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Letter

A disease resistance assay in *Nicotiana benthamiana* reveals the immune function of Response to HopBA1

Keiichi Hasegawa,[1] Ton Timmers,[2] Jijie Chai,[1,3,†] and Takaki Maekawa[3,4,*]

[1]Institute for Biochemistry, University of Cologne, 50674 Cologne, Germany
[2]Central Microscopy, CEMIC, Max Planck Institute for Plant Breeding Research, 50829 Cologne, Germany
[3]Cluster of Excellence on Plant Sciences (CEPLAS), Cologne, Germany
[4]Institute for Plant Sciences, University of Cologne, 50674 Cologne, Germany
*Author for correspondence: tmaekawa@uni-koeln.de
[†]Present address: School of Life Sciences, Westlake University, Zhejiang 310024, China.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/General-Instructions) is Takaki Maekawa (tmaekawa@uni-koeln.de).

Dear Editor,

Resistance (R) proteins, such as intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) or plasma membrane proteins with an extracellular leucine-rich repeat domain, are integral components of the plant innate immune system (van Wersch et al. 2020). R protein–mediated host responses include the generation of reactive oxygen species, sustained increases in cytosolic Ca^{2+}, transcriptional reprogramming, and, typically, rapid host cell death at sites of pathogen infection, which together ultimately should lead to pathogen growth restriction (Morel and Dangl 1997; Wang et al. 2023). To assess the activity of a given R protein or its mutant variants, agroinfiltration-mediated transient gene expression assays have been widely used in *Nicotiana* species (e.g. *Nicotiana benthamiana*). In these transient assays, overexpression of *R* genes often leads to constitutive activation of downstream host responses in the absence of pathogens, i.e. autoimmunity (Chakrabarti et al. 2009; Nishimura et al. 2017; van Wersch et al. 2020), and host cell death is often chosen as an indicator of R protein activity from the host responses mentioned above, in part because of the ease of experimentation. However, the extent to which host cell death is a proxy for disease resistance signaling has long been debated, as host cell death and pathogen growth restriction can be uncoupled in several cases (Bendahmane et al. 1999; Coll et al. 2010; Maekawa et al. 2023). To assess the disease resistance activity of R proteins, for example, bacterial growth assays have been employed in combination with transient *R* gene expression in *N. benthamiana* (Buscaill et al. 1999). Bacterial growth assays, however, require multiple experimental procedures, including agroinfiltration, pathogen infection, and bacterial counts, which hinders high-throughput studies of *R* gene–mediated disease resistance. Here, we report a simple plate reader–based assay to assess *R* gene–mediated disease resistance activity by quantifying the growth of Potato virus X (PVX) that produces yellow fluorescent protein (YFP) (PVX-YFP). As a proof of concept, we used this assay to investigate whether a previously identified noncell death–inducing mutant of the R protein of Response to HopBA1 (RBA1), which lacks 2′,3′-cAMP/cGMP synthetase activity but retains NADase activity (Nishimura et al. 2017; Wan et al. 2019; Tian et al. 2022; Yu et al. 2022), still has the ability to mediate an immune response. We found that the cell death–defective RBA1 variant suppressed viral growth, indicating a cell death–independent immune function of RBA1.

PVX is a single-stranded RNA virus and expression of full-length PVX coding sequence via agroinfiltration-mediated expression allows PVX replication in *Nicotiana* species (Larsen and Curtis 2012). Insertion of a coding sequence of a fluorescent protein (e.g. GFP and YFP) under a duplicated copy of the viral coat protein promoter allows tracking of PVX replication in plants (Peart et al. 2002). Unlike bacterial growth assays in *Nicotiana* species, where the pathogen inoculation step takes place a few days after agroinfiltration, in the PVX disease resistance assay, 2 agroinfiltrations can be performed simultaneously, 1 with an *Agrobacterium* (*Agrobacterium tumefaciens*) strain containing a binary vector carrying an *R* gene, the other strain carrying a binary vector for expression of the infectious PVX RNA (Collier et al. 2011).

In this study, we generated a binary vector that allows for PVX replication along with monomeric YFP production and used YFP intensity as a proxy for PVX viral load. To quantify PVX-produced YFP in *N. benthamiana* leaf extracts, we optimized a fluorescence plate reader–based measurement (Fig. 1A), which is simpler than quantification by western blot. Briefly, in this method, agrobacteria carrying the PVX-YFP binary vector and agrobacteria carrying an expression construct for an R protein were coinfiltrated into *N. benthamiana* leaves. Four days after infiltration, leaf discs were collected from the infiltrated areas using a biopsy punch. The leaf protein extracts were used to measure YFP intensity (detailed method is described in the Supplementary Methods S1). First, we determined the emission spectra of leaf extracts containing PVX-YFP or PVX without fluorescent protein (Fig. 1B). The emission spectrum of PVX-produced YFP in the leaf extract matched the canonical spectrum of YFP (Fig. 1B), indicating that PVX replication in leaves did not produce fluorescence that overlapped with the YFP spectrum. The intensity plot of YFP in a dilution series of lysate from leaves infected with PVX-YFP, diluted with a leaf protein extract from nontransformed leaves,

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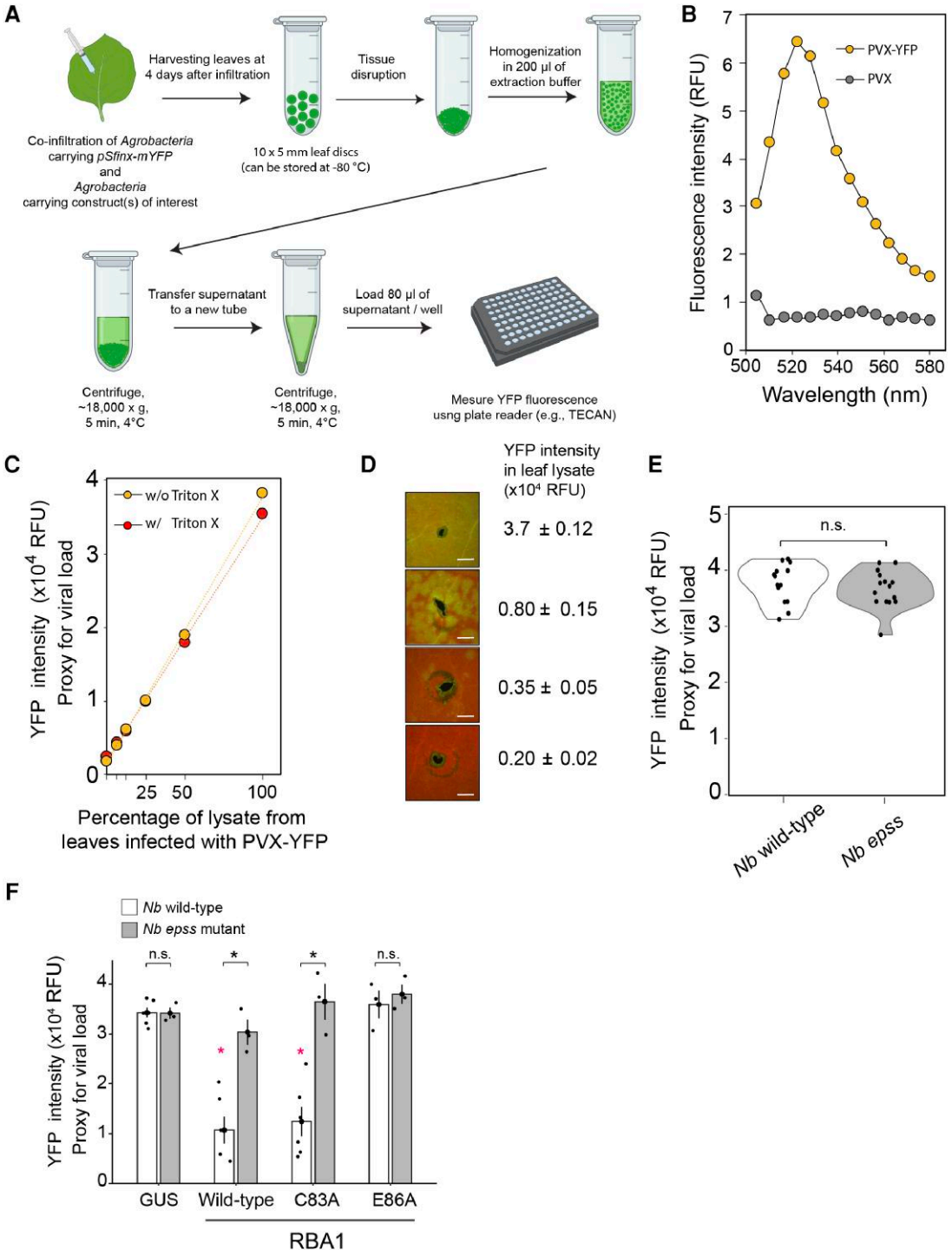

Figure 1. Disease resistance assay using Potato virus X expressing YFP in *N. benthamiana*. **A)** Schematic representation of disease resistance assay using Potato virus X expressing YFP (PVX-YFP). **B)** The emission spectra for leaf extracts expressing PVX-YFP or PVX. The *y* axis shows relative fluorescence units. The emission spectra were determined by a confocal microscope. **C)** A linear correlation between YFP fluorescence and dilution factor in a leaf lysate containing PVX-YFP in the presence and in the absence of 0.1% Triton X-100 in the extraction buffer. The *x* axis indicates percentage of lysate from leaves infected with PVX-YFP. Leaf lysate from nontransformed plants was used for dilution of lysate from leaves infected with PVX-YFP. The R^2 values with the linear regression model are >0.99 for both conditions (with or without Triton X-100). **D)** Representative stereomicroscopic fluorescence images of *N. benthamiana* leaves infiltrated with the *Agrobacteria* carrying the pSfinx-mYFP binary vector with OD_{600} values of 0.01, 0.001, 0.0001, or 0 (from top to bottom). Means and standard errors of YFP intensity of *N. benthamiana* leaf lysate infiltrated with the *Agrobacteria* carrying the pSfinx-mYFP binary vector measured by plate reader are shown ($n = 4$ for OD_{600} values of 0.01, 0.001, and 0.0001; $n = 3$ for OD_{600} value of 0). Samples were examined 4 d after infiltration. Scale bars indicate 5 mm. **E)** Violin plots showing the degree of dispersion of the quantified YFP intensities from leaves coinfiltrated with the *Agrobacteria* carrying the pSfinx-mYFP binary vector and the *Agrobacteria* carrying a *GUS* gene expression binary vector. The Wilcoxon rank sum test was used to compare the 2 groups ($P = 0.67$). **F)** The R protein of the Response to HopBA1 (RBA1) variant (C83A), which lacks 2′,3′-cAMP/cGMP synthetase activity but retains NADase activity (Yu et al. 2022), does not induce cell death but confers resistance to PVX. Bar plots describe the results of 4 independent replicates for wild type and 3 independent replicates for wild type and the *eds1 pad4 sag101a sag101b* (*epss*) mutant of *N. benthamiana*. Red asterisks indicate significant differences from the GUS control and black asterisks depict significant differences between the 2 genotypes as determined by analysis of variance followed by Tukey's honest significant difference test ($P < 0.05$). Error bars indicate standard error.

showed that this method has a high dynamic range with a linear correlation between YFP fluorescence and dilution factor (Fig. 1C). The addition of a detergent to the extraction buffer generally enhances protein extraction, but it may also release substances from endomembrane compartments or organelles, which could potentially affect YFP detection in the lysate. However, no apparent difference in YFP recovery or the YFP intensity plot was observed between samples with and without Triton X-100 (Fig. 1C). Therefore, Triton X-100 can be omitted from the extraction buffer.

Furthermore, the quantified YFP fluorescence intensities in the plate reader–based measurement corresponded to some extent with the fluorescence images (Fig. 1D). Consistent with a previous study (Peart et al. 2002), PVX-YFP proliferation was comparable in wild type and the *enhanced disease susceptibility 1* (*eds1*), *phytoalexin deficient 4* (*pad4*), and *senescence associated gene 101* (*sag101a* and *sag101b*) (*epss*) mutant of *N. benthamiana* (Fig. 1E). Coexpression of a wheat (*Triticum monococcum*) *NLR*, *stem rust 35* (*Sr35*), and its cognate effector, *AvrSr35* (Förderer et al. 2022), caused host cell death in *N. benthamiana* in the presence of PVX-YFP (Supplementary Fig. S1A), suggesting that *Agrobacterium*-mediated delivery of PVX-YFP T-DNA does not suppress NLR-induced host cell death. Furthermore, the low YFP intensity of leaf lysates from *Sr35*- and *AvrSr35*-coexpressing samples (mean relative fluorescence unit ± standard error = 1,991 ± 402, $n = 4$) is nearly equivalent to the YFP intensity of leaf protein extract from nontransformed leaves (Fig. 1C; refer to the data obtained when 0% of the lysate from leaves infected with PVX-YFP was used), implying that host cell death does not result in the production of metabolites that show autofluorescence in the YFP assay.

An Arabidopsis (*Arabidopsis thaliana*) R protein, RBA1, which possesses a Toll/interleukin-1 receptor–like domain (TIR domain) is responsible for the recognition of the bacterial-type III effector protein HopBA1 in *A. thaliana* but is able to induce host cell death in the absence of the effector upon transient expression in *N. benthamiana* (Nishimura et al. 2017; Yu et al. 2022). The TIR domain of RBA1 harbors NADase and nuclease activities, which are responsible for the generation of 2′-(5″-phosphoribosyl)-5′-adenosine monophosphate (pRib-AMP)/2′-(5″-phosphoribosyl)-5′-adenosine diphosphate (pRib-ADP) and ADP-ribosylated ADPR (diADPR)/ADP-ribosylated adenosine tri-phosphate (ADPr-ATP) and cyclic nucleotide monophosphates such as 2′,3′-cAMP/cGMP, respectively (Wan et al. 2019; Yu et al. 2022). The 2 enzymatic activities of RBA1 require 1 of the 2 catalytic residues, namely, Cys83 or Glu86. The RBA1 Cys83, which is a highly conserved among TIR-containing proteins, is responsible for nuclease activity but not for NADase activity. In contrast, RBA1 Glu86 is required for both catalytic activities of RBA1. The RBA1(C83A) variant that is compromised in nuclease activity but retains NADase activity is unable to elicit cell death in *N. benthamiana* (Yu et al. 2022). Therefore, both NADase and nuclease activities of RBA1 are required for RBA1-mediated cell death. However, it remains unknown whether the C83A substitution in RBA1, which impairs cell death induction, also impairs disease resistance activity.

To address this question, we used the PVX-YFP–based assay to examine the disease resistance activity of the RBA1(C83A) variant and the RBA1(E86A) variant as a control. Interestingly, we found that the RBA1(C83A) variant retained the same disease resistance activity as wild-type RBA1, whereas RBA1(E86A) showed no disease resistance activity to PVX (Fig. 1F). In the presence of PVX, wild-type RBA1 caused host cell death, while the RBA1(C83A) variant did not (Supplementary Fig. S1B). These data suggest that the 2′,3′-cAMP/cGMP synthase activity (i.e. nuclease activity) of RBA1 is required for host cell death but that the residual NADase activity is sufficient to confer immunity to PVX. Furthermore, the disease resistance activity of the RBA1(C83A) variant was significantly impaired in the *epss* mutant (Lapin et al. 2019) of *N. benthamiana* (Fig. 1F). As EDS1 functions with PAD4 or SAG101 (Feys et al. 2005), the *epss* mutant is completely defective in the EDS1 signaling pathway. Therefore, our result is consistent with the model that TIR NADase products mediate immunity via the EDS1 signaling pathway (Huang et al. 2023).

In summary, our results have uncoupled the disease resistance activity of RBA1 from the host cell death that is induced by 2′,3′-cAMP/cGMP synthetase activity. Similar to RBA1, the *Arabidopsis* TIR–containing NLR Suppressor of *npr1-1* constitutive 1 (SNC1) does not seem to require 2′,3′-cAMP/cGMP synthetase activity (i.e. nuclease activity) for disease resistance (Tian et al. 2022). Therefore, it is possible that there are cell death–signaling receptors that perceive 2′,3′-cAMP/cGMP. However, for cell death signaling, it is essential that EDS1 receptor complexes perceive the TIR NADase products. Another possibility is that there may be a concentration threshold beyond which TIR NADase products trigger cell death. In this scenario, both TIR NADase and 2′,3′-cAMP/cGMP synthase activities are required to reach the threshold for cell death induction. In particular, although the RBA1(C83A) variant retains NADase activity (Yu et al. 2022), it remains to be seen whether this variant can produce TIR NADase–derived products that exceed the aforementioned "threshold" *in planta*.

In contrast to bacterial infection, PVX proliferation in *N. benthamiana* is not restricted by the intrinsic activity of the EDS1 signaling pathway as previously shown by virus-induced *NbEDS1* gene silencing (Peart et al. 2002) and as we consistently show in this study using a *Nbeds1* gene knockout mutant (Fig. 1E). These results suggest that PVX proliferation in leaves is not accompanied by generation of signaling molecules derived from TIR enzymatic activities. Therefore, immunity to PVX via the EDS1 pathway is negligible, and this feature means that this method can be used to test a weak-to-moderate disease resistance activity of given R proteins without masking by immunity mediated by the EDS1 pathway. Finally, we would like to emphasize that disease resistance assays are not intended as a replacement for the widely used cell death assay in the *N. benthamiana* and *N. benthamiana*–transient gene expression system; rather, these 2 assays should be seen as playing complementary roles in illuminating the cell death–independent immunity of R proteins.

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Author contributions

K.H., J.C., and T.M. conceived the project. K.H. and T.T. performed the investigations. K.H., T.T., J.C., and T.M. validated the data. J.C. and T.M. supervised the work. K.H. and T.M. wrote the paper with the coauthor contributions.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. Host cell death response caused by a coiled-coil domain containing Sr35 and TIR domain containing

RBA1 in the absence or presence of YFP-expressing Potato virus X (PVX-YFP) in *N. benthamiana*.

Supplementary Methods S1. Detailed pathogen infection assays using YFP-expressing Potato virus X.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

References

Bendahmane A, Kanyuka K, Baulcombe DC. The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell*. 1999:11(5):781–792. https://doi.org/10.1105/tpc.11.5.781

Buscaill P, Sanguankiattichai N, Lee YJ, Kourelis J, Preston G, van der Hoorn RAL. Agromonas: a rapid disease assay for *Pseudomonas syringae* growth in agroinfiltrated leaves. *Plant J*. 1999:105(3): 831–840. https://doi.org/10.1111/tpj.15056

Chakrabarti A, Panter SN, Harrison K, Jones JD, Jones DA. Regions of the Cf-9B disease resistance protein able to cause spontaneous necrosis in *Nicotiana benthamiana* lie within the region controlling pathogen recognition in tomato. *Mol Plant Microbe Interact*. 2009:22(10):1214–1226. https://doi.org/10.1094/MPMI-22-10-1214

Coll NS, Vercammen D, Smidler A, Clover C, Van Breusegem F, Dangl JL, Epple P. Arabidopsis type I metacaspases control cell death. *Science*. 2010:330(6009):1393–1397. https://doi.org/10.1126/science.1194980

Collier SM, Hamel L-P, Moffett P. Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein. *Mol Plant Microbe Interact*. 2011:24(8):918–931. https://doi.org/10.1094/MPMI-03-11-0050

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A, Parker JE. Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell*. 2005:17(9):2601–2613. https://doi.org/10.1105/tpc.105.033910

Förderer A, Li E, Lawson AW, Deng Y-N, Sun Y, Logemann E, Zhang X, Wen J, Han Z, Chang J, *et al.* A wheat resistosome defines common principles of immune receptor channels. *Nature*. 2022:610(7932): 532–539. https://doi.org/10.1038/s41586-022-05231-w

Huang S, Jia A, Ma S, Sun Y, Chang X, Han Z, Chai J. NLR signaling in plants: from resistosomes to second messengers. *Trends Biochem Sci*. 2023:48(9):776–787. https://doi.org/10.1016/j.tibs.2023.06.002

Lapin D, Kovacova V, Sun X, Dongus JA, Bhandari D. A coevolved EDS1-SAG101-NRG1 module mediates cell death signaling by TIR-domain immune receptors. *Plant Cell*. 2019:31(10): 2430–2455. https://doi.org/10.1105/tpc.19.00118

Larsen JS, Curtis WR. RNA viral vectors for improved *Agrobacterium*-mediated transient expression of heterologous proteins in *Nicotiana benthamiana* cell suspensions and hairy roots. *BMC Biotechnol*. 2012:12(1):21. https://doi.org/10.1186/1472-6750-12-21

Maekawa T, Kashkar H, Coll NS. Dying in self-defence: a comparative overview of immunogenic cell death signalling in animals and plants. *Cell Death Differ*. 2023:30(2):258–268. https://doi.org/10.1038/s41418-022-01060-6

Morel JB, Dangl JL. The hypersensitive response and the induction of cell death in plants. *Cell Death Differ*. 1997:4(8):671–683. https://doi.org/10.1038/sj.cdd.4400309

Nishimura MT, Anderson RG, Cherkis KA, Law TF, Liu QL, Machius M, Nimchuk ZL, Yang L, Chung EH, El Kasmi F, *et al.* TIR-only protein RBA1 recognizes a pathogen effector to regulate cell death in Arabidopsis. *Proc Natl Acad Sci U S A*. 2017:114(10):E2053–E2062. https://doi.org/10.1073/pnas.1620973114

Peart JR, Cook G, Feys BJ, Parker JE, Baulcombe DC. An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. *Plant J*. 2002:29(5):569–579. https://doi.org/10.1046/j.1365-313X.2002.029005569.x

Tian L, Lu J, Li X. Differential requirement of TIR enzymatic activities in TIR-type immune receptor SNC1-mediated immunity. *Plant Physiol*. 2022:190(4):2094–2098. https://doi.org/10.1093/plphys/kiac452

van Wersch S, Tian L, Hoy R, Li X. Plant NLRs: the whistleblowers of plant immunity. *Plant Commun*. 2020:1(1):100016. https://doi.org/10.1016/j.xplc.2019.100016

Wan L, Essuman K, Anderson RG, Sasaki Y, Monteiro F, Chung EH, Osborne Nishimura E, DiAntonio A, Milbrandt J, Dangl JL, *et al.* TIR domains of plant immune receptors are NAD+-cleaving enzymes that promote cell death. *Science*. 2019:365(6455):799–803. https://doi.org/10.1126/science.aax1771

Wang J, Song W, Chai J. Structure, biochemical function, and signaling mechanism of plant NLRs. *Mol Plant*. 2023:16(1):75–95. https://doi.org/10.1016/j.molp.2022.11.011

Yu D, Song W, Tan EYJ, Liu L, Cao Y, Jirschitzka J, Li E, Logemann E, Xu C, Huang S, *et al.* TIR domains of plant immune receptors are 2′,3′-cAMP/cGMP synthetases mediating cell death. *Cell*. 2022:185-(13):2370–2386.e18. https://doi.org/10.1016/j.cell.2022.04.032