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Supplemental Information

Intra-strain Elicitation and Suppression of Plant Immunity by *Ralstonia solanacearum* Type-III Effectors in *Nicotiana benthamiana*

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Yuying Sang^{1,#}, Wenjia Yu^{1,2,#}, Haiyan Zhuang¹, Yali Wei^{1,2}, Lida Derevnina³, Gang Yu¹, Jiamin Luo^{1,2} and Alberto P. Macho^{1*}.

Includes:

Supplemental table (Table S1)

Supplemental figures (S1-S9)

Table S1: Primers used in this study for RipE1 cloning and qRT-PCR.

Gene	Forward primers	Reverse primers
Primers for site-directed amino acid mutation		
<i>RipE1-C172A</i>	AGGGGCGGGGAACGCCGGCGA ACACGCC	GGCGTGTCGCCGGCGTTCCC CGCCCCT
<i>RipE1 ΔAD</i>	GATACTGACGCACATCGACGCC ACCCA	TGGGTGGCGTCGATGTGCGTCA GTATC
Primers for qRT-PCR in <i>N. benthamiana</i>		
<i>NbEF1a</i>	CCCAAGAGGCCCTCAGACA	CACACGACCAACAGGGACAGT
<i>NbPR-1</i>	GGTCAACACGGCGAAAACC	GCCTTAGCAGCCGTCATGA
<i>NbICS1</i>	GTGTGCGCTCTGCTGTCTTCT	CTGCGTATAGCACGCCAATC
<i>NbPAL05</i>	AAGGGAGCTGAAATCGCCAT	TCCGCACTTTGGACATGGTT
<i>NbPAL08</i>	TATCACCCCATGCTTGCCCTC	AGTGGCCTTGGAATTGGGTC
<i>NbPAL10</i>	GTCACTCCATGTTTGCCCCT	GACCTGTGAGTAAACCGGCA
<i>NbLOX2</i>	TCTTGGGTGGCTCCTCTGACT	TGTTGGAGGTCTGCCTGTTCT
<i>NbAOS</i>	CTGGGGTCAAACCTCCACACT	TTGTGATGCAACTGGTGGTT
Primers for qRT-PCR in <i>A. thaliana</i>		
<i>AtACTIN2</i>	TGCTGGACGTGACCTTACTG	TTCTCGATGGAAGAGCTGGT
<i>AtPR1</i>	TGATCCTCGTGGGAATTATGT	TGCATGATCACATCATTACTTCAT
<i>AtICS1</i>	GCGTCGTTCCGGTTACAGG	ACAGCGAGGCTGAATCTCAT
<i>AtPAL1</i>	TATCCCGAACAGGATCAAGG	TCTCCGGTCAAAGCTCTGT
<i>AtPDF1.2</i>	ACTATGTCTTCCCAGCACAC	AACAACAACGGGAAAATAAA
<i>AtVSP2</i>	GTTTGGATCTTTGACCTAGACGA	CTCTAACCACGACCAGTACGC
Primers for cloning RipE1 to sXVE:GFPc:Bar estradiol inducible vector		
<i>RipE1</i>	TCAGGATCCATGCCGCCCGTCC TGCCGT	ACTCTCGAGGCTTTCCGTGGCG GGCGGCT

Figure S1

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Xs_XopE1 1 MRRSEAEERSPPNLLQSLQEIKMGLCSSKPSVAGSPVAGSPEHYLTHTEQTTPSTP-SS
Rs_RipE1 1 -----MPPVLPSTILRCF-----RPAVSR-PEAETAAPS--SSQENRPGSPERS
Ps_HopX1 1 MRIHSAGHSLPAPGPSVETTE-----K-AVQS-SSAQNPAACS-SQTERPEAGSTQVR

Xs_XopE1 60 PEAPMSPSLHGLVALGSSGTRRDRFR----OPTLQPHEVQQAAYQLGMRLSGRPIEDAS
Rs_RipE1 42 PRRR-PAALQGLTPRAGSSRRQAAPEAPAGPARFLIDGERQFGGYLMARDVDQRPVHGEP
Ps_HopX1 51 ENYF-YSS-----VKTRLEFP--VSSTGQAISDTPSSLPFGYLLLRRLDRPLDEDS

Xs_XopE1 115 DRQRLADATETVHETRLALHRGRGNVSDLRLSNGRSATYSSLSYCLGE-----
Rs_RipE1 101 -IDTLRSANETLLQTRRILTHGRGNVEDDIDATHGLSTHIAQGGRSIQESMWRRAH----
Ps_HopX1 98 -TKALVPADEAVREARRALPFRGNIDVDAQRTHLQSCARAVAAKRLRKDABRAGHEPMP

Xs_XopE1 164 -N---DENLLAGSALAAGAGNCDHNAAINARRHAVRMEDEGGQ----MMNVRDYEQTHL
Rs_RipE1 155 -PKPVVWA---AIAMVAGAGNCGEHADLAIFLHAAKKEGEA---VDNVHIDDFDHF
Ps_HopX1 157 GNDEMNVHVLVAMSEQVFGAGNCGEHARIASFAYGALAQESGRSPREKIHLAEQPGKDHV

Xs_XopE1 214 YALYQPPSSAAEAESPVVLDSWGDPVAVLLRDSHWAETYGTSTNVIERFDKRDATDALAR
Rs_RipE1 205 WAIIVHR--AEPDLERDVIIDAWGKGPATFAVDGMPTYREGERRTKI--GYDKASGEFAHA-
Ps_HopX1 217 WAETDN--SSA-GSSPVMDFWNSGAAIILAE DSREFAKDRSAVERTY-SFTLAMAAEAGKV

Xs_XopE1 274 TNAFRAEIEDPQTDLHANARDLETAFLANPAPGDI FSAMEVIAP-----
Rs_RipE1 261 -----DMEML-----ATVLAIRVGGISNTMRRLGPD--YRYPPEERVWAVTP
Ps_HopX1 273 T---RETAEVV-----LTHTTSRLQKRLADQLPNVSPLEGGRYQQE-----KS

Xs_XopE1 318 ---ELAQSTRQRIQEYS-----PRTRQALAA--DAAR--QA
Rs_RipE1 301 IVAQRFTDRVKAEMSKPADLGKLMVPPDCATPSSVEPPVTNERLMQFLRHEIHATRIART
Ps_HopX1 313 VLDEAFARVSDK-----LNSDDPRRALQMEIEAVGVAMS

Xs_XopE1 347 YGLDNAQPTSPRTTAATIQDAERLD-----ALGR
Rs_RipE1 361 LGAHSVDTMAH-AARRIVAVASDLQGYPIEAHPLQAKKDAEDIAAAERRRRARRAALGKGE
Ps_HopX1 348 LGAEGVKTVAR-QAPKVVRRARS-----VASSKGM

Xs_XopE1 376 PPLSW-
Rs_RipE1 420 PPATES
Ps_HopX1 377 PPRR--

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Figure S1. Phylogenetic analysis of RipE1

Alignment of the amino acid sequence of RipE1 from *R. solanacearum* GM1000 (Rs_RipE1), XopE1 from *Xanthomonas campestris* pv. *vesicatoria* (Xs_XopE1), and HopX1 from *Pseudomonas syringae* pv. *tabaci* 11528 (Ps_HopX1). Residues forming the predicted catalytic triad are indicated in red, and the conserved domain A is indicated in blue. The black shaded amino acids are identical among the three effectors.

Figure S2

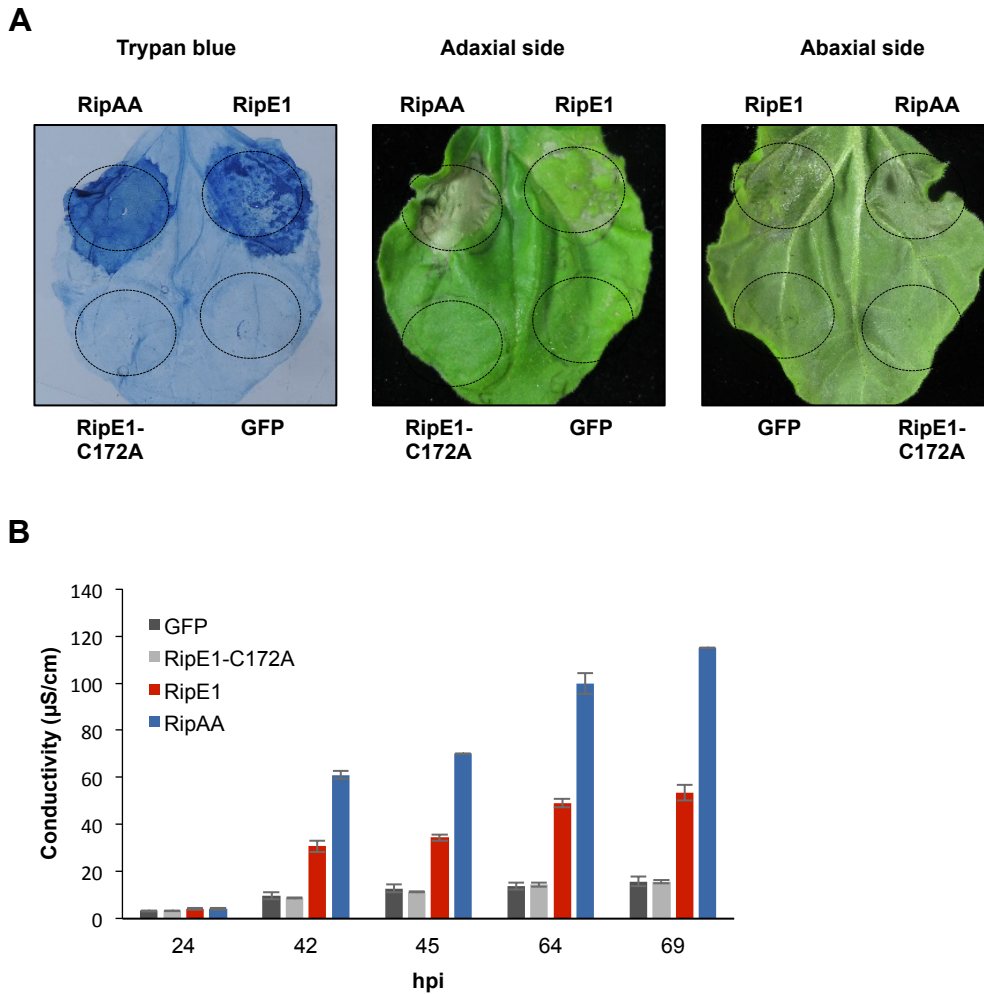


Figure S2. RipE1 induces cell death in *N. benthamiana*.

RipE1-GFP, RipE1-C172A-GFP, RipAA-GFP (as positive control), and GFP (as negative control) were expressed in the same leaf of *N. benthamiana* using *Agrobacterium* with an OD_{600} of 0.5. (a) Trypan blue staining was performed 4 days post-inoculation, and additional photos of an independent unstained leaf is shown for reference. (b) Ion leakage measured in leaf discs taken from *N. benthamiana* tissues expressing the indicated constructs, at the indicated time points. Values indicate mean \pm SE ($n=3$ biological replicates). These experiments were repeated 3 times with similar results.

Figure S3

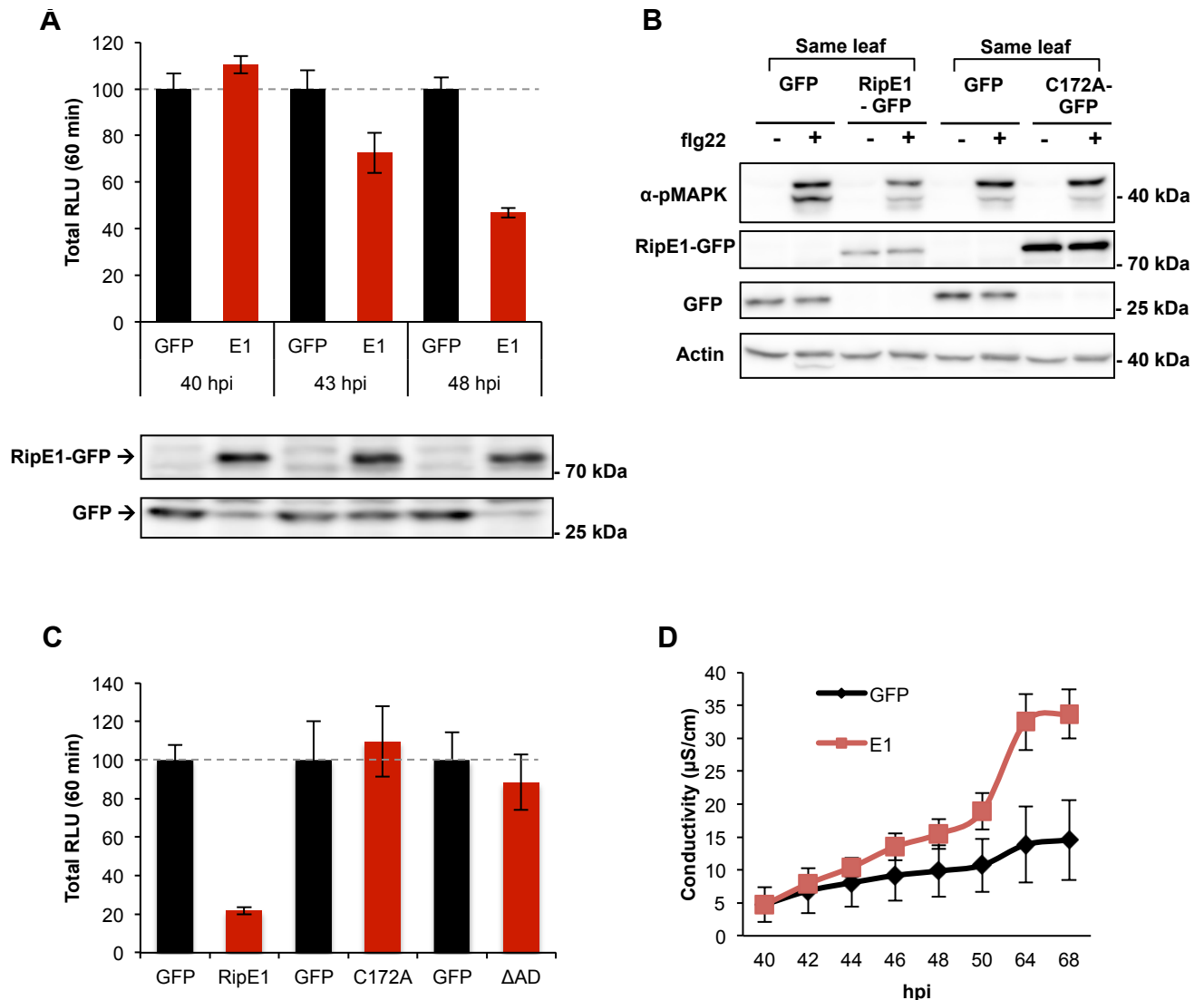


Figure S3. RipE1 expression inhibits PTI responses in *N. benthamiana*, which correlates with the induction of cell death.

Agrobacterium was used to induce the transient expression of RipE1-GFP in half of the leaf and GFP in the other half. (a) Oxidative burst triggered by 50 nM flg22 in *N. benthamiana* tissues at the indicated time points, measured in a luminol-based assay as relative luminescence units (RLU). Values are average \pm SE (n=24), and are represented as % of the GFP control in each time point. Western blot with anti-GFP is shown for reference of protein accumulation at each time point. (b) MAPK activation was induced 40 hours after Agrobacterium infiltration with 100 nM flg22 and analysed 15 minutes after flg22 treatment using anti-phosphorylated MAPK antibody (anti-pMAPK). Immunoblots were also analysed using anti-GFP antibody to verify protein accumulation. Anti-actin was used to verify equal loading. Molecular weight (kDa) marker bands are indicated for reference. (c) Oxidative burst was induced as in (a) and measured 2 days post-Agrobacterium infiltration. Mutant variants are described in the Figure 1. (d) Ion leakage was measured as in the Figure 1. Measurement over time after RipE1 expression reflects that the induction of cell death correlates in time with the suppression of PTI responses. The experiments were repeated three times with similar results.

Figure S4

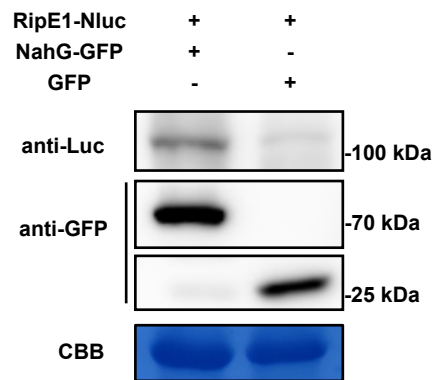


Figure S4. Protein accumulation upon co-expression of RipE1 and NahG.

Western blot showing protein accumulation in the experiments shown in the figure 2. Molecular weight (kDa) marker bands are indicated for reference.

Figure S5

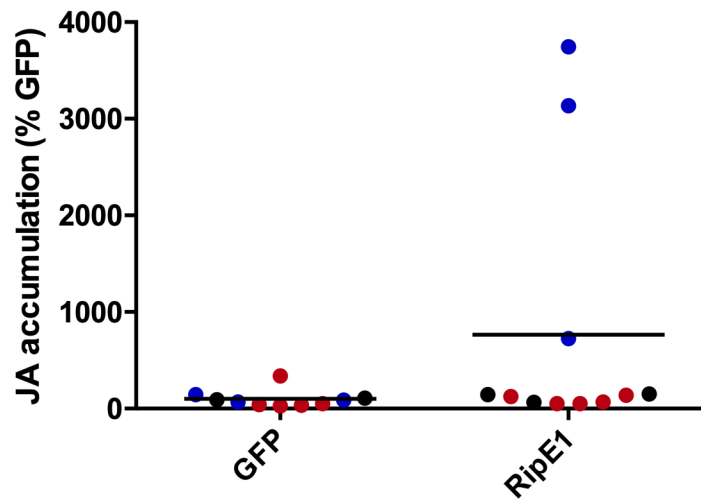


Figure S5. RipE1 expression leads to an increase in JA contents.

Measurement of JA accumulation in *N. benthamiana* tissues expressing GFP or RipE1, using *Agrobacterium* with an OD_{600} of 0.5. Samples were taken 42 hours after *Agrobacterium* infiltration. Three independent biological repeats were performed, and the different colors indicate values from different replicates. Values are represented as % of the GFP control in each replicate.

Figure S6

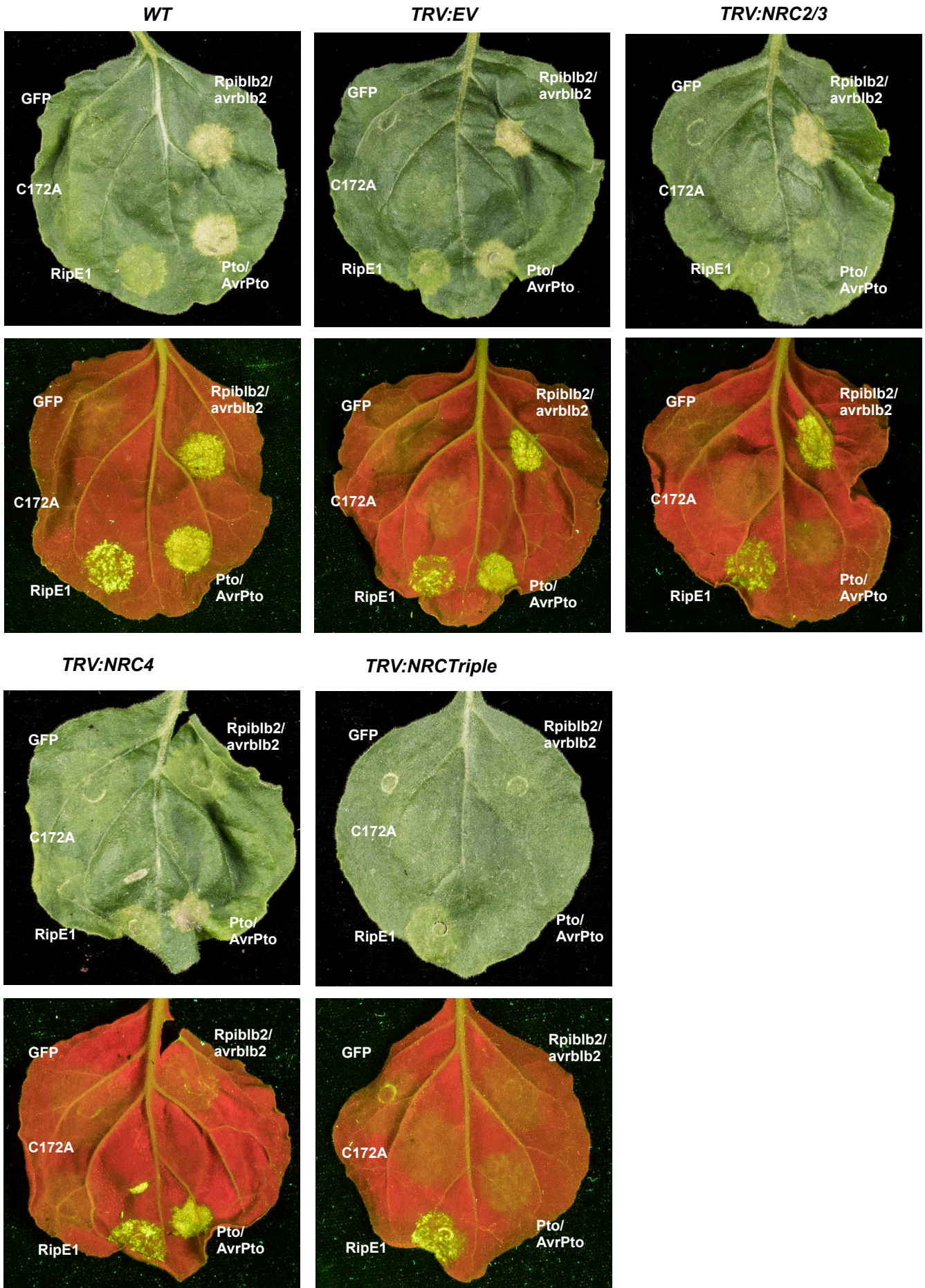


Figure S6. RipE1-triggered cell death does not require NRC proteins.

RipE1-GFP, RipE1-C172A-GFP or GFP (as control) were transiently expressed using agrobacterium into wild type (WT) *N. benthamiana*, leaves silenced with EV (as control) and those silenced with different NRC homologs (NRC2/3, NRC4 and NRC2/3/4-Triple), using VIGS. For RipE1-GFP, RipE1-C172A-GFP and GFP an OD₆₀₀ of 0.5 was used. RpiIb2 (OD₆₀₀ 0.2)/AVRbIb2 (OD₆₀₀ 0.1) and Pto (OD₆₀₀ 0.6)/AVRPto (OD₆₀₀ 0.1), which are NRC4 and NRC2/3 dependent, respectively, were included as controls. Photos were taken 5 days post inoculation under natural or UV light. UV images were taken from the abaxial side and flipped horizontally for representation.

Figure S7

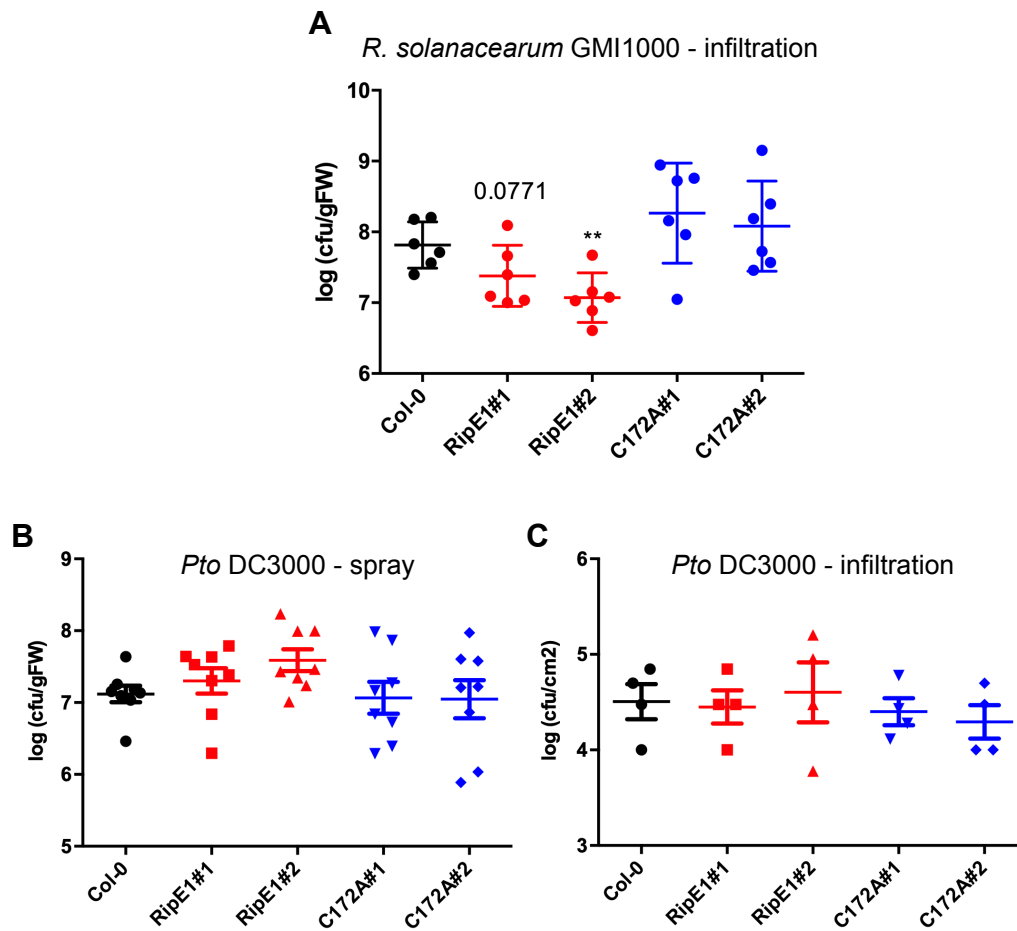


Figure S7. Leaves of RipE1-expressing Arabidopsis plants show enhanced resistance against inoculation with *R. solanacearum*, but not *P. syringae* pv *tomato* (*Pto*) DC3000.

(a) Growth of *R. solanacearum* GMI1000 (inoculated at 10^7 cfu/ml) infiltrated with a needleless syringe into wild-type Col-0 and RipE1-expressing Arabidopsis plants. Four week-old plants were sprayed with 100 μ M estradiol and then covered for 2 days prior inoculation. Bacterial colony-forming units (cfu) were determined 2 days post-inoculation (dpi). Values indicate mean \pm SE (n=6 biological replicates). Asterisks indicate significant differences compared to Col-0 WT plants according to a Student's t test (** p < 0.01). A specific p value is indicated for the RipE1#1 plant to indicate a reproducible (but not statistically significant) difference in bacterial growth compared to Col-0 WT plants. (b) Growth of surface (spray)-inoculated *Pto* DC3000 (inoculated at 10^8 cfu/ml) in wild-type Col-0 and RipE1-expressing Arabidopsis seedlings. Three week-old seedlings were sprayed with 100 μ M estradiol and then covered for 2 days prior inoculation. Bacterial colony-forming units (cfu) were determined 3 days post-inoculation (dpi). Values indicate mean \pm SE (n=8 biological replicates). (c) Growth of *Pto* DC3000 (inoculated at 10^5 cfu/ml) infiltrated with a needleless syringe into wild-type Col-0 and RipE1-expressing Arabidopsis plants. Four week-old plants were sprayed with 100 μ M estradiol and then covered for 2 days prior inoculation. Bacterial colony-forming units (cfu) were determined 3 days post-inoculation (dpi). Values indicate mean \pm SE (n=4 biological replicates). Experiments were repeated more than three times with similar results.

Figure S8

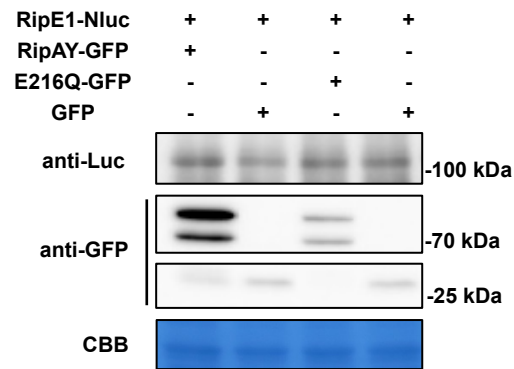


Figure S8. Protein accumulation upon co-expression of RipE1 and RipAY. Western blot showing protein accumulation in the experiments shown in the figure 6. Molecular weight (kDa) marker bands are indicated for reference.

Figure S9

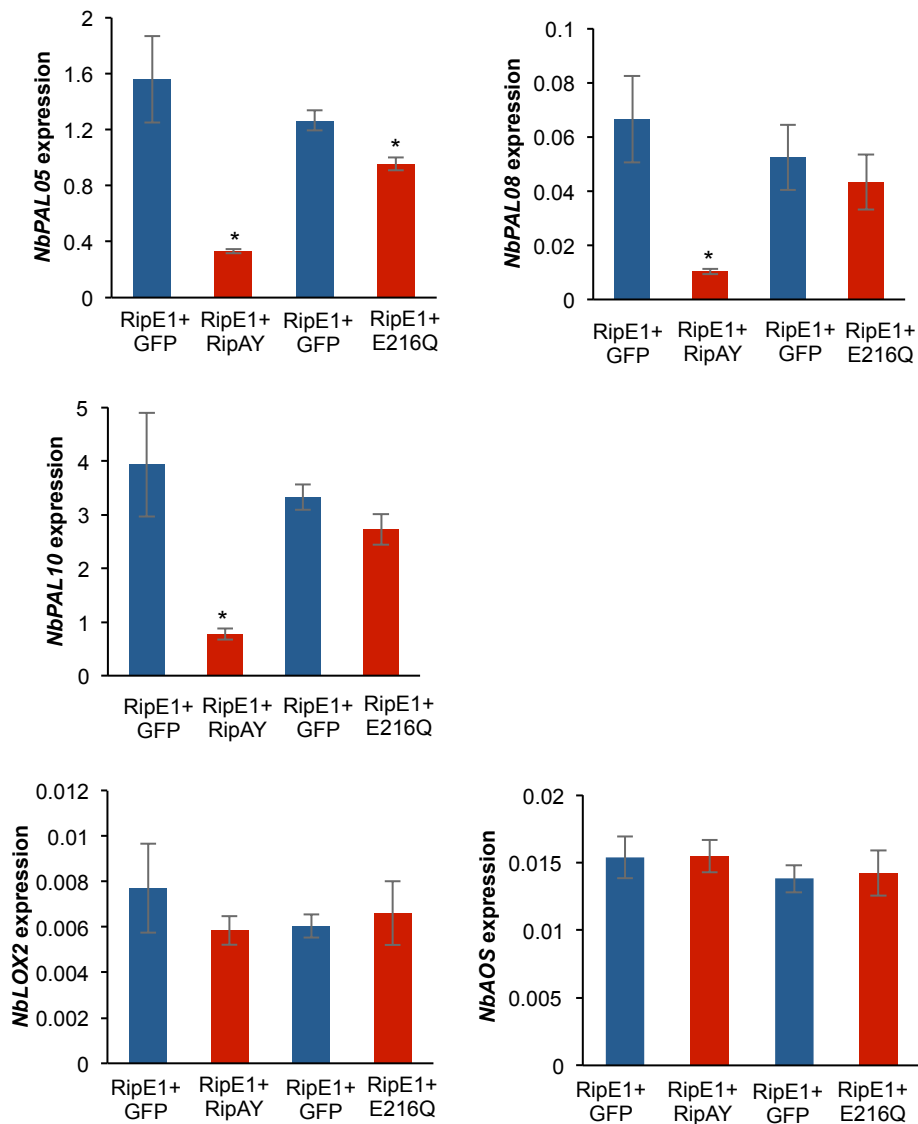


Figure S9. RipAY suppresses the overexpression of SA-related genes triggered by RipE1.

Quantitative RT-PCR to determine the expression of *NbPAL05*, *NbPAL08*, *NbPAL10*, *NbLOX2*, and *NbAOS* in *N. benthamiana* tissues 48 hours after Agrobacterium infiltration. Expression values are relative to the expression of the housekeeping gene *NbEF1a*. Values indicate mean \pm SE (n=3 biological replicates). Asterisks indicate significant differences compared to the mock control according to a Student's t test (* p < 0.05).