

Expanded View Figures

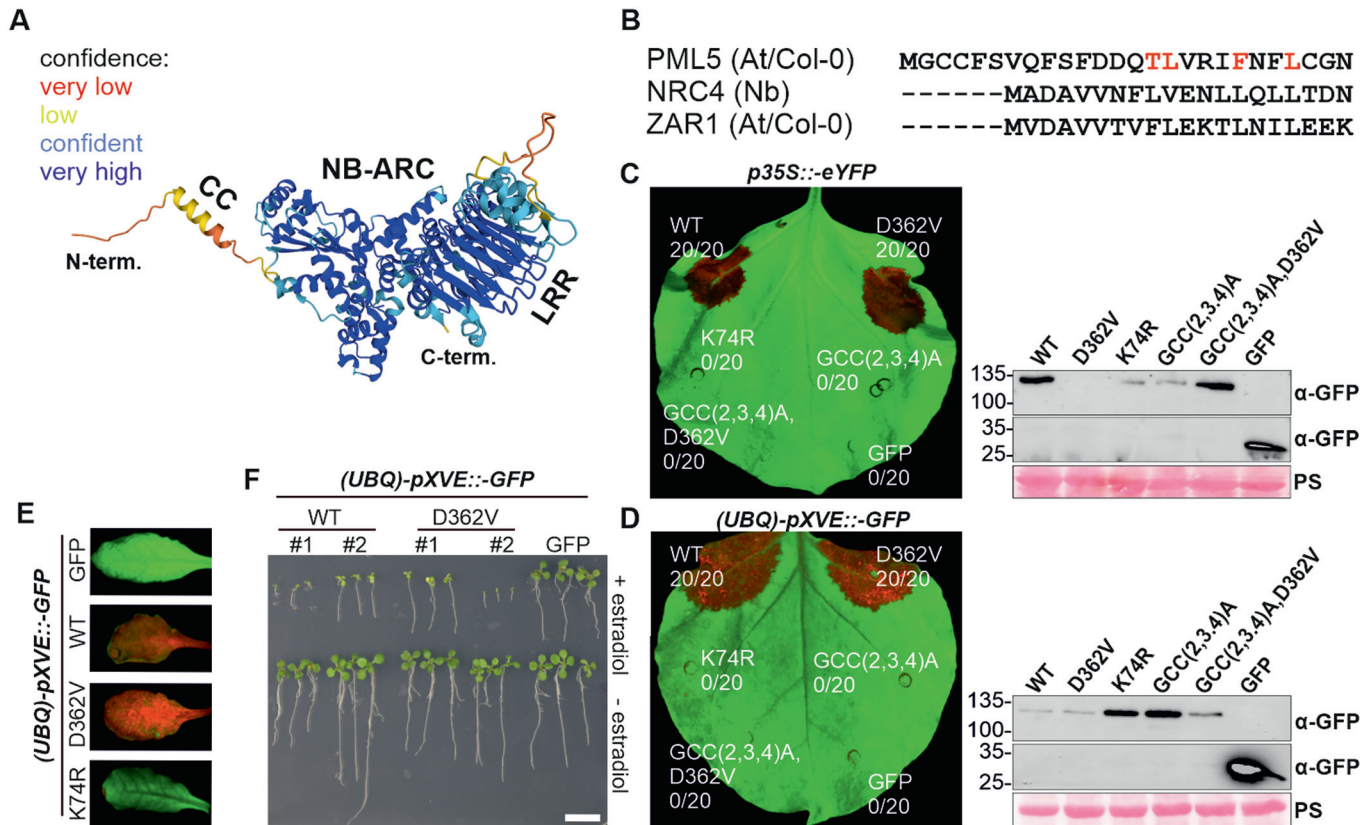


Figure EV1. PML5 functions as a canonical cell death inducing NLR.

(A) AlphaFold 2 structural prediction of PML5. CC, NB-ARC, and LRR domains are indicated. A potential alpha 1 helix is predicted between Asp/D13 and Ile/I 27. (B) Sequence alignment of the first 26 N-terminal amino acids of Col-0 PML5, ZAR1 and *N. benthamiana* (Nb) NRC4, highlighting the hydrophobic and polar uncharged amino acids mutated in PML5 (see Fig. 1D). (C, D) Cell death induced by transiently expressed wild-type PML5 and mutant variants (C) constitutively under 35S promoter (top left) or conditionally expressed under a 35S promoter-controlled estradiol inducible system (bottom left). GFP (C) and Citrine-HA (D) served as negative controls. Total protein extracts were immunoblotted and detected with an anti-GFP (α -GFP) antibody. Protein expression analyses of constructs infiltrated are shown on right side with single GFP (C) and Citrine-HA (D) in the respective lower blot around 30 kDa. Cell death images were taken 2 days post-infiltration (C) or 3 days post-induction (D). Leaves are shown in false color, red indicates cell death and green healthy/alive tissue. WT = wild-type PML5; D362V = MHD mutant; K74R = P-loop mutant; GCC(2,3,4)A = N-myristoylation and S-acylation (PTM) mutant; GCC(2,3,4)A, D362V = PTM/MHD quadruple mutant. (E) Cell death phenotype in a single rosette leaf of 4-week-old Arabidopsis plant 18 h post-induction with 20 μ M estradiol is shown. Leaves are shown in false color, red indicates cell death in wild-type PML5 and D362V lines, and green healthy/alive tissue in PML5 K74R and GFP negative control lines. (F) Growth restriction/cell death phenotype of two independent transgenic Arabidopsis lines conditionally overexpressing PML5-GFP (WT), D362V-GFP. A 35::GFP plant line was used as a control. 10-day-old Arabidopsis seedlings grown on $\frac{1}{2}$ MS plates supplemented with or without 20 μ M estradiol are shown. Scale bars, 1 cm.

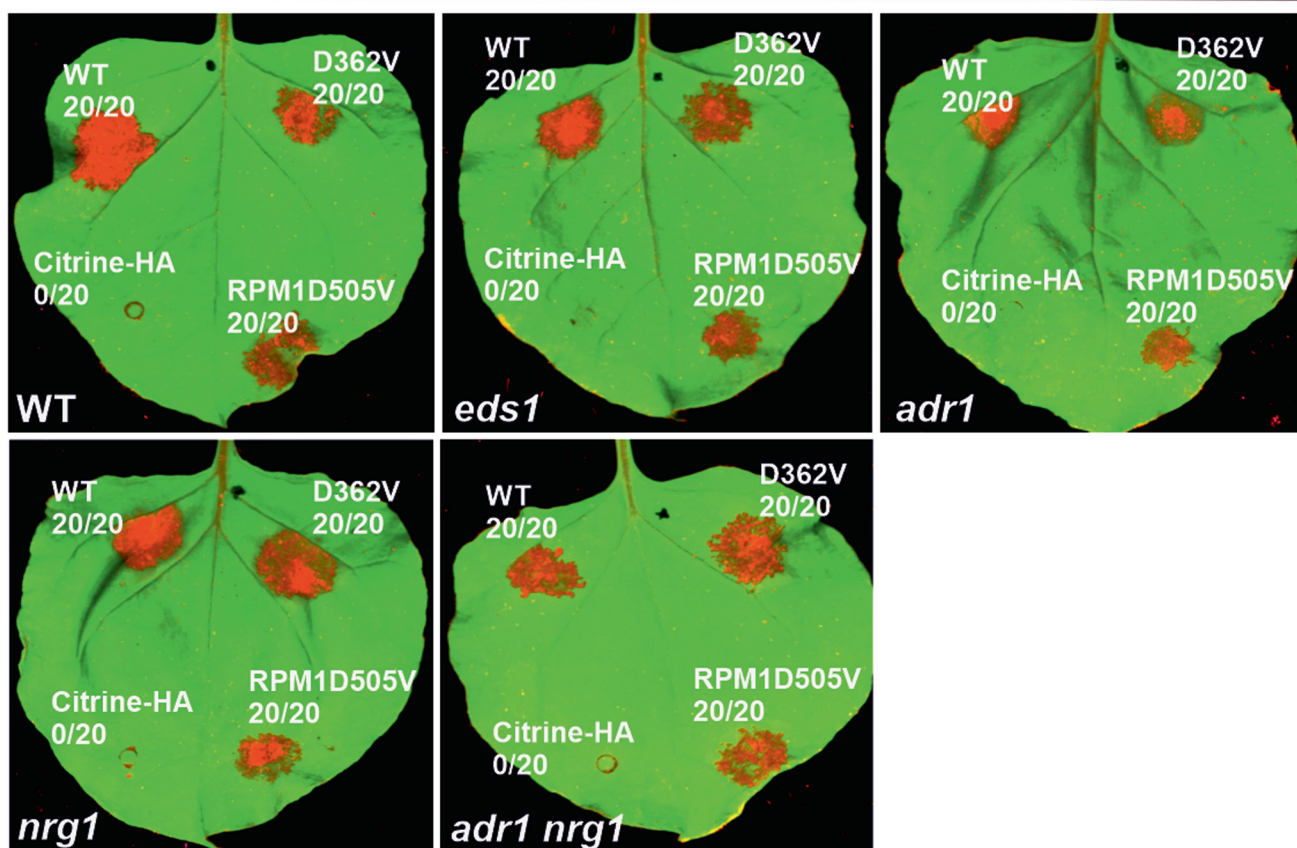
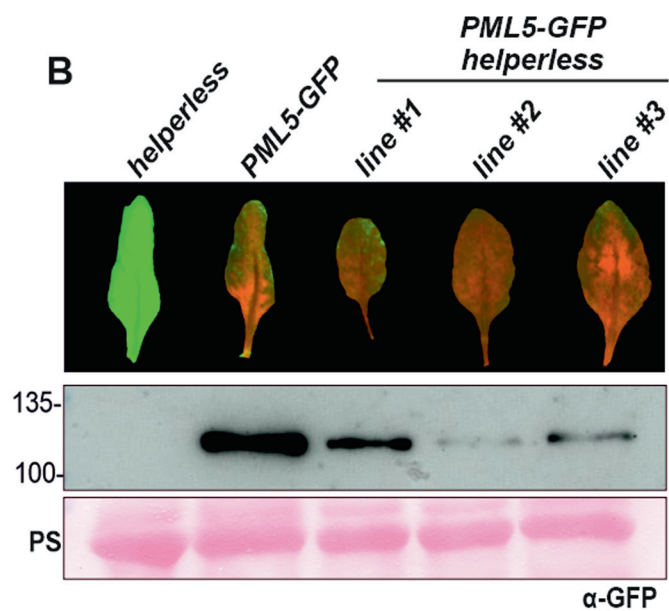
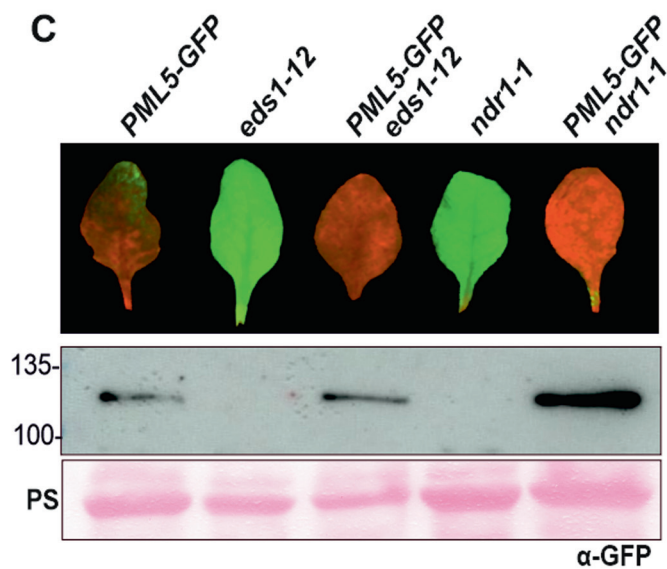
A**(35S)-pXVE::Citrine-HA****B****C**

Figure EV2. PML5-induced cell death is independent of EDS1, RNL helper NLRs, and NDR1.

(A) Cell death induced by transiently expressed wild-type PML5 (WT) and D362V mutant in WT, *eds1*, *adr1*, *nrg1*, *adr1 nrg1* *N. benthamiana* mutant lines. The RPM1 D505V auto-active mutant was used as a positive control and Citrine-HA as a negative control. Images were taken 1 day post-induction with 20 μ M estradiol. Leaves are shown in false color, red indicates cell death and green healthy/alive tissue. (B) Cell death in three independent transgenic *Arabidopsis helperless* mutant lines conditionally expressing PML5-GFP. PML5-GFP in Col-0 (PML5-GFP) served as the positive control. Protein blot (bottom) shows the expression of PML5-GFP detected with an anti-GFP (α -GFP) antibody in the positive control and three independent transgenic PML5-GFP *helperless* lines. Ponceau S (PS) staining is shown as a loading control. Images and samples for protein blot were taken 48 h post-induction with 20 μ M estradiol. Leaves are shown in false color, red indicates cell death and green healthy/alive tissue. (C) Cell death in transgenic *Arabidopsis eds1-12* and *ndr1-1* mutant conditionally expressing PML5-GFP. PML5-GFP in Col-0 (PML5-GFP) served as the positive control. Protein blot (below) shows the expression of PML5-GFP detected with anti-GFP (α -GFP) antibody in the positive control and transgenic PML5-GFP *eds1-12* and *ndr1-1* mutants. Ponceau S (PS) staining is shown as a loading control. Samples for protein blot were taken 18 h post-induction (hpi) with 20 μ M estradiol, cell death images were taken 48 hpi. Leaves are shown in false color, red indicates cell death and green healthy/alive tissue.

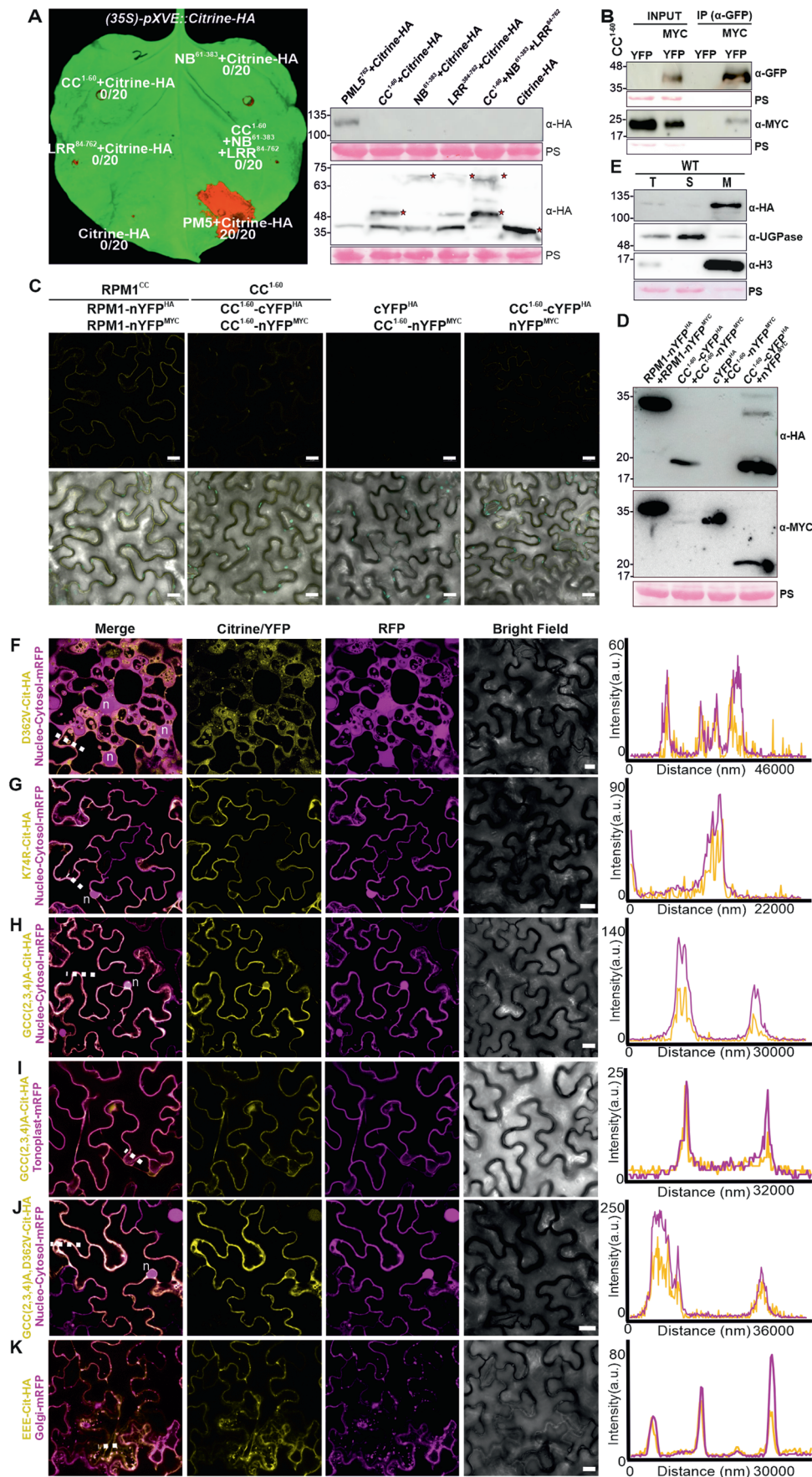


Figure EV3. PML5 N-terminal 60 amino acids are sufficient for cell death induction.

(A) Combination of PML5 CC¹⁻⁶⁰, NB⁶¹⁻³⁸³, and LRR³⁸⁴⁻⁷⁶² domains is not sufficient to induce a WT-like cell death response. PML5 domains were transiently expressed alone or in co-infiltrations in *N. benthamiana*. Image was taken 2 days post-induction with 20 μ M estradiol. (Right panel) Total protein extracts were immunoblotted and detected with an anti-HA (α -HA) antibody. Ponceau S (PS) staining is shown as a loading control. The red asterisks indicate the different fragments expressed. Leaves are shown in false color, red indicates cell death and green healthy/alive tissue. (B) PML5 CC¹⁻⁶⁰ self-associates in *N. benthamiana* transient expression. C-terminally YFP and MYC tagged CC¹⁻⁶⁰ were co-expressed. Total proteins were immunoprecipitated using anti-GFP (α -GFP) beads and immunoblotted using an anti-GFP (α -GFP) and anti-Myc (α -myc) antibody. Ponceau S (PS) staining is shown as a loading control. (C) Bimolecular fluorescence complementation (BiFC) experiment of PML5 CC¹⁻⁶⁰ fusion proteins, CC¹⁻⁶⁰-cYFP^{HA} and CC¹⁻⁶⁰-nYFP^{MYC}, transiently expressed in *N. benthamiana*. No YFP complementation different to the negative control could be detected for the PML5 CC¹⁻⁶⁰ domain. RPM1 CC¹⁻¹⁵⁵ served as positive control showing YFP complementation. Co-expression of cYFP^{HA} and nYFP^{MYC} with PML5 CC¹⁻⁶⁰-nYFP^{MYC} and PML5 CC¹⁻⁶⁰-cYFP^{HA}, respectively, served as negative controls. Scale bars, 20 μ m. (D) Protein-blot analysis of total proteins from the transiently expressed proteins of the BiFC assay shown EV3C. Proteins were detected using anti-Myc (α -MYC) and anti-HA (α -HA) antibodies. Ponceau S (PS) staining is shown as a loading control. (E) Subcellular fractionation of transiently expressed PML5-Citrine-HA protein indicates a strong microsomal/membrane association. Total protein extracts were immunoblotted and detected with an anti-HA (α -HA) antibody for PML5, anti-UGPase (α -UGPase) as a cytosolic marker, and anti-Histone H3 (α -Histone H3) as a microsomal marker. T = total protein fraction; S = soluble protein fraction; M = microsomal protein fraction. (F-K) Confocal laser scanning microscopy showing subcellular localization of transiently expressed PML5-Citrine-HA mutants in comparison to different marker proteins: (F) D362V with nucleo-cytoplasmic mRFP; (G) K74R with nucleo-cytoplasmic mRFP; (H, I) GCC(2,3,4)A with nucleo-cytoplasmic mRFP and tonoplast mRFP; (J) GCC(2,3,4)A,D362V with nucleo-cytoplasmic mRFP; (K) T15E,L16E,F20E (EEE) with Golgi localized mRFP. White dotted lines indicate the area used for colocalization profile analysis and the corresponding profiles are shown on the right. *n* = nucleus. Scale bars, 10 μ m; for information on compartment marker proteins, see the Methods section. Protein expression was induced with 20 μ M estradiol and confocal imaging was performed at 6–8 h post-induction.

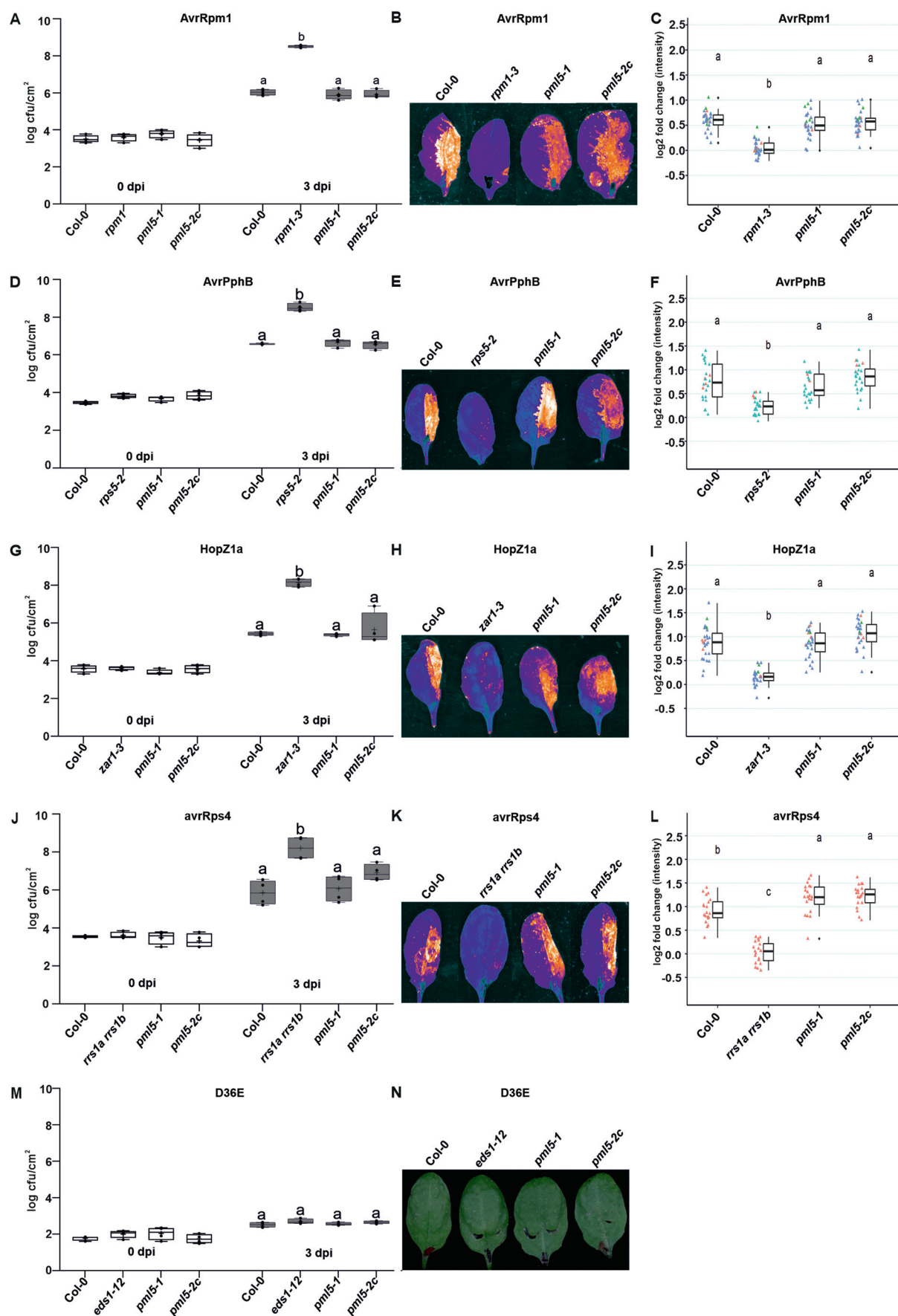
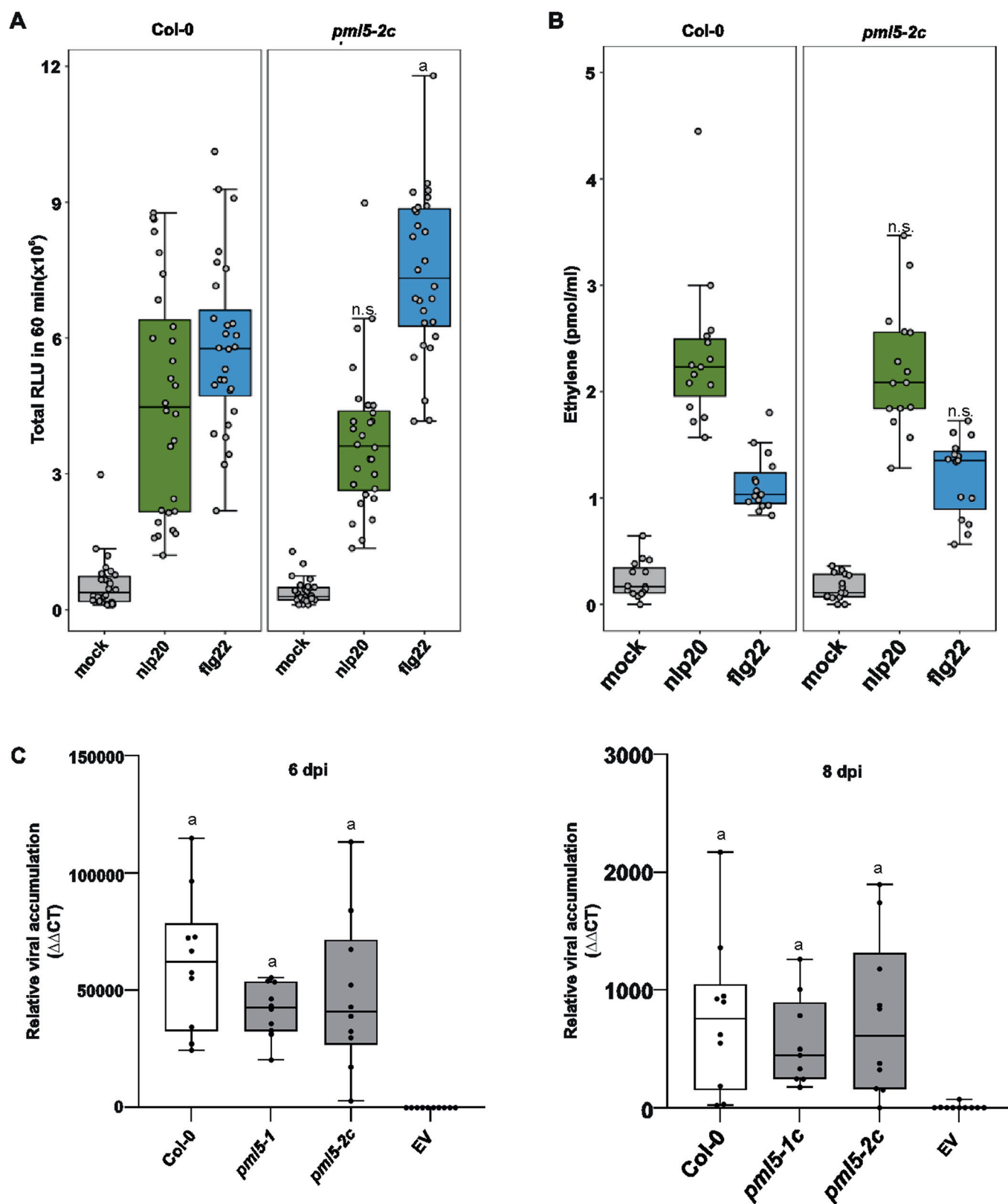


Figure EV4. *PML5* does not contribute to resistance against avirulent *Pst* DC3000.

(A–M) Resistance and cell death induced upon infection with different avirulent *Pst* DC3000 strains is not altered in *pml5* mutants. Rosette leaves of 6-week-old *Arabidopsis* plants were hand infiltrated with various *Pst* DC3000 strains using an $OD_{600} = 0.001$: (A) *Pst* DC3000 *AvrRpm1*, (D) *Pst* DC3000 *AvrPphB*, (G) *Pst* DC3000 *HopZ1a*, (J) *Pst* DC3000 *AvrRps4*, (M) *Pst* DC3000 D36E and bacterial growth was determined on day 0 and day 3 post infiltration. For (A, D, G, J, M), data are shown as boxplots and data points (colony forming units per square cm—cfu/cm²) are indicated as black dots and represent 1 biological replicate with 4 technical replicates each ($n = 4$, with 6 leaf samples each). (B, E, H, K) Induction and (C, F, I, L) strength of cell death is not affected in *pml5* mutants. The right side of the leaves was hand infiltrated with *Pst* DC3000: *AvrRpm1* (B, C), *AvrPphB* (E, F), and *HopZ1a* (H, I) at an $OD_{600} = 0.1$ and with *Pf0-1 AvrRps4* (K, L) at an $OD_{600} = 0.2$. Infiltrated leaves were imaged with a Typhoon laser scanner 5 h post infiltration (hpi) for *AvrRpm1* (B), 22 hpi for *AvrPphB* (E), 24 hpi for *HopZ1a* (H), and *AvrRps4* (K). The leaf images are shown in false color: Purple/blueish parts indicate non-infiltrated healthy tissue, and orange/yellowish dead cells. (C, F, I, L) Quantification of cell death intensity measured of infiltrated leaves similar to leaves shown in (B, E, H, K). Details on the methodology can be found in Methods section. Data are presented as boxplots. Results shown are from 1 biological replicate with 20 technical replicates for (L), 2 biological replicates with 24 technical replicates for (F) and 3 biological replicates with 28 technical replicates for (C, I). Data points of the different biological replicates are indicated by differently colored triangles. (N) Infiltration of *Pst* DC3000 D36E does not induce visible disease symptoms on *pml5-1*, *pml5-2c* or Col-0 and *eds1-12*. Data information: For (A, D, G, J, M) data are presented as boxplots (center line, median; bounds of box, the first and the third quartiles; whiskers, 1.5 times the interquartile range; error bar, minima and maxima), similarly for (C, F, I, L). There, black dots additionally represent outliers. Data points with different letters indicate significant differences of $P \leq 0.05$ (one-way ANOVA with a post hoc Tukey's HSD test). Exact *P* values for all experiments are provided in Dataset EV1.



◀ **Figure EV5. Loss of *PML5* has no effect on PRR-induced responses and TRV resistance.**

(A) Total ROS production in leaf discs of Col-0 and *pml5-2c* mutant treated with water (mock), 1 μ M nlp20, or 100 nM flg22 over 60 min. RLU, relative light unit. Data points are indicated as gray dots from four biological replicates each with seven technical replicates ($n = 28$) and plotted as boxplots. The gray color box indicates mock control, green indicates nlp20 treatment and blue indicates flg22 treatment in Col-0 and *pml5-2c* plants. (B) Ethylene accumulation in Col-0 and *pml5* mutant after 4 h treatment with water (mock), 1 μ M nlp20, or 1 μ M flg22. Data points are indicated as gray dots from five biological replicates each with 3 technical replicates ($n = 15$). (C) TRV viral accumulation was determined by qRT-PCR at 6 days post infection (dpi) (left) and 8 dpi (right) in infected Col-0, *pml5-1c*, and *pml5-2c* plants. Col-0 infected with an Empty Vector (EV) control served as negative control. Data points are from 10 different independent plants and the experiment was repeated 3 times (3 technical replicates) with similar results. Data information: Data in (A–C) are represented as boxplots (center line, median; bounds of box, the first and the third quartiles; whiskers, 1.5 times the interquartile range; error bar, minima and maxima). Statistical differences compared to the same treatment (A, B) in Col-0 were analyzed by two-sided Student's t-test ($\alpha = 0.05$) and are indicated with letters (a, $P < 0.01$, 'n.s.' not statistically different). In (C), data points with different letters indicate significant differences of $P \leq 0.05$ (one-way ANOVA with a post hoc Tukey's HSD test). Exact P values for all experiments are provided in Dataset EV1.