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Supplemental information

NLR immune receptor RB is differentially targeted by two homologous but functionally distinct effector proteins

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1	Supplemental Information
2	NLR Immune Receptor RB Is Differentially Targeted by Two Homologous but
3	Functionally Distinct Effector Proteins
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18 Supplemental Figure 1. Microscopic Analysis of Subcellular Localization of RB Fusion 19 Proteins.

Microscopic analysis of subcellular localization of RB fusion proteins. RB constructs carrying a C-terminal GFP tag together with WT or mutated nuclear localization signal (NLS/nls) or nuclear export signal (NES/nes) were expressed in *N. benthamiana*, and subcellular localization was determined at 48 hpi (left). DAPI staining depicts nuclei in blue (middle). An overlay of GFP and DAPI fluorescence signals is shown on the right. Scale bars represent 50 μm. The
 experiments were repeated three times with similar results.



Supplemental Figure 2. Microscopic Analysis of Subcellular Localization of IPI-O1 Fusion Proteins.

29 Microscopic analysis of subcellular localization of IPI-O1 fusion proteins. IPI-O1 constructs

30 carrying a C-terminal GFP tag and WT or mutated NLS/nls or NES/nes were expressed in N.

31 *benthamiana*, and subcellular localization was determined at 48 hpi (left). DAPI staining depicts

32 nuclei in blue (middle). An overlay of GFP and DAPI fluorescence signals is shown on the right.

33 Scale bars represent 50 µm. The experiments were repeated three times with similar results.



Supplemental Figure 3. Western Blot Detection of the RB and IPI-O1 Fusion Proteins with
 a WT or Mutated Nuclear Localization Signal (NLS/nls) or Nuclear Export Signal
 (NES/nes).

39 The fusion proteins were extracted from leaves collected at 36 hpi and detected by Western 40 blotting with an anti-GFP antibody. Ponceau S staining of immunoblots served as loading 41 controls.

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46 Supplemental Figure 4. RB and IPI-O1 Trigger Cell Death in the Cytoplasm.

(A) Cell death mediated by RB-GFP-GR in the presence of Myc-IPI-O1. The indicated 47 constructs were co-expressed with IPI-O1 in N. benthamiana. (B) Cell death induced by IPI-O1-48 GFP-GR or IPI-O4-GFP-GR. The indicated constructs were each expressed in the *RB* transgenic 49 50 N. benthamiana. Cell death induced at 48 hpi was visualized before (left) and after ethanol destaining (right). Infiltrated area is shown by a black circle and HR by a red circle. Scale bars 51 52 represent 1 cm. The experiments were repeated six times with similar results. (C, D) Western blot detection of the RB (C) and IPI-O1 (D) fusion proteins with a GR tag. The fusion proteins 53 54 were extracted from leaves collected at 36 hpi and detected by Western blotting with an anti-GFP antibody. Ponceau S staining of immunoblots served as loading controls. 55





58 Supplemental Figure 5. IPI-O1 and IPI-O4 but not RB Are Partially Localized to the 59 Plasma Membrane.

RB, IPI-O1, or IPI-O4 fused to GFP at the C-terminus was each co-expressed with the plasma membrane-localized Hir3.1-mCherry in *N. benthamiana*. Confocal images were taken at 48 hpi (left). The mCherry depicts plasma membrane in red (middle). An overlay of GFP and mCherry fluorescence signals is shown on the right. Scale bars represent 10 μ m. Fluorescence intensity profiles of the GFP and mCherry channels along the direction of the arrows are shown in the plots. The experiments were repeated three times with similar results.

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69 Supplemental Figure 6. Membrane-Associated RB and IPI-O1 Are Unable to Induce Cell
70 Death.

(A) Membrane-associated RB failed to mediate cell death in the presence of IPI-O1. RB-GFP 71 fused to Rop10 or mRop10, or RB-GFP alone was co-expressed in N. benthamiana with Myc-72 IPI-O1 or Myc-IPI-O4. (B) Membrane-associated IPI-O1 failed to induce cell death in RB 73 transgenic N. benthamiana plants. IPI-O1 or IPI-O4 fused to Rop10 or mRop10 were each 74 expressed in the RB transgenic N. benthamiana plants. Cell death induced at 48 hpi was 75 visualized before and after ethanol destaining. Infiltrated area is shown by a black circle and HR 76 by a red circle. Scale bars represent 1 cm. The experiments were repeated six times with similar 77 78 results. (C, D) Western blot detection of RB (C) and IPI-O1 (D) fused to a Rop10 or mRop10 tag. 79 The fusion proteins were extracted from leaves collected at 36 hpi and detected by Western blotting with an anti-GFP antibody. Ponceau S staining of immunoblots served as loading 80 81 controls.

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85			20 40 60		
86	MLA10	:	MDIVTGAISNLIPKLGELLTEEFKLHKGVKKNIEDLGKELDSMNAALIKIGEVPREQLDS	:	60
87	Sr33	:	MDIVTGAIAKLIPKLGELLVGEYKLHKGVKKNIEDLLKELKTMNAALIKIGEVPPDQLDS	:	60
88	Rx	:	MAYAAVTSLMRTIHQSMELTGCDLOPFYEKLKSLRAILEKSCNIMGDHE	:	49
89	ZAR1	:	MVDAVVTVFLEKTLNILEEKGRTVSDYRKOLEDLOSELKYMOSFLKDAEROKRTNE	:	56
90	RB	:	MAEAFIOVLLDNLTSFLKGELVLLFGFODEFORLSSMFSTIOAVLEDAOEKOLNNK	:	56
91			6161 2116aL		
92			$\alpha_1 \qquad \alpha_2 \qquad \alpha_3 \qquad \alpha_3 \qquad \alpha_4 \qquad \alpha_4 \qquad \alpha_4 \qquad \alpha_5 \qquad \alpha_5 \qquad \alpha_6 $		
93					
94			80 100 120		
95	MLA10	:	ODKLWADEVRELSYVIEDVVDKFLVOVDGIKSDDNNNKFKGLMKRTTELLKK	:	112
96	Sr33	:	ODKLWADEVRELSYVIEDAVDKFLVRVHGVEPDDNTNGFKGLMKRTTKLLKK	:	112
97	Rx	:	GLTILEVEIVEVAYTTEDMVDSESRNVFLAONLEERSRAMWEIFFVLEOALECIDSTVKO	:	109
98	ZAR1	:	TLRTLVADLRELVYEAEDILVDCOLADGDDGNE-ORSSNAWLSRLHPARVPLOYKK	:	111
99	RB	:	PLENWLOKLNAATYEVDDILDEYKTKATRFSOSEYGRYHPKVIP	:	100
100		•	6 e Y eD 6d k	•	
101			α3		
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103			140 160 180		
104	MLA10	:	VKHKHGIAHAIKDIOEOLOKVADRRDRNKVFVPHPTRTIAIDPCLRALYA-EATEIVGIY	:	171
105	Sr33	:	VVDKHGIAHAIKDIKKELOEVAARRDRNKFDGIASIPTEAIDPRLRALYI-EAAEUVGIY	:	171
106	Rx	:	WMATSDSMKDLKPOTSSLVSLPEHDVEOPENIMVG	:	144
107	ZAR1	:	SKRLOEINERITKIKSOVEPYFEFITPSNVGRDNGTDRWSSPVYDHTOVVG	:	162
108	RB	:	FRHKVGKRMDOVMKKLKAIAEERKNFHLHEKIVEROAVRRETGSVL-TEPOVYG	:	153
109			6 6 6 6vG		
110			α4		
111					
112			200		
113	MLA10	:	GKRDOGLMRLLSMEGDDASNKRUKKVSIVCF : 202		
114	Sr33	:	GKRDOELMSLLSLEGDDASTKKUKKWSIVGF : 202		
115	Rx	:	RENEFEMMLDOLARGGREVEVISIVGM : 171		
116	7AR1		LEGDKRKUKEWLFRSNDSOULTMAEVCM : 190		
117	RB	:	RDKEKDEUVKTLIN-NVSDAOHUSVUPTLGM : 183		
118		•	6 I. 6 i6G		
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Supplemental Figure 7. Sequence and Secondary Structure Alignment of the CC Domains from RB and Other NLRs Proteins.

124 The amino acid sequences of the CC domains from RB and other NLRs, MLA10, Sr33, Rx, and 125 ZAR1, are aligned. The heptad repeats of hydrophobic amino acids within CC domains are 126 highlighted in yellow, the EDVID motifs in pink, and other conserved amino acids in light grey 127 to dark corresponding to the levels of identity. The predicted α -helices of the CC domains are 128 shown as colored cylinders.

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Supplemental Figure 8. Ribbon Diagrams of the Predicted Monomeric, Dimeric, and
Pentameric Structures for RB CC Domain.

The predicted structures of RB CC monomer (A), dimer (B), and pentamer (C) are represented as ribbons. The four hydrophobic residues (F31, L34, F38, and I41) of the heptad repeats in the α 2 helix are highlighted in magenta. The conserved EDVID (DDILD in RB) motif is highlighted in purple. In the dimeric structure, the two monomers are shown in green and cyan. The four hydrophobic residues (F31, L34, F38, and I41) are located on the same side of the RB CC dimer.

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142 Supplemental Figure 9. Mutations in the Heptad Repeats of RB Abolish Its Function.

RB and its variants carrying mutations in the heptad repeats from the predicted $\alpha 1$, $\alpha 3$, and $\alpha 4$ helices were each co-expressed with IPI-O1 or IPI-O4 in *N. benthamiana*. Cell death induced at 48 hpi was visualized before (left) and after ethanol destaining (right). Infiltrated area is shown by a black circle and HR by a red circle. Scale bars represent 1 cm. The experiments were repeated six times with similar results.



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149 Supplemental Figure 10. Cell Death-Inducing Activities of RB MHD Mutants.

(A) Cell death mediated by the RB MHD mutants in the absence of effectors. The indicated RB MHD variants were each expressed in *N. benthamiana*. (B) Cell death mediated by the RB MHD mutants in the presence of effectors. RB variants harboring mutations in the MHD motif were co-expressed with Myc-IPI-O1 or Myc-IPI-O4 in *N. benthamiana*. Cell death induced at 48 hpi was visualized before (left) and after ethanol destaining (right). Infiltrated area is shown by a black circle and HR by a red circle. Scale bars represent 1 cm. The experiments were repeated six times with similar results.



158 Supplemental Figure 11. Western Blot Detection of the RB Variant Fusion Proteins.

The GFP-tagged RB variants carrying mutations in the heptad repeats from the second α -helix (A), the F31E/L34E/I41E triple mutant (RB EEE) (B), or the autoactive D475V mutation in the MHD motif in combination with mutations in the heptad repeats of RB CC (C) were each expressed in *N. benthamiana*. The RB variant fusion proteins were extracted from leaves collected at 36 hpi and detected by Western blotting with an anti-GFP antibody. Ponceau S staining of immunoblots served as loading controls.

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Supplemental Figure 12. Disruptive Effect of IPI-O4 on the RB CC Self-Association Positively Correlated with the IPI-O4 Expression Level.

172 Total proteins were extracted from *N. benthamiana* plants expressing RB CC-3×FLAG and RB

173 CC-4 \times Myc together with 3 \times HA-tagged IPI-O4 at an OD₆₀₀ of 0.001, 0.01, or 0.1. 174 Immunoprecipitation was carried out with an anti-Myc antibody, and immunoblots were probed 175 with anti-Myc, anti-FLAG, or anti-HA antibodies. Ponceau S staining of immunoblots served as 176 loading controls. The experiments were repeated twice with similar results.

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181 Supplemental Figure 13. Self-Association of IPI-O1 Is Perturbed by IPI-O4 *In Planta*.

Total proteins were extracted from *N. benthamiana* plants expressing $3 \times$ FLAG-IPI-O1 and 4×Myc-IPI-O1 together with 3×HA-tagged IPI-O1 or IPI-O4. Immunoprecipitation was carried out with anti-FLAG M2 magnetic beads, and immunoblots were probed with anti-Myc, anti-FLAG, or anti-HA antibodies. Ponceau S staining of immunoblots served as loading controls. The experiments were repeated twice with similar results.

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189	Supplemental Table 1. Oligonucleotide Sequences for Site-Directed Mutagenesis	and
190	Plasmid Construction.	
191	Supplemental Table 1 was submitted in an Excel file.	
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