the antioxidant markers such as catalase (CAT), ascorbate
247 peroxidase (APX) and glutathione reductase (GR) were significantly higher in *R. solani*
248 (**Fig. 7H-J**) and *R. solanacearum* (**Fig. 8H-J**) infected OE lines while the MDA content,
249 H_2O_2 content and ion leakage were reduced in these lines (**Fig. 7E-G** and **8E-G**).
250 We further observed the OE lines to have high level of tolerance against Tomato Leaf Curl
251 Joydepur Virus (*ToLCJoV*) infection. The leaf curling symptom was negligible in the OE
252 lines but severe in case of WT as well as EV plants (**Fig. S15A**). ROS accumulation (**Fig.**
253 **S15B**), disease severity index (**Fig. S15C**) and total chlorophyll content (**Fig. S15D**) further
254 reinforced enhanced tolerance against *ToLCJoV* infection in OE lines.
255

256 **Discussion**
257 Plants are susceptible to various bacterial, fungal and viral diseases. Controlling them in an
258 eco-friendly manner is a challenge for sustainable agriculture. We endeavoured to identify
259 gene(s) that can provide broad spectrum disease resistance in plants. Initially, while
260 studying the defense protein interaction network, we identified RAV1, an ethylene
261 responsive transcription factor as a master transcriptional regulator of defense genes in
262 *Arabidopsis thaliana*. Previous studies had shown that overexpression of pepper RAV1

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263 provides resistance against *Pseudomonas syringae* pv. tomato DC3000 (a hemi-biotrophic

264 bacterial pathogen) infection in *A. thaliana* by induction of PR genes (40). Here we

265 observed that overexpression of *AtRAV1* confers remarkable resistance against fungal

266 (*Rhizoctonia solani*) infection in *A. thaliana*. Similarly, overexpression of *SlRAV1* (the

267 *AtRAV1* ortholog in tomato) imparted remarkable level of protection against fungal (*R.*

268 *solani*), bacterial (*R. solanacearum*) and viral (Tomato leaf curl virus) diseases in tomato.

269 We observed that AtRAV1 acts as a master transcriptional activator of various key defense

270 genes (that are topologically central to defense protein interaction network) as well as JA,

271 SA and ET responsive defense marker genes in *A. thaliana*. The enhanced expression of

272 *AtMPK3*, *AtMPK4* and *AtMPK6* transcripts as well as their proteins in the pathogen infected

273 OE lines suggests the activation of MAP kinase signalling. The AtMPK3/AtMPK6

274 signalling seems essential for mediating AtRAV1 mediated defense; as knocking out

275 AtMPK3 or AtMPK6 (but not AtMPK4) rendered the *AtRAV1* OE lines hyper susceptible to

276 *R. solani* infection. The MPK3/MPK6 mediated signalling plays an important role in

277 elucidation of pathogen triggered immunity (PTI) and effector triggered immunity (ETI) as

278 well as production of defense associated phytoalexin, camalexin in plants (41–44). Also the

279 MPK3/MPK6 are known to phosphorylate some plant transcription factors and regulate

280 important cellular response including defense response (45). Notably, the

281 AtMPK3/AtMPK6 mediated phosphorylation of some members of ERF family

282 (ERF6/ERF104) assist in elucidation of plant defense response (46). In this study, we

283 observed that AtMPK3 but not AtMPK6 can phosphorylate the AtRAV1 under in-vitro

284 condition. Recently, it has been reported that AtMPK3 but not AtMPK6 regulates

285 submergence tolerance by phosphorylation of SUB1A1 (Submergence1A1) transcription

286 factor (47). We anticipate that the AtMPK3 mediated phosphorylation of AtRAV1 may

287 stabilize the protein during pathogen attack and activates it to stimulate the defense

288 response. Thus on one hand, the AtRAV1 binds to the promoter of different MAP kinases

289 (AtMPK3/AtMPK4/AtMPK6) and induces their signalling while on the other hand AtMPK3

290 phosphorylates AtRAV1 and modulates its function. In deed we observed that phospho-

291 defective variants of AtRAV1 (SDM2 and SDM5) are unable to impart disease resistance

292 against *R. solani* infection in *A. thaliana*.

293 There has been trade-off between disease resistance and plant growth (48). The constitutive

294 activation of defense genes can negatively impact plant growth and development (49).

295 However in this study, we did not observe any apparent growth or developmental defects in

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296 the RAV1 overexpressing *A. thaliana* as well as tomato lines. We attribute this may be due

297 to observed dynamics of induction of defense genes expression in the OE lines. Although

298 several defense genes including MAP kinases were overexpressed in OE lines, the extent of

299 their up-regulation was further enhanced upon pathogen infections. Similarly, the

300 antioxidant machinery of the host (tomato) was significantly enhanced upon pathogen (*R.*

301 *solani* and *R. solanacearum*) infection in OE lines. Such dynamic expression of defense

302 genes may ensure strong protection during pathogen attack while averting the negative

303 effect of induced defense responses under control (uninfected) conditions. Further, due to a

304 relatively short (<60 min) half-life (50) and potential ability to auto-regulate its own

305 transcription, the level of RAV1 is limited under normal conditions. Conversely with

306 pathogen inducible nature, the RAV1 level gets enhanced in the infected OE lines and upon

307 potential phosphorylation by AtMPK3 the protein gets stabilized, leading to sustained

308 activation of MPK3/MPK6 signalling and thereby induction of defense genes. In support of

309 this, we observed limited induction of defense genes including MAPKs in the *A. thaliana*

310 lines overexpressing the phosphor-defective variants of AtRAV1 (**Fig. 6**).

311 Overall, the present study reports RAV1, an ethylene responsive transcription factor as a

312 master regulator of plant defense and is a novel phosphorylation target of AtMPK3. A cross

313 talk between RAV1 and MAPKs is required for inducing disease resistance against *R. solani*

314 infection in *A. thaliana*. Furthermore, the overexpression of tomato RAV1 provides

315 remarkable level of protection against bacterial, fungal and viral infections in tomato.

316 Considering that RAV1 orthologs are conserved in different monocot and dicot plants and

317 we do not observed any trade-off between enhanced disease resistance with apparent

318 growth/developmental defects in the overexpression *A. thaliana* and tomato lines, our study

319 emphasizes that RAV1 can be gainfully deployed as a biotechnological intervention to

320 develop broad spectrum disease resistance in a variety of crops.

321

322 **Methods**

323 **Identification of key defense proteins in *Arabidopsis thaliana*.** Mukhtar and colleagues

324 have reported the list of plant (*A. thaliana*) and pathogen proteins (*Pseudomonas syringae*

325 and *Hyaloperonospora arabidopsidis*) that are involved in plant pathogen interactions (51).

326 The plant proteins (n=392 proteins) potentially involved in defense responses were

327 obtained from that list (**Table S3**) and were mapped on to AtPIN (*A. thaliana* Protein

328 Interaction Network) database (52) to construct a Arabidopsis Defense Protein Interaction

329 Network (ADPIN). Visualization of protein-protein interaction network was performed
330 using Cytoscape (53). Furthermore the ADPINv1 (The Arabidopsis Defense Proteins
331 Interaction Network vicinity 1) was constructed by extending ADPIN to include its first
332 interacting partners.

333 Network centrality measures were computed for ADPINv1 proteins by graph-theoretical
334 analysis. Degree (hubs), betweeness (bottlenecks) and average shortest path (swift
335 communicators) were studied to identify proteins that are central to the interactome and
336 might be critical for executing plant defense. Top 10 proteins with the best values for each
337 of these network parameters, were selected. Notably few proteins were commonly
338 predicted to have best values for each of the network parameter. After removing these
339 redundant proteins from the list, 16 unique proteins, topologically and dynamically central
340 to the network, were obtained (**Table S3**). These proteins are considered as key defense
341 proteins. Gene Investigator tool (https://genevestigator.com/) revealed that genes encoding
342 these proteins are induced during biotic stress (**Table S4**).

343 **Identification of *AtRAV1* as master transcriptional regulator of key defense genes.** The
344 Arabidopsis Information Resources (TAIR) id of each of the 16 identified key defense gene
345 was used as query in Plant PAN database (Plant PAN; http://PlantPAN2.itps.ncku.edu.tw).
346 The transcription start site/5'UTR-End limit was fixed as 100bp, while transcription stop
347 site/3'UTR-End was fixed as 1000bp. The analysis predicted putative transcription binding
348 sites present in the 5'UTR (promoter) region in each of the genes. The *AtRAV1*
349 (At1G13260) transcriptional factor binding sites were common in each of them.

350 **Plant Materials and Growth Conditions.** Different *A. thaliana* (Colombia-0; wild type,
351 transgenic and Salk_ 021865 mutant) lines used in this study were grown on soilrite at
352 22ºC with 8/16-h photoperiod, 70 % relative humidity in growth chamber. Similarly,
353 different tomato (*S. lycopersicum;* cultivar Pusa ruby) plants and tobacco (*N. benthamiana*)
354 plants were grown on soilrite at 26ºC with 12/12-h photoperiod, 70 % relative humidity in
355 growth chamber.

356 **Generation of *AtRAV1* overexpressing *A. thaliana* and *SlRAV1* overexpressing tomato.**
357 The CDS of *AtRAV1* (AT1G13260) gene was cloned in pGJ100 (a modified pBin19 binary
358 vector having MCS of pBSKS vector) and transformed into GV3101 strain of
359 Agrobacterium. The Agrobacterium harbouring 35S:*RAV1* construct was inoculated into
360 three weeks old *A. thaliana* (Col-0) plants by floral dip method (54). Presence of transgene
361 was reconfirmed by PCR using *CaMV35S F* and *RAV1OX-R* primer. The *NptII-F* and

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362   *NptII-R* primer pair was further used to confirm the presence of vector in both OE and EV

363   lines. The expression of *AtRAV1* gene in different *A. thaliana* lines was verified by qRT

364   PCR using primers mentioned in **Table S5**.

365   Similarly, the full length gene sequence of *SlRAV1* (EU164416) was cloned into gateway

366   binary vector (pGWB408) using SlRAV1OX-F and SlRAV1OX-R primer pairs (**Table S5**)

367   and transgenic tomato lines (OE:L1 and OE:L2) were generated through Agrobacterium

368   (GV3101 strain) mediated transformation. The presence of transgene was confirmed by

369   PCR using CaMV35S F and SlRAV1OX-R primer while T-DNA was verified by NptII-*F*

370   and NptII-*R* primer. The expression of *SlRAV1* was validated by qRT-PCR using gene

371   specific (SlRAV1 RTF and SlRAV1 RTR) primer. Total crude protein was isolated from

372   100 mg of plant leaves using P-PER plant protein extraction kit (Thermo scientific: 89803)

373   and the western blot was carried out by electro blotting the 20µg of total crude protein onto

374   polyvinylidene fluoride (PVDF) membrane and probed with mouse polyclonal anti-His-

375   antibody (1:30000 dilutions). The blot was developed as per manufacturer's protocol

376   (Sigma-Aldrich, Japan).

377   **Generation of MAP kinase mutants in AtRAV1 overexpressing OE1 lines.** The

378   *AtMPK3* (*atmpk3*, SALK_100651), *AtMPK4* (*atmpk4-2*, SALK_056245) and *AtMPK6*

379   (*atmpk6-2*, SALK_073907) mutants were obtained from ABRC stock centre. Presence of

380   T-DNA insertion in mutant (*atmpk3, atmpk4-2 and atmpk6-2)* was individually conformed

381   by PCR using T-DNA border primer and MAP kinase gene specific reverse primer

382   (BP+RP) (**Table S5**). The cross between the MAP kinase mutant plants (as male plant) and

383   the AtRAV1 OE1 (as female) was set up and obtained seeds were grown on soilrite. Upon

384   PCR   validation   of   presence   of   AtRAV1   overexpression   construct   (using

385   CaMV35S_forwerd and AtRAV1 reverse primer) and T-DNA insertion in particular MAP

386   kinase (using BP+RP primer), we propagated the seeds to T2 generation and used them for

387   further studies.

388   **Yeast one hybrid assay.** Y1H assay was performed using Matchmaker Gold Yeast One-

389   Hybrid Library Screening System (Clontech, USA). The nucleotide sequences of promoter

390   region of selected key defense genes having potential RAV1 binding motifs were retrieved

391   by using online tool (https://bioinformatics.psb.ugent.be/plaza/). The RAV1 motif enriched

392   promoter sequence of each of the gene (**Table S1**) was cloned in pAbAi bait vector

393   (Clontech, USA) in the upstream of Aureobasidine A. The plasmid was linearized with

394   *Bst*BI   restriction   enzyme   and   transformed   into   Y1H   Gold   yeast   strain,   as   per

395 manufacturer's protocol and the positive transformants (Y1H-Bait) was selected on AbA
396 (200mg ml^{-1} AbA) plate. Subsequently the full-length copy of *AtRAV1* was cloned in
397 pGADT7-AD (Clontech, USA) prey vector as GAL4 transcription activation domain
398 (GAL4 AD) fusion protein. It was transformed in the Y1H-Bait strain and positive
399 transformants (Y1H-Bait+Prey) were selected by growing serially diluted (10^{-1}, 10^{-2}, 10^{-3}
400 and 10^{-4}) cells at 30°C for 3 days on AbA (200 mg/ml) containing double drop out (SD-
401 URA-LEU) plates.

402 **GUS based reporter assay.** The promoter region of the selected key defense genes (as
403 described above) was fused with *GUS* gene in pBI101 (pBI101:promoter:*GUS*) and
404 *AtRAV1* full length gene (AT1G13260) was cloned under CaMV35S promoter in pGJ100
405 (pGJ100:AtRAV1). The primer used for cloning is enlisted in **Table S5**. Both the
406 recombinant plasmids were individually transformed into GV3101 strain of Agrobacterium
407 and were co-infiltrated into the leaves of *N. benthamiana*. After 48 hours of infiltration,
408 GUS expression was analyzed by staining the leaves with GUS solution (1 mg/ml) for 16
409 hours at 37°C. Upon destaining in a solution (1:3 ratio of glacial acetic acid: ethanol) for 3
410 to 4 hrs at room temperature and washing with distilled water (55).

411 **cDNA synthesis and expression analysis.** Total RNA was isolated from plant tissues
412 using RNeasy Plant RNA isolation kit (Qiagen, Valencia, CA). 1μg of total RNA was used
413 for cDNA synthesis using Verso cDNA synthesis kit (Thermo Fisher Scientific Inc, USA),
414 as per the manufacturer's protocol. qRT-PCR of 16 key defence genes and various defense
415 marker genes (SA, JA and ET) was performed using primers mentioned in **Table S5**. The
416 relative fold change was calculated by using $2^{-\Delta\Delta Ct}$ method (56).

417 **Pathogen infection assays.** *R. solani* AG1-IA (BRS1) strain (18) was used to infect *A.*
418 *thaliana* and tomato. The *R. solani* sclerotia pre-germinated in potato dextrose broth (PDB,
419 Himedia, Mumbai, India) at 28ºC for 6 hours were used to infect the leaves of *A. thaliana*
420 plants. A minimum of three leaves per plant and minimum 10 plants of each line per
421 experiment was infected. However for tomato infection, the detached leaves (n=3) of at
422 least 10 plants were used in each experiments (57). On the basis of observed symptom
423 patterns (severe or mild or no symptoms), we categorised percentage of leaves having
424 particular disease symptom as disease index. Total chlorophyll was calculated per square
425 cm area according to Arnon's equation as mentioned earlier (58). Further, the *R. solani*
426 biomass in the infected samples was estimated by monitoring the expression of its 18S
427 rRNA gene through qRT-PCR using primers mentioned in **Table S5**.

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428 The deadly bacterial pathogen *Ralstonia solanacearum* strain F1C1 was also used to infect

429 tomato. The pathogen was grown in BG media (Peptone 10g, yeast extract 1g, casamino

430 acid 1g, agar 1.5g per litre) at 28°C for 48 h and 2×10^8 cfu/ml bacterial cells were drench

431 inoculated in the 3 weeks grown nursery pots of tomato as per method described in (16). A

432 minimum of 25 plants of each lines were infected in each experiment and the experiment

433 was independently repeated twice. Disease symptoms were monitored at 7 dpi and

434 percentage of plants with wilting symptoms was plotted as bar chart. The abundance of *R.*

435 *solanacearum* was estimated by serial dilution plating method and counting the CFU per

436 gram fresh weight of leaf as described earlier (59).

437 Also, the Tomato leaf curl Joydebpur virus (*ToLCJoV*) was infected to the 3 weeks old

438 tomato leaves as described (60). The disease symptoms were observed at 21 dpi and the

439 disease index, DAB staining and total chlorophyll content were estimated.

440 **Microscopic analysis.** The infected leaves were harvested and stained with WGA-FITC as

441 described earlier to monitor the growth of *R. solani* mycelia (61). The samples were

442 observed under GFP filter of Confocal Laser Scanning Microscope (AOBS TCS-SP5,

443 Leica, Germany). The images were analysed using LAS AF Version: 2.6.0 build 7266

444 software.

445 **Biochemical assays.** DAB staining was used for detection of ROS while trypan blue

446 staining was performed to detect cell death assay in *R. solani* infected leaves of *A. thaliana*

447 and tomato leaves (62).

448 Further, the MDA, H_2O_2 content and ion leakage (%) were quantified using the earlier

449 described method (61). Similarly the activities of various antioxidant enzymes (CAT, APX

450 and GR) were estimated using the protocol described in (62).

451 **Expression and purification of AtRAV1 protein.** The *AtRAV1* gene was cloned in

452 pET28a bacterial expression vector using *RAV1OX-F* and *RAV1OX-R* gene specific

453 primers and transformed into *E. coli* (BL-21 strain, DE3-codon$^+$) cells. The protein was

454 purified using affinity chromatography (Ni^{+2}-NTA) following the method described earlier

455 (57). Similarly, the different variants of *AtRAV1*, having different phosphorylation residues

456 mutated (SDM1: Ser310Ala; SDM2: Thr19Ala; SDM3: Thr23Ala; SDM4: Thr193Ala;

457 SDM5: having all four potential phosphorylation sites mutated) were synthesized

458 commercially (Gene Universal Inc; http://www.geneuniversal.com/) and cloned in pET28a

459 to purify different variant proteins. The western blot was performed by electro blotting

460 protein onto polyvinylidene fluoride (PVDF) membrane and probed with mouse polyclonal

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461 anti-His-antibody (1:30000 dilutions). Also western blotting of AtMPK3, AtMPK4 and

462 AtMPK6 was performed using anti-MAP kinase-antibody (1:20000 dilutions) as primary

463 antibody and anti-mouse IgG (Sigma) protein (1:15000 dilutions) as secondary antibody.

464 ***In-vitro* phosphorylation of AtRAV1 by MAP Kinases.** The bacterially purified AtRAV1

465 protein and its variants (as described above) were used for in-vitro phosphorylation assay.

466 The CDS of *AtMPK3* and *AtMPK6* were cloned in pGEX4t2 vector in-frame with amino-

467 terminal GST tag and transformed into *E. coli* (BL21). Upon 1mM IPTG induction for 4h,

468 the proteins were purified using GST-beads as per manufacturer's protocols (63). The in-

469 vitro kinase assay was performed as described in (47). Briefly, the MAP kinases and RAV1

470 variant proteins (1:10) were incubated in a 20µl kinase reaction buffer (25 mM Tris-Cl (pH

471 7.5), 10 mM MgCl2, 5 mM MnCl2, 1 mM DTT, 1 mM β-glycerol-phosphate, 1 µM

472 Na3VO4, 0.5 mg/ml MBP, 25 µM ATP and 1 µCi [γ^{-32} P] ATP) at 30°C for 30 minutes.

473 The reaction was stopped by addition of 2x-SDS- loading buffer and heating at 95°C for 5

474 minutes. The samples were fractionated in 10% to 12% SDS-PAGE. The phosphorylation

475 signals were detected by Typhoon phosphor imaging system (GE Health Care, Life

476 Sciences, USA).

477 **Overexpression of phospho-defective variants of *AtRAV1* in *A. thaliana*.**

478 The SDM2 and SDM5 variants of *AtRAV1* which were defective in *in-vitro*

479 phosphorylation by AtMPK3 were PCR amplified and cloned in the pGJ100 plant

480 transformation vector. The constructs were subsequently transformed into GV3101 strain

481 of Agrobacterium and the recombinant bacterial strains were used for transformation in the

482 WT (Col-0) and atrav1 (*Salk_021865)* mutant lines. The transgenic lines were confirmed

483 through PCR and sequence analysis.

484 **Statistical analysis.** One-way analysis of variance was performed using Sigma Plot 12.0

485 (SPSS, Inc. Chicago, IL, USA) with *P ≤ 0.05* considered statistically significant. The

486 statistical significance is mentioned in the figure legend, wherever required.

487 **Acknowledgements**

15

Authors' contributions

500 GJ conceived the work and coordinated its progress, GJ as well as GB planned while GB
501 supervised the network analysis which leads to identification of key defense proteins. RKC
502 carried out the detail characterization of *AtRAV1* as well as *SlRAV1* to establish its role in
503 providing broad spectrum disease resistance against bacterial, fungal and viral infection. RK
504 assisted in making various constructs and performed YIH. DMS performed protein
505 purification, antioxidant assays and western blot analysis. SG assisted in confocal
506 microscopic analysis and bioinformatics analysis. PKB performed the *in-vitro*
507 phosphorylation assays, AKS contributed different MAPK mutants and antibodies, SP had
508 provided valuable comments on the manuscript. GJ, RKC and DMS had written the
509 manuscript and all authors have approved the final manuscript.

Competing interests

511 The authors declare that they have no competing interests. Material distribution footnote:
512 The author responsible for distribution of materials integral to the findings presented in this
513 article in accordance with the policy described in the instructions for authors.
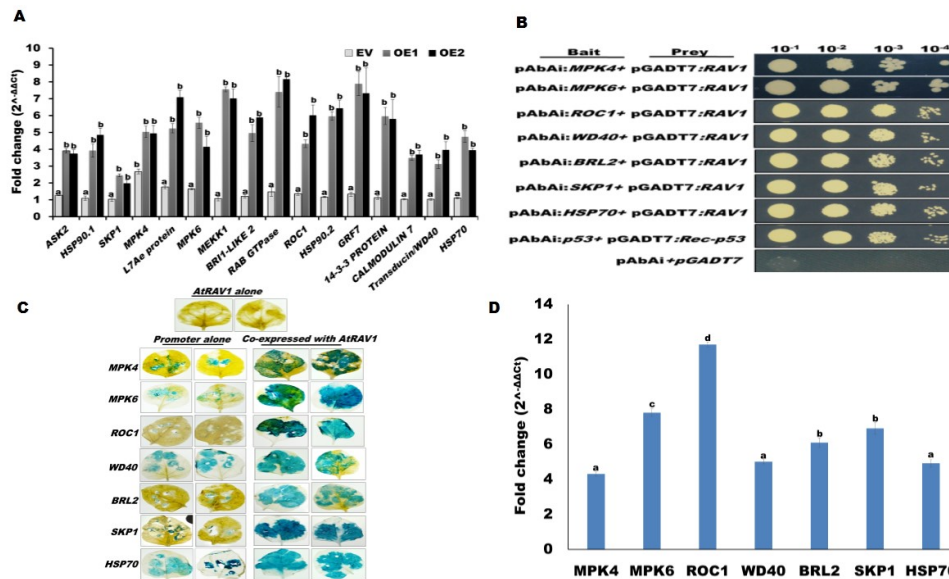
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521 **Figure legends**

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524 **Fig. 1.** *AtRAV1* up-regulates the expression of key defence genes. (A) Relative expression
525 pattern of sixteen key defense genes in *A. thaliana*. The differential expression of these
526 genes in *AtRAV1* overexpression lines (OE1 and OE2) with respect to the wild type (WT)
527 plants was calculated using beta actin gene as endogenous control. (B) Yeast one hybrid
528 (Y1H) based transactivation assay. The full-length *AtRAV1* were ligated into the pGADT7
529 (Prey) vector and promoters of selected key defence genes were cloned in pAbAi bait
530 vectors in upstream of Aureobasidine A (AbA). The growth of co-transformed (Prey+Bait)
531 yeast strain on SD/-Leu/-Ura/AbA verifies the transactivation activity. (C) GUS reporter
532 assay. Transcriptionally fused *GUS* under the promoter of selected key defense genes was
533 found induced (appearance of blue color) when co-expressed with AtRAV1 in *N.*
534 *benthamiana*. (D) qRT-PCR quantification of GUS gene expression in *N. benthamiana*
535 leaves. The *Npt*II gene was used as internal control and relative expression was quantified
536 upon normalization with promoter: GUS infiltrated samples. Graph shows mean values ±
537 standard error of at least three technical replicates. For each gene, different letters indicate
538 significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were
539 obtained in at least three biological repeats.

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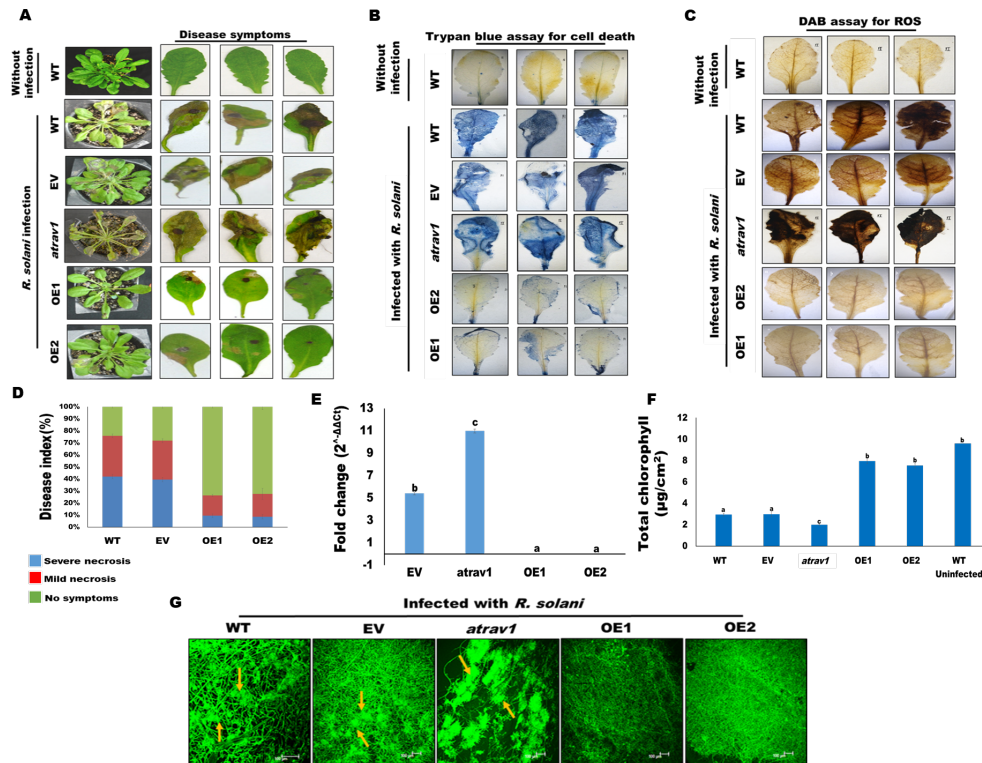
Fig. 2. Overexpression of *AtRAV1* provides disease resistance against *R. solani* infection in *A. thaliana*. (A) Disease symptoms observed as brown necrotic lesions in infected leaves at 4 dpi. (B) Trypan blue staining for visualization of cell death and (C) DAB staining for ROS accumulation (brown coloration) in the infected leaves. (D) Disease index (based upon observed necrotic symptoms). (E) Bar graph showing qRT-PCR quantification of 18S RNA gene of *R. solani* (reflecting pathogen load). (F) Total chlorophyll content in *R. solani* infected *A. thaliana* leaves. (G) Confocal imaging of WGA-FITC stained *R. solani* mycelium in the infected leaves. The arrows indicate infection cushions in WT and EV plants. Graph shows mean values ± standard error of at least three technical replicates. Values with different letters are significantly different at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in at least three biological repeats.

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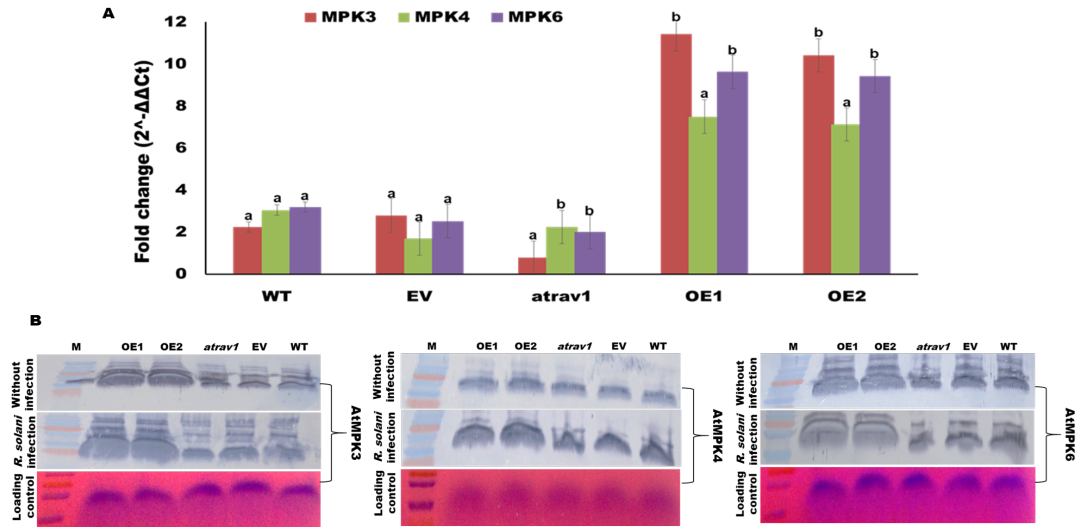
Fig. 3. The MAP kinases are induced in *R. solani* infected *AtRAV1* overexpressing lines. (A) Bar graph represents qRT-PCR based expression analysis of different *MAP* kinase genes in *R.solani* infected *A. thaliana* leaves. The relative expression was quantified by normalizing the expression with uninfected samples using beta actin as endogenous control. (B) Western blot analysis showing the expression of AtMPK3, AtMPK4 and AtMPK6 proteins in different *A. thaliana* plants with or without *R. solani* infection. Graph shows mean values ± standard error of at least three technical replicates. For each gene, different letters indicate significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in at least three biological repeats.
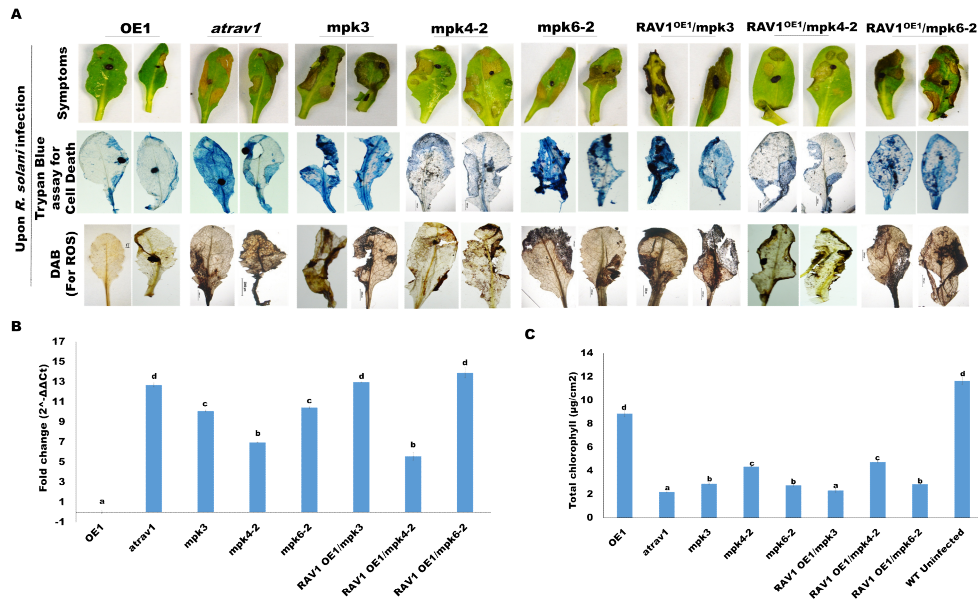
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568 **Fig. 4.** AtRAV1 mediated disease resistance requires functional AtMPK3 and AtMPK6
569 proteins. (A) The disease symptoms, cell death (trypan blue staining) and ROS
570 accumulation (DAB staining) in the *R. solani* infected leaves of *A. thaliana* plants at 4 dpi.
571 (B) Bar graph showing qRT-PCR based quantification of 18S ribosomal RNA of *R. solani*
572 (reflecting pathogen load) in the infected plants. (C) Total chlorophyll content in the
573 infected *A. thaliana* leaves. Data are reflected as mean ± SE of at least three technical.
574 Values with different letters are significantly different at *P<0.05* (estimated using one-way
575 ANOVA). Similar results were obtained in three biological repeats.

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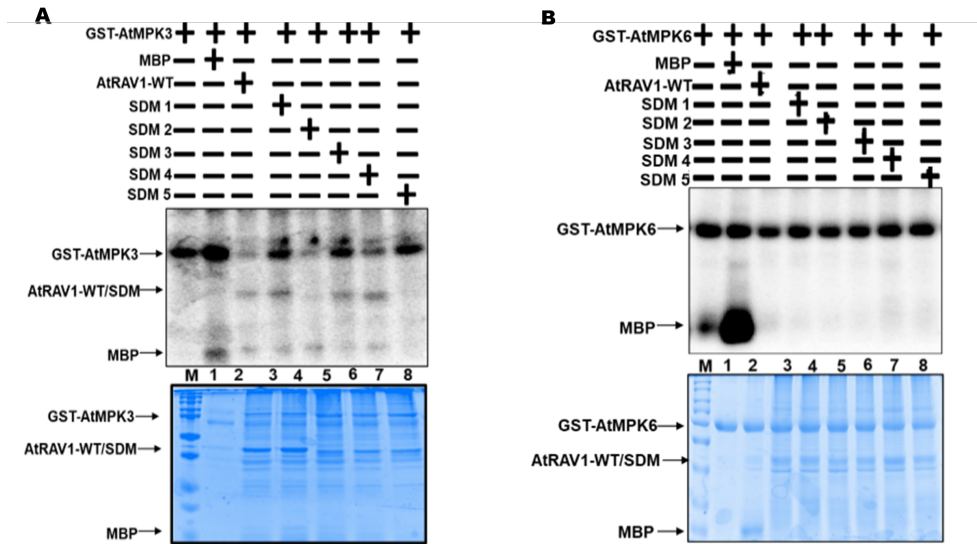
Fig. 5. AtRAV1 is phosphorylated by AtMPK3 under in-vitro condition. (A) and (B) Upper panel: Autoradiogram showing in-vitro phosphorylation of bacterially expressed AtRAV1 with AtMPK3-GST and AtMPK6-GST. The phosphorylation of MBP was used as a positive control. (A) and (B) Lower panel: coomassie brilliant blue stained gel (12%) with positions of different proteins indicated by arrows. The plus and minus signs indicate the presence and absence of proteins, respectively during the assay.

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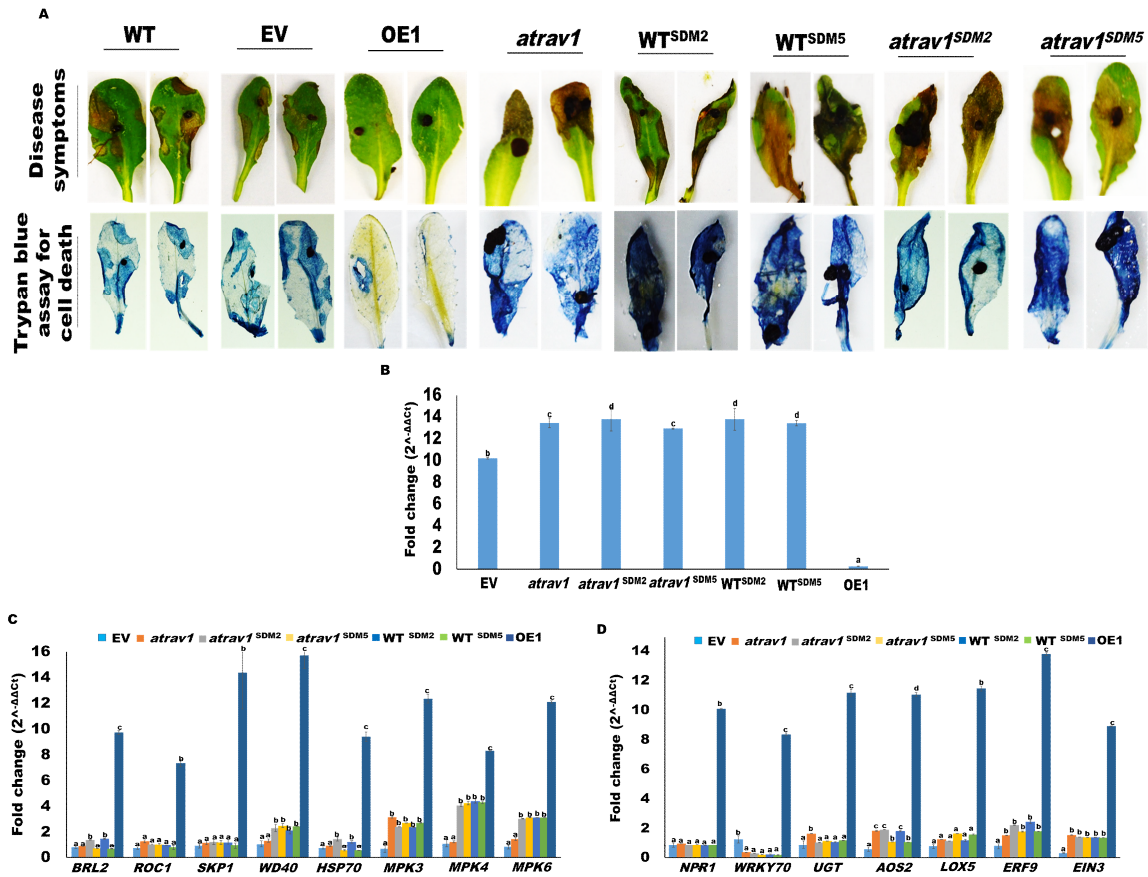
Fig. 6. The overexpression of phospho-defective variants of *AtRAV1* fail to impart resistance against *R. solani* infections in *A. thaliana*. (A) The disease symptoms and extent of host cell death (trypan blue staining) in the *R. solani* infected leaves of different A. *thaliana* plants at 4 dpi. (B) Bar graph showing qRT-PCR based quantification of 18S ribosomal RNA of *R. solani* (reflecting pathogen load) in the infected plants. The expression of (C) selected key defense genes and (D) Defense marker genes in *R. solani* infected lines. The relative expression was quantified by normalizing the expression with that of *R. solani* infected wild type plants using beta actin as endogenous control. Graph shows mean values ± standard error of at least three technical replicates. For each gene, different letters indicate significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in two different biological repeats.

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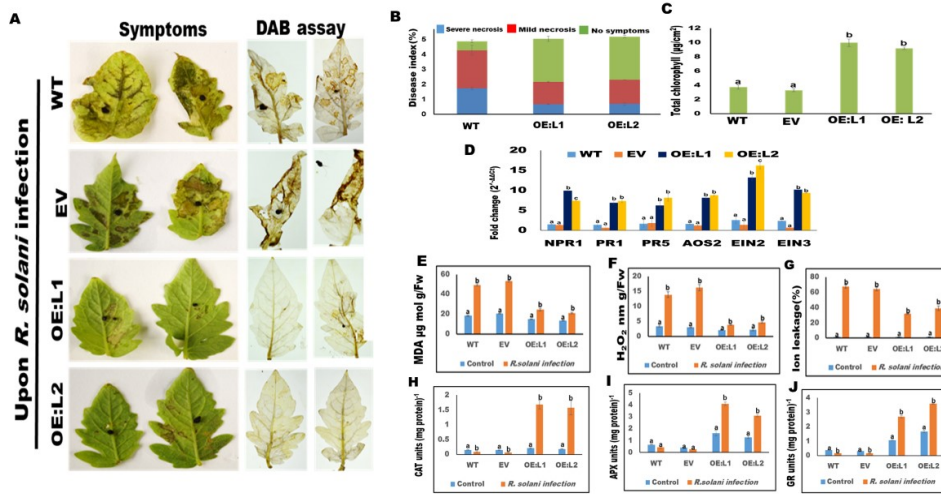
Fig. 7. Overexpression of *SlRAV1* provides resistance against *R. solani* infection in tomato. (A) Disease symptoms, (B) Disease index (in terms observed necrotic symptoms) and (C) Total chlorophyll content in the *R. solani* infected tomato leaves at 4 dpi. (D) Expression analysis of SA, JA and ET mediated marker genes in the infected samples. The relative expression was quantified with respect to uninfected samples using beta actin as internal control. (E) MDA content, (F) H_2O_2 content, (G) ion leakage (%) and the enzymatic activities of various antioxidant markers, (H) CAT, (I) APX and (J) GR in the infected plants. Graph shows mean values ± standard error of at least three technical replicates. For each gene, different letters indicate significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in at least three biological repeats.

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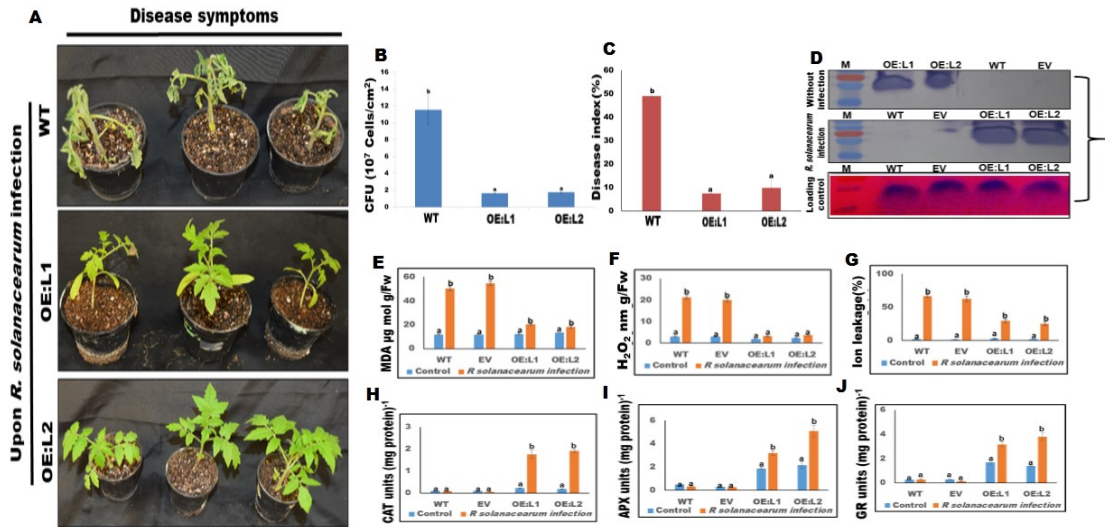
Fig. 8. Overexpression of *SlRAV1* provides resistance against *R. solanacearum* infection in tomato. (A) Disease symptoms, (B) The pathogen load (CFU/ml) and (C) disease index (% of plants with wilting symptoms) in drench inoculated *R. solanacearum* infected tomato plants at 7dpi. (D) Western-blot analysis reflecting the accumulation of His-tagged SlRAV1 protein in tomato plants with or without *R. solanacearum* infection. (E) MDA content, (F) H_2O_2 content, (G) ion leakage (%) and the enzymatic activities of various antioxidant markers, (H) CAT, (I) APX and (J) GR in the infected plants. Graph shows mean values ± standard error of at least three technical replicates. For each gene, different letters indicate significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in three biological repeats.

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Supplementary information:

A cross talk of AtRAV1, an ethylene responsive transcription factor with MAP kinase imparts broad spectrum disease resistance in plants

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Short title: RAV1 promotes disease resistance in plants

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Fig. S1. Centrality of key defense proteins in Arabidopsis defense protein interaction network. Protein-protein interaction network of Arabidopsis defense proteins and their immediate interacting partners: ADPINv1. The Arabidopsis defense proteins were mapped onto AtPIN and the interactome of these proteins and their immediate interactors was extracted using Cytoscape. A complex network with 6051 interactions amongst 1343 proteins was observed. Central nature of key plant defense interactome proteins was identified using network metrics. Sixteen key defense proteins are highlighted onto ADPINv1 in red.

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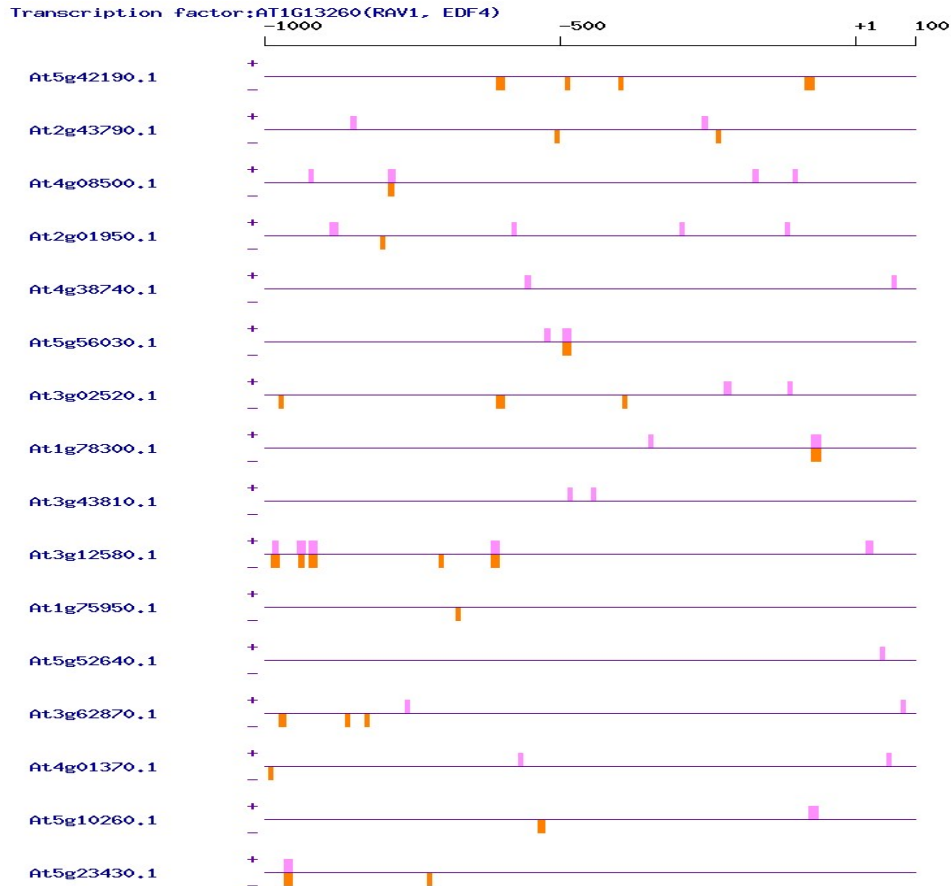
Fig. S2. The AtRAV1 transcription factor (AT1G13260) binding sites in the promoter region of key defense genes. The AtRAV1 transcription factor (AT1G13260) binding sites in the promoter region in each of the 16 key defense genes are represented as vertical bars. The violet colour indicates binding sites in the negative strand while pink colour indicates binding sites in the positive strand.

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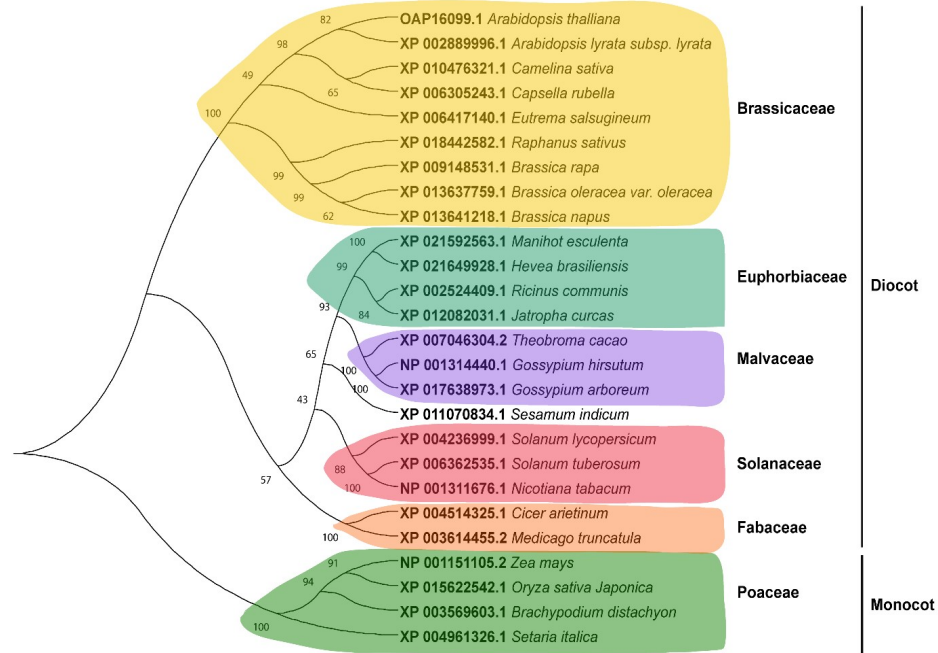
Fig. S3. Phylogenetic analysis of RAV1 proteins. The bootstrap values are indicated at each branch node. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The evolutionary analysis was conducted in MEGA X using Neighbor-Joining method.

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A Transcription factor:AT1G13260(RAV1; EDF4)

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TTGGA**TTTCT**ATTGACCAGT**AGAAA**TACTTGTTATGATGTCGGTCGCTCGTTGGCTAAGTA**TTTCT**TATAAATATATAAGACAT
ATTTAA**AATCT**TTTTACATTACATACACCCACTTATG**AGAAA**CAAATATAATAAAATGTTGATACCAAATATATATATATATATATA
ATAAAATGGGATAATTACAAGAACAACAAAAGAGAGATTTTTTATGAATTTGGAATTG**GAATCT**ATCGAGTGCATATATTATAC
GGACAATAATCCTCATAGCAATACACATTT**TTTCT**ATAAAA**AGAAA**CGTAGGATAAGATTAT**AGAAA**GAAGAATAAAAACCT**G**
GAAATG**GAAA**ATAAAGAA**GATATATATC**ATGAGTTCATGACTAAACAACTATTTATAGCTTTTTCAATAATA**ATATC**ATTCAAG
AGTGAAGAATAACAATCAGATTTGGAAG**AGAAA**AAAATTTAAAGAGAGACACCATTTAACATCGGTAGGATCTGAACATGGT
TATCA**AATCT**AATAC**TTTCT**ATTTTATTATTTTTCATATAGTTATTATCGTTACTATAGTATTATAGTGAATTAAAATTTTAAGCATC
AAAA**CTCAG**GGGGGATGAACTTCACTAAAATGATGTCA**ATATC**TCACGTGGACCCTT**TATCC**ATTCCATGGCCCACACATG
GGTCCCACTTCCCCATGCACACAGCCCACAACATATTCA**TTTCT**ATATTTGTTCCTATACTCTCT**TTTCT**CTT**TTTCT**TACATA
AATAAAATACTAACTTATTATAAGGATTATTATTGCTTCGTGTGT**TTTCT**TCTATAAGTTACATCAACAGTTCATGAAATAGACC
CTAATATAGTAACCTAAATTAAATGCCGTATATTTCATTATGTTTCCACGAAGCTTCCTTTGTGTTCTTTGAGTTGACCCATAC
ACACCCATCTTACTTCCCATGCACCTGCACATATACTATAATTACTCCCCTCAAACACGCATTCTTACAAATAT

Fig. S4. Potential RAV1 binding sites present in the promoter of *AtRAV1*. (A) Schematic view depicting the presence of potential RAV1 transcription factor binding sites in the *AtRAV1* promoter. The violet colour indicates binding sites in negative strand while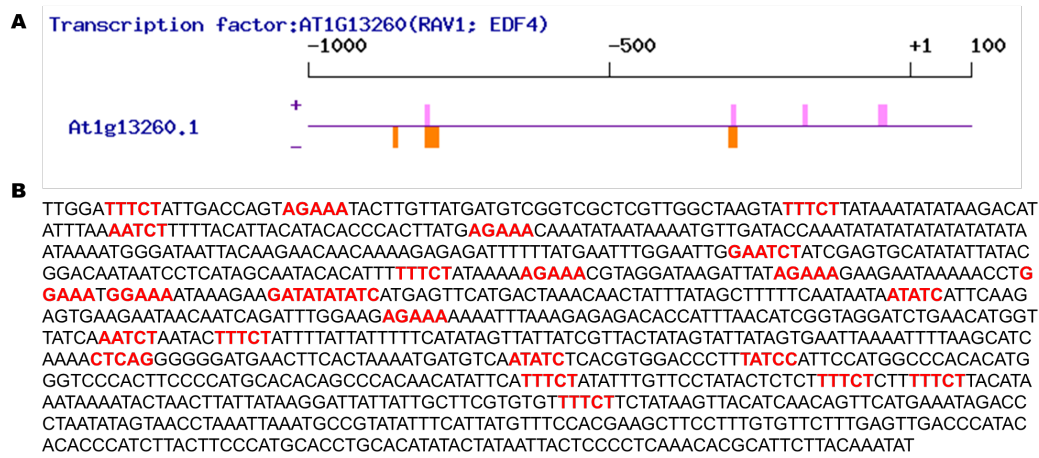 pink colour indicates binding sites in the positive strand. (B) The potential RAV1 binding motifs are highlighted in the *AtRAV1* promoter region. Eight distinct RAV1 binding motifs [AGAAA (5), TTTCT (8), AATCT (3), GGAAA (2), GATAT (1), ATATC (3), CTCAG (1) and TATCC (1)], are highlighted.
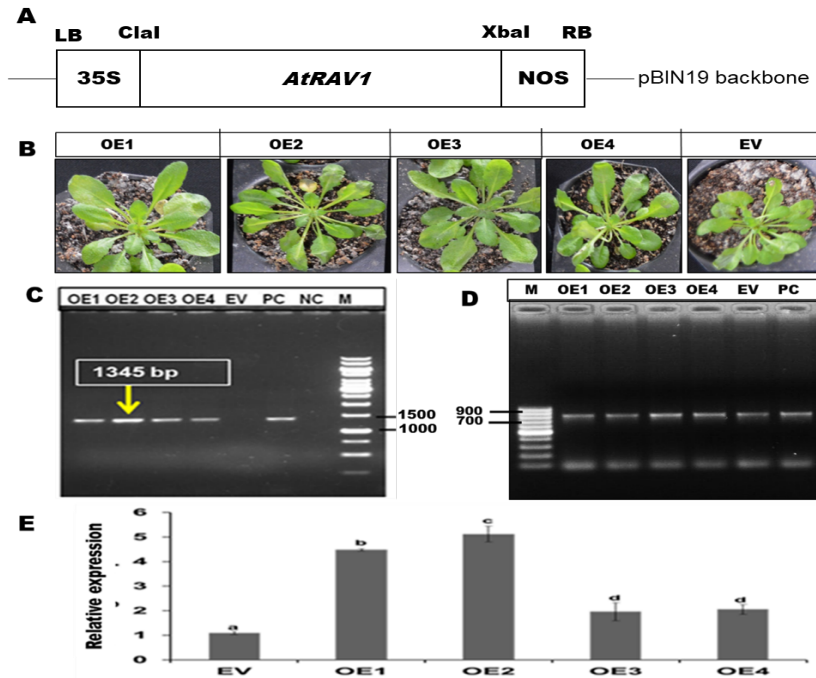
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Fig. S5. Characterization of transgenic *A. thaliana* lines. (A) The pGJ100 binary vector map that was used for generating transgenic lines. (B) The representative photographs of *AtRAV1* overexpressing (OEs) and empty vector (EV) transgenic plants. (C) PCR product using CaMV 35S F and RAV1OX-R primer pair. PC (positive control) represents PCR product obtained using plasmid of pGJ100 containing 35S:AtRAV1; NC (negative control) reflects no template control and M: represents DNA marker. (D) PCR product using NptII-F and NptII-R primers, highlighting the integration of T-DNA in both OE and EV transgenic lines. (E) Relative gene expression of *AtRAV1* in different OE and EV lines, when normalized with the expression in wild type (WT) plants using beta-actin as housekeeping gene. Graph shows mean values ± standard error of at least three biological replicates. Values with different letters are significantly different at *P < 0.05* (estimated using one-way ANOVA)
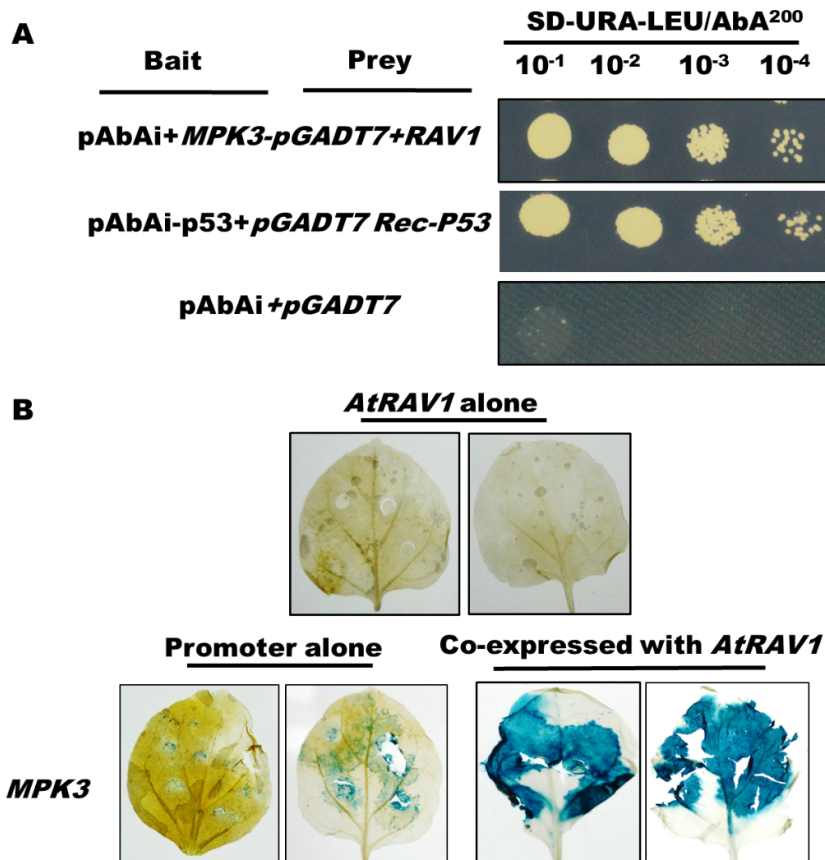
Fig. S6. *AtRAV1* induces the expression of AtMPK3 by potentially binding to its promoter. (A) Yeast one hybrid assay: growth of yeast cells on SD-URA-LEU medium in presence of AbA (Aureobasidin A) reflects that AtRAV1 transactivates the expression of AbA gene under *AtMPK3* promoter. The pAbAi-p53+pGADT7Rec-P53 was used as a positive control while the empty vector of pAbAi + pGADT7 was used as negative control. (B) GUS based reporter assay suggesting that AtRAV1 activates GUS expression driven through the promoter of *AtMPK3* gene in *N. benthamiana*.

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946 **Fig. S7.** Validation of *atrav1* mutant line (Salk_021865). (A) The position of T-DNA

947 insertion (marked by inverted triangles) in *AtRAV1* gene in. Red arrow indicates the

948 positions of primers used to validate T-DNA insertion. (B) PCR product showing T-DNA

949 insertion in *atrav1* mutant. *ACT2* (beta actin) gene of *A. thaliana* was used as loading

950 control.

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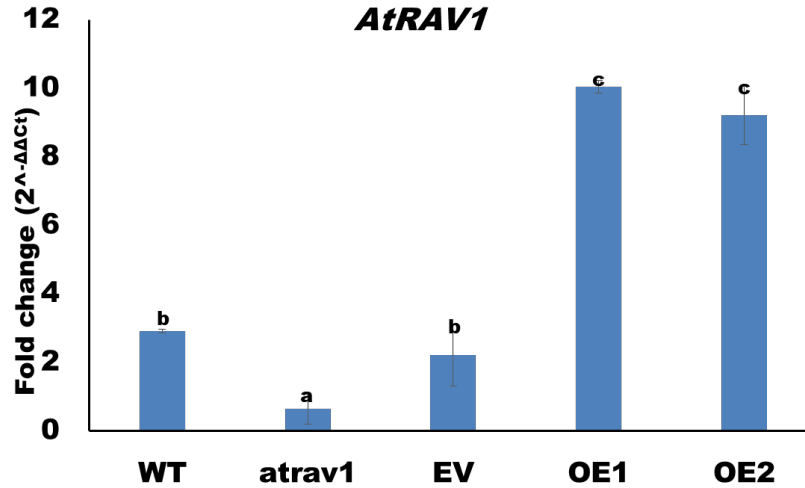955

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Fig. S8. The pathogen infection induces the expression of *AtRAV1*. The relative expression of *AtRAV1* gene in *R. solani* infected *A. thaliana* plants are summarized as bar chart. The relative expression was quantified by normalizing the expression with the uninfected samples using beta actin as endogenous control. Graph shows mean values ± standard error of at least three technical replicates. Values with different letters are significantly different at *P < 0.05* (estimated using one-way ANOVA). Similar results were obtained in three biological repeats.

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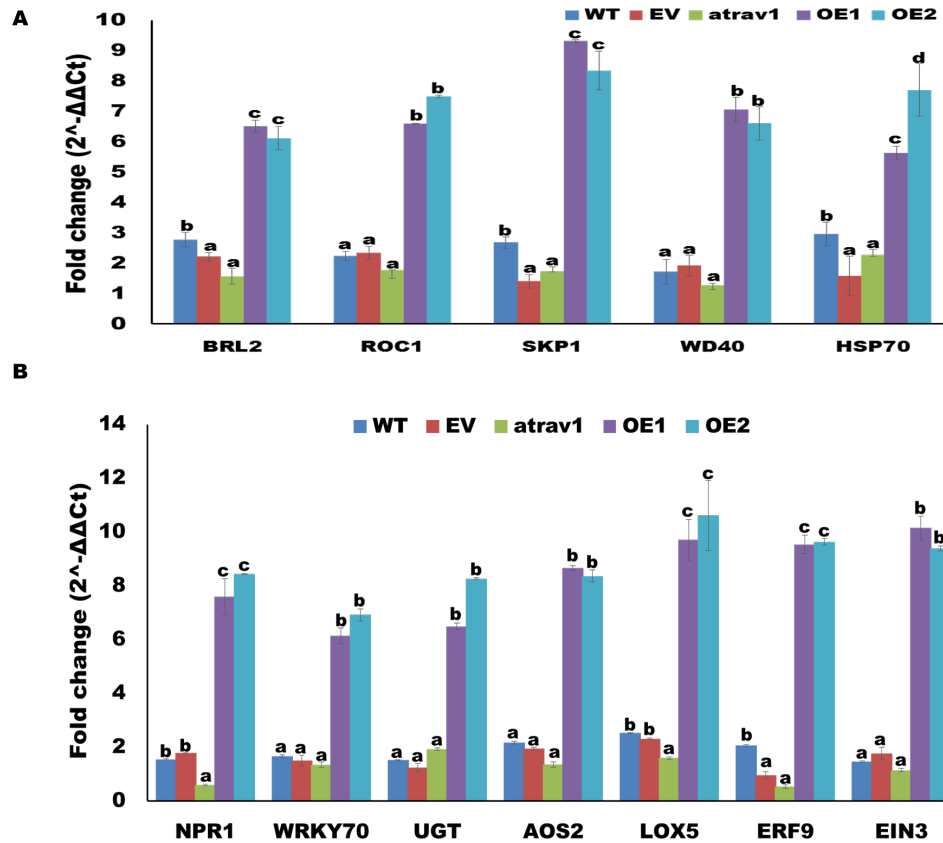
Fig. S9. *R. solani* infection enhances the expression of key defense and defense marker genes in *AtRAV1* overexpression lines. The expression of (A) selected key defense genes and (B) Defense marker genes in *R. solani* infected lines. The relative expression was quantified by normalizing the expression with uninfected samples using beta actin as endogenous control. Graph shows mean values ± standard error of at least three technical replicates. For each gene, different letters indicate significant difference at *P<0.05* (estimated using one-way ANOVA). Similar results were obtained in three biological repeats.
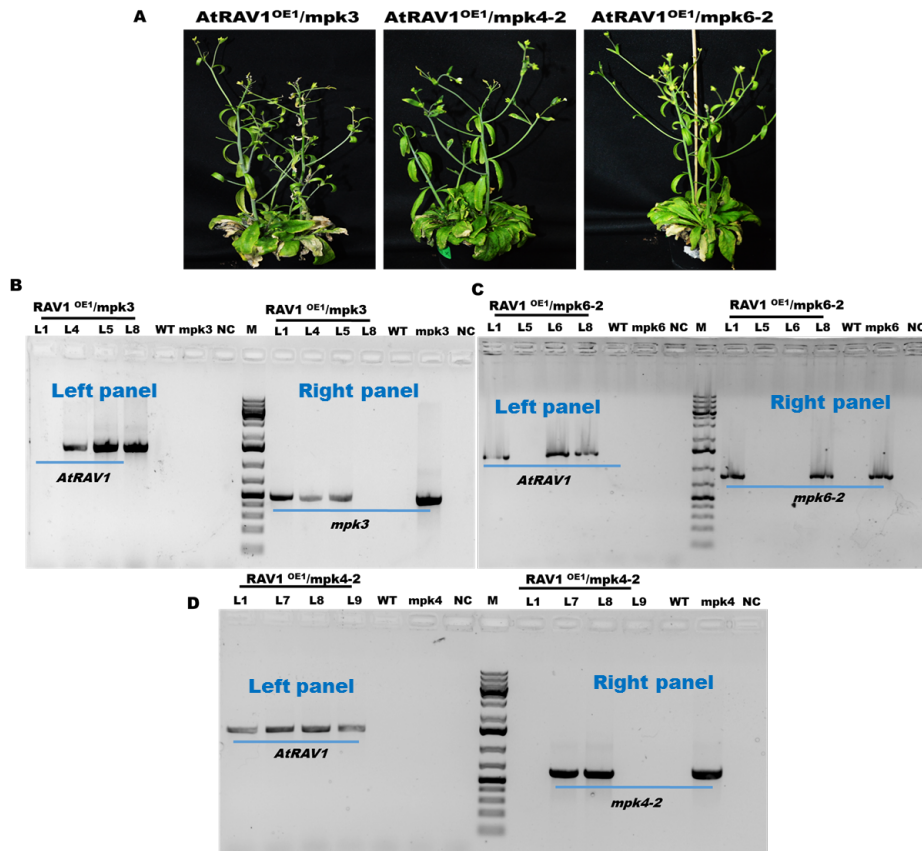
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Fig. S10. Validation of AtRAV1^{OE1}/mpk3, AtRAV1^{OE1}/mpk4-2 and AtRAV1^{OE1}/mpk6-2 lines in *A. thaliana.* (A) The representative images of different plants (AtRAV1^{OE1}/mpk3, AtRAV1^{OE1}/mpk4-2 and AtRAV1^{OE1}/mpk6-2). B, C and D right panel represent PCR based validation of T-DNA insertion of different MAP kinase knockout mutant (mpk3, mpk4-2 and mpk6-2) in *AtRAV1* OE1 lines using T-DNA border primer and gene specific reverse primer (BP+RP). B, C and D left panel represent presence of *AtRAV1* transgene was confirmed by PCR using CaMV 35S F and RAV1OX-R primer. Negative control (NC) reflects no template control.

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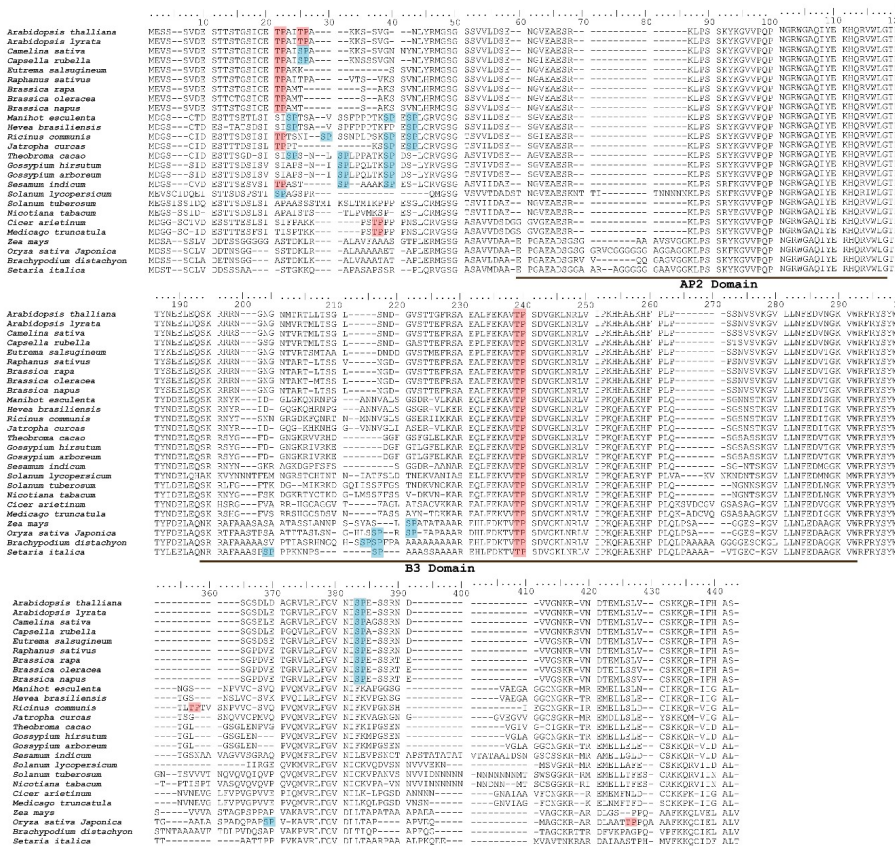
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Fig. S11. ClustalW alignment of RAV1 protein sequences in different plants. Presence of conserved AP2 and B3 domain region and potential MAP kinase phosphorylation sites (TP in red and SP in blue) in RAV1 amino acid sequence is highlighted.
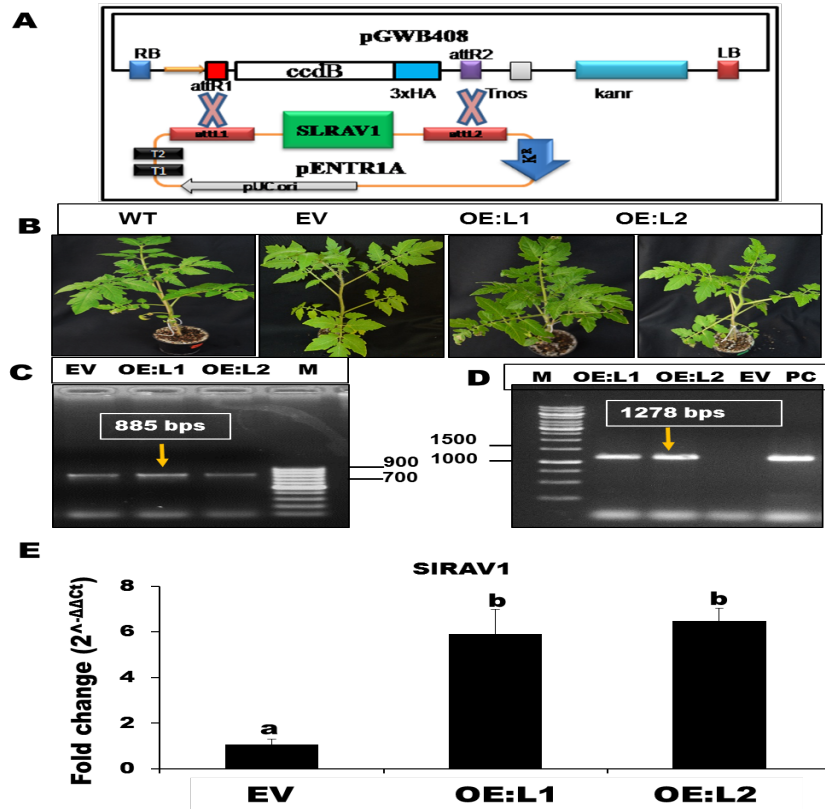
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Fig. S12. Characterization of transgenic tomato lines. (A) T-DNA map of pGWB408 gateway binary vector that was used for generating transgenic lines in tomato. (B) The representative images of empty vector (EV) and *SlRAV1* overexpressing (OEs) transgenic plants. (C) Presence of T-DNA (885 bp) confirmed by PCR using NptII-F and NptII-R primer. (D) Presence of *SlRAV1* transgene (1278bp) confirmed by PCR using CaMV 35S F and SlRAV1OE-R primer. Positive control (PC) represents PCR product obtained from recombinant plasmid (pGWB408 containing 35S:*SlRAV1*). (E) Relative gene expression of *SlRAV1* in different OE and EV lines, upon normalization with respect to WT plants. Graph shows mean values ± standard error of at least three technical replicates. Values with different letters are significantly different at $P < 0.05$ (estimated using one-way ANOVA). Similar results were obtained in three biological repeats.
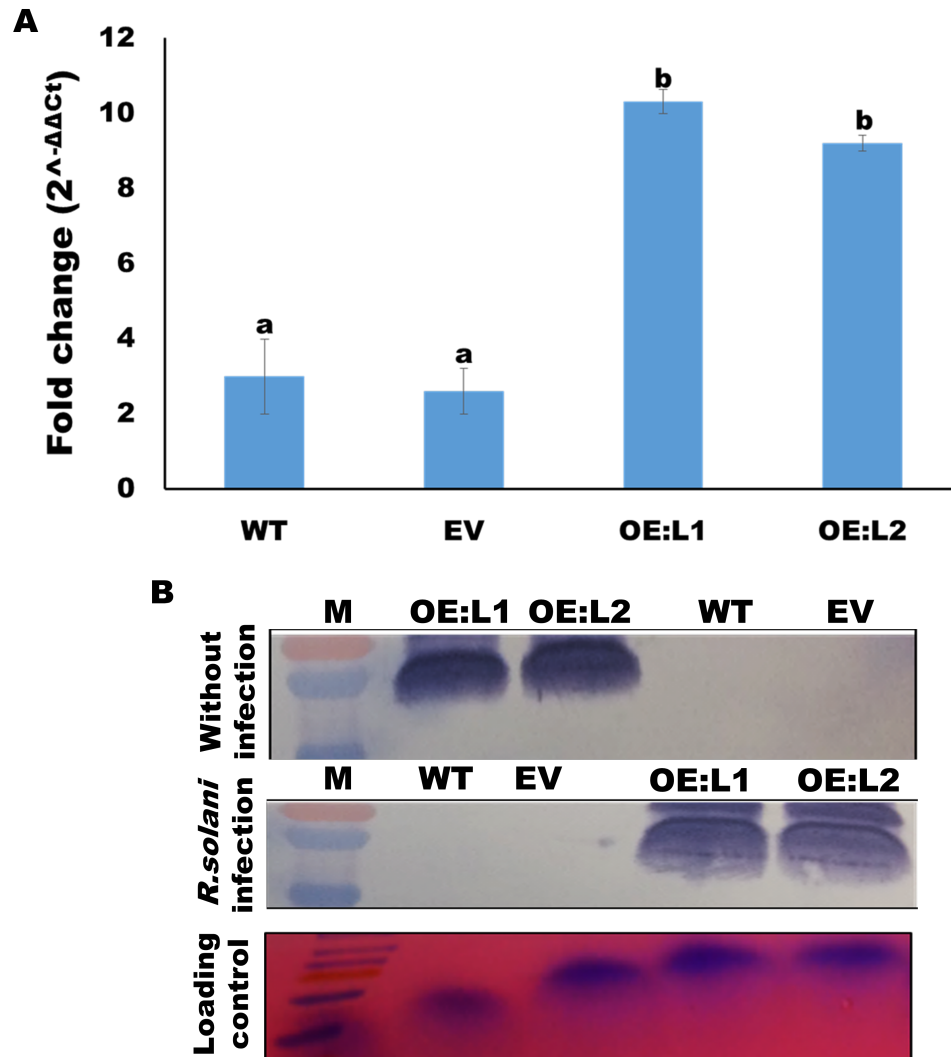
Fig. S13. *R. solani* infection upregulates the expression of SlRAV1 in tomato. (A) The relative expression of *SlRAV1* gene in *R. solani* infected (4 dpi) tomato leaves are summarized as bar chart. The relative expression was quantified by normalizing the expression with uninfected samples using beta actin as endogenous control. Graph shows mean values ± standard error of at least three technical replicates. Values with different letters are significantly different at *P < 0.05* (estimated using one-way ANOVA). (B) Western-blot analysis reflecting the accumulation of His-tagged version of SlRAV1 protein in different tomato plants with or without *R. solani* infection. Similar results were obtained in three biological repeats.

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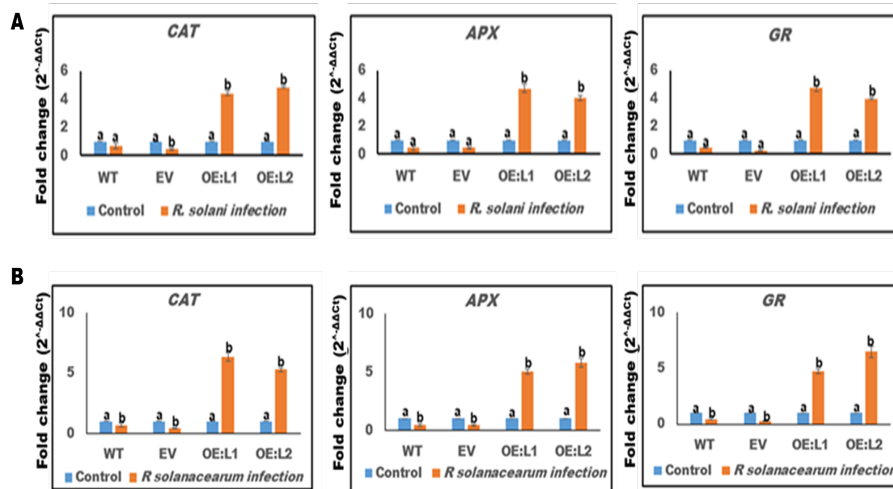
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1040 **Fig. S14.** Expression analysis of different antioxidant marker genes upon *R. solani* and *R.*
1041 *solanacearum* infection. The relative expression of various antioxidant marker genes
1042 (*CAT*, *APX* and *GR*) in (A) *R. solani* (4 dpi) and (B) *R. solanacearum* (7 dpi) infected
1043 tomato leaves. The relative expression was quantified by normalizing the expression with
1044 uninfected samples using beta actin as endogenous control. Graph shows mean values ±
1045 standard error of at least three technical replicates. Values with different letters are
1046 significantly different at *P < 0.05* (estimated using one-way ANOVA). Similar results
1047 were obtained in three biological repeats.

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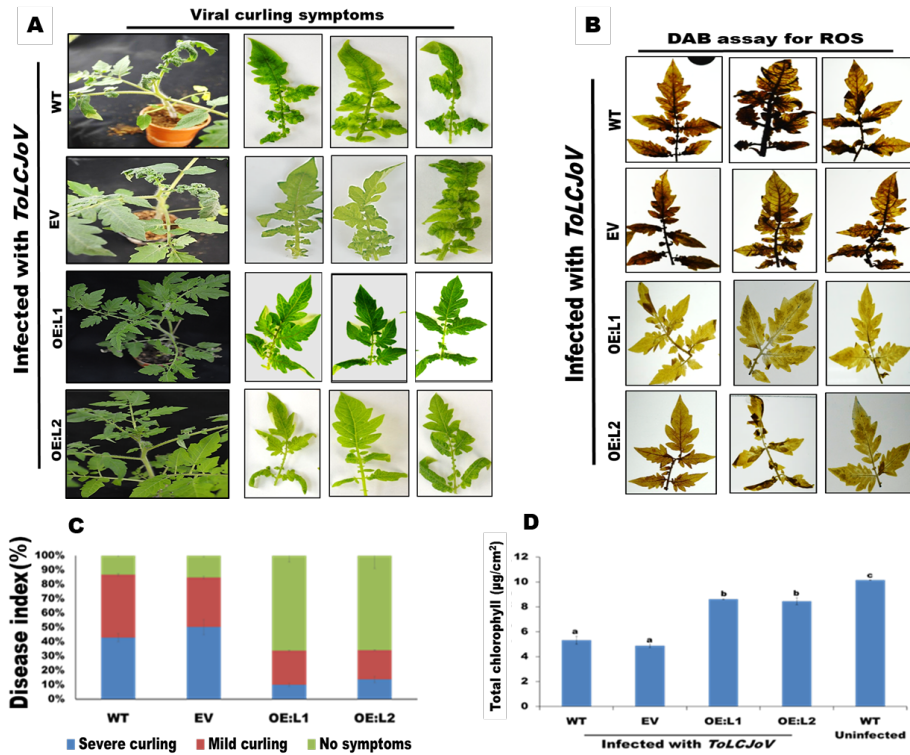
Fig. S15. *SlRAV1* overexpression provides tolerance against *Tomato leaf curl Joydepur virus (ToLCJoV)* infections in tomato. (A) Disease symptoms (leaf curling) in *ToLCJoV* infected tomato at 21 dpi. (B) DAB staining of *ToLCJoV* infected tomato leaves. (C) Observed disease symptoms in *ToLCJoV* infected tomato leaves plotted as disease index. (D) Total chlorophyll content in *ToLCJoV* infected tomato leaves at 21 dpi. Graph shows mean values ± standard error of at least three technical replicates. Values with different letters are significantly different at *P < 0.05* (estimated using one-way ANOVA). Similar results were obtained in three biological repeats.

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1074 **Table S1.** RAV1 binding motifs in the promoter region of selected key

1075 defence genes.

Gene name	Promoter region having potential AtRAV1 binding sites	Number of RAV1 binding motifs
MPK4	-124 to -1858	3
ROC1	-169 to -1812	11
WD40	-133 to -1775	6
BRL2	-40 to -1916	9
SKP1	-167 to-1906	6
HSP70	-103 to -1806	11

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1077 **Table S2.** List of Salicylic acid, Jasmonic acid and Ethylene (SA, JA and

1078 ET) mediated defense marker gene used in this study.

Sl. no.	TAIR ID.	Gene name	Function	Reference
1.	*At1g64280*	*AtNPR1*	Induced systemic resistance against *Botrytis cinerea* by *Bacillus cereus.* Key regulator of SA-mediated signaling.	(1, 2)
2.	*At3g56400*	*AtWRKY70*	Function as activator of SA-dependent defense genes and a repressor of JA-regulated genes. *WRKY70* controlled suppression of JA-signaling is partly executed by *NPR1*.	(3)
3.	*At1g05675*	*AtUGT superfamily*	Mediates abscisic acid homeostasis in	(4)

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			Arabidopsis	
4.	*At5g42650*	*AtAOS2*	JA biosynthetic pathway. Defense response	(4, 5)
5.	*At3g22400*	*AtLOX5*	Activate Brassinosteroid signaling to promote cell wall based defense and limit pathogen infection	(6)
6.	*At5g44210*	*AtERF9*	Participates in resistance against necrotrophic fungi.	(7)
7.	*At3g20770*	*AtEIN3*	Modulate plant salt tolerance. EIN3 interferes with the sulfur deficiency signaling in *Arabidopsis thaliana.*	(8, 9)

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1080 **Table S3.** As XLS sheet (attached separately)

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1082 **Table S4.** List of 16 key defense genes.

Sl. no.	TAIR Id.	Gene name	Function of gene	Expression pattern as per Gene investigator analysis*	Refer ences
1	*At5g4 2190*	*SKP-LIKE 2, ASK2, SKP1B*	Involved in mitotic cell cycle control and ubiquitin mediated	Highly expressed	(10)

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			proteolysis.		
2	*At5g52640*	*ATHSP90.1*	Interacts with disease resistance signalling components SGT1b and RAR1 and is required for RPS2-mediated resistance.	Highly expressed	(11)
3	*At1g75950*	*S PHASE KINASE-ASSOCIATED PROTEIN 1, SKP1*	Component of the SCF family of E3 ubiquitin ligases. Predominately expressed from leptotene to pachytene. Negatively regulates recombination.	Highly expressed	(12, 13)

4	*At4g0 1370*	*ATMPK 4, MAP KINASE 4*	Negatively regulates systemic acquired resistance. Required for male-specific meiotic cytokinesis.	Highly expressed	(14, 15)
5	*At3g6 2870*	*Ribosom al protein L7Ae/L3 0e/S12e/ Gadd45 family protein*	Structural constituent of ribosome, involved in translation, located in cytosolic ribosome.	Highly expressed	(16)
6	*At2g4 3790*	*ATMAP K6, ATMPK 6*	Involved in seed formation and modulation of primary and lateral root developme nt. Differentia lly	Highly expressed	(17, 18)

			regulates growth and pathogen defense in *Arabidopsis thaliana.*		
7	*At4g08500*	*MAPK/ ERK KINASE KINASE 1, ATM EKK1*	Activate in response to flagellin receptor FLS2 WRKY53 transcription factor. Mediates function during cold acclimation in *Arabidopsis thaliana.*	Moderate expression	(19, 20)
8	*At2g01950*	*BRI1- LIKE 2, BRL2,*	Auxin-activated signalling pathway, Brassinosteroid mediated signalling pathway. Regulates	Low to moderate expression	(21, 22)

			the containment of microbial infection-induced cell death.		
9	*At5g1 0260*	*ATRAB H1E, RAB GTPAS E HOMOL OG H1E*	Involved in: protein transport, small GTPase mediated signal transductio n. Vesicle Trafficking in Arabidopsi s pollen tubes.	Low to moderate expression	(23, 24)
10	*At4g3 8740*	*ROC1, ROTAM ASE CYP 1*	Blue light signalling pathway, Brassinoste roid mediated signalling pathway.	Highly expressed	(25)
11	*At5g5 6030*	*ATHSP9 0.2,*	Important for	Highly expressed	(26, 27)

		EARLY-RESPONSIVE TO DEHYDRATION	stomatal closure and modulate abscisic acid-dependent physiological responses. Required for NLR immune receptor accumulation.		
12	*At3g02520*	*GENERAL REGULATORY FACTOR 7.GRF7*	Contribute to polarity of PIN auxin carrier and auxin transport-related development. Important for plant development.	Highly expressed	(28, 29).

13	*At1g7 8300*	*GENER AL REGUL ATORY FACTO R 2, GF14 OMEGA , GRF2, 14-3-3*	Brassinoste roid mediated signalling pathway. Contribute to polarity of PIN auxin carrier and auxin transport-related developme nt.	Highly expressed	(28, 30)
14	*At3g4 3810*	*ATCAM 7, CALMO DULIN 7*	Inhibition of the Arabidopsi s BRASSIN OSTEROI D-INSENSIT IVE 1 receptor kinase. Promote photo morphogen esis. Regulate root growth	Highly expressed	(31, 32)

			and abscisic acid responses.		
15	*At5g23430*	*Transducin/WD40 repeat-like superfamily protein*	Controls seed germination, growth and biomass accumulation in *Arabidopsis thaliana.*	Moderate expression	(33)
16	*At3g12580*	*ARABIDOPSIS HEAT SHOCK PROTEIN 70, ATHSP70*	Regulate development and abiotic stress. Required for protection against oxidative stress.	Moderate expression	(34, 35)

1083   *: The expression during various biotic stress were consider during the

1084   analysis

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1089   **Table S5.** List of primer used in this study.

Primer name.	GeneID.	Primer sequence (5' to 3')
For full length gene amplification and cloning (Highlighted region in primer represent restriction sites)		
At1G132 60.1	RAV1OX -F	5'**ATCGAT**ATGGAATCGAGTAGCGTTGATGAGA3'
	RAV1OX -R	5'**TCTAGA**TTACGAGGCGTGAAAGATGCGTTGCT 3'
EU1644 16	SlRAV1O X-F	5'**GGATCC**ATGGAGGTAAGTTGCATAG 3'
	SlRAV1O X-R	5' **CTCGAG**TCAAGGCATCAATTATTACCCT 3'
For Expression study by Real time q-RT PCR		
At5g421 90.1	AtASK2 RTF	5'CGAAATTGACGAAGCGGTGG3'
	AtASK2 RTR	5'GCAGCTTCGACATGTCTCTT3'
At5g526 40.1	AtHSP90 RTF	5'ACGGTACCACTCCACAAAGAG3'
	AtHSP90 RTR	5'ACCGCCTTTTTGCTTTCACC3'
At1g759 50.1	AtSKP1 RTF	5'GGGACTGTTGGACTTGACTT3'
	AtSKP1 RTR	5'CGGCGAACCTCTTCTTCTT3'
At4g013 70.1	AtMPK4 RTF	5'CATGGTGGTAGCTATGTTCAGT3'
	AtMPK4 RTR	5'GCAGCACAGACAATTCCATAAG3'
At3g628 70.1	AtL7Ae RTF	5'TTGAGAGACGCCCAAAGCAA 3'

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	AtL7Ae RTR	5'AGGATACGCTTCTGCCTTTGA3'
At2g437 90.1	AtMPK6 RTF	5'TGAACGAAAACGCAAAGCGA3'
	AtMPK6 RTR	5'CAGTGATGGATTGGCGAGGA3'
At4g085 00.1	AtMEKK 1 RTF	5'TAAGGTTCAGGGTCAGGATTTG3'
	AtMEKK 1RTR	5'TCTACCACACCATCAGCTACTA3'
At2g019 50.1	AtBRI1 RTF	5'AAACCTCGTACCGCTCTTGG3'
	AtBRI1 RTR	5'ATCCTCCGTTTTTCGCCTGT3'
At5g102 60.1	AtRABH1 RTF	5'ACCAGCATCATCACTCGTTT3'
	AtRABH1 RTR	5'GTTGGAGACGAACAGTCCTATC3'
At4g387 40.1	AtROC1 RTF	5'TTTCACCGTGTGATCCCTAAC3'
	AtROC1 RTR	5'GGTGTGCTTCCTCTCGAAAT3'
At5g560 30.1	AtHSP90 RTF	5'CTGCTAGGATTCACAGGATGTT3'
	AtHSP90 RTR	5'CTTCCTCCATCTTGCTCTCTTC3'
At3g025 20.1	AtGRF7 RTF	5'CTGCTGAGAGCACTCTGGTT3'
	AtGRF7 RTR	5'CAGGCACGATCAGGTGAGTT3'
At1g783 00.1	At14-3-3 RTF	5'AGAGCTTGCTCCAACACACC3'

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	At14-3-3 RTR	5'AGGTTACAGGCACGATCAGG3'
At3g438 10.1	AtCAM7 RTF	5'AGGAGCTTGGGACTGTGATG3'
	AtCAM7 RTR	5'CTATTGTCCCGTTCCCGTCT 3'
At5g234 30.1	AtWD40 RTF	5'CCAGAGCAGACCCTAGAATAGA3'
	AtWD40 RTR	5'TAGGTGACCTTCGGGAATCA3'
At3g125 80.1	AtHSP70 RTF	5'GGCAGATGAGTTCGAGGATAAG3'
	AtHSP70 RTR	5'AGGTGTGTCGTCATCCATTC3'
At3G187 80.1	AtACT RTF	5' GACCTTTAACTCTCCCGCTATG3'
	AtACT RTR	5'GAGACACACCATCACCAGAAT3'
At1g642 80.1	AtNPR1 RTF	5'CGGTTTCGATTCGGTTGTG3'
	AtNPR1 RTR	5'TCGTCTGCGCATTCAGAAACT3'
At3g564 00.1	AtWRKY 70 RTF	CAAGGGTGCAAGGCAACAA3'
	AtWRKY 70 RTR	5'TTGGGAGTTTCTGCGTTGGT3'
At1g056 75.1	AtUGT RTF	5'GCCGTGGCTTCTGGATGTAG3'
	AtUGT RTR	5'AGAACGAGCCCTTGAATACATGA3'
At5g426 50.1	AtAOS2 RTF	5'CGGGCGGGTCATCAAGT3'

	AtAOS2 RTR	5'AATCGCTCCCATCGTGAGTT3'
At3g224 00.1	AtLOX5 RTF	5'TGCGGTCAATGACTCTGGTTAT3'
	AtLOX5 RTR	5'ACCACGCTGAGCTGCCTATT3'
At5g442 10.1	AtERF RTF	5'GGTTATGCTTCTGCTGGTTTTTTC3'
	AtERF RTR	5'ATCAAACCGAACCGGACAAA3'
At3g207 70.1	AtEIN3 RTF	5'GTTCCACAAGCTGAGCCTGAT3'
	AtEIN3 RTR	5'TCTCCACATCCTCCTCTCCAA3'
At1G132 60.1	AtRAV1 RTF	5'TACCGAAACATCACGCAGAG3'
	AtRAV1 RTR	5'TAACGGAACCTCCACACTTTC3'
X04879. 1	CaMV 35S F	5'GCGATAAAGGAAAGGCTATCG3'
U55761. 1	NptII F	5'TGATTGAACAAGATGGATTGC3'
	NptII R	5'GAACTCGTCAAGAAGGCGATA3'
U60480. 1	SlActin_F	TGGCATCATACTTTCTACAATG
	SlActin_R	CTAATATCCACGTCACATTTCAT
Primer used for GUS based Reporter assay (Highlighted region in primer represent restriction sites)		
At1g759 50.1	SKP1_GU S_F	**AAGCTT**CTGATAAGACTCAGTATCTTTAA
	SKP1_GU S_R	**GGATCC**AGTCTTAACCTAATTAGGT
At4g013 70.1	MPK4_G US_F	**GTCGAC**ATTATCGCCAAAGCTTCTCTC

	MPK4_G US_R	**GGATCC**TCATGGTTAAACAACTTATA
At2g019 50.1	BRL2_G US_F	**AAGCTT**ATCTTTGTGGTATACTGTATTTA
	BRL2_G US_R	**GGATCC**AGTGTAGTATTATATAAACT
At4g387 40.1	ROC1_G US_F	**AAGCTT**CAGATTTCTTCTACAGA
	ROC1_G US_R	**GGATCC**TATAAATAAACAAGGATTA
At5g234 30.1	WD40_G US_F	**GTCGAC**ATTCCTATTCTCATAAACT
	WD40_G US_R	**GGATCC**TATATAATGGAATTATGAAAC
At3g125 80.1	HSP70_G US_F	**GTCGAC**AAGAAATATGGGTGAGACT
	HSP70_G US_R	**GGATCC**ATTCGGTGTTTAGGCAC
Primer used for Y1H assay (Highlighted region in primer represent restriction sites)		
At1g759 50.1	SKP1_BA IT_F	**GAGCTC**CTGATAAGACTCAGTATCTTTAA
	SKP1_BA IT_R	**CTCGAG**AGTCTTAACCTAATTAGGT
At4g013 70.1	MPK4_B AIT_F	**GAGCTC**ATTATCGCCAAAGCTT
	MPK4_B AIT_R	**GTCGAC**TCATGGTTAAACAACTTATA
At2g019 50.1	BRL2_BA IT_F	**GAGCTC**ATCTTTGTGGTATACTGTATTTA
	BRL2_BA IT_R	**GTCGAC**AGTGTAGTATTATATAAACT

At4g387 40.1	ROC1_B AIT_F	**GAGCTC**CAGATTTCTTCTACAGA
	ROC1_B AIT_R	**GTCGAC**TATAAATAAACAAGGATTA
At5g234 30.1	WD40_B AIT_F	**GAGCTC**ATTCCTATTCTCATAAACT
	WD40_B AIT_R	**GTCGAC**TATATAATGGAATTATGA
At3g125 80.1	HSP70_B AIT_F	**CCCGGG**AAGAAATATGGGTGAGACT
	HSP70_B AIT_R	**GTCGAC**ATTCGGTGTTTAGGCAC
At1G132 60.1	ATRAV_ PREY_F	**TCCCCC**CGGGTATGGAATCGAGTAGCG
	ATRAV_ PREY_R	**CCGCTC**GAGCCGAGGCGTGAAAGATGC
Primer used for *atrav1* mutant validation		
At1G132 60.1	RAV1_ R (RP)	GTGAAGATGGACGAAGACGAG
	TDNA_B order (BP)	ATTTTGCCGATTTCGGAAC
Primer used for *MAP kinase* mutant validation		
AT3G45 640 (SALK_ 100651) mpk3	TDNA_B order (BP)	ATTTTGCCGATTTCGGAAC
	mpk3_RP	TTGGTGTTTTTGTTGTCATGG
AT4G01 370 (SALK_ 056245) mpk4-2	TDNA_B order (BP)	ATTTTGCCGATTTCGGAAC
	mpk4_RP	GTCTTAGAGATCAGCGGGGAC

AT2G43790 (SALK_073907) mpk6-2	TDNA_B order (BP)	ATTTTGCCGATTTCGGAAC
	mpk6_RP	ATCTATGTTGGCGTTTGCAAC
Primer used for ATRAV1 protein purification		
At1G13260.1	RAV1_PET_F	CATATGATGGAATCGAGTAGCGTTGATG
	RAV1_PET_R	CTCGAGCGAGGCGTGAAAGATGCGTTGCTT

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1091   **References**

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