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Supplemental information

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NLR Immune Receptor RB Is Differentially Targeted by Two Homologous but Functionally Distinct Effector Proteins

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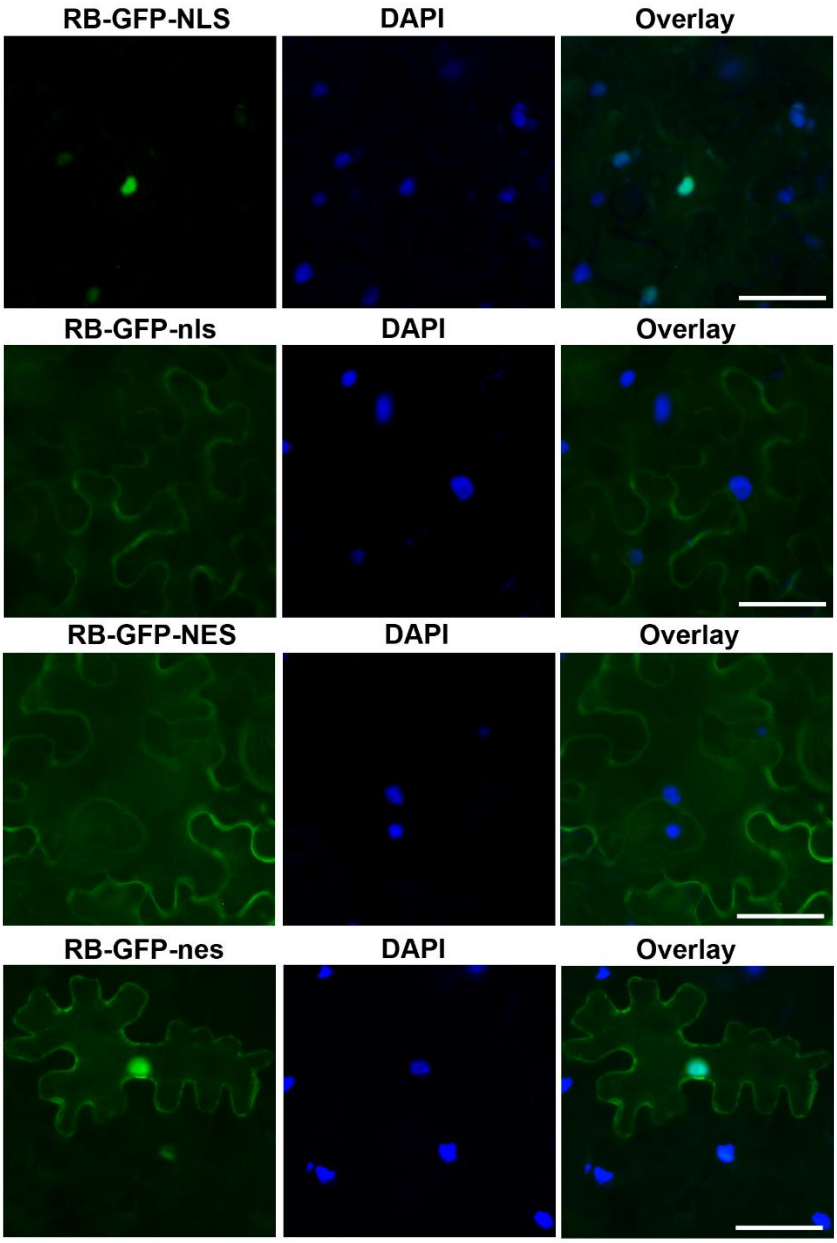
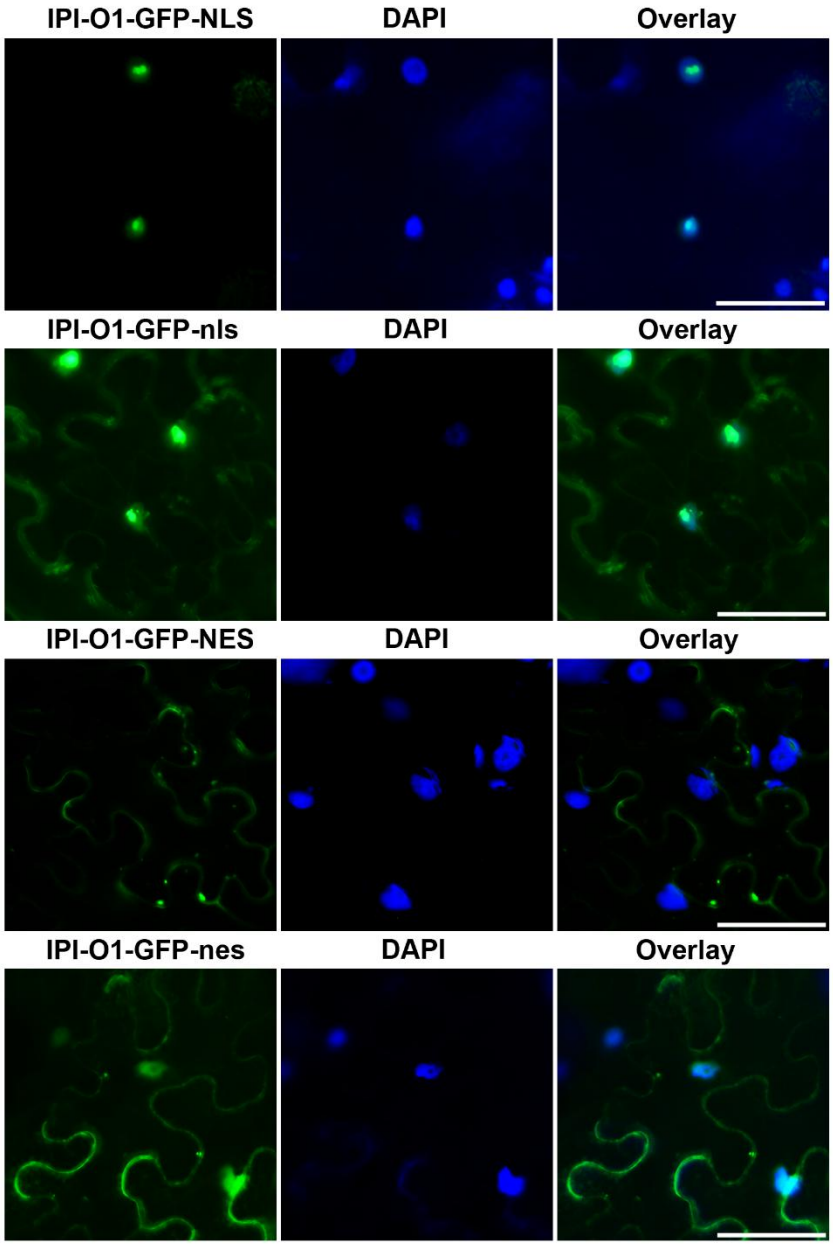


Supplemental Figure 1. Microscopic Analysis of Subcellular Localization of RB Fusion Proteins.

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.

Microscopic analysis of subcellular localization of IPI-O1 fusion proteins. IPI-O1 constructs carrying a C-terminal GFP tag and WT or mutated NLS/nls or 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.

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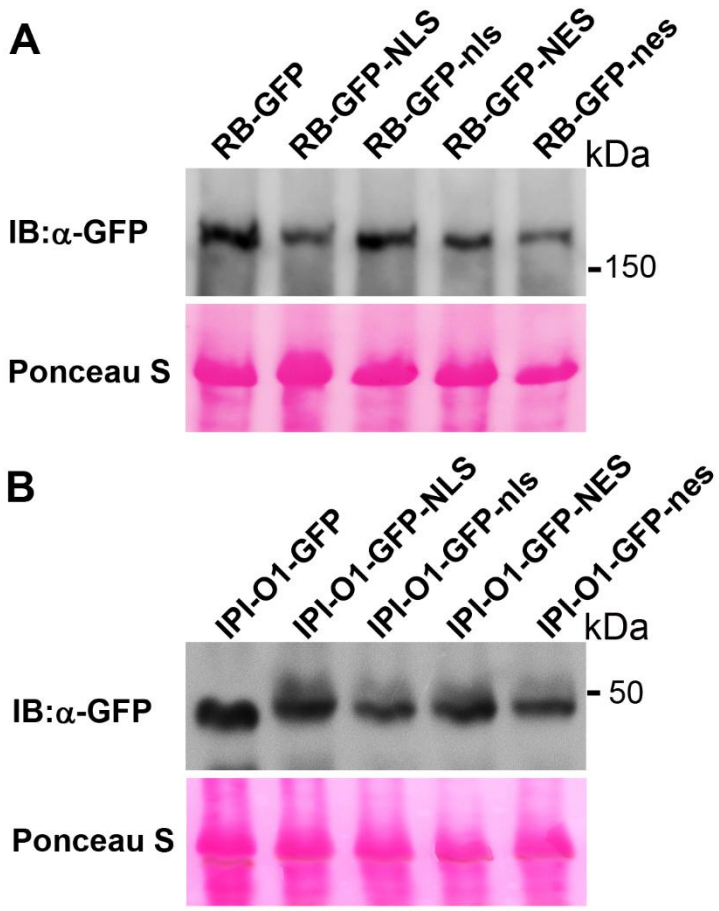
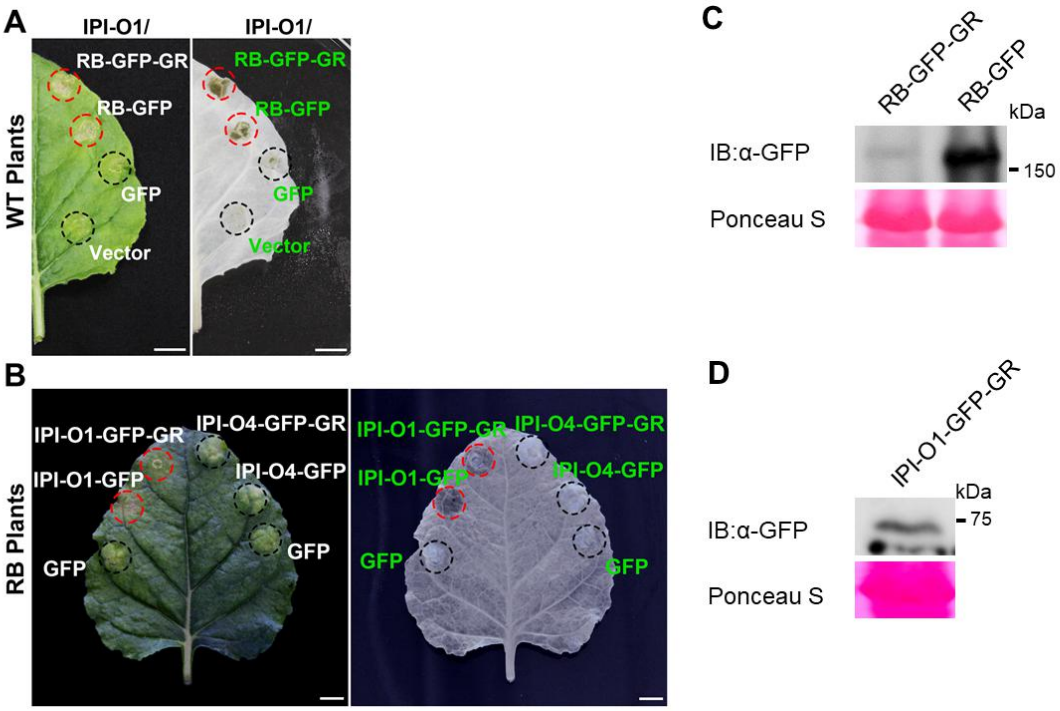


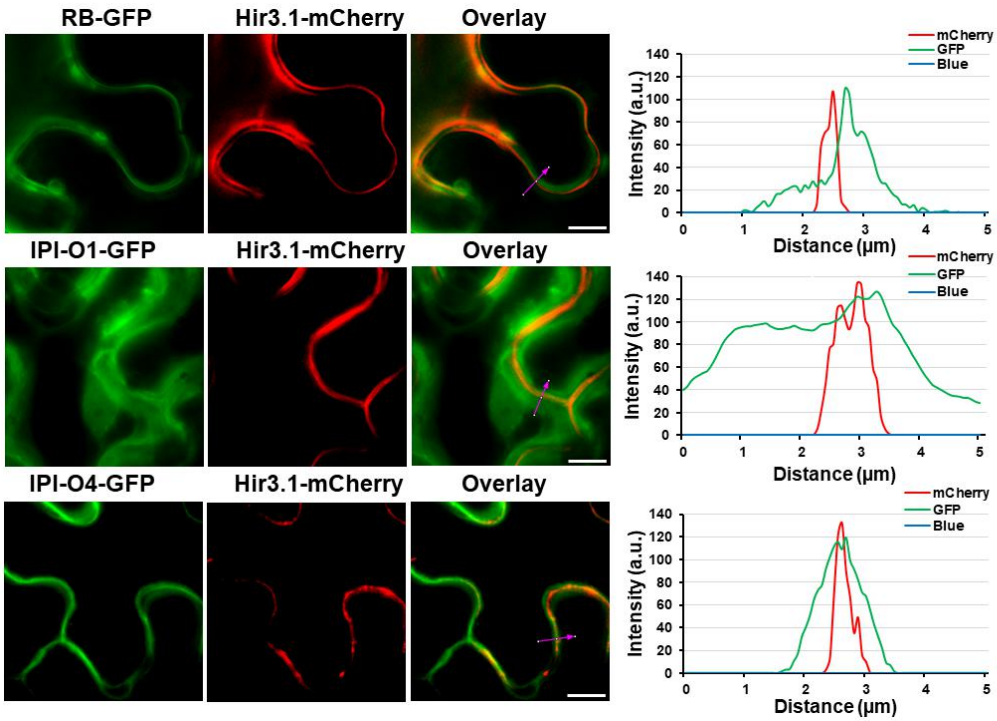
Supplemental Figure 3. Western Blot Detection of the RB and IPI-O1 Fusion Proteins with a WT or Mutated Nuclear Localization Signal (NLS/nls) or Nuclear Export Signal (NES/nes).

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 controls.



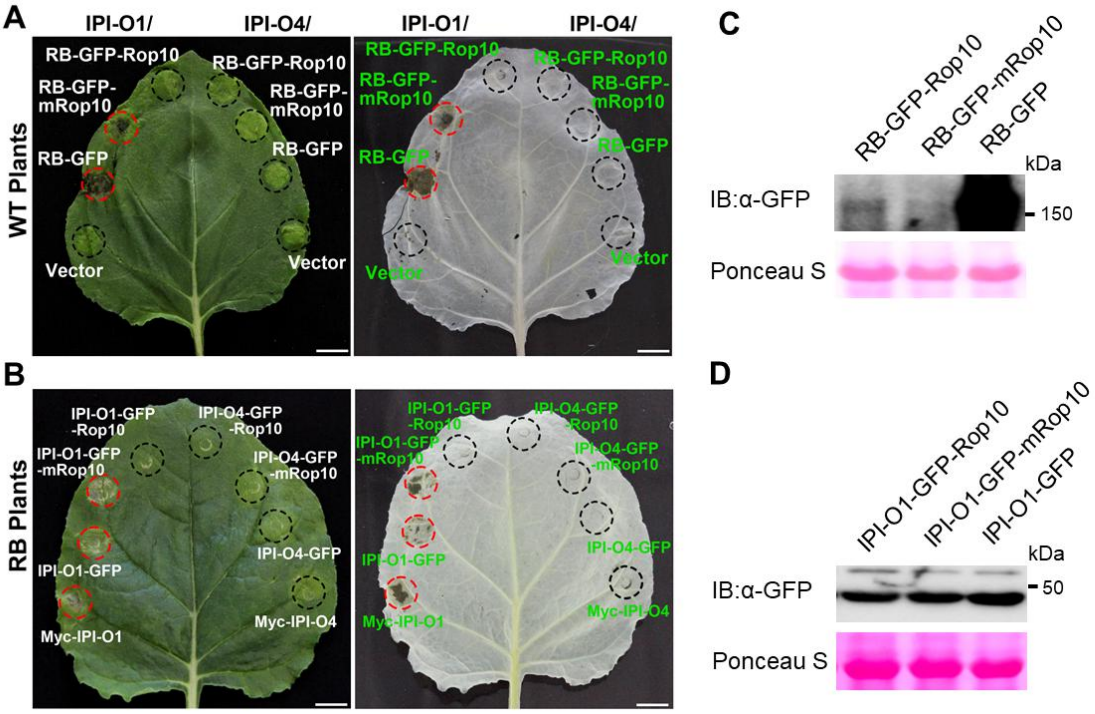
Supplemental Figure 4. RB and IPI-O1 Trigger Cell Death in the Cytoplasm.

(A) Cell death mediated by RB-GFP-GR in the presence of Myc-IPI-O1. The indicated constructs were co-expressed with IPI-O1 in *N. benthamiana*. (B) Cell death induced by IPI-O1-GFP-GR or IPI-O4-GFP-GR. The indicated constructs were each expressed in the *RB* transgenic *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. (C, D) Western blot detection of the RB (C) and IPI-O1 (D) fusion proteins with a GR tag. 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 controls.



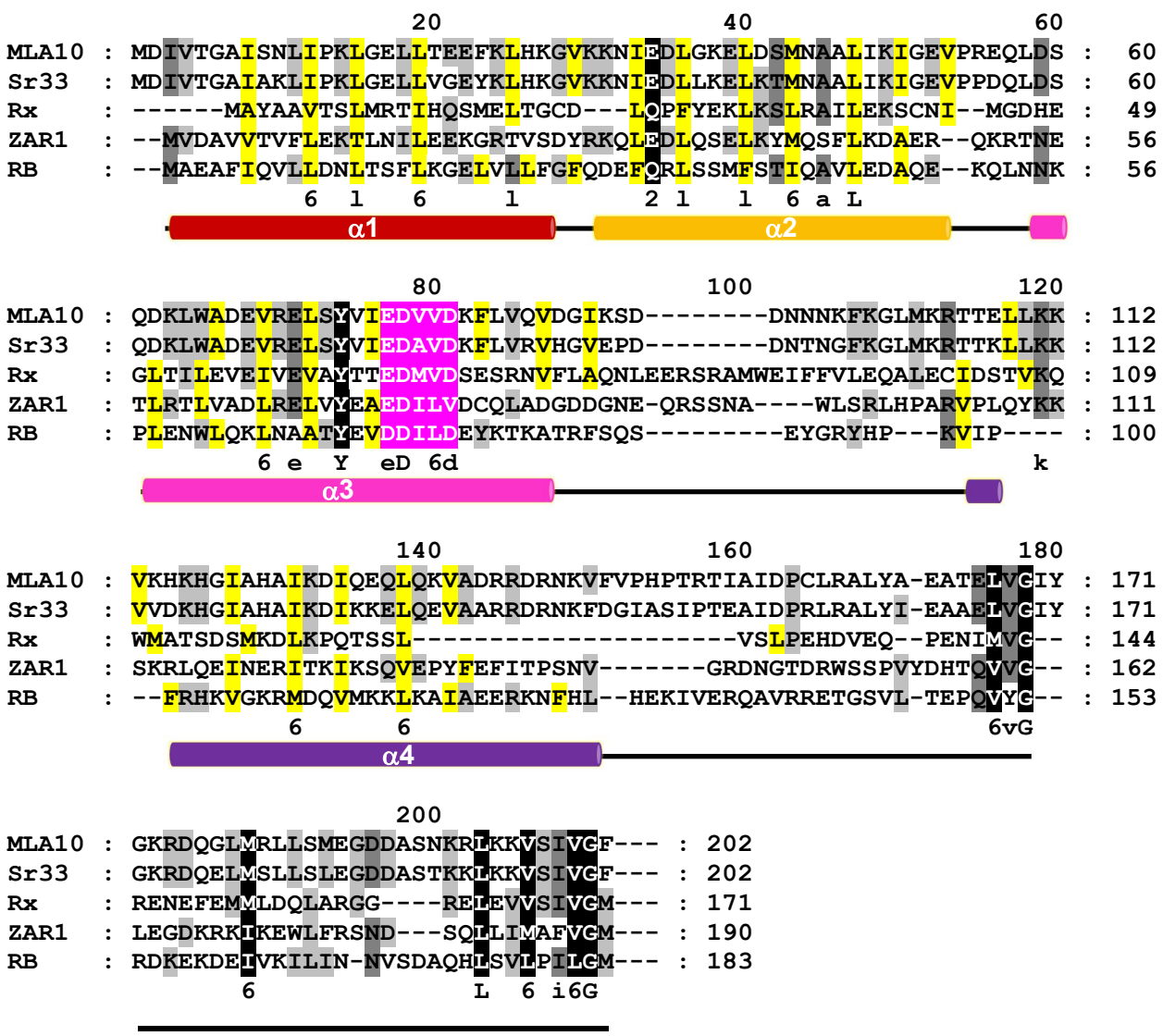
Supplemental Figure 5. IPI-O1 and IPI-O4 but not RB Are Partially Localized to the Plasma Membrane.

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 μm. 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.



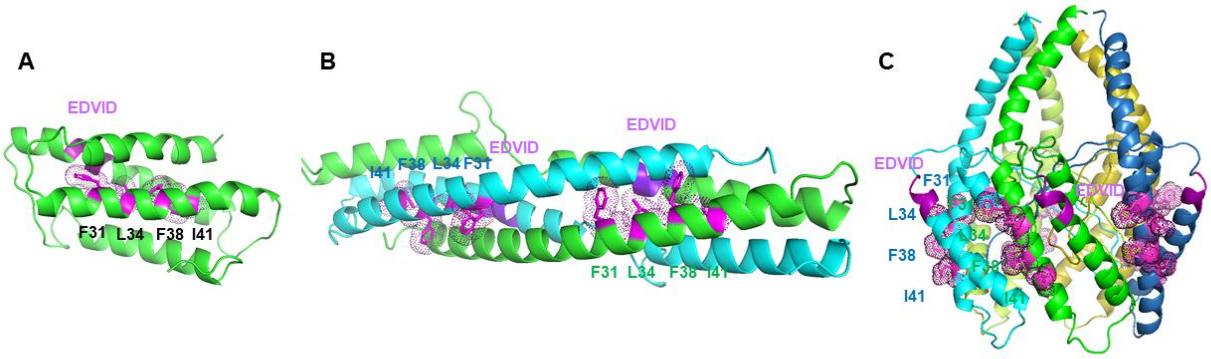
Supplemental Figure 6. Membrane-Associated RB and IPI-O1 Are Unable to Induce Cell Death.

(A) Membrane-associated RB failed to mediate cell death in the presence of IPI-O1. RB-GFP fused to Rop10 or mRop10, or RB-GFP alone was co-expressed in *N. benthamiana* with Myc-IPI-O1 or Myc-IPI-O4. (B) Membrane-associated IPI-O1 failed to induce cell death in *RB* transgenic *N. benthamiana* plants. IPI-O1 or IPI-O4 fused to Rop10 or mRop10 were each expressed in the *RB* transgenic *N. benthamiana* plants. Cell death induced at 48 hpi was visualized before and after ethanol destaining. 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. (C, D) Western blot detection of RB (C) and IPI-O1 (D) fused to a Rop10 or mRop10 tag. 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 controls.



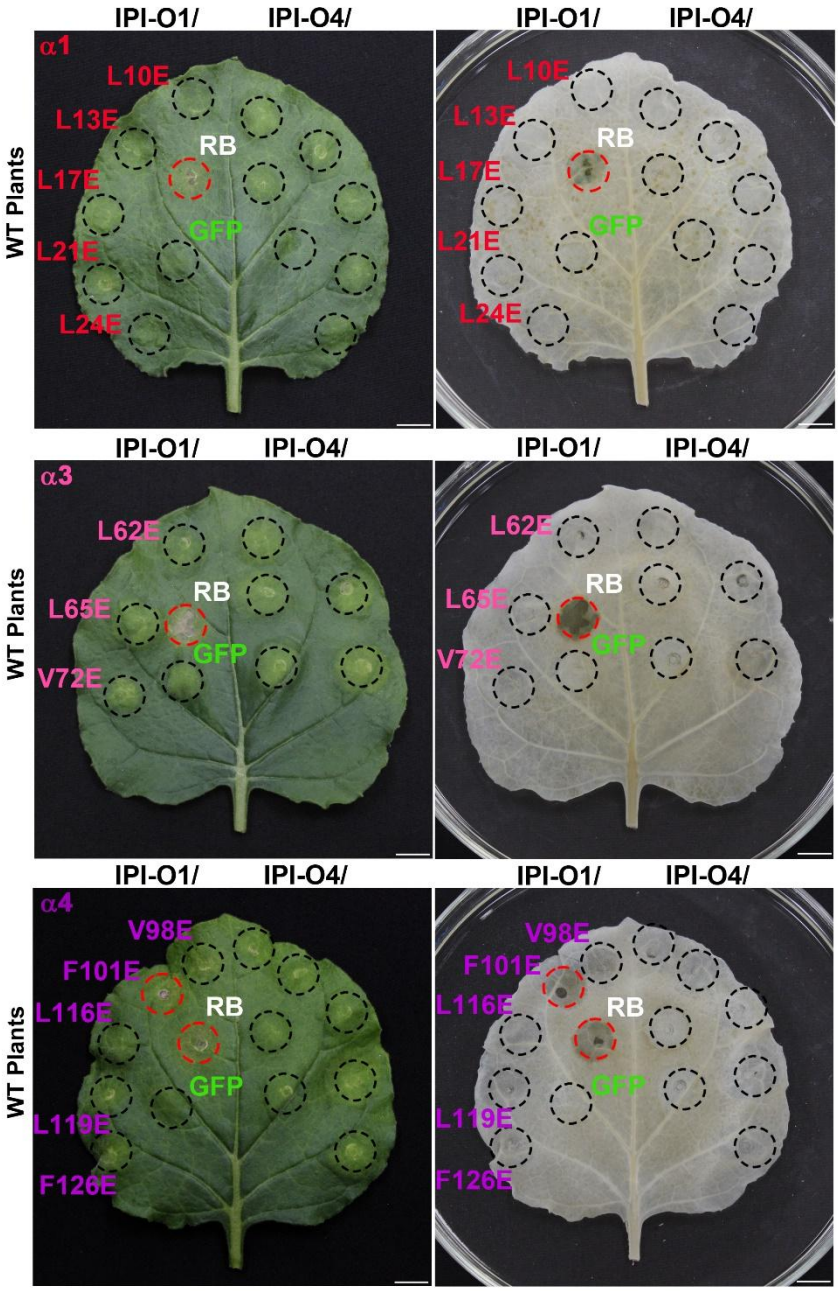
Supplemental Figure 7. Sequence and Secondary Structure Alignment of the CC Domains from RB and Other NLRs Proteins.

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



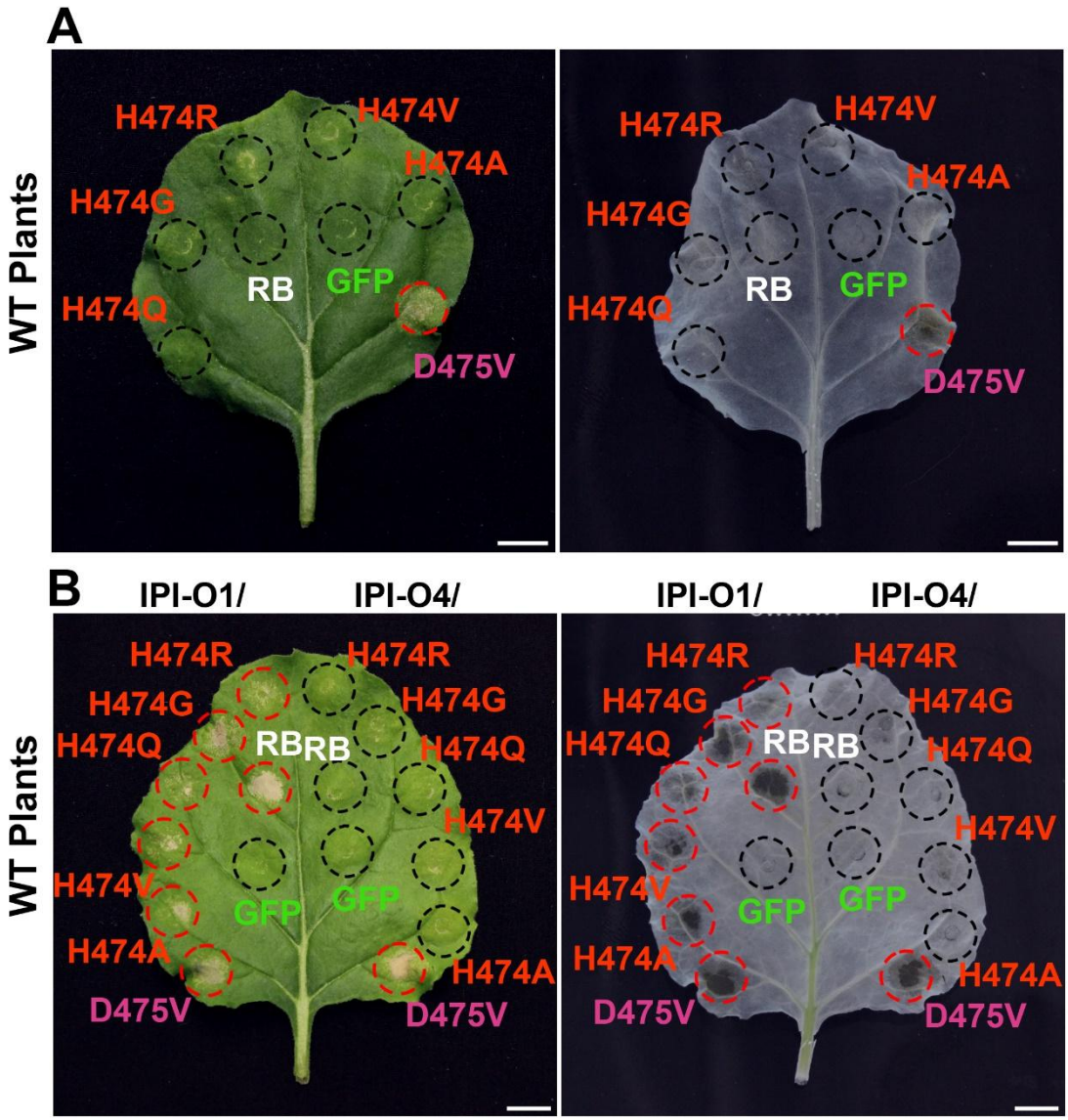
Supplemental Figure 8. Ribbon Diagrams of the Predicted Monomeric, Dimeric, and Pentameric Structures for RB CC Domain.

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 α2 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.



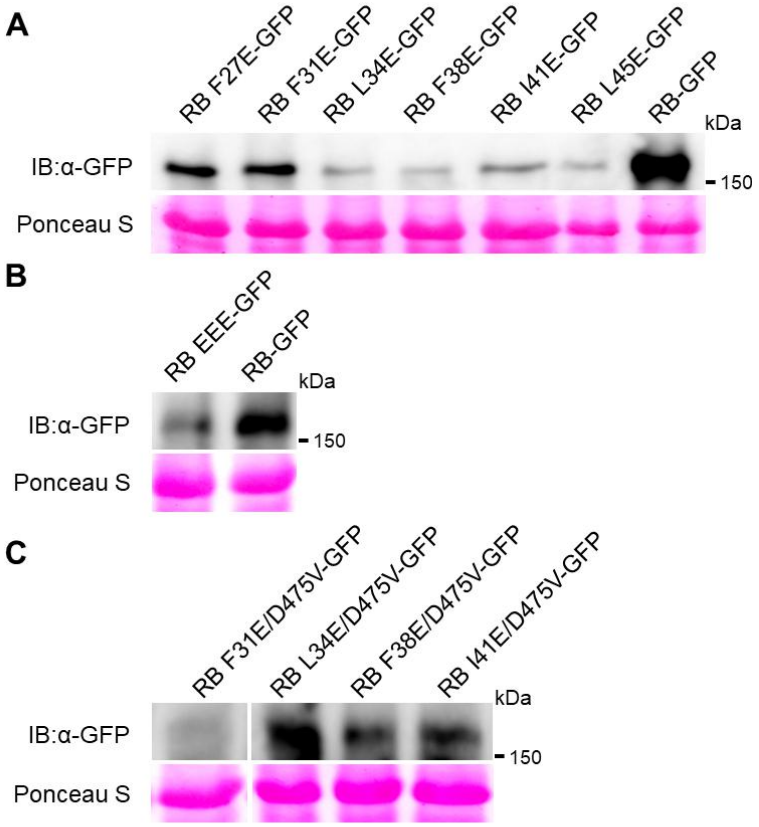
Supplemental Figure 9. Mutations in the Heptad Repeats of RB Abolish Its Function.

RB and its variants carrying mutations in the heptad repeats from the predicted α1, α3, and α4 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.



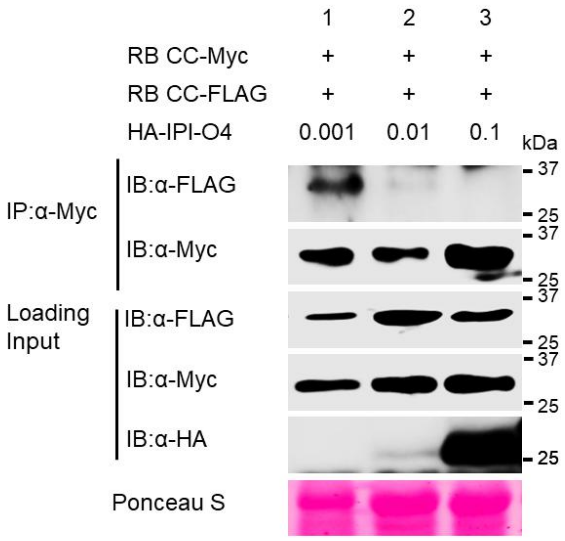
Supplemental Figure 10. Cell Death-Inducing Activities of RB MHD Mutants.

(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.



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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170 **Supplemental Figure 12. Disruptive Effect of IPI-O4 on the RB CC Self-Association**
171 **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 × Myc together with 3 × HA-tagged IPI-O4 at an OD_{600} 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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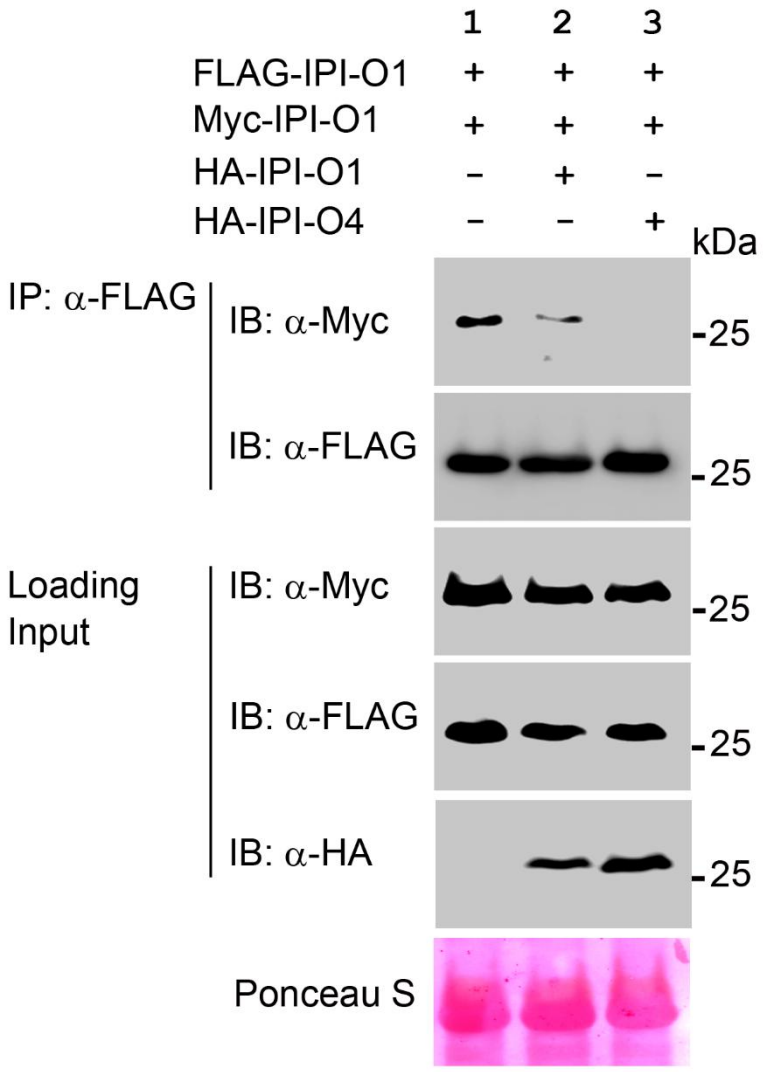

Supplemental Figure 13. Self-Association of IPI-O1 Is Perturbed by IPI-O4 *In Planta*.

Total proteins were extracted from *N. benthamiana* plants expressing 3 × 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.

Supplemental Table 1. Oligonucleotide Sequences for Site-Directed Mutagenesis and Plasmid Construction.

Supplemental Table 1 was submitted in an Excel file.