

2019_JJPD_SETI_paper [for BioRxiv and JXB]

1 **Estradiol-inducible AvrRps4 expression reveals distinct properties of**
2 **TIR-NLR-mediated effector-triggered immunity**

3

4 Bruno Pok Man Ngou^{1,2}, Hee-Kyung Ahn^{1,2}, Pingtao Ding^{1,2,*}, Amey Redkar^{1,3},
5 Hannah Brown¹, Yan Ma¹, Mark Youles¹, Laurence Tomlinson¹, Jonathan DG
6 Jones^{1,*}

7

8 1 The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, UK

9 2 These authors contributed equally to this work

10 3 Department of Genetics, University of Córdoba, Córdoba 14071, Spain

11

12 * For correspondence: pingtao.ding@tsl.ac.uk; jonathan.jones@tsl.ac.uk

13

14 **Highlight**

15 Inducible expression of AvrRps4 activates RRS1/RPS4-mediated effector-
16 triggered immunity without the presence of pathogens, allowing us to
17 characterise downstream immune responses triggered by TIR-NLRs without
18 cell-surface receptor-mediated immunity.

19

20 **Abstract**

21 Plant nucleotide-binding domain, leucine-rich repeat receptor (NLR) proteins
22 play important roles in recognition of pathogen-derived effectors. However,
23 the mechanism by which plant NLRs activate immunity is still largely unknown.
24 The paired *Arabidopsis* NLRs RRS1-R and RPS4, that confer recognition of
25 bacterial effectors AvrRps4 and PopP2, are well studied, but how the
26 RRS1/RPS4 complex activates early immediate downstream responses upon
27 effector detection is still poorly understood. To study RRS1/RPS4 responses
28 without the influence of cell-surface receptor immune pathways, we generated
29 an *Arabidopsis* line with inducible expression of effector AvrRps4. Induction
30 does not lead to hypersensitive cell death response (HR) but can induce
31 electrolyte leakage, which often correlates with plant cell death. Activation of
32 RRS1 and RPS4 without pathogens cannot activate mitogen-associated

2019_JYPD_SETI_paper [for BioRxiv and JXB]

33 protein kinase cascades, but still activates upregulation of defense genes, and
34 therefore resistance against bacteria.

35

36 **Keywords**

37 Plant innate immunity, NLR activation, protein complex, hypersensitive
38 response, cell death, MAP kinase, defense gene expression, estradiol-
39 inducible expression system, Golden Gate modular cloning

40

41 **Introduction**

42 To investigate plant immunity, researchers routinely conduct pathogen
43 inoculations on plants in a controlled environment. Upon pathogen attack,
44 plants activate innate immune responses via both membrane-associated and
45 intracellular receptors, which makes it difficult to unravel the distinct
46 contribution of each component. Most plasma-membrane localized receptors
47 perceive conserved pathogen-associated molecular patterns (PAMPs) or
48 host-cell-derived damage-associated molecular patterns (DAMPs) and
49 activate PAMP-triggered immunity (PTI) or DAMP-triggered immunity (DTI).

50 Plant intracellular immune receptors belong to a family of nucleotide-binding
51 leucine-rich repeat (NB-LRR) proteins, also known as NLRs. NLRs recognize
52 pathogen effectors and activate effector-triggered immunity (ETI), which often
53 leads to accumulation of reactive oxygen species (ROS) and a hypersensitive
54 cell death response (HR). Most plant NLRs carry either coiled-coil (CC) or
55 Toll/interleukin-1 receptor (TIR) N-terminal domains. Both CC and TIR
56 domains are believed to function in signaling upon activation of NLRs, but the
57 detailed mechanisms are unknown. Many CC-NLRs localize at and function in
58 association with the plasma membrane, whereas TIR-NLRs can function in
59 diverse locations, including the nucleus. Regardless of the distinct localization
60 patterns between CC- and TIR-NLRs, their downstream outputs culminate in
61 elevated resistance, but have never been directly compared side-by-side. To
62 study the specific immune outputs generated by ETI, inducible expression
63 tools have been applied (McNellis *et al.*, 1998; Tornero *et al.*, 2002; Allen *et*
64 *al.*, 2004; Porter *et al.*, 2012).

65 In *Arabidopsis*, functionally paired NLRs RRS1-R and RPS4 confer resistance
66 against a soil-borne bacterial pathogen *Ralstonia solanacearum* through the

2019_JYPD_SETI_paper [for BioRxiv and JXB]

67 recognition of an effector PopP2 secreted via Type III secretion system and a
68 hemibiotrophic ascomycetous fungal pathogen *Colletotrichum higginsianum*
69 (Narusaka *et al.*, 2009). They can also confer resistance against bacteria
70 *Pseudomonas syringae* pv. *tomato* DC3000 carrying AvrRps4, an effector
71 protein from *Pseudomonas syringae* pv. *pisi*, causing bacterial blight in *Pisum*
72 *sativum* (pea) (Sohn *et al.*, 2009; Narusaka *et al.*, 2009). Previously, it was
73 known that the 135th to 138th residues of AvrRps4, KRVY, are required for
74 the recognition of AvrRps4 by RRS1 and RPS4 (Sohn *et al.*, 2009). Crystal
75 structure information of RRS1 and RPS4 on the TIR domains and the co-
76 crystal structures between RRS1-R WRKY domain and effector PopP2 have
77 indicated some structural basis of how RRS1/RPS4 have been activated
78 (Williams *et al.*, 2014; Zhang *et al.*, 2017). However, it is still unknown how the
79 protein complex assembles and functions.
80 Here we report tools for studying the immune complex of RRS1-R and RPS4
81 *in vivo*. We established a set of transgenic *Arabidopsis* lines to study
82 RRS1/RPS4-mediated ETI in the absence of pathogens. Using these lines,
83 we show that some but not all immune outputs induced by the conditionally
84 expressed AvrRps4 resemble other reported effector-inducible lines.

85

86 **Materials and Methods**

87 Plant material and growth conditions

88 *Arabidopsis thaliana* accessions Wassilewskija-2 (Ws-2) and Columbia-0
89 (Col-0) were used as wild type in this study. The *eds1-2* mutant used has
90 been described previously (Falk *et al.*, 1999). Seeds were sown on compost
91 and plants were grown at 21°C with 10 hours under light and 14 hours in dark,
92 and at 70% humidity. Tabaco plants were grown at 22°C with 16 hours under
93 light and 8 hours in dark, and at 80% constant humidity. The light level is
94 approximately 180-200 µmols with fluorescent tubes.

95

96 FastRed selection for transgenic *Arabidopsis*

97 Seeds harvested from the Agrobacteria-transformed *Arabidopsis* are
98 resuspended in 0.1% Agarose and exposed under fluorescence microscope
99 with DsRed (red fluorescent protein) filter. Seeds with bright red fluorescence
100 are selected as the positive transformants.

2019_JJPD_SETI_paper [for BioRxiv and JXB]

101

102 GUS staining

103 *Nicotiana benthamiana* (*N. b.*) leaves were infiltrated with Agrobacteria
104 carrying constructs with β -glucuronidase (GUS) reporter gene expressed
105 under selected *Arabidopsis* promoters (Table S1). Leaves were collected at 2
106 days post infiltration (dpi), and vacuum-infiltrated with GUS staining buffer (0.1
107 M sodium phosphate pH 7.0, 10 mM EDTA pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5
108 mM $K_4Fe(CN)_6$, 0.76 mM 5-Bromo-4-chloro-3-indolyl- β -D-glucuronide
109 cyclohexylamine salt or X-Gluc, 0.04% Triton X-100). After vacuum-infiltration,
110 the leaves were incubated at 37°C overnight in the dark. The leaves were
111 rinsed with 70% ethanol until the whole leaf de-stains to a clear white.

112

113 Immunoblotting

114 *N. b.* leaves were infiltrated with Agrobacteria carrying our stacking constructs
115 (Table S2). At 2 dpi, same leaves were infiltrated with either DMSO or 50 μ M
116 β -estradiol (E2) diluted in water. Samples were collected at 6 hpi of DMSO or
117 E2 treatment, and snap-frozen in liquid nitrogen. Proteins were extracted
118 using GTEN buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM
119 NaCl) with 10 mM DTT, 1% NP-40 and protease inhibitor cocktail
120 (cCompleteTM, EDTA-free; Merck). For *Arabidopsis* seedlings, seedlings grown
121 for 8 days after germination were treated with DMSO or E2 with indicated time
122 points and snap-frozen in liquid nitrogen. After centrifugation at 13,000 rpm for
123 15 minutes to remove cell debris, protein concentration of each sample was
124 measured using the Bradford assay (Protein Assay Dye Reagent Concentrate;
125 Bio-Rad). After normalization, extracts were incubated with 3 \times SDS sample
126 buffer at 95°C for 5 minutes. 6% SDS-PAGE gels were used to run the protein
127 samples. After transferring proteins from gels to PVDF membranes (Merck-
128 Millipore) using Trans-Blot Turbo System (Bio-Rad), membranes were
129 immunoblotted with HRP-conjugated Flag antibodies (Monoclonal ANTI-
130 FLAG[®] M2-Peroxidase HRP antibody produced in mouse, A5892; Merck-
131 Millipore), HRP-conjugated HA antibodies (12013819001; Merck-Roche) or
132 Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP[®] Rabbit
133 monoclonal antibody (4370; Cell Signaling Technology). Anti-Rabbit IgG
134 (whole molecule)-Peroxidase antibody produced in goat (A0545; Merck-

2019_JJPDI_SETI_paper [for BioRxiv and JXB]

135 Sigma-Aldrich) was used as secondary antibody following the use of
136 Phospho-p44/42 MAPK antibody.

137

138 Bacterial growth assay

139 *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying pVSP61 empty
140 vector was grown on selective King's B (KB) medium plates containing 15%
141 (w/v) Agar, 25 µg/ml rifampicin and 50 µg/ml kanamycin for 48 h at 28°C.
142 Bacteria were harvested from the plates, resuspended in infiltration buffer (10
143 mM MgCl₂) and the concentration was adjusted to an optical density of 0.001
144 at 600 nm (OD₆₀₀=0.001, representing approximately 5×10⁵ colony forming
145 units [CFU] ml⁻¹). Bacteria were infiltrated into abaxial surfaces of 5-week-old
146 Arabidopsis leaves with a 1-ml needleless syringe. For quantification, leaf
147 samples were harvested with a 6-mm-diameter cork borer (Z165220; Merck-
148 Sigma-Aldrich), resulting in leaf discs with an area of 0.283 cm². Two leaf
149 discs per leaf were harvested as a single sample. For each condition, four
150 samples were collected immediately after infiltration as 'day 0' samples to
151 ensure no significant difference introduced by unequal infiltrations and six
152 samples were collected at 3 dpi as 'day 3' samples to compare the bacteria
153 growth between different genotypes, conditions and treatments. For 'day 0',
154 samples were ground in 200 µl of infiltration buffer and spotted (10 µl per spot)
155 on selective KB medium agar plates to grow for 48 h at 28°C. For 'day 3',
156 samples were ground in 200 µl of infiltration buffer, serially diluted (5, 50, 500,
157 5000 and 50000 times) and spotted (6 µl per spot) on selective KB medium
158 agar plates to grow for 48 h at 28°C. The number of colonies (CFU per drop)
159 was monitored and bacterial growth was represented as in CFU cm⁻² of leaf
160 tissue. All results are plotted using ggplot2 in R (Wickham, 2009), and
161 detailed statistics summary can be found in the supplemental materials.

162

163 HR assay in Arabidopsis

164 *Pseudomonas fluorescens* engineered with a type III secretion system (Pf0-1
165 'EtHAn' strains) expressing one of wild-type or mutant effectors, AvrRps4,
166 AvrRps4^{KRY135-138AAAA}, PopP2, PopP2^{C321A}, AvrRpt2 or pVSP61 empty vector
167 were grown on selective KB plates for 24 h at 28°C (Thomas *et al.*, 2009;
168 Sohn *et al.*, 2014). Bacteria were harvested from the plates, resuspended in

2019_JJPDI_SETI_paper [for BioRxiv and JXB]

169 infiltration buffer (10 mM MgCl₂) and the concentration was adjusted to
170 OD₆₀₀= 0.2 (10⁸ CFU ml⁻¹). The abaxial surfaces of 5-week-old Arabidopsis
171 leaves were hand infiltrated with a 1-ml needleless syringe. Cell death was
172 monitored 24 h after infiltration.

173

174 Electrolyte leakage assay

175 Either 50 µM E2 or DMSO were hand infiltrated in 5-week-old Arabidopsis
176 leaves with a 1-ml needleless syringe for electrolyte leakage assay. Leaf discs
177 were taken with a 2.4-mm-diameter cork borer from infiltrated leaves. Discs
178 were dried and washed in deionized water for 1 hour before being floated on
179 deionized water (15 discs per sample, three samples per biological replicate).
180 Electrolyte leakage was measured as water conductivity with a Pocket Water
181 Quality Meters (LAQUAtwin-EC-33; Horiba) at the indicated time points. All
182 results are plotted using ggplot2 in R (Wickham, 2009) , and detailed statistics
183 summary can be found in the supplemental materials.

184

185 Trypan blue staining

186 Either 50 µM E2 or DMSO were hand infiltrated in 5-week-old Arabidopsis
187 leaves with a 1-ml needleless syringe for trypan blue staining. 6 leaves per
188 sample were collected 24 hours after infiltration. Leaves were boiled in trypan
189 blue solution (1.25 mg/ml trypan blue dissolved in 12.5% glycerol, 12.5%
190 phenol, 12.5% lactic acid and 50% ethanol) in a boiling water bath for 1 min
191 and de-stained by chloral hydrate solution (2.5 g/ml). De-stained leaves were
192 mounted, and pictures were taken under on Leica fluorescent
193 stereomicroscope M165FC. All images were taken with identical settings at
194 2.5x magnification. Scale bar=0.5mm.

195

196 Gene expression measurement by reverse transcription-quantitative
197 polymerase chain reaction (RT-qPCR)

198 For gene expression analysis, RNA was isolated from 5-week-old Arabidopsis
199 leaves and used for subsequent RT-qPCR analysis. RNA was extracted with
200 Quick-RNA Plant Kit (R2024; Zymo Research) and treated with RNase-free
201 DNase (4716728001; Merck-Roche). Reverse transcription was carried out
202 using SuperScript IV Reverse Transcriptase (18090050; ThermoFisher

2019_JYPD_SETI_paper [for BioRxiv and JXB]

203 Scientific). qPCR was performed using a CFX96 TouchTM Real-Time PCR
204 Detection System. Primers for qPCR analysis of *Isochorismate Synthase1*
205 (*ICS1*), *Pathogenesis-Related1* (*PR1*), *AvrRps4* and *Elongation Factor 1*
206 (*EF1 α*) are listed in Table S4. Data were analyzed using the double
207 delta Ct method (Livak and Schmittgen, 2001). All results are plotted using
208 ggplot2 in R (Wickham, 2009), and detailed statistics summary can be found
209 in the supplemental materials.

210

211 Confocal laser scanning microscopy (CLSM) imaging
212 Transgenic plant materials were imaged with the Leica DM6000/TCS SP5
213 confocal microscopy (Leica Microsystems) for confirmation of expression of
214 inducible AvrRps4 fused with monomeric yellow-green fluorescent protein,
215 mNeonGreen or mNeon (Shaner *et al.*, 2013). Roots from 3-week-old
216 Arabidopsis seedlings were sprayed with 50 μ M E2 and imaged at 1 day post
217 spray. Fluorescence of mNeon was excited at 500 nm and detected at
218 between 520 and 540 nm. CLSM images of root cells from Arabidopsis
219 seedlings are recorded via the camera. The images were analyzed with the
220 Leica application Suite and Fiji software (Schindelin *et al.*, 2012).

221

222 Co-immunoprecipitation

223 Arabidopsis transgenic seedlings, and the background ecotype Col-0 grown
224 for 7 days after germination (DAG) were treated with 0.1% DMSO or 50 μ M E2
225 for 3 hours. Proteins from seedlings were extracted using IP buffer (10%
226 Glycerol, 50mM Tris-Cl pH 6.8, 50mM KCl, 1mM EDTA, 5Mm MgCl₂, 1% NP-
227 40, 10mM DTT, 1mM dATP). Crude extract of the seedlings was centrifuged
228 and supernatants were incubated with Anti-HA-conjugated beads (EZviewTM
229 Red Anti-HA Affinity Gel; E6779; Sigma). A small portion of supernatants
230 were taken for input samples. At 2 hours after incubation of the extract with
231 beads, beads were washed three times with IP buffer containing 0.1% NP-40.
232 Proteins bound to beads were eluted by boiling the beads with SDS sample
233 buffer. Immunoblotting of the input and eluted samples were performed as
234 described above.

235

236 **Results**

237 **RRS1 over-expression can compromise RPS1/RPS4 function**

238 Overexpression of *RPS4* leads to autoimmunity and dwarfism under standard
239 growth condition (see methods) (Heidrich *et al.*, 2013). This autoimmunity is
240 both temperature- and RRS1-dependent. In contrast, elevated expression of
241 *RRS1-R* from ecotype Ws-2 in Col-0, an ecotype expressing a dominant allele
242 of *RRS1-S*, does not trigger auto-immunity (Huh *et al.* 2017). Furthermore,
243 high level RRS1-R expression does not confer recognition of effector PopP2
244 (Fig 1). Overexpression of *RRS1* in an *RPS4* overexpression line attenuates
245 dwarfism and autoimmunity (Huh *et al.*, 2017). Here, we found that only
246 simultaneously over-expressing both *RRS1* and *RPS4* can lead to the gain-of-
247 recognition of PopP2 in the susceptible ecotype Col-0 (Fig 1). Thus, we
248 propose that a balanced protein expression of RRS1 and RPS4 is required for
249 both suppressing autoimmunity and functional recognition of the
250 corresponding effectors.

251

252 **A survey of leaf-expressed genes reveals promoters for moderate and**
253 **balanced expression levels of RRS1 and RPS4**

254 Genome-wide expression profiling has revealed numerous genes altered by
255 PTI alone or PTI plus ETI at early time points of RRS1/RPS4-mediated
256 immune activation (Sohn *et al.*, 2014). This analysis also enabled the
257 discovery of genes that are moderately and constitutively expressed without
258 changing their transcript abundance during immune activation. In plants, gene
259 expression patterns and levels are usually specified by their promoters. Based
260 on the endogenous expression relative transcript abundance in the 'stable
261 gene set', we selected six promoters with 'moderate' expression (Table S1).
262 We define the 'moderate' expression based on two criteria: (1) the gene
263 transcript abundance with those promoters are at least 100 times more than
264 the endogenous transcript abundance of *RRS1* and *RPS4*; (2) the gene
265 transcript abundance with those promoters is lower than that with the 35S
266 promoter. The selected genes encode proteins that are involved in essential
267 biological processes that we expect to be expressed in most mesophyll cells,
268 including a delta-tonoplast intrinsic protein (our name; At1, locus identifier
269 AT3G16240, protein symbol name TIP2-1), a ribosomal protein S16 (At2,
270 AT4G34620, RPS16-1), a cysteine synthase isomer CysC1 (At3, AT3G61440,

2019_JYPD_SETI_paper [for BioRxiv and JXB]

271 CYSC1), a photosystem II subunit Q (At4, AT4G21280, PSBQ1), a xyloglucan
272 endotransglucosylase/hydrolase 6 (At5, AT5G65730, XTH6), and a ubiquitin-
273 like protein 5 (At6, AT5G42300, UBL5) (Table S1).

274 To test the strength of the selected *Arabidopsis* promoters (pAt1-pAt6) for
275 driving gene expression *in planta*, constructs were designed and generated to
276 use them to express β -glucuronidase (GUS) (pAt:GUS). Agrobacterium
277 strains carrying each pAt:GUS construct was infiltrated in tobacco leaves with
278 the infiltration buffer as negative control and GUS expressed under the CaMV
279 35S promoter (35S:GUS) as positive control. GUS expressed under pAt4
280 shows similar level of activity to that with 35S, whereas GUS activities
281 detected from other pAt promoters are significantly weaker (Fig S1).

282

283 **A T-DNA construct expresses RPS4, RRS1 and inducible AvrRps4**

284 We designed a binary vector to reconstruct the effector ligand AvrRps4 and its
285 receptors RRS1 and RPS4, using the Golden Gate Modular Cloning Toolbox
286 (Fig 2A) (Engler *et al.*, 2014). We chose moderate and balanced promoters
287 pAt2 and pAt3 from our promoter survey experiment for expressing RRS1 and
288 RPS4, respectively. We have also cloned *RRS1-R* full-length coding
289 sequences (CDS) from Ws-2 and *RPS4* full-length CDS from Col-0 for the
290 expression of RRS1-R and RPS4 proteins. We chose synthetic C-terminal-
291 fusion epitope tags His₆-TEV-FLAG₃ (HF) and HA₆ for detecting RRS1 and
292 RPS4 protein expressions, respectively (Fig 2A, Table S2) (Gauss *et al.*, 2005;
293 Soleimani *et al.*, 2013). We have used an E2-inducible system for AvrRps4
294 expression (Zuo *et al.*, 2000). We named this multi-gene stacking binary
295 construct 'Super ETI', or SETI. All restriction enzyme sites for Bsal and Bpil in
296 modules for promoters, CDSs for genes or epitope tags and the terminators
297 were synonymously eliminated (Fig 2A, Table S1). More detailed information
298 for the cloning can be found in supplemental materials.

299 To verify the SETI construct, we used a transient expression system in
300 *Nicotiana benthamiana* by infiltrating Agrobacteria that deliver the SETI T-
301 DNA. Protein accumulation of RRS1-R-HF and RPS4-HA was detected (Fig
302 S2).

303

304 **The single-locus lines carrying the SETI T-DNA show inducible growth
305 arrest**

306 We generated transgenic *Arabidopsis* lines using the SETI construct
307 expressing wild-type *AvrRps4* (SETI_WT). With the FastRed selection module,
308 we have selected several positive SETI_WT lines (see Materials and Methods,
309 Table S2, Fig S3). We confirmed protein expression of RRS1-R-HF and
310 RPS4-HA (Fig 2B). We also tested the expression of inducible *AvrRps4*-
311 mNeon under fluorescence microscope upon the treatment with E2.
312 mNeonGreen signal was detected at 24 hours post spray on transgenic
313 seedlings, consistent with the mRNA accumulation of *AvrRps4* at 4 hours post
314 E2-infiltration in leaves (Fig 2D, Fig S2C). On E2-containing growth medium,
315 SETI_WT transgenic lines display severe growth arrest (Fig 2C, Fig S3). We
316 selected one of the lines (SETI_WT) for subsequent experiments (Fig 2C, Fig
317 S3).

318

319 **RRS1-R and RPS4 form pre-activation complexes in *Arabidopsis***

320 The SETI lines enable detection of epitope-tagged RRS1-R and RPS4 (Fig
321 2B). We investigated *in vivo* interaction of tagged RRS1-R and RPS4 by co-
322 immunoprecipitation (co-IP). When RPS4-HA was immunoprecipitated using
323 HA beads, we found RRS1-R and RPS4 stay in association with each other
324 both before and 3 hours after the induction of *AvrRps4* expression (Fig 3).
325 There were no significant differences of RRS1-R and RPS4 association upon
326 *AvrRps4* induction. While all previous studies in interactions of RRS1-R and
327 RPS4 was tested only using *N. benthamiana* transient expression system
328 (Huh *et al.*, 2017), generation of SETI line enabled the detection of RRS1-R
329 and RPS4 interaction in its native system in *Arabidopsis*.

330

331 **Some but not all defense responses are induced by E2 in SETI lines**

332 The induced expression of multiple effectors, such as *AvrRpt2*, *AvrRpm1* and
333 *ATR13* can induce cell death or named macroscopic HR in *Arabidopsis*
334 leaves (McNellis *et al.*, 1998; Tornero *et al.*, 2002; Allen *et al.*, 2004). We
335 therefore tested whether induced expression of *AvrRps4* can trigger
336 macroscopic HR in *Arabidopsis*. As seen in Fig 4A, no HR can be observed
337 after *AvrRps4* expression is induced in the SETI leaves. However, only the

2019_JYPD_SETI_paper [for BioRxiv and JXB]

338 expression of AvrRps4 but not mutant AvrRps4 (here referred to as
339 AvrRps4_KRVYmut_t, with residues KRVY 135 to 138 mutated into AAAA)
340 leads to electrolyte leakage (Fig 4C). We also observed slightly stronger
341 trypan blue stains in the SETI leaves treated with E2 compared to mock
342 treatment; suggesting that the expression of AvrRps4 causes microscopic or
343 weak but not macroscopic or strong HR in contrast to other known inducible
344 effector lines (Fig 4B).

345 Salicylic acid induction is another hallmark of ETI (Castel *et al.*, 2019).
346 Enzymes such as Isochorismate Synthase 1 (ICS1), Enhanced Disease
347 Susceptibility 5 (EDS5) and AvrPphB Susceptible 3 (PBS3) are involved in the
348 biosynthesis of salicylic acid and the expression of these genes is also highly
349 induced during ETI (Sohn *et al.*, 2014). The expression of *ICS1* after the
350 *AvrRps4* induction was tested by quantitative real-time PCR. *ICS1* was highly
351 induced 4 hours after the induction of *AvrRps4* by E2 (Fig 5A). In contrast,
352 *Pathogenesis-Related protein 1 (PR1)* was highly induced only 8 hours after
353 the induction of *AvrRps4* (Fig 5B). This shows that ETI triggered by
354 RRS1/RPS4 is sufficient for the induction of *ICS1* and the biosynthesis of
355 salicylic acid, which subsequently leads to expression of *PR1*.

356 Activation of mitogen-activated protein kinases (MAPKs) by PTI has been
357 reported under many cases and happens within a few minutes of the
358 activation of PTI. However, the activation of MAPKs by ETI is slower and lasts
359 longer than PTI-induced MAPK activation (Tsuda *et al.* 2013). We tested
360 whether the induced expression of *AvrRps4* can lead to MAPK activation in
361 SETI_WT and control lines. In contrast to AvrRpt2-inducible transgenic
362 Arabidopsis plants, induced expression of *AvrRps4* does not activate MAPKs
363 (Fig 5C) (Tsuda *et al.* 2013).

364 We further tested if the induction of ETI would elevate resistance. We
365 infiltrated the leaves with E2 or mock solution one day before we infiltrated
366 plants with *Pst* DC3000 (Materials and Methods). SETI_WT plants pre-treated
367 with E2 are more resistant to the bacteria than those pre-treated with mock,
368 while there was no significant difference between E2 and mock pre-treatment
369 in Col-0 (Fig 6).

370

371 **Discussion**

2019_JYPD_SETI_paper [for BioRxiv and JXB]

372 To facilitate studying the functional complex of *RRS1* and *RPS4* *in vivo*, we
373 generated an expression construct of E2-inducible *AvrRps4* stacked with
374 epitope-tagged *RRS1* and *RPS4*. To achieve balanced expression levels
375 higher than endogenous expression of *RRS1* and *RPS4*, we surveyed
376 constitutively expressed gene promoters. Here, we report 6 new and tested
377 promoter modules that are compatible with the Golden Gate Modular Cloning
378 toolkit. We used two of the promoters to express *RRS1* and *RPS4*, and we
379 avoided autoimmunity induced by excessive expression of *RPS4* or non-
380 recognition of PopP2 cause by excessive expression of *RRS1-R*. We were
381 also able to generate inducible *AvrRps4* expression to activate *RRS1/RPS4*-
382 mediated ETI under the control of E2 treatment. We thus were able to stack
383 genes for inducible expression of a pathogen effector and its NLR receptors in
384 one construct. In addition, with the epitope tags, we are able to monitor
385 effector-dependent changes in the NLR proteins without interference from
386 using a pathogen effector-delivery system. We could thus express any
387 effectors or pathogen ligands that will trigger immunity in plant cells with the
388 E2-inducible module, and their immune receptors using the same gene
389 stacking strategy.

390 There are multiple advantages to enabling investigation of ETI without the
391 complication of co-activating PTI. Firstly, we could test the contribution of
392 other genes to ETI activation by introducing mutants into the SETI
393 background, either using conventional crossing or using genome-editing such
394 as CRISPR/Cas9. These lines can also help investigating downstream
395 signaling from plant NLRs. Multiple forward genetic screens have been
396 conducted, but few novel components have been found, and most mutations
397 are either in the NLRs or regulatory elements rather than signaling
398 components (van Wersch *et al.*, 2016). Another plausible explanation is that
399 the signaling path downstream of plants NLRs is very short, but this is
400 debatable, because several significant steps are required for immunity. EDS1,
401 PAD4 and SAG101 are required for TIR-NLR signaling (Falk *et al.*, 1999;
402 Gantner *et al.*, 2019). NRC family proteins in Solanaceae species required for
403 many NLRs, and NRG1/ADR1s in *Arabidopsis* required for TIR-NLRs and
404 ADR1s for some CC-NLRs (Bonardi *et al.*, 2011; Dong *et al.*, 2016; Wu *et al.*,
405 2017, 2019; Castel *et al.*, 2019). NRG1s and ADR1s seem to function

2019_JJPD_SETI_paper [for BioRxiv and JXB]

406 downstream of EDS1 and may function distinctly with SAG101 and PAD4,
407 respectively (Lapin *et al.*, 2019). SETI lines carry heterologously expressed
408 RRS1-R/RPS4 and also endogenous RRS1-S/RPS4, RRS1B/RPS4B, which
409 together provide three redundant copies of NLR pairs that can recognize
410 AvrRps4. In theory, in an EMS-mutagenesis forward genetic screen to identify
411 suppressors of immunity induced by AvrRps4, there should be a reduced
412 background of mutations in the receptor(s), improving prospects to reveal
413 mutations in genes that are functionally important in NLR signaling and
414 regulation.

415 With SETI, we are able to assess pure ETI response mediated by the TIR-
416 NLRs, RRS1 and RPS4. E2 induction provoked rapid transcriptional changes
417 in activation of defense genes and also ion leakage. AvrRps4-induced ETI
418 enhanced resistance against bacterial pathogens. However, neither MAPK
419 activation nor macroscopic HR, in contrast to other inducible ETI examples
420 (Tornero *et al.*, 2002; Tsuda *et al.*, 2013). This indicates that outputs of plant
421 NLRs might differ. Both TIR and CC domains alone are sufficient to activate
422 plant immunity. However, whether they signal through similar or different
423 downstream components is still unknown.

424 In diverse multicellular eukaryotes, immune complexes are assembled into
425 oligomeric complexes to signal downstream. The mammalian inflammasome,
426 assembled in response to bacterial peptide recognition by NAIP proteins and
427 subsequent activation and binding of NLRC4 proteins, is a classic example
428 (Zhang *et al.*, 2015). The plant CC NLR ZAR1 forms an effector-dependent
429 resistosome, which is a pentamer of ZAR1 assembled together with cofactors
430 PBL2 and RKS1 (Wang *et al.*, 2019). The structure of TIR domains implies
431 that activation might require the disassociation of the RRS1 and RPS4 TIR
432 domains and the oligomerization of RPS4 TIR domains (Williams *et al.*, 2014).
433 In SETI lines, RRS1 and RPS4 form a pre-activation complex in the absence
434 of pathogen effector. However, co-IP data cannot distinguish the ratio of
435 which RRS1 and RPS4 bind to each other. It will be interesting to check via
436 various non-denaturing methods if RRS1-R and RPS4 form a dimer or a
437 higher order oligomerization *in vivo*, or whether there is a conformational
438 change in complex upon effector recognition. Furthermore, with the SETI lines

2019_JYPD_SETI_paper [for BioRxiv and JXB]

439 generated in this study, we can ask what other co-factors are required for the
440 activation of RRS1-R and RPS4 at native conditions.

441 The availability of SETI lines also will enable us to study how PTI and ETI
442 interact with each other, especially in the context of RRS1- and RPS4-
443 mediated immunity. Some models have been proposed in discussing on this
444 topic (Tsuda *et al.*, 2009; Cui *et al.*, 2015). From the zig-zag model, PTI and
445 ETI holds in different threshold on activating immunity (Jones and Dangl,
446 2006). With SETI line, we could specifically ask how physically PTI and ETI
447 can influence each other. A lot of evidence shows that the PTI receptors
448 PRRs usually have very specific post-translational modification events at early
449 time points, there is also some evidence showing ETI can activate somewhat
450 overlapping but different PTMs on immune-related proteins (Withers and
451 Dong, 2017; Kadota *et al.*, 2019). It will be interesting to know how the
452 activation of RRS1/PRS4 leads to the changes of PTMs and how those
453 changes contribute to the robustness of immunity. In addition, transcriptional
454 changes are not the only process reported as the early changes of ETI but
455 also the changes in translations (Meteignier *et al.*, 2017; Xu *et al.*, 2017). Both
456 work using inducible AvrRpm1 or AvrRpt2 reveal interesting observations on
457 trade-off between defense and growth, and the specific regulatory element in
458 the genome (Meteignier *et al.*, 2017; Xu *et al.*, 2017). Both effectors are
459 recognized by CC-type NLRs, so it will be interesting to know what changes in
460 translations will be induced by TIR-NLRs using SETI line. One can also use
461 proteomics tools to generate complex information using inducible SETI to fish
462 for ETI-specific interaction networks.

463 Recently it has been shown plant NLRs can also form higher order protein
464 complex, similar to inflammasome in mammalian immune system. However, it
465 is unknown if all plant NLRs form the same kind of complex or using the same
466 mechanism to activate defense. It was noted that NLRs have evolved to
467 partner with other NLRs to function genetically, but if this model is also true
468 biochemically is still unknown (Adachi *et al.*, 2019). Unlike ZAR1, RRS1 and
469 RPS4 requires each other to function, and they localized and function
470 exclusively in the nuclei but not the cell membrane, so it will be interesting to
471 compare them once the transmission electron cryomicroscopic (Cryo-EM)
472 structure of RRS1 and RPS4 complex is resolved. SETI line could be a very

2019_JJPD_SETI_paper [for BioRxiv and JXB]

473 good toolkit to make mutagenesis to verify the function based on the structural
474 information.

475 We have observed the activation of ETI alone in the absence of pathogens is
476 sufficient to prime the resistance against bacterial pathogens in *Arabidopsis*
477 (Fig 6). Previously, we have reported a group of upregulated genes at the
478 early time point of activation of RRS1-R/RPS4 are related to salicylic acid
479 pathway, so it will be interesting to know if the elevated or primed resistance
480 against bacteria induced in SETI lines are due to the activation of salicylate
481 pathway (Sohn *et al.*, 2014).

482 Another major question regarding the signaling pathways is that SAG101 and
483 PAD4 seems to be redundant but functionally equivalent to EDS1 (Wagner *et*
484 *al.*, 2013; Lapin *et al.*, 2019). They also have been shown to be genetically
485 linked to helper NLRs NRG1s and/or ADR1s to function (Castel *et al.*, 2019;
486 Wu *et al.*, 2019). Using SETI line, one can test their function more specifically
487 in ETI in the absence of PTI and many other unwanted pathogen
488 interferences.

489

490 **Acknowledgements**

491 We thank the Gatsby Foundation (United Kingdom) for funding to the JDGJ
492 laboratory. BN was supported by the Norwich Research Park (NRP)
493 Biosciences Doctoral Training Partnership (DTP) from the Biotechnology and
494 Biological Sciences Research Council (BBSRC) (grant agreement
495 BB/M011216/1). HA were supported by European Research Council
496 Advanced Grant “ImmunitybyPairDesign”. PD acknowledges support from the
497 European Union’s Horizon 2020 Research and Innovation Program under
498 Marie Skłodowska-Curie Actions (grant 656243) and a Future Leader
499 Fellowship from BBSRC (BB/R012172/1). AR acknowledge support from the
500 EMBO Long Term Fellowship (ALTF-842-2015). HB was supported by the
501 NRP DTP funding from BBSRC (grant agreement BB/M011216/1). YM were
502 supported by Biotechnology and Biological Sciences Research Council
503 (BBSRC) Grant BB/M008193/1. LT, MY and JDGJ were supported by the
504 Gatsby Foundation funding to the Sainsbury Laboratory.

505

506 **References**

2019_JJPD_SETI_paper [for BioRxiv and JXB]

507 **Adachi H, Derevnina L, Kamoun S.** 2019. NLR singletons, pairs, and networks:
508 evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of
509 plants. *Current Opinion in Plant Biology* **50**, 121–131.

510 **Allen RL, Bittner-Eddy PD, Grenville-Briggs LJ, Meitz JC, Rehmany AP, Rose
511 LE, Beynon JL.** 2004. Host-parasite coevolutionary conflict between *Arabidopsis*
512 and downy mildew. *Science* **306**, 1957–1960.

513 **Bonardi V, Tang S, Stallmann A, Roberts M, Cherkis K, Dangl JL.** 2011.
514 Expanded functions for a family of plant intracellular immune receptors beyond
515 specific recognition of pathogen effectors. *Proceedings of the National Academy of
516 Sciences of the United States of America* **108**, 16463–16468.

517 **Castel B, Ngou P-M, Cevik V, Redkar A, Kim D-S, Yang Y, Ding P, Jones JDG.**
518 2019. Diverse NLR immune receptors activate defence via the RPW8-NLR NRG1.
519 *The New Phytologist* **222**, 966–980.

520 **Cui H, Tsuda K, Parker JE.** 2015. Effector-triggered immunity: from pathogen
521 perception to robust defense. *Annual review of plant biology* **66**, 487–511.

522 **Dong OX, Tong M, Bonardi V, El Kasmi F, Woloshen V, Wünsch LK, Dangl JL,
523 Li X.** 2016. TNL-mediated immunity in *Arabidopsis* requires complex regulation of the
524 redundant ADR1 gene family. *The New Phytologist* **210**, 960–973.

525 **Engler C, Youles M, Gruetzner R, Ehnhert T-M, Werner S, Jones JDG, Patron NJ,
526 Marillonnet S.** 2014. A golden gate modular cloning toolbox for plants. *ACS
527 synthetic biology [electronic resource]* **3**, 839–843.

528 **Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE.** 1999. EDS1, an
529 essential component of R gene-mediated disease resistance in *Arabidopsis* has
530 homology to eukaryotic lipases. *Proceedings of the National Academy of Sciences of
531 the United States of America* **96**, 3292–3297.

532 **Gantner J, Ordon J, Kretschmer C, Guerois R, Stuttmann J.** 2019. An EDS1-
533 SAG101 Complex is Essential for TNL-mediated Immunity in *Nicotiana benthamiana*.
534 *The Plant Cell*.

535 **Gauss R, Trautwein M, Sommer T, Spang A.** 2005. New modules for the repeated
536 internal and N-terminal epitope tagging of genes in *Saccharomyces cerevisiae*. *Yeast*
537 **22**, 1–12.

538 **Heidrich K, Tsuda K, Blanvillain-Baufumé S, Wirthmueller L, Bautista J, Parker
539 JE.** 2013. *Arabidopsis* TNL-WRKY domain receptor RRS1 contributes to
540 temperature-conditioned RPS4 auto-immunity. *Frontiers in plant science* **4**, 403.

541 **Huh SU, Cevik V, Ding P, Duxbury Z, Ma Y, Tomlinson L, Sarris PF, Jones JDG.**
542 2017. Protein-protein interactions in the RPS4/RRS1 immune receptor complex.
543 *PLoS Pathogens* **13**, e1006376.

544 **Jones JDG, Dangl JL.** 2006. The plant immune system. *Nature* **444**, 323–329.

545 **Kadota Y, Liebrand TWH, Goto Y, et al.** 2019. Quantitative phosphoproteomic
546 analysis reveals common regulatory mechanisms between effector- and PAMP-
547 triggered immunity in plants. *The New Phytologist* **221**, 2160–2175.

548 **Lapin D, Kovacova V, Sun X, et al.** 2019. A coevolved EDS1-SAG101-NRG1

2019_JJPDI_SETI_paper [for BioRxiv and JXB]

549 module mediates cell death signaling by TIR-domain immune receptors. *BioRxiv*.

550 **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using
551 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–
552 408.

553 **McNellis TW, Mudgett MB, Li K, Aoyama T, Horvath D, Chua NH, Staskawicz BJ.**
554 1998. Glucocorticoid-inducible expression of a bacterial avirulence gene in
555 transgenic *Arabidopsis* induces hypersensitive cell death. *The Plant Journal: for Cell*
556 and *Molecular Biology* **14**, 247–257.

557 **Meteignier L-V, El Oirdi M, Cohen M, Barff T, Matteau D, Lucier J-F, Rodrigue S,**
558 **Jacques P-E, Yoshioka K, Moffett P.** 2017. Translatome analysis of an NB-LRR
559 immune response identifies important contributors to plant immunity in *Arabidopsis*.
560 *Journal of Experimental Botany* **68**, 2333–2344.

561 **Narusaka M, Shirasu K, Noutoshi Y, Kubo Y, Shiraishi T, Iwabuchi M, Narusaka**
562 **Y.** 2009. RRS1 and RPS4 provide a dual Resistance-gene system against fungal and
563 bacterial pathogens. *The Plant Journal: for Cell and Molecular Biology* **60**, 218–226.

564 **Porter K, Shimono M, Tian M, Day B.** 2012. *Arabidopsis* Actin-Depolymerizing
565 Factor-4 links pathogen perception, defense activation and transcription to
566 cytoskeletal dynamics. *PLoS Pathogens* **8**, e1003006.

567 **Schindelin J, Arganda-Carreras I, Frise E, et al.** 2012. Fiji: an open-source
568 platform for biological-image analysis. *Nature Methods* **9**, 676–682.

569 **Shaner NC, Lambert GG, Chammas A, et al.** 2013. A bright monomeric green
570 fluorescent protein derived from *Branchiostoma lanceolatum*. *Nature Methods* **10**,
571 407–409.

572 **Sohn KH, Segonzac C, Rallapalli G, Sarris PF, Woo JY, Williams SJ, Newman**
573 **TE, Paek KH, Kobe B, Jones JDG.** 2014. The nuclear immune receptor RPS4 is
574 required for RRS1SLH1-dependent constitutive defense activation in *Arabidopsis*
575 *thaliana*. *PLoS Genetics* **10**, e1004655.

576 **Sohn KH, Zhang Y, Jones JDG.** 2009. The *Pseudomonas syringae* effector protein,
577 AvrRPS4, requires in planta processing and the KRVY domain to function. *The Plant*
578 *Journal: for Cell and Molecular Biology* **57**, 1079–1091.

579 **Soleimani VD, Palidwor GA, Ramachandran P, Perkins TJ, Rudnicki MA.** 2013.
580 Chromatin tandem affinity purification sequencing. *Nature Protocols* **8**, 1525–1534.

581 **Thomas WJ, Thireault CA, Kimbrel JA, Chang JH.** 2009. Recombineering and
582 stable integration of the *Pseudomonas syringae* pv. *syringae* 61 *hrp/hrc* cluster into
583 the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1. *The Plant Journal:*
584 *for Cell and Molecular Biology* **60**, 919–928.

585 **Tornero P, Chao RA, Luthin WN, Goff SA, Dangl JL.** 2002. Large-scale structure-
586 function analysis of the *Arabidopsis* RPM1 disease resistance protein. *The Plant Cell*
587 **14**, 435–450.

588 **Tsuda K, Mine A, Bethke G, Igarashi D, Botanga CJ, Tsuda Y, Glazebrook J,**
589 **Sato M, Katagiri F.** 2013. Dual regulation of gene expression mediated by extended
590 MAPK activation and salicylic acid contributes to robust innate immunity in
591 *Arabidopsis thaliana*. *PLoS Genetics* **9**, e1004015.

2019_JJPD_SETI_paper [for BioRxiv and JXB]

592 **Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F.** 2009. Network properties
593 of robust immunity in plants. *PLoS Genetics* **5**, e1000772.

594 **Wagner S, Stuttmann J, Rietz S, Guerois R, Brunstein E, Bautor J, Niefind K,**
595 **Parker JE.** 2013. Structural basis for signaling by exclusive EDS1 heteromeric
596 complexes with SAG101 or PAD4 in plant innate immunity. *Cell Host & Microbe* **14**,
597 619–630.

598 **Wang J, Hu M, Wang J, Qi J, Han Z, Wang G, Qi Y, Wang H-W, Zhou J-M, Chai J.**
599 2019. Reconstitution and structure of a plant NLR resistosome conferring immunity.
600 *Science* **364**.

601 **van Wersch R, Li X, Zhang Y.** 2016. Mighty dwarfs: arabidopsis autoimmune
602 mutants and their usages in genetic dissection of plant immunity. *Frontiers in plant*
603 *science* **7**, 1717.

604 **Wickham H.** 2009. *ggplot2 - Elegant Graphics for Data Analysis* . New York, NY:
605 Springer New York.

606 **Williams SJ, Sohn KH, Wan L, et al.** 2014. Structural basis for assembly and
607 function of a heterodimeric plant immune receptor. *Science* **344**, 299–303.

608 **Withers J, Dong X.** 2017. Post-translational regulation of plant immunity. *Current*
609 *Opinion in Plant Biology* **38**, 124–132.

610 **Wu C-H, Abd-El-Haliem A, Bozkurt TO, Belhaj K, Terauchi R, Vossen JH,**
611 **Kamoun S.** 2017. NLR network mediates immunity to diverse plant pathogens.
612 *Proceedings of the National Academy of Sciences of the United States of America*
613 **114**, 8113–8118.

614 **Wu Z, Li M, Dong OX, Xia S, Liang W, Bao Y, Wasteneys G, Li X.** 2019.
615 Differential regulation of TNL-mediated immune signaling by redundant helper CNLs.
616 *The New Phytologist* **222**, 938–953.

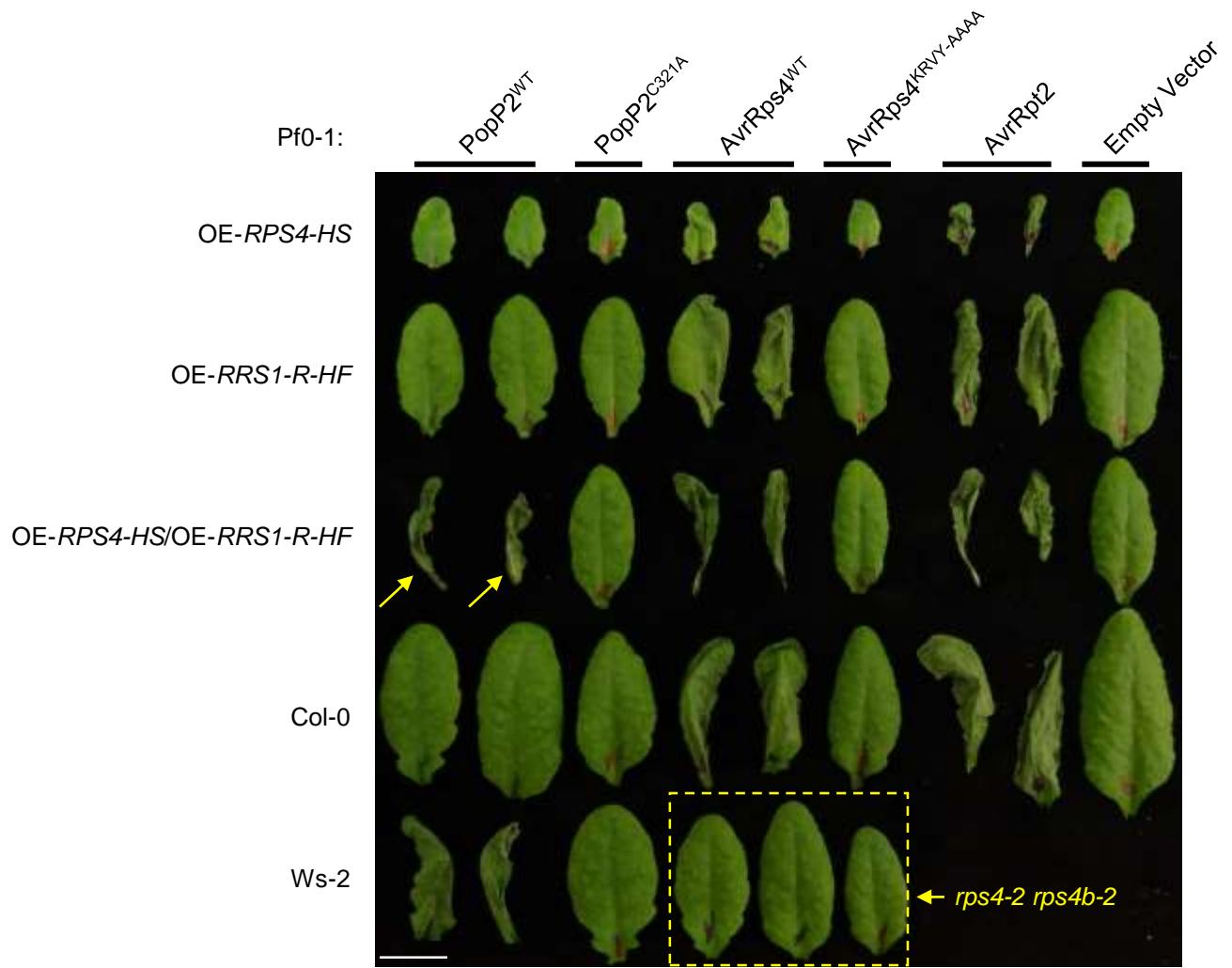
617 **Xu G, Greene GH, Yoo H, Liu L, Marqués J, Motley J, Dong X.** 2017. Global
618 translational reprogramming is a fundamental layer of immune regulation in plants.
619 *Nature* **545**, 487–490.

620 **Zhang L, Chen S, Ruan J, et al.** 2015. Cryo-EM structure of the activated NAIP2-
621 NLRC4 inflammasome reveals nucleated polymerization. *Science* **350**, 404–409.

622 **Zhang Z-M, Ma K-W, Gao L, Hu Z, Schwizer S, Ma W, Song J.** 2017. Mechanism
623 of host substrate acetylation by a YopJ family effector. *Nature Plants* **3**, 17115.

624 **Zuo J, Niu Q-W, Chua N-H.** 2000. An estrogen receptor-based transactivator XVE
625 mediates highly inducible gene expression in transgenic plants. *The Plant Journal: for Cell and Molecular Biology* **24**, 265–273.

627



*Fig. 1. Over-expression of RPS4 and RRS1-R reconstruct the recognition of PopP2 in Col-0. Arabidopsis transgenic lines overexpressing RPS4 (OE-RPS4-HS), RRS1-R (OE-RRS1-R-HF) or both generated by crossing (OE-RPS4-HS/OE-RRS1-R-HF) in the Col-0 background, with Col-0 and Ws-2 accession were tested for hypersensitive response (HR). 5-week old leaves were infiltrated with *Pseudomonas* fluorescence (Pf) 0-1 strains carrying empty vector (EV), wild-type (WT) AvrRps4, mutant AvrRps4^{KRVY-AAAA}, WT PopP2, mutant PopP2^{C321A}, and WT AvrRpt2. Leaves were collected 1 day post infiltration (dpi) for imaging. Scale bar = 1cm. Yellow arrows indicate reconstructed PopP2 recognition of Col-0 background overexpressing RRS1-R and RPS4. Yellow dashed box highlights loss of AvrRps4 recognition in the double mutant rps4-2 rps4b-2. Infiltration of EV and AvrRpt2 serve as negative and positive controls of HR, respectively.*

Figure 2

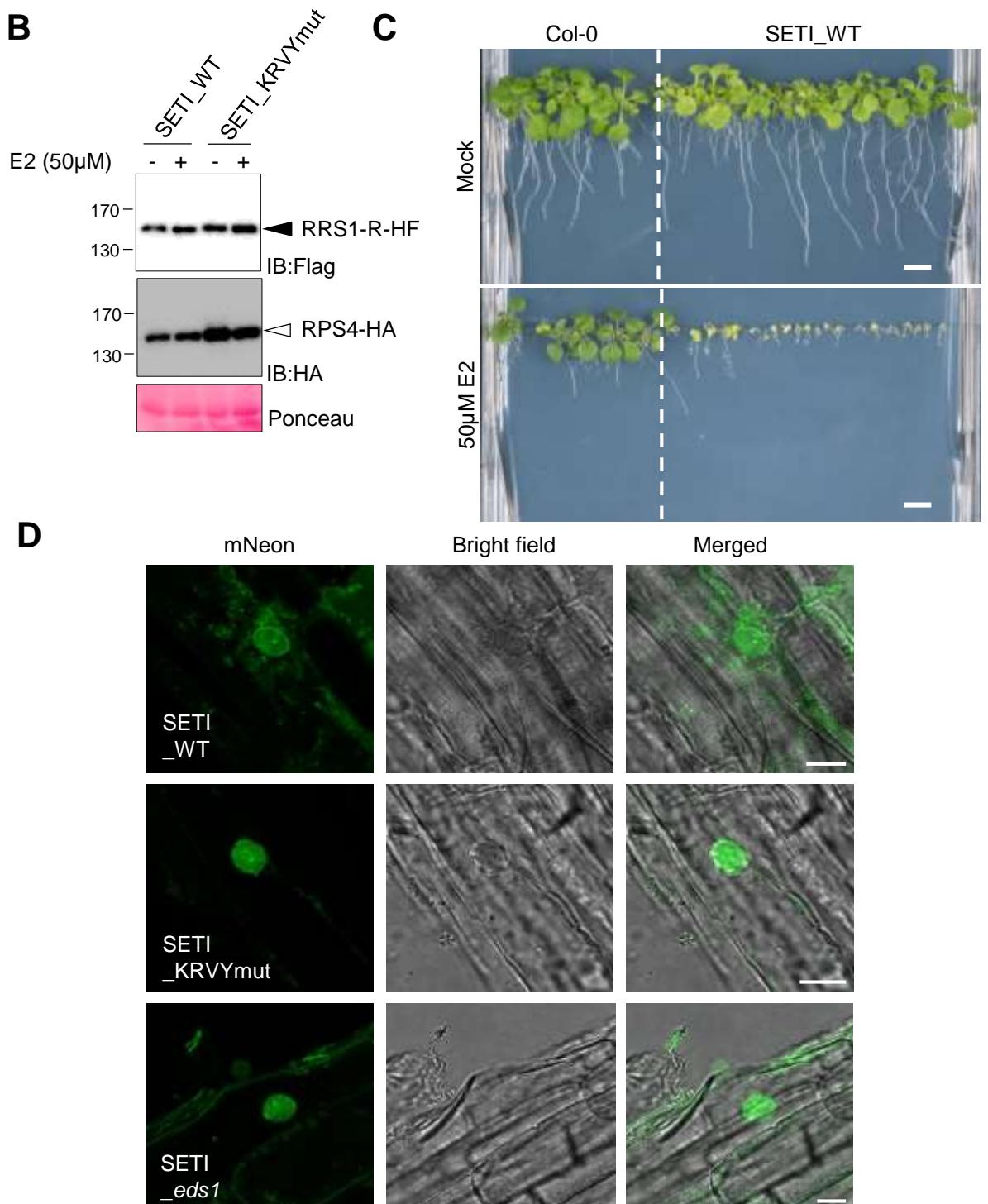
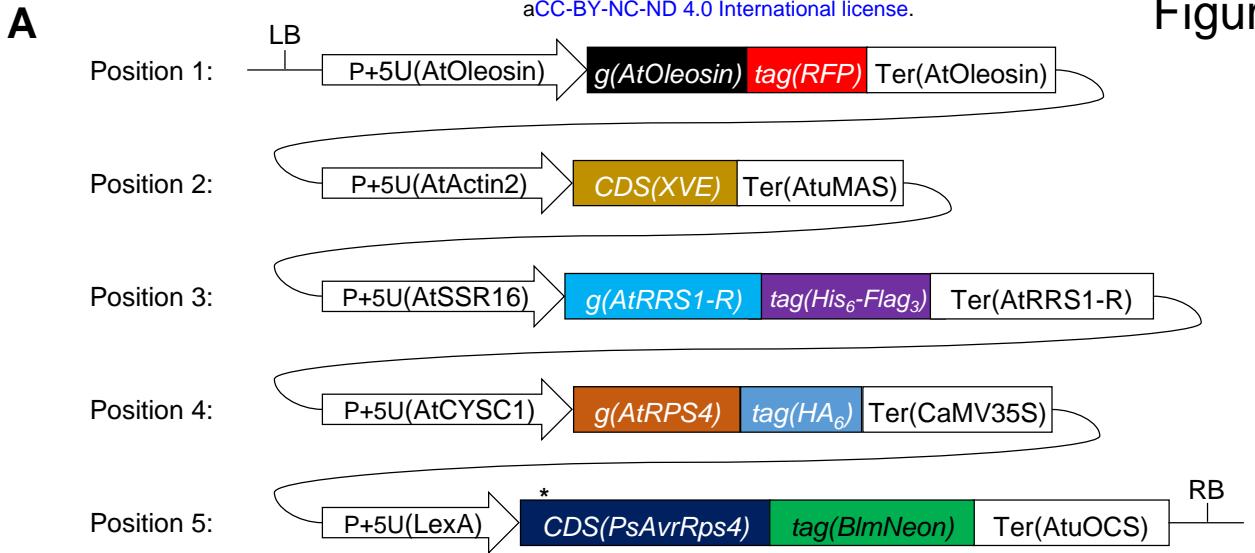


Fig 2. Single T-DNA expresses RRS1-R-HF, RPS4-HA and inducible wild-type AvrRps4 or AvrRps4 mutant variants

(A) Illustrative layout of the SUPER-ETI (SETI) construct. There are five individual expression units or Golden Gate Level 2 positional components listed, which are indicated position 1 to position 5. Position 1; expression unit of the FastRed selection marker (Shimada et al. 2010). Position 2, 5; chimeric transactivator XVE (LexA-VP16-ER) and the corresponding LexA inducible system to express AvrRps4 or its mutant variants under the control of β -estradiol (E2) treatment. Position 3, 4; full-length RRS1-R and RPS4 proteins with epitope tags His₆-Flag₃ and HA₆, respectively. All cloning details can be found in Methods and Materials. All individual units used for construct assembly can be found in the Supplemental Table 2 and 3.

(B) Protein accumulation of RRS1-R-HF (IB:Flag, black arrowhead) and RPS4-HA (IB:HA, white arrowhead) of SETI lines expressing AvrRps4 (SETI_WT) or mutant AvrRps4 KRVY-AAAA (SETI_KRVYmut). Seedlings were grown in liquid culture and induced with 50 μ M E2 for 2 hours at 7 days after germination (DAG). Ponceau staining of Rubisco large subunits were used as loading control.

(C) Seedling phenotype of SETI Arabidopsis transgenic line at 14 DAG in GM media containing Mock (0.1% DMSO) or 50 μ M E2. Col-0 was sown as control for the effect of E2 on seedling growth. Scale bar = 0.5cm

(D) Confocal images of SETI_WT, SETI_KRVYmut, SETI_eds1 root cells expressing AvrRps4-mNeon and AvrRps4^{KRVY-AAAA}-mNeon induced by 50 μ M E2 for 24h. mNeon channel shows nucleo-cytoplasmic localization of AvrRps4-mNeon and AvrRps4^{KRVY-AAAA}-mNeon. Bright field channel and merged image of mNeon and Bright field channel are shown together. Bars = 10 μ m.

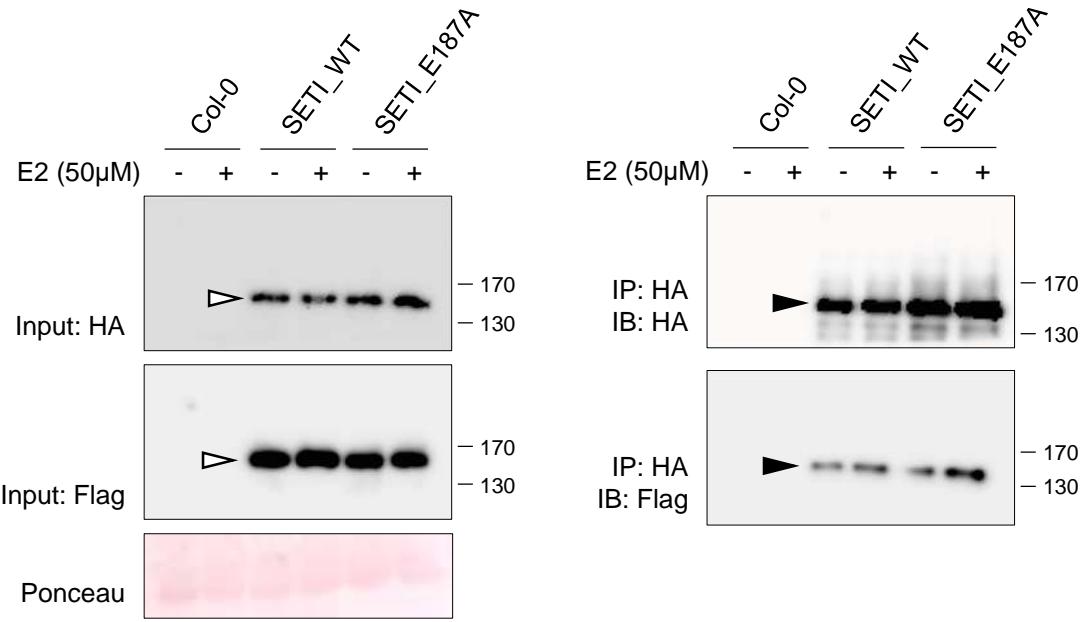


Fig 3. RRS1-R and RPS4 interact *in vivo*.

Co-immunoprecipitation of RRS1-R-HF with RPS4-HA. Col-0, SETI_WT, and SETI_E187A seedlings at DAG7 were treated with 50 μ M E2 for 3hours. Crude extracts were centrifuged and RPS4-HA proteins were immunoprecipitated with Anti-HA-conjugated beads. Immunoprecipitation of RPS4-HA, and co-immunoprecipitation of RRS1-R-HF were determined by immunoblot analysis with HA (IB:HA) or Flag (IB:Flag). Ponceau staining indicates equal loading of the input samples. RRS1-R-HF (black arrowhead), and RPS4-HA (white arrowhead) are indicated.

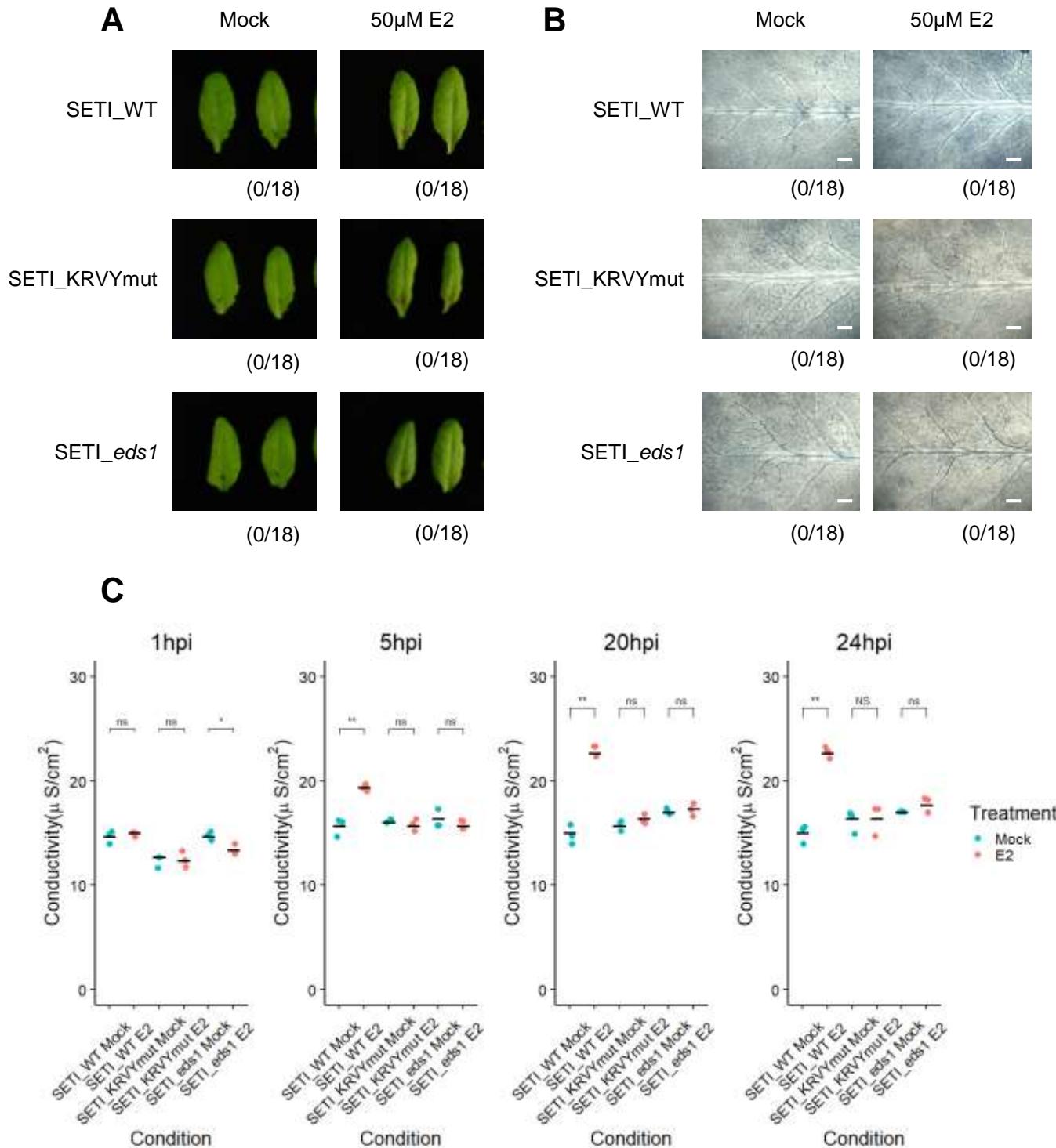


Fig. 4. Induced expression of AvrRps4 in *Arabidopsis* cause microscopic but not macroscopic cell death.

(A) HR phenotype assay in *Arabidopsis*. 5-week old SETI_WT, SETI_KRVYmut and SETI_eds1 leaves were infiltrated with Mock (1% DMSO) or 50 μ M E2. Images were taken at 1dpi. Numbers indicate the number of leaves displaying cell death from the total number of infiltrated leaves (18 for each genotype and treatment).

(B) Trypan blue staining. 5-week old SETI_WT, SETI_KRVYmut and SETI_eds1 were infiltrated with Mock (1% DMSO) or 50 μ M E2. Leaves were stained with trypan blue solution at 1dpi. After destaining, leaves were imaged using stereoscopic microscope. Scale bar = 0.5mm

(C) Electrolyte leakage assay. 5-week old SETI_WT, SETI_KRVYmut and SETI_eds1 leaves were infiltrated with Mock (1% DMSO) or 50 μ M E2. Fifteen leaf discs were collected for each data point. Conductivity was measured at 1, 5, 20 and 24 hours post infiltration (hpi). Each data point represents one technical replicate and three technical replicates are included per treatment and genotype for one biological replicate. Black line represents the mean of the technical replicates. This experiment was repeated three times independently with similar results (Supplemental Figure 2). Significant differences relative to the mock treatment in each genotype was calculated with t-test and the P-values are indicated as ns (non-significant), P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 5

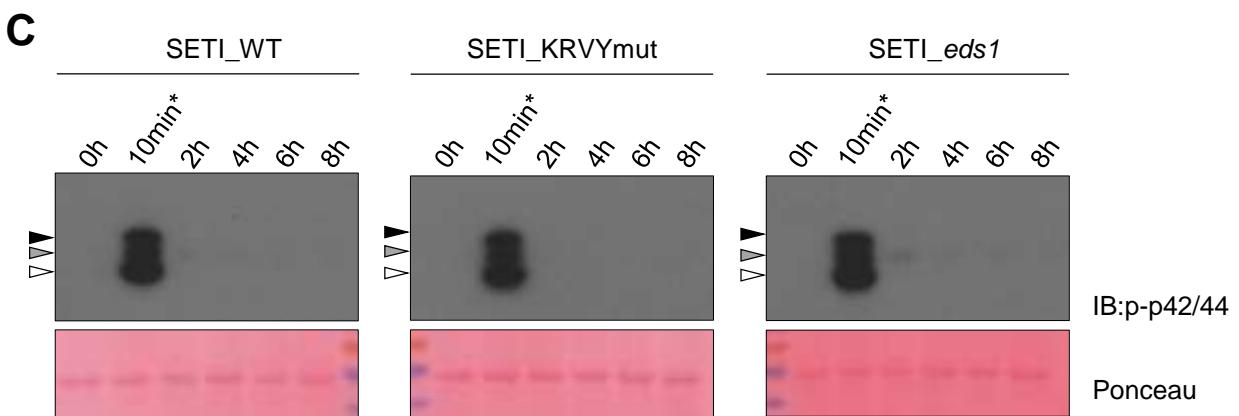
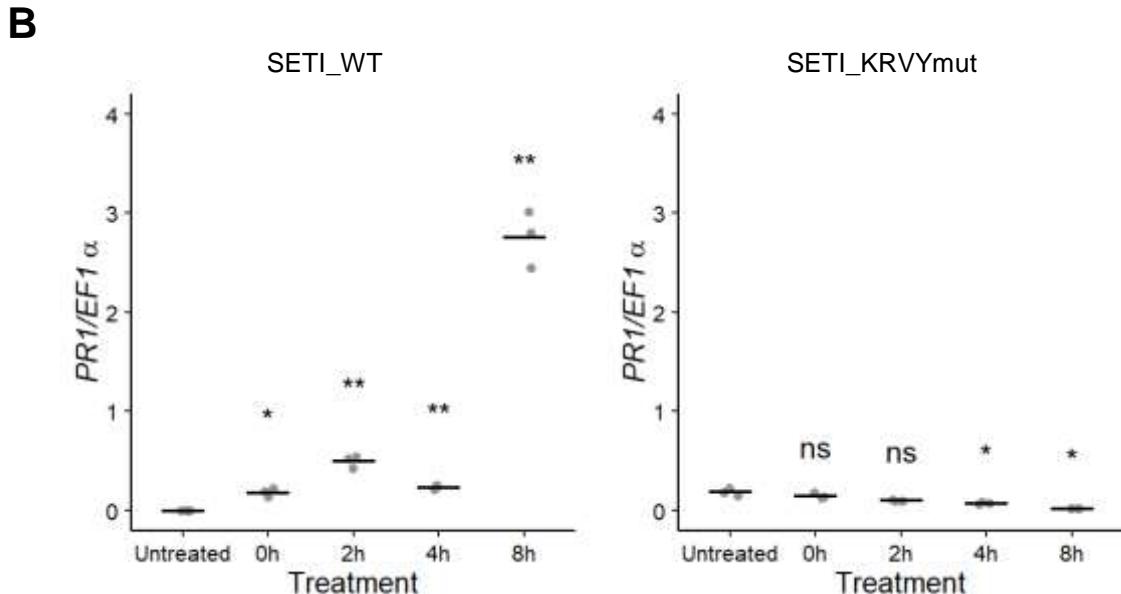
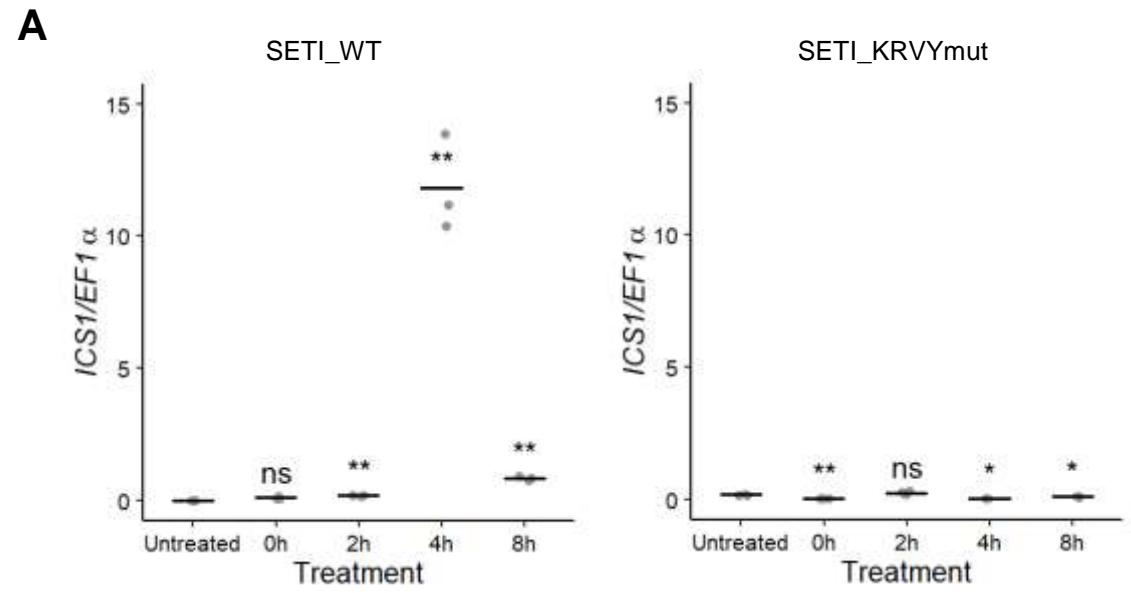


Fig. 5. Induced expression of AvrRps4 in *Arabidopsis* leads to *ICS1* and *PR1* expression, but not MAPK activation.

(A and B) *ICS1* (A) and *PR1* (B) expression after induction with E2 for 2, 4, and 8h in SETI (left panel) and SETI_KRVYmut (right panel) leaf samples. 5-week old SETI and SETI_KRVYmut leaves were infiltrated with 50 μ M E2. Samples were collected at 0, 2, 4 and 8hpi for RNA extraction and subsequent qPCR. Expression level is presented as relative to *EF1 α* expression. Each data point represents one technical replicate. Black line represents the mean of the technical replicates. This experiment was repeated three times independently with similar results. Significant differences relative to the Untreated samples was calculated with t-test and the P-values are indicated as ns (non-significant), $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(C) Activation of MAP kinases in SETI_WT, SETI_KRVYmut and SETI_eds1 seedlings by E2-induction of effector AvrRps4 or mutant AvrRps4^{KRVY-AAAA}. Seedlings grown in liquid culture at 7 dag were treated with 50 μ M E2 for indicated time points (0, 2, 4, 6, 8h) and collected for samples. SETI_WT, SETI_KRVYmut and SETI_eds1 seedlings treated with 100nM flg22 for 10 minutes (10min*) were used as positive control. Proteins were extracted from these seedlings and phosphorylated MAP kinases were detected using p-p42/44 antibodies. Arrowheads indicate phosphorylated MAP kinases (black; pMPK6, grey; pMPK3, white; pMPK4/11). Ponceau staining were used as loading control.

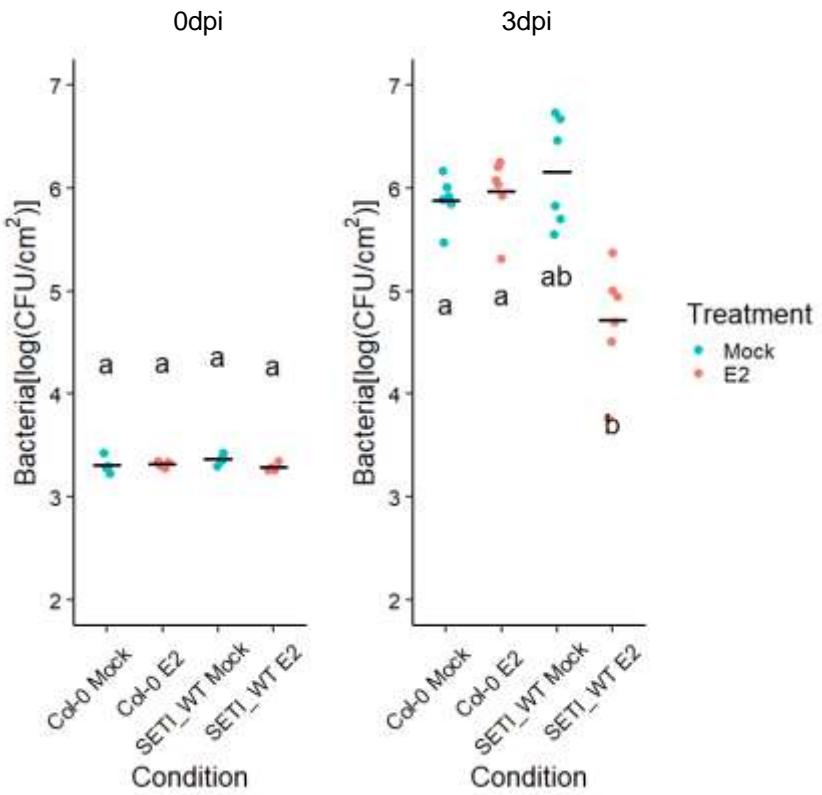


Fig. 6. Effector-triggered immunity triggered by the expression of AvrRps4 leads to resistance against *Pseudomonas syringae* pv. *tomato* strain DC3000.

5-week old SETI_WT and Col-0 leaves were infiltrated with Mock (1% DMSO) or 50µM E2. At 1dpi, leaves were inoculated with *Pst*DC3000 (OD600=0.001). Bacteria in the leaves were then quantified as colony-forming units (CFU) at 0dpi and 3dpi. Each data point represents two leaves collected from one individual plant. Samples from four individual plants were collected for 0 dpi and samples from six individual plants were collected for 3 dpi. Black line represents the mean of the technical replicates. This experiment was repeated three times independently with similar results. Biological significance of the values were determined by one-way ANOVA followed by post hoc TukeyHSD analysis. Letters above the data points indicate significant differences (P<0.05).