Induced proximity of a TIR signaling domain on a plant-mammalian NLR chimera activates defense in plants

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Plant and animal intracellular nucleotide-binding, leucine-rich repeat (NLR) immune receptors detect pathogen-derived molecules and activate defense. Plant NLRs can be divided into several classes based upon their N-terminal signaling domains, including TIR (Toll-like, Interleukin-1 receptor, Resistance protein)- and CC (coiled-coil)-NLRs. Upon ligand detection, mammalian NAP1 and NLRCA NLRC4 NLRs oligomerize, forming an inflammasome that induces proximity of its N-terminal signaling domains. Recently, a plant CC-NLR was revealed to form an inflammasome-like hetero-oligomer. To further investigate plant NLR signaling mechanisms, we fused the N-terminal TIR domain of several plant NLRs to the N terminus of NLRC4. Inflammasome-dependent induced proximity of the TIR domain in plants initiated defense signaling. Thus, induced proximity of a plant TIR domain imposed by oligomerization of a mammalian inflammasome is sufficient to activate authentic plant defense. Ligand detection and inflammasome formation is maintained when the known components of the NLRC4 inflammasome are transferred across kingdoms, indicating that NLRC4 complex can robustly function without any additional mammalian proteins. Additionally, we found NADase activity of a plant TIR domain is necessary for plant defense activation, but NADase activity of a mammalian or a bacterial TIR is not sufficient to activate defense in plants.

NLR immune receptors | plant immunity | inflammasome | effector-triggered immunity

Plants lack an adaptive immune system and rely on innate immunity to defend against pathogens (1, 2). Upon perception of pathogen effector proteins by plant nucleotide-binding, leucine-rich repeat (NLR) intracellular receptors, immune signaling is initiated that often culminates in a programmed cell death called the hypersensitive response (HR) (3). NLRs carry an N-terminal signaling domain, a nucleotide-binding (NB) domain, and a C-terminal leucine-rich repeat (LRR) domain (2). The P-loop (or Walker A) motif contained in the NB domain binds ATP or ADP. NLRs typically bind ADP in their resting state and exchange it for ATP when activated (1, 2, 4). The two major classes of plant NLRs are defined by the N-terminal signaling domain they contain: either a TIR (Toll-like, Interleukin-1 receptor, Resistance protein) or a CC (coiled-coil) domain (2). Ectopic expression of TIR and CC domains can activate immune signaling (1, 5–7). Self-association interfaces are required for TIR-mediated immune signaling (8–10), but the mechanism of signaling activation is unknown.

Animal NLR domain architecture resembles plant NLRs (1, 2, 11). NLRC4, a mammalian NLR, contains an N-terminal

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caspase activation and recruitment domain (CARD), a NACHT NB domain, and a C-terminal LRR. NLRC4 cooperates with NAIP NLRs to detect bacterial PAMPs (pathogen-associated molecular patterns); in mice, NAIP1 and NAIP2 detect type III secretion system (T3SS) needle and rod components, respectively, and NAIP5 detects flagellin (12, 13). The PAMP binds to the NAIP protein, altering its conformation and provoking recruitment of an NLRC4 molecule, which initiates stepwise recruitment of additional NLRC4 molecules, forming a wheel-like oligomer called an inflammasome (14–16). This complex brings the N-terminal CARDs into close proximity, allowing recruitment and activation of caspases (1, 17). Hence, activation of NLRC4-mediated immune signaling occurs via induced proximity of N-terminal signaling domains.

Recently, the structure of *Arabidopsis* ZAR1, an NLR with a CC N-terminal signaling domain, was resolved in complex with the pseudokinase RKS1 and the decoy kinase PBL2 (18, 19). ZAR1 associates with RKS1, and the effector AvrAC uridylylates PBL2 and induces its recruitment to the ZAR1-RKS1 heterodimer (20). Subsequently, a wheel-like structure forms, termed a “resistosome,” consisting of five heterotrimeric ZAR1-RKS1-PBL2 protomers, and activates an immune response (18, 19, 21). Similar to the NLRC4-inflammasome, induced proximity is imposed on the N-terminal CC signaling domains leading to a

![Diagram of domain architecture of TIRRPS4 drawn to scale.](https://example.com/diagram)

**Fig. 1.** Induced proximity of TIRRPS4 triggers HR-like cell death when fused to NLRC4. (A) Schematic diagram of domain architecture of TIRRPS4-NLRC4 drawn to scale. TIR domain, Toll-like, interleukin-1 receptor, Resistance protein domain; CARD, caspase activation and recruitment domain; NACHT, NAIP, CIITA, HET-E, TP1; LRR, leucine rich repeat. TIRRPS4 contains amino acids residues 1–236 of RPS4. (B and C) Oligomerization assay to test the formation of inflammasome-like complexes in plants. *N. benthamiana* leaves were transiently cotransfected with combinations of TIRRPS4-NLRC4, NAIP, PAMP (as indicated by a + or symbol), and silencing suppressor p9 by *A. tumefaciens* infiltration. After 3 d, leaves were harvested, proteins tagged with a FLAG epitope were immunoprecipitated from SDS-PAGE (B) and BN-PAGE (C), and immunoblotted for V5, FLAG or Myc. Results shown are representative of at least three independent replicates. See also SI Appendix, Fig. S1. Arrowhead indicates predicted inflammasome complex. HF, (His)6-(FLAG)3 tag. SDS-PAGE (D) and BN-PAGE (E) of mutant versions of TIRRPS4-NLRC4 or NAIPs. TIRRPS4-NLRC4, NAIP, and FlaA were immunoprecipitated from extracts from coinfiltrated *N. benthamiana* leaves. Results shown are representative of at least three independent replicates. D/A and L/D indicates TIRRPS4 mutations R116A; ∆P indicates NAIP5 with a deleted P-loop (amino acids 464–487; NAIP5∆Ploop). Arrowhead indicates predicted inflammasome complex.
significant structural change in this domain. This suggests that induced proximity of N-terminal signaling domains may be a conserved mechanism of signaling activation in NLRs, although it has not yet been observed in TIR-domain containing NLRs. Here, we fused the TIR domain from RPS4, a well-characterized Arabidopsis NLR (22–24), to NLRC4 to investigate whether induced proximity imposed by an animal NLR is sufficient to activate an N-terminal TIR signaling domain of a plant NLR in plants.

Some but not all TIR domains can hydrolyze NAD$^+$ to nicotinamide and various forms of ADP ribose (ADPR) (25–28). A conserved catalytic glutamate is required for NAD$^+$ hydrolysis (25, 26). This catalytic glutamate is also required for defense activation for plant TIRs (22, 27). Plant and bacterial TIRs, in contrast to the TIR domain of mammalian SARM1, can make a variant cyclic ADPR (v-cADPR) (25, 27, 28). Here, we use the TIRRPS4-NLRC4 platform to demonstrate that while NADase activity of plant TIRs is necessary for their activation of cell death, the in vivo generation of v-cADPR or cADPR is not sufficient to induce cell death.

Results and Discussion

We examined whether NLRC4-imposed induced proximity is sufficient to activate defense mediated by the RPS4 TIR domain (TIR$^{RPS4}$). We generated a TIRRPS4-NLRC4 chimera under control of the CaMV 35S promoter for expression in plant leaves (Fig. 1A). To determine if this construct could form a TIRRPS4-NLRC4/NAIP/PAMP inflammasome in plants, we transiently coexpressed epitope-tagged TIRRPS4-NLRC4, NAIP2, NAIP5, and either Legionella pneumophila flagellin (FlaA) or Salmonella typhimurium T3SS rod protein PrgJ in Nicotiana benthamiana. To detect inflammasomes, we separated proteins extracted from infiltrated leaves by blue native polyacrylamide gel electrophoresis (BN-PAGE) (SI Appendix, Fig. S1). We additionally purified the complex by immunoprecipitating NAIPs to remove donor surface mutants (L435D) of TIRRPS4-NLRC4 with donor surface of other NLRC4s and NAIPs (14).

We next asked whether a mammalian inflammasome signaling platform could activate TIR-dependent plant responses, like HR. Transient coexpression of the following inflammasome-forming combination triggered HR in N. tabacum leaves: TIR$^{RPS4}$-NLRC4, NAIP1, and the T3SS needle protein from Yersinia pestis (YscF); TIR$^{RPS4}$-NLRC4/NAIP2/PrgJ; and TIR$^{RPS4}$-NLRC4/NAIP5/FlaA (Fig. 2 A and B; HR index scale shown in SI Appendix, Fig. S2F). No cell death was observed when combinations that do not form inflammasomes were coexpressed (Fig. 2 A and B). TIR$^{RPS4}$-NLRC4 donor and acceptor surface mutants, which are incapable of forming inflammasomes, coexpressed with NAIP5 and FlaA did not trigger HR (Fig. 2C); this indicates that a higher-order complex containing multiple TIR domains is required, and not just a conformational change in TIR$^{RPS4}$-NLRC4 induced by interaction with NAIP5/FlaA. Similarly, NAIP5 P-loop mutants did not trigger HR when expressed with TIR$^{RPS4}$-NLRC4 and FlaA (SI Appendix, Fig. 2C).

In contrast, the TIR domain of mammalian SARM1, can make a multiple TIR domains is required, and not just a conformational change in TIR$^{RPS4}$-NLRC4 induced by interaction with NAIP5/FlaA.

We designed the N-terminal TIR signaling domain of a plant NLR that activates plant TIR-dependent responses in a proof-of-principle experiment. In this study, we show that a TIR domain from RPS4, a well-characterized Arabidopsis NLR, can activate an N-terminal TIR signaling domain of a plant NLR in plants. However, the in vivo generation of cyclic ADPR (cADPR) or v-cADPR is not sufficient to induce cell death.

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An oligomeric complex of >1,200 kDa, consistent with an inflammasome, also appeared upon coexpression in leaves of TIR$^{RPS4}$-NLRC4 and cognate NAIP-PAMP pairs (Fig. 1C).

NLRC4 has two interaction surfaces that mediate oligomerization: the “donor” surface, which recruits NLRC4 monomers to the complex, and the “acceptor” surface, which interacts with the donor surface of other NLRC4s and NAIPs (14–16). We transiently coexpressed either an acceptor surface mutant (D125A) or a donor surface mutant (L435D) of TIR$^{RPS4}$-NLRC4 with NAIP5 and FlaA. As expected, the acceptor surface mutant TIR$^{RPS4}$-NLRC4(D125A) did not immunoprecipitate with NAIP5 (Fig. 1D), nor was it capable of forming an inflammasome, because the acceptor surface was prevented from associating with the donor surface of NAIP5 (Fig. 1E). The donor surface mutant TIR$^{RPS4}$-NLRC4(L435D) did immunoprecipitate with NAIP5 but did not form a >1,200 kDa oligomers (Fig. 1E). NAIP5 and FlaA immunoprecipitated with TIR$^{RPS4}$-NLRC4(L435D) at a lower apparent affinity than with TIR$^{RPS4}$-NLRC4 (Fig. 1D). This may be due to an avidity defect: A fully assembled inflammasome ring likely increases the apparent affinity of all members of the complex. Therefore, the TIR$^{RPS4}$-NLRC4(L435D)/NAIP5/FlaA trimer may dissociate more readily than the full 13-mer inflammasome. Consistent with previous results (12), a P-loop motif deletion in NAIP5 prevented in planta inflammasome assembly (Fig. 1 D and E). Taken together, these results indicate that TIR$^{RPS4}$-NLRC4/NAIP/PAMP can form an authentic inflammasome complex in plants..

We next asked whether a mammalian inflammasome signaling platform could activate TIR-dependent plant responses, like HR. Transient coexpression of the following inflammasome-forming combination triggered HR in N. tabacum leaves: TIR$^{RPS4}$-NLRC4, NAIP1, and the T3SS needle protein from Yersinia pestis (YscF); TIR$^{RPS4}$-NLRC4/NAIP2/PrgJ; and TIR$^{RPS4}$-NLRC4/NAIP5/FlaA (Fig. 2 A and B; HR index scale shown in SI Appendix, Fig. S2F). No cell death was observed when combinations that do not form inflammasomes were coexpressed (Fig. 2 A and B). TIR$^{RPS4}$-NLRC4 donor and acceptor surface mutants, which are incapable of forming inflammasomes, coexpressed with NAIP5 and FlaA did not trigger HR (Fig. 2C); this indicates that a higher-order complex containing multiple TIR domains is required, and not just a conformational change in TIR$^{RPS4}$-NLRC4 induced by interaction with NAIP5/FlaA. Similarly, NAIP5 P-loop mutants did not trigger HR when expressed with TIR$^{RPS4}$-NLRC4 and FlaA (SI Appendix, Fig. 2C).
S24). These data suggest that induced proximity of the RPS4 TIR domain is sufficient to activate immune sensing and HR.

Structure-function analyses of TIR domains revealed two surfaces of the RPS4 TIR essential for RPS4-mediated immunity: The AE interface surface, required for heterodimerization and homodimerization, and the DE surface, predicted to be a self-interaction surface (8, 9). To test whether inflammasome-mediated signaling acts by promoting self-association through these interfaces, we introduced an AE interface mutation (S33A, H34A; SH/AA) and a DE interface mutation (R116A) into separate TIR-RPS4-NLRC4 constructs, NAIP5 and FlaA formed inflammasome-like TIR-RPS4(NH2/SH)-NLRC4 and TIR-RPS4(R116A)-NLRC4 (Fig. 1 D and E, and SI Appendix, Fig. S2 B and C). Inflammasomes containing TIR-RPS4(NH2/SH)-NLRC4 exhibited no HR, and TIR-RPS4(R116A)-NLRC4 exhibited occasional, weak HR (SI Appendix, Fig. S2 D and E). Taken together, these data demonstrate that oligomerization via the NLRC4-NACHT was not sufficient for TIR-RPS4 HR and that both TIR self-association interfaces are required. Wan et al. and Horsecfield et al. reached similar conclusions by fusing TIR-RPS4 to the SAM oligomerization domain from SARM1 (27, 28). Hence, induced proximity promotes formation of a TIR domain oligomer that is sufficient for signaling through the AE and DE interfaces.

Induced proximity of N-terminal signaling domains may be a general mechanism of TIR-NLR signaling activation. To test this, we fused to NLRC4 the TIRs of the plant NLRs SNC1 from Arabidopsis; N from tobacco; L6, M, and P from flax; and RRS1, an NLR that acts with RPS4 to confer effector perception in Arabidopsis (refs. 23, 24, and 29; alignment of TIRs shown in SI Appendix, Fig. S3). Two fragments of TIR-SNC1 activated HR when fused to NLRC4 coexpressed with NAIP5 and FlaA: TIR-SNC1(1-179), the minimal TIR domain containing amino acid residues 1–179, and TIR-SNC1(1-226), an autoactive fragment (Fig. 3 A and B) (9). TIRL6 activated HR when fused to NLRC4 coexpressed with NAIP5 and FlaA (Fig. 3 C) but was also active in the absence of the appropriate ligand, suggesting autoactivity of this construct. TIR-P2/L6, an L6 construct with its N-terminal Golgi membrane anchor replaced with the N terminus of the flax NLR P2 (30), showed HR only in the presence of the correct ligand (Fig. 3 D). AE and DE interface mutations in these TIRs also abolished HR (Fig. 3 E), except for TIR-SNC1(1-179) (SI Appendix, Fig. S3 B). Although this mutation reduces the visible HR induced by TIR-SNC1, it does not affect self-association in solution and has only a minor effect on ion leakage, suggesting that it only partially impairs function (9). TIR-RPS4-NLRC4 did not trigger inflammasome-dependent HR (SI Appendix, Fig. S2 E), consistent with RRS1’s “sensor” function in the RRS1/RPS4 pair: RPS4 monitors RRS1 and initiates immune signaling when RRS1’s WRKY domain interacts with effectors (24).

In contrast to TIR-SNC1 and TIRL6, the TIRs from N, M, and P did not trigger HR when fused to NLRC4 and coexpressed with FlaA and NAIP5 (SI Appendix, Fig. S4). Tobacco leaf sections infiltrated with TIR-NLRC4, NAIP5, and FlaA displayed yellowing, but TIR-M-NLRC4 and TIR-P-NLRC4 fusions were green and healthy (SI Appendix, Fig. S4). Therefore, other factors likely influence TIR domain capacity to trigger HR upon induced proximity.

TIR-mediated immunity is dependent on proteins that act as hubs for signaling, including the lipase-like nucleotycplasmic protein, EDS1, and the RPW8-NLR, NRG1 (2, 31, 32). TIR-RPS4-NLRC4/NAIP5/FlaA did not trigger HR in an EDS1-silenced tobacco RNA interference (RNAi) line but did trigger HR in nonsilenced tobacco (SI Appendix, Fig. S5A). We also tested the activation of HR by TIR-RPS4-NLRC4/NAIP5/FlaA in N. benthamiana nrg1 mutants. TIR-RPS4-NLRC4/NAIP5/FlaA triggered HR in WT N. benthamiana but not in the nrg1 mutant (SI Appendix, Fig. S5B). Furthermore, we demonstrated the requirement of EDS1 for HR triggered by other TIR-NLRC4 constructs by coinfiling NAIP5, FlaA, and NLRC4 fusions to either TIRL6, TIR-P2/L6, or TIR-SNC1 in N. benthamiana eds1 mutants (SI Appendix, Fig. S5C). Complementing these mutants transiently with N. benthamiana EDS1 rescued HR triggered by these TIR-NLRC4 constructs (SI Appendix, Fig. S5C) (33). The genetic requirement for the immune signaling hubs EDS1 and NRG1, as well as the requirement for intact TIR interaction interfaces, demonstrates that HR triggered by TIR-RPS4-NLRC4/NAIP5/FlaA mimics RPS4/RRS1-mediated HR.

The TIRs used in this study (except for that of RRS1) contain a conserved glutamate, which is at position 88 in RPS4 (E88)
(SI Appendix, Fig. S3). The glutamate at this position is required for TIR NADase activity (26–28). Mutating this residue in full-length RPS4 or TIRPS4 abolishes HR (22, 27, 34). An E88A substitution to TIRRPS4-NLRC4 abolished HR upon inflammasome formation (SI Appendix, Fig. S2 B, C, and E). We hypothesized that the induced inflammasome would activate plant TIR NADase activity, which is required for HR in our system. To test this hypothesis, we coexpressed the inflammasome components in N. benthamiana and purified the TIR-NLRC4 proteins or complexes by immunoprecipitation. Beads with the purified TIR-NLRC4 proteins were then incubated with NAD+ (5 μM), and metabolites were extracted and analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). NAD+ could be cleaved into nicotinamide (Nam), ADPR, and cADPR (25). A TIRSARM1-NLRC4/NAIP5/FlaA inflammasome complex showed strong production of Nam as well as a reduction in NAD+ levels in the reaction mix (SI Appendix, Fig. S6 A and B). However, when purified from leaves expressing TIRSARM1-NLRC4/NAIP5/FlaA, the monomeric TIRSARM1-NLRC4 did not show NADase activity (SI Appendix, Fig. S6 A and B), consistent with a requirement for oligomerization for SARM1-TIR enzymatic activity (26). In contrast, we detected no significant loss of NAD+ and no detectable production of Nam with TIRRPS4-NLRC4/NAIP5/FlaA or TIRP2L6-NLRC4/NAIP5/FlaA (SI Appendix, Fig. S6 A and B). TIRL6 has been reported to have higher enzymatic activity than TIRRPS4, but both proteins have much lower activity than detected for SARM1 (28).

Recent reports revealed that plant TIR domains, like certain prokaryotic TIR domains, cleave NAD+ into a variant cADPR (v-cADPR), and v-cADPR may serve as a signaling molecule to trigger plant HR (35). We sought to test if TIRRPS4-NLRC4/NAIP5 inflammasome formation but HrpB75F did not induce TIRRPS4-NLRC4/NAIP2 inflammasome formation (Fig. 4B and SI Appendix, Fig. S8). Therefore, the TIRRPS4-NLRC4/NAIP inflammasome can assemble and trigger HR in plants in response to flagellin from plant-pathogenic bacteria, but not in response to rod components. However, despite reports that bacterial flagellin from Pst DC3000 can enter plant cells (35), we were unable to detect enhanced bacterial resistance in transgenic Arabidopsis lines carrying TIRRPS4-NLRC4 and NAIP5 (SI Appendix, Fig. S9). The discrepancy between the observation of HR in transient assays and the lack of an immune response in stably transformed Arabidopsis may be due to the different amounts of flagellin present in plant cells in each assay: Overexpression of flagellin in transient assays is likely to result in much more intracellular flagellin accumulation than occurs during Pst DC3000 infection.

By fusing plant NLR TIR domains to the N terminus of the mammalian NLR NLRC4, we showed here that induced proximity of plant NLR TIRs is sufficient for immune activation, resembling the activation mechanism of mammalian NLR CARDs. These data emphasize the modular nature of NLRs, where the NB domain provides a regulated mechanism for oligomerization and inducing proximity of N-terminal domains, and N-terminal domains can be swapped between NLRs and retain functionality. Similarly, our data demonstrate that the known components of the NAIP/NLRC4 inflammasome can detect ligands and functionally assemble when transplanted across kingdoms, suggesting that there are no additional unidentified mammalian-specific components required for NAIP/NLRC4 activation.

TIR activation required authentic oligomerization at the center of the assembled inflammasome. The linear helix hypothesized to form in an active TIR oligomer (9), or another signaling active conformation, must assemble in the TIR-NLRC4/NAIP inflammasome. Although the structure of the NLRC4 CARDs in an inflammasome has not been resolved, cryoelectron tomography predicts a helix at the center of an inflammasome (36), while purified NLRC4 CARDs in vitro form a tetramer that comprises the base of a helical filament (37, 38). Steric constraints imposed by CARD oligomerization may have prevented the assembly into a signaling-active conformation of some of the plant TIRs we tested.

The assembly of the ZAR1/RKS1/PBL2 resistosome coordinates the ZAR1-CCs into a pore-like structure. This structure resembles a pore-forming toxin, leading to the hypothesis that the ZAR1 resistosome triggers HR cell death through integration and pore formation in the plant plasma membrane (18). Although ZAR1 similarly requires induced proximity of N-terminal signaling domains, the TIR domains of RPS4 and other TNLs are not predicted to form pores.

We were unable to detect plant TIR domain NADase activity in immunopurified plant TIR-NLRC4 complex, perhaps due to its low enzymatic activity and abundance. However, NADase...
enzymatic activity is a conserved function across prokaryotic and eukaryotic TIR domain proteins (25–28), and the key catalytic glutamate residue is widely present in plant TIR domains (SI Appendix, Fig. S3). We infer that NADase activity of plant TIRs and the production of v-cADPR may be necessary but not sufficient for cell death and defense activation. Our data (SI Appendix, Fig. S5) and other reports show that plant TIR domain-induced HR requires EDS1, SAG101, and NRG1 (31, 32, 39–42). EDS1-SAG101-NRG1 have coevolved as a module to mediate cell death signaling by TIR-domain immune receptors within plant species (42, 43). Future work will focus on the additional components required for v-cADPR activation of immune signaling.

Materials and Methods

Plant Material and Growth Conditions. Nicotiana tabacum cv. “Pette Gerard” or “Samsun” and N. benthamiana were grown on soil at 24 °C, 55% relative humidity with a 16/8 h light/dark photoperiod. Arabidopsis thaliana accession “Col-0” was grown at 21 °C, 70% humidity with 10/14-h light/dark photoperiod. The N. tabacum cv. “Samsun” EDS1 RNAi line was provided by Barbara Baker, Department of Plant and Microbial Biology, University of California, Berkeley, CA, the N. benthamiana eds1 line was provided by Brian Staskawicz, Department of Plant and Microbial Biology, University of California, Berkeley, CA (33), and we previously generated the N. benthamiana nrg1 line (32).

Plasmid Construction. Plasmids were constructed using Golden Gate cloning, as described in refs. 24 and 44. The TIR domain of RPS4 was PCR amplified with primers containing 6bp recognition sites and a specific 4-bp overhang, then cloned into the pICSL01002. All other TIR domains were similarly cloned. The TIRsARM1 was PCR amplified from pGW1-Myc-Sarm1 (45), a gift from Yi-Ping Hsueh, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan (Addgene plasmid no. 50707; http://www.addgene.org/50707/; RRID:Addgene_50707). NLRC4, NAIPs, and PAMPs were similarly cloned into the coding sequence module pICSL01005. Modules in pICSL01005 and pICSL01002 were released with BsaI digestion and assembled in pICSLB8900 with the CamV35 promoter, Ocs terminator, and N-terminal and/or C-terminal epitope tags.

Leaf Infiltration. Transient transformation by agroinfiltration of N. tabacum lamina sections between veins for HR and whole N. benthamiana leaves for protein analyses was performed on 4- to 5-wk-old plants. Agrobacterium tumefaciens strains were mixed at 10:10:10 in infiltration medium (10 mM Tris, 10 mM 2-(N-morpholino)ethanesulfonic acid [MES], pH 5.6) each at an OD$_{600}$ of 0.5, and hand-infiltrated with a 1-mL needle-less syringe.

Protein Assays. Protein was extracted from transiently transformed N. benthamiana leaves 72 h postinfiltration (hpi) with A. tumefaciens as previously described (23). Briefly, leaves were harvested and ground in liquid nitrogen, and extracted in GTEN buffer (100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 5 mM 1,4-dithiothreitol [DTT], 1× complete protease inhibitor mixture [Roche] and 0.2% [vol/vol] Nonidet P-40). Cleared samples were separated into subsamples for input for BN-PAGE, input for SDS-PAGE, and samples for immunoprecipitation. Immunoprecipitations were performed for 4 h at 4 °C with gentle agitation, in the presence of 10 μL per 1 mL of protein extract of anti-FLAG M2 affinity Gel (A2220 Sigma-Aldrich), anti-V5 (A7345 Sigma-Aldrich) or anti-Myc (9E10 Thermofisher). Beads were washed four times in GTEN buffer. FLAG beads were incubated with 150 ng/μL 3XFLAG peptide (Sigma-Aldrich) for 30 min at 4 °C. Other samples were eluted by boiling in SDS-PAGE loading buffer. For BN-PAGE, IP-FLAG eluate and input samples were mixed with 10× BN-PAGE loading buffer, loaded onto Invitrogen NativePAGE 3–12% Bis-Tris Protein Gels, and electrophoresed according to the manufacturer’s instructions. For SDS-PAGE, anti-V5 and anti-Myc beads, as well as IP-FLAG eluate and input samples, were mixed with 3× SDS-PAGE loading buffer and heated for 20 min at 80 °C. After electrophoresis, separated proteins were transferred to Immunobilon- P PVDF (Merck Millipore) membranes for immunoblotting. Membranes were blocked for 2 h in 5% nonfat milk, probed with horseradish peroxidase (HRP)-conjugated antibodies overnight and imaged.

Fig. 4. TIR$^{RPS4}$-NLRC4 and NAIP5 confer perception of flagellin from plant-pathogenic bacteria in plants. (A) Transient coexpression of TIR$^{RPS4}$-NLRC4 and plant-pathogenic bacterial flagellin and NAIP5, but not NAIP2 and T3SS rod components, triggers HR-like cell death when coexpressed with TIR$^{RPS4}$. N. tabacum leaf sections were coinfected with A. tumefaciens strains (each at OD$_{600} = 0.5$) carrying TIR$^{RPS4}$-NLRC4, a NAIP, and a PAMP. HR was visually assessed and photographed after 3 dpi. The numbers in parentheses are the number of leaves displaying HR equivalent to the image shown out of the total number of leaves infiltrated. Superscript abbreviations indicate plant-pathogenic bacterial origin of genes encoding each PAMP: Pst, Pst DC3000; Xeu, X. euvesicatoria; Rso,Ralstonia solanacearum. (B) BN-PAGE of immunoprecipitated combinations of TIR$^{RPS4}$-NLRC4, NAIP5, and Pst DC3000 PAMPs. Arrowheads indicate inflammasome oligomer. See also SI Appendix, Fig. S8.

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**NADase Assay and LC-MS/MS Metabolite Measurement.** Transiently expressed proteins were extracted from *N. benthamiana* as described above. NADase activity was measured as described in Esposito et al. (26). To be brief, 20 μL anti-FLAG beads with bound protein was mixed with 5 μL of reaction buffer (924 mM NaCl and 6.4 μM phosphate buffered saline (PBS)), 5 μL of 100 μM NAD⁺ and 20 μL of water. Reactions were either terminated immediately, or after 30 min at 20 °C with periodic vortexing, by the addition of 50 μL of 1 M perchloric acid and incubation on ice for 10 min. Terminated reactions were neutralized by the addition of 16.7 μL of 3 M K₂CO₃. Samples were then centrifuged, and 10 μL of supernatant was frozen in liquid nitrogen and stored at −80 °C. NAD⁺ and nicotinamide (Nam) were measured in reaction supernatants by LC-MS/MS. Samples were separated on 100 × 2.1 mm 2.6 μM Kinetex EVO C18 column with guard, whose runoff was attached to a 50 × 2.1 mm 2.6 μM Kinetex F5 column. The high performance liquid chromatography (HPLC) was run with an aqueous solvent of 0.1% formic acid adjusted to pH 6.02 by addition of ammonium hydroxide, with a gradient to 60% methanol. Target compounds were detected by electrospray MS using the 2020 single quadrupole’s dual ion source in ESI mode, with spray chamber conditions of 200 °C heat block, 250 °C desorption line, 1.5 L.min⁻¹ nebulizer gas, and 15 L.min⁻¹ drying gas. The instrument collected positive mode scan data from m/z 100–800 and single ion monitoring data for masses 123.1, 664, 560, and 542 (positive, total even time 0.1 s).

Plant extracts were prepared and analyzed according to Won et al. (27). Briefly, after transiently transforming *N. benthamiana* leaves 45 hpi with *A. tumefaciens*, leaves were harvested and ground in liquid nitrogen. Samples were extracted with 50% methanol in water and deproteinized with chloroform. The aqueous phase was lyophilized and reconstituted in 5 mM ammonium formate, centrifuged at 13,000 rpm for 10 min, and the supernatant analyzed by LC-MS/MS. Analysis of v-cADPR was on an Acquity ultra performance liquid chromatography (UPLC) attached to a Xevo TQS tandem quadrupole mass spectrometer. Chromatography was exactly the same as for the single quadrupole. *E. coli* lysate of AbTIR was used as a reference for v-cADPR. Spray chamber conditions were 500 °C desolvation temperature, 900 L.hr⁻¹ desolvation gas, 150 L.hr⁻¹ cone gas, 7 bar nebulizer pressure.

**Bacterial Growth Assay.** Pst DC3000 strains were grown on selective King’s B (KB) medium agar plates for 48 h at 28 °C. Bacteria were harvested from the plates and resuspended in infiltration buffer (10 mM MgCl₂, pH 5.6), and the OD₆₀₀ of the resuspended cell was adjusted to 0.001. Leaves were hand-infiltrated by a needleless syringe. Leaf discs were harvested using a 6-mm cork borer. Two leaf discs per seedling were used as a single treatment, with four replicates sampled after infiltration and eight replicates after 3 days postinfiltration (dpi). Samples were ground and the lysate was diluted in infiltration buffer, and then spotted on selective KB medium plates.

**Data Availability.** All data are contained in the manuscript, supplemental figures, or Dataset S1.

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