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Deciphering the Role of *Arabidopsis* TIR-NBS 2 Receptor in Cell Death Signaling in *Nicotiana benthamiana*

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Abstract

The truncated Toll/interleukin-1 receptor-NBS (TIR-NBS) proteins act significant role in immunity. Previous articles reported an *Arabidopsis thaliana* resistance protein TIR-NBS 2 (TN2) can arise violent cell death in tobacco (*Nicotiana benthamiana*) leaves. Nevertheless, the specific mechanism by which this protein strikes cell death is generally poor. Here, we preliminarily dissected the activation mechanism of TN2 immune receptor in *N. benthamiana*. The outcomes of the transient analysis attested that the TN2-TIR domain itself is enough to cell-death induction. The yellow fluorescent protein (YFP)-tagged TN2 and TN2-TIR were major localized to the cytoplasm, which was considered essential to the function of the TN2 and TN2-TIR. Additionally, the cell-death-inducing activity of TN2 can be inhibited by the full-length EXO70B1 instead of its truncated fragments. These research findings provided some clues for understanding the activation mechanism of TN2.

Keywords

Cell Death, TIR-NBS 2, EXO70B1, *Nicotiana benthamiana*

1. Introduction

In plants, the two-level innate immune system has evolved to discern potential pathogens and touch off efficacious defense responses [1]. The first type of immune response is triggered by pattern recognition receptors (PRRs) localized at the cell membrane. PRRs identify and response to pathogen-associated molecular

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pattern (PAMP), known as pattern-triggered immunity (PTI). Certain pathogens secrete virulence effectors to combat PTI. Plants activate the second layer of innate immune system to monitor virulence proteins, named effector-triggered immunity (ETI). ETI is usually sparked by resistance (R) genes and give rise to local necrosis of plants to restrict the sustained spread of pathogens, namely hypersensitive response (HR) [2].

The majority of disease-resistance genes encode nucleotide-binding sites and leucine-rich repeat proteins (NLRs). A typical NLR receptor typically comprises a specific N-terminal domain, a conserved NBS domain, and a highly variable C-terminal LRR domain. According to the differences in its N-terminus, NLRs are predominantly classified into three categories: CC-NBS-LRR (CNL), TIR-NBS-LRR (TNL), and RPW8-NBS-LRR (RNL) [3]. Transient overexpression of the CC or TIR domains of certain NLR receptors is enough to spark off cell death [4]-[9], demonstrating that N-terminal CC and TIR domains are critical for initiating the death signal. In addition, plants also possess a number of truncated NLRs. Such as, a total of 21 TIR-NBS (TN) proteins have been identified in *Arabidopsis thaliana* (Col-0) [3]. Increasingly studies proclaimed that TN proteins also play a very important role in immunity. For instance, the functional acquisition mutant of TN1, also called CHILLING SENSITIVE 1 (CHS1), emerges cold sensitivity at low temperatures, nevertheless, the cold sensitivity phenotype of the *chs*1-2 mutant at low temperatures relies on the full-length TNL protein SUPPRESSOR OF chs1-2, 3 (SOC3) [10]. Further experiments demonstrated that SOC3 can associate with TN1 and TN2 respectively to monitor the steady state of *Arabidopsis* E3 ubiquitin ligase SENESCENCE-ASSOCIATED E3 ubiquitin LIGASE1 (SAUL1) [11]. Additionally, TN13 is reported to be necessary for resistance against *Pto* DC3000 lacking the effectors AvrPto or AvrptoB [12].

Previous articles reported an *Arabidopsis thaliana* resistance protein TIR-NBS 2 (TN2) can arise violent cell death in tobacco (*Nicotiana benthamiana*) leaves [13]. Nevertheless, the specific mechanism by which this protein strikes cell death is generally poor. Here, we preliminarily dissected the activation mechanism of TN2 immune receptor in *N. benthamiana*. The outcomes of the transient analysis attested that the TN2-TIR domain itself is enough to cell-death induction. The yellow fluorescent protein (YFP)-tagged TN2 and TN2-TIR were major localized to the cytoplasm, which was considered essential to the function of the TN2 and TN2-TIR. Additionally, the cell-death-inducing activity of TN2 can be inhibited by the full-length EXO70B1 instead of its truncated fragments. These research findings provided some clues for understanding the activation mechanism of TN2.

2. Materials and Methods

2.1. Plant Growth Environment

A. thaliana and *N. benthamiana* plants were cultivated in a greenhouse under a 16

hours light/8 hours dark cycle (24˚C). Plants were used for transient expression assays at 4 - 5 weeks of age.

2.2. Construction of Recombinant Vectors

To acquire cDNA clones of *Arabidopsis* TN2 genes, total RNA was isolated from 4 - 5 weeks old wild-type *Arabidopsis* leaves using the Trizol reagent, and cDNA was generated using the cDNA Synthesis Kit following the manufacturers' instruction. For full-length TN2 gene, PCR product was recombined with the binary vector the modified pUC19 (containing attL1 and attL2 feature sequences) using ClonExpress II One Step Cloning Kit. For truncated domain fragments of TN2 gene, two different domains TIR (1 - 178 aa) and NBS (179 - 380 aa) were identified by TAIR website, and the PCR product was cloned into the modified pUC19 vector. After complete sequencing, they were transferred to the expression vector through Gateway cloning technology (Thermo Fisher Scientific), such as pEarleygate101 for YFP-HA or YFP-HA-Rop or YFP-HA-mutRop tagged protein expression. Electro-transfer the expression plasmid into *Agrobacterium tumefaciens* strain GV3101.

2.3. Transient Expression

Agrobacterium tumefaciens GV3101 strains carrying recombinant binary vectors were used to infiltrate *N. benthamiana* leaves. Liquid cultures of all *Agrobacterium* strains were initially grown at 28˚C with agitation in Luria–Bertani (LB) media supplemented with the appropriate antibiotics. The bacterial cells were pelleted by centrifugation at 11,000 rpm for 1 min, resuspended in infiltration buffer (10 mM MgCl2, 10 mM MES, and 200 μm AS) and adjusted to an appropriate OD_{600} for infiltration.

2.4. Subcellular Localization

Subcellular localization was executed following a previously mentioned method, with minor modifications [9]. In one word, a fluorescence microscope (Olympus BX53) was used to observe photographs of live cells on the abaxial sides of *N. benthamiana* leaves at 28 or 40 h post target protein expression.

3. Results

3.1. Membrane and Cytoplasmic Localization of TN2 Triggers Cell Death in *N. benthamiana*

We previously cloned a range of TIR-NBS (TN) genes from *Arabidopsis*, and transiently expressed them in *N. benthamiana* leaves to plumb the subcellular localization and function of these receptors. We found that several TN proteins, including TIR-NBS 2 (TN2), can trigger cell death *N. benthamiana* (**Figure 1(A)**), a characteristic function of activating a TN protein. This discovery is consistent with the previous findings published in scientific papers [13]. Subcellular localization of proteins is an important aspect of protein function study. Therefore, we

employed a fluorescence microscope based on C-terminal YFP tagging to investigate the subcellular localization of TN2, and observed that the cellular localization of TN2-YFP seemed to exhibit a dynamic process. Specifically, in the early stage of transient expression, the fluorescence signal of TN2-YFP appears in the plasma membrane and cytoplasm, and over time, a large amount of aggregated fluorescence signals appears in the cytoplasm (**Figure 1(B)**).

Thus, the membrane and cytoplasmic localization of TN2 receptor was autoactive in the leaves of *N. benthamiana.*

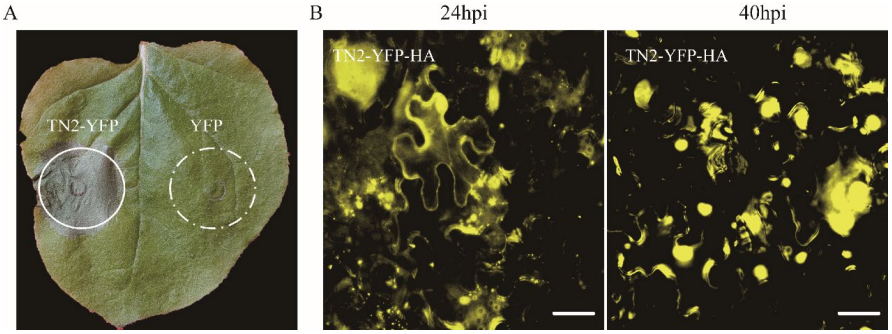

Figure 1. Membrane and cytoplasmic localization of TN2 triggers cell death in *N. benthamiana*. (A) Transiently expressed TN2 induces cell death. 35*S*::*TN2-YFP-HA* was transiently expressed in *N. benthamiana* by *Agrobacterium* infiltration (OD_{600} = 0.8). 35*S*::*YFP-HA* was used as a negative control. Picture was photographed at 72 h post infiltration (hpi). The experiment was repeated independently three times, and similar results were obtained in each replicate; (B) Observation the subcellular localization of TN2 with fluorescence microscope. TN2-YFP-HA was transiently expressed in *N. benthamiana* (OD_{600} = 0.8). Images were observed at 24 hpi and 40 hpi, respectively. Bar = 20 μm. The experiments were repeated at least three times with minimum three technical replicates and similar results were obtained.

3.2. The TIR Domain of TN2 Is Sufficient for Cell-Death Induction

The results of sequence alignment showed that TN2 encoded a potentially resistant protein harboring two domains. Consequently, two truncated fragments were generated [14], and both fused with the YFP-HA tag to determine whether these two domains possess cell-death-inducing activity (**Figure 2(A)**). On overexpressing two segments in *N. benthamiana*, the TN2-TIR domain caused sever cell death, and NBS domain was defective in causing macroscopic cell death, and we detected that the expression levels of different truncated proteins were at comparable levels, ruling out the possibility that their functional differences were caused by changes in protein expression (data not displayed) (**Figure 2(B)**). Fluorescence microscope results showed that TN2-TIR domain was also membrane and cytoplasmic-localized, but NBS module exhibited apparent cytoplasmic and nuclear distribution (**Figure 2(C)**).

In summary, TN2-TIR domain exhibited the almost identical localization pattern as full-length TN2, and the former is enough to trigger cell death in *N. benthamiana* leaves.

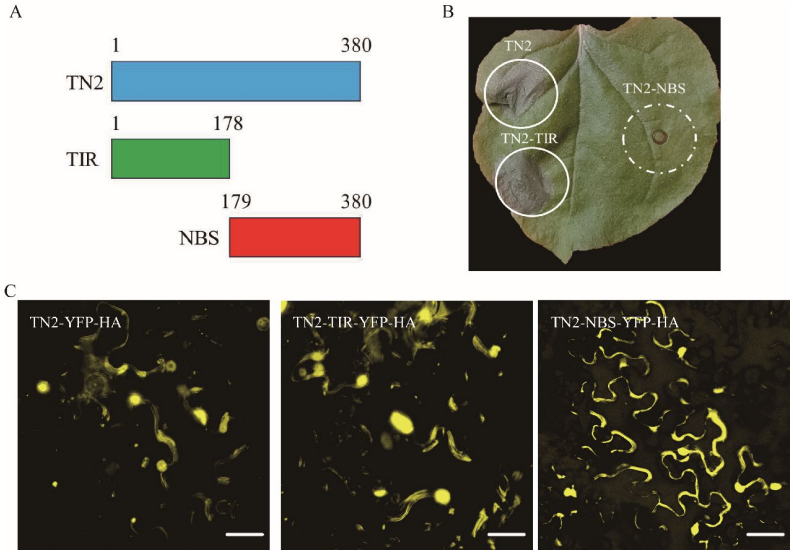

Figure 2. The TIR domain of TN2 can induce cell death in *N. benthamiana*. (A) Schematic diagram of TN2 receptor domain structure and the derived fragments. The two domains of TN2 are rendered in different colors. The numbers in the picture indicate the length of the protein; (B) Analysis of cell-death-inducing ability of TN2 fragments (OD_{600} = 0.8). The presented data are the average of three technical replicates, and experiments were biologically repeated at least three times with similar results; (C) Observation the subcellular localization of TN2 fragments with fluorescence microscope. Images were observed at 40 hpi. Bar = 20 μm. The experiment was repeated at least three times, each time minimum three technical replicates were analyzed.

3.3. Cytoplasmic Localization Is Crucial for the Function of Full-Length TN2 and Its Tir Domain

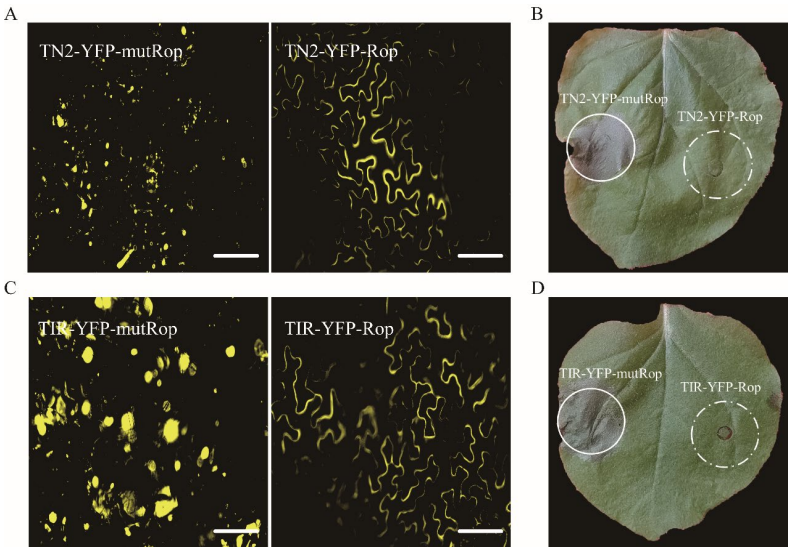

Figure 3. Cytoplasmic localization is crucial for the function of full-length TN2 and its TIR domain. (A, C) Rop tag can indeed anchor TN2 and TN2-TIR proteins separately to the PM. (B, D) Analysis of cell-death-inducing activity of TN2-YFP-HA-Rop and TIR-YFP-HA-Rop, respectively (OD_{600} = 0.8). Each experiment was performed at least three times with similar results and three technical replicates were performed for each sample. Fluorescent images were observed at 40 hpi. Bar = 20 μm.

Considering that both full-length TN2 and its TIR domain represent partial membrane localization, and both proteins can strike cell death in *N. benthamiana*. We want to validate whether these two proteins appear in large quantities in the cytoplasm after initiating the death signal on the plasma membrane (PM), or whether they need to dissociate from the PM into the cytoplasm before starting signal in the cytoplasm. In other words, are TN2 and TN2-TIR activated on the PM or in the cytoplasm? Full-length TN2 and its TIR domain were fused with the Rop tag, respectively, which could tether fusion protein on PM [15], to further assess whether the TN2 and its TIR domain worked on the PM. The TN2-YFP-Rop was found to be successfully anchored to PM and could not mediate cell death in *N. benthamiana* (**Figure 3(A)** and **Figure 3(B)**). Not surprising, TN2-TIR domain forcibly anchored to the PM has also completely lost its ability to trigger cell death (**Figure 3(C)** and **Figure 3(D)**). In conclusion, cytoplasmic localization is important for the function of full-length TN2 and its TIR domain.

3.4. Cell-Death-Inducing Activity of TN2 Can Be Inhibited by Full-Length EXO70B1 Instead of Its Truncated Form

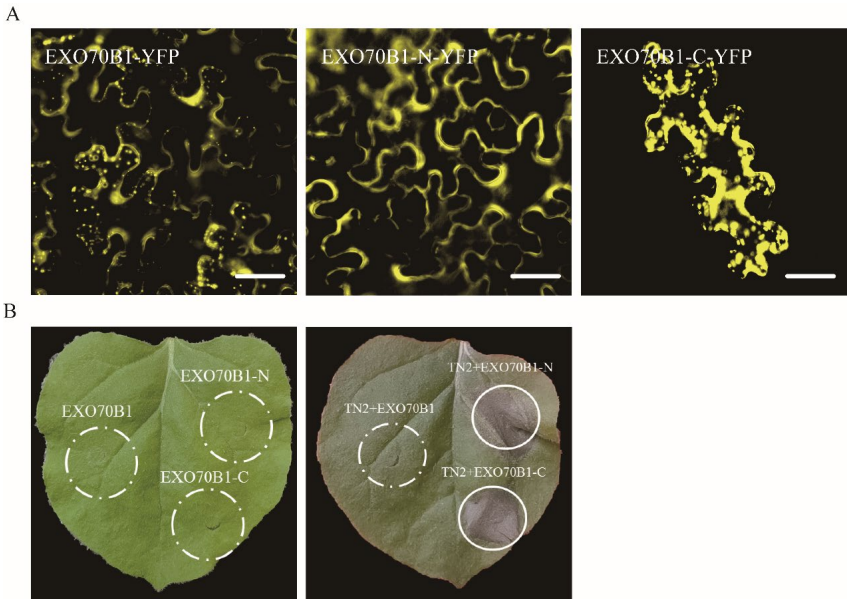

Figure 4. Cell-death-inducing activity of TN2 can be inhibited by full-length EXO70B1 instead of its truncated form. (A) Observation the subcellular localization of EXO70B1 and its truncated fragments with fluorescence microscope (OD_{600} = 0.8). Images were observed at 40 hpi. Bar = 20 μm; (B) Cell-death-inducing activity of TN2 can be suppressed by full-length EXO70B1 rather than its truncated form. Each experiment was performed at least three times with similar results and three technical replicates were performed for each sample.

Wang *et al.* demonstrated that EXO70B1 can suppresses TN2- or TN2-TIR-triggered cell death in tobacco (*N. tabacum* and *N. benthamiana*) [13]. We constructed recombinant plasmids expressing full-length EXO70B1 and two truncated forms, EXO70B1-N and EXO70B1-C, which represent the N- and C-terminal halves of

the protein, respectively. Fluorescence microscopy manifested that EXO70B1-YFP exhibited accumulation in PM, cytoplasm, and nucleus, whereas, EXO70B1-N and EXO70B1-C only exhibited GFP signals in PM and cytoplasm (**Figure 4(A)**).

Furthermore, we co-expressed full-length EXO70B1, EXO70B1-N or EXO70B1-C with EXO70E2 in *N. benthamiana*, respectively. The results indicated that the cell-death-inducing ability of TN2 was abrogated when co-expressed with full-length EXO70B1, while the other fragments of EXO70B1 did not affect the cell-death-inducing activity of TN2 (**Figure 4(B)**). These results suggested that the cell-death-inducing ability of TN2 can be abolished by full-length EXO70B1 rather than its truncated form.

4. Discussion

In the absence of pathogens, ectopic expression of full-length or truncated TIR domains of some NLR proteins can trigger cell death in tobacco [16]. In this study, we demonstrate that transient expression of TN2 or TN2-TIR alone triggers strong and rapid cell death in *N. benthamiana*, proclaiming its act as a pro-cell death signaling domain. However, such a role differed from the N-termini of several TNs, such as TN10, suggesting that TNs exploited diverse domains for mediating downstream cell death signaling [16]. Our results confirmed that cytoplasmic localization was crucial for the functioning of TN2, but we cannot rule out the possibility that TN2 needs to enter the nucleus to initiate cell death response, that is to say, activated TN2 was likely to undergo re-localization, which was confirmed in NLR receptors including MLA10, RPS4, etc. [17] [18]. Additionally, Zhao *et al.* confirmed that EXO70B1 can interact with TN2 [19], we observed that cell-death-inducing activity of TN2 was suppressed by co-expression of full-length EXO70B1, not its truncated form (**Figure 4(B)**), indicating that TN2 is likely to only interact with the full-length EXO70B1.

The genome of *Arabidopsis* (Col-0) encodes approximately 20 TN proteins, some of which have been reported to play a role in plant immunity. For example, TN13 is a modifier of SNC1 and a 6 (MOS6) interaction partner required to resist *Pto* DC3000 lacking AvrPto and AvrPtoB [12]. Recent studies have shown that TN13 also interact with the CNL protein RPS5 and contribute to RPS5 mediated disease resistance. Nevertheless, it is still unclear how truncated NLR proteins activate disease resistance. Future studies are needed to determine whether TN2-activated resistance depends on full-length NLR proteins.

Previous studies have shown that the TIR domains of certain plant NLR receptors, such as SNC1-TIR (1-226), and RPS4-TIR (1-236), can induce cell death in *N. benthamiana*, and these TIR domains all possess NAD^+ cleavage activity [20]. In this study, we found that the TN2-TIR domain also has cell-death-inducing activity. Future work should focus on exploring the impact of highly conserved sites in TN2-TIR on its function, and further analysis is needed to determine whether this domain also possesses NAD^+ cleavage activity similar to SNC1-TIR

and RPS4-TIR.

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Conflicts of Interest

The authors declared that they have no known competing financial interests, which seem to affect the work reported in this article.

References

[1] Jones, J.D.G. and Dangl, J.L. (2006) The Plant Immune System. *Nature*, **444**, 323-329. https://doi.org/10.1038/nature05286

[2] Coll, N.S., Epple, P. and Dangl, J.L. (2011) Programmed Cell Death in the Plant Immune System. *Cell Death & Differentiation*, **18**, 1247-1256. https://doi.org/10.1038/cdd.2011.37

[3] Wang, W., Feng, B., Zhou, J. and Tang, D. (2020) Plant Immune Signaling: Advancing on Two Frontiers. *Journal of Integrative Plant Biology*, **62**, 2-24. https://doi.org/10.1111/jipb.12898

[4] Maekawa, T., Cheng, W., Spiridon, L.N., Töller, A., Lukasik, E., Saijo, Y., *et al.* (2011) Coiled-Coil Domain-Dependent Homodimerization of Intracellular Barley Immune Receptors Defines a Minimal Functional Module for Triggering Cell Death. *Cell Host & Microbe*, **9**, 187-199. https://doi.org/10.1016/j.chom.2011.02.008

[5] Wang, G., Ji, J., EI-Kasmi, F., Dangl, J.L., Johal, G. and Balint-Kurti, P.J. (2015) Correction: Molecular and Functional Analyses of a Maize Autoactive NB-LRR Protein Identify Precise Structural Requirements for Activity. *PLOS Pathogens*, **11**, e1004830. https://doi.org/10.1371/journal.ppat.1004830

[6] Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., *et al.* (2011) Structural and Functional Analysis of a Plant Resistance Protein TIR Domain Reveals Interfaces for Self-Association, Signaling, and Autoregulation. *Cell Host & Microbe*, **9**, 200-211. https://doi.org/10.1016/j.chom.2011.02.009

[7] Lee, H., Seo, Y., Lee, J.H., Lee, S.E., Oh, S., Kim, J., *et al.* (2022) Plasma Membrane-Localized Plant Immune Receptor Targets H^+-ATPase for Membrane Depolarization to Regulate Cell Death. *New Phytologist*, **233**, 934-947. https://doi.org/10.1111/nph.17789

[8] Bolus, S., Akhunov, E., Coaker, G. and Dubcovsky, J. (2020) Dissection of Cell Death Induction by Wheat Stem Rust Resistance Protein Sr35 and Its Matching Effector Avrsr35. *Molecular Plant-Microbe Interactions®*, **33**, 308-319. https://doi.org/10.1094/mpmi-08-19-0216-r

[9] Huang, J., Wu, X., Sun, K. and Gao, Z. (2021) Structure and Function Analysis of a CC-NBS-LRR Protein At1g12290. *Biochemical and Biophysical Research Communications*, **534**, 206-211. https://doi.org/10.1016/j.bbrc.2020.11.111

[10] Zhang, Y., Wang, Y., Liu, J., Ding, Y., Wang, S., Zhang, X., *et al.* (2017) Temperature-Dependent Autoimmunity Mediated by *Chs1* Requires Its Neighboring *TNL* Gene

SOC3. *New Phytologist*, **213**, 1330-1345. https://doi.org/10.1111/nph.14216

[11] Liang, W., van Wersch, S., Tong, M. and Li, X. (2018) TIR-NB-LRR Immune Receptor SOC3 Pairs with Truncated TIR-NB Protein CHS1 or TN2 to Monitor the Homeostasis of E3 Ligase saul1. *New Phytologist*, **221**, 2054-2066. https://doi.org/10.1111/nph.15534

[12] Roth, C., Lüdke, D., Klenke, M., Quathamer, A., Valerius, O., Braus, G.H., *et al.* (2017) The Truncated NLR Protein TIR-NBS13 Is a MOS6/Importin-α3 Interaction Partner Required for Plant Immunity. *The Plant Journal*, **92**, 808-821. https://doi.org/10.1111/tpj.13717

[13] Wang, W., Liu, N., Gao, C., Rui, L. and Tang, D. (2019) The Pseudomonas Syringae Effector AvrPtoB Associates with and Ubiquitinates Arabidopsis Exocyst Subunit EXO70B1. *Frontiers in Plant Science*, **10**, Article ID: 1027. https://doi.org/10.3389/fpls.2019.01027

[14] Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, **35**, 1547-1549. https://doi.org/10.1093/molbev/msy096

[15] Chen, T., Liu, D., Niu, X., Wang, J., Qian, L., Han, L., *et al.* (2017) Antiviral Resistance Protein Tm-2^2 Functions on the Plasma Membrane. *Plant Physiology*, **173**, 2399-2410. https://doi.org/10.1104/pp.16.01512

[16] Chen, Y., Zhong, G., Cai, H., Chen, R., Liu, N., Wang, W., *et al.* (2021) A Truncated TIR-NBS Protein TN10 Pairs with Two Clustered TIR-NBS-LRR Immune Receptors and Contributes to Plant Immunity in Arabidopsis. *International Journal of Molecular Sciences*, **22**, Article 4004. https://doi.org/10.3390/ijms22084004

[17] Shen, Q., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., *et al.* (2007) Nuclear Activity of MLA Immune Receptors Links Isolate-Specific and Basal Disease-Resistance Responses. *Science*, **315**, 1098-1103. https://doi.org/10.1126/science.1136372

[18] Wirthmueller, L., Zhang, Y., Jones, J.D.G. and Parker, J.E. (2007) Nuclear Accumulation of the Arabidopsis Immune Receptor RPS4 Is Necessary for Triggering Eds1-Dependent Defense. *Current Biology*, **17**, 2023-2029. https://doi.org/10.1016/j.cub.2007.10.042

[19] Zhao, T., Rui, L., Li, J., Nishimura, M.T., Vogel, J.P., Liu, N., *et al.* (2015) A Truncated NLR Protein, TIR-NBS2, Is Required for Activated Defense Responses in the Exo70b1 Mutant. *PLOS Genetics*, **11**, e1004945. https://doi.org/10.1371/journal.pgen.1004945

[20] Horsefield, S., Burdett, H., Zhang, X., Manik, M.K., Shi, Y., Chen, J., *et al.* (2019) NAD^+ Cleavage Activity by Animal and Plant TIR Domains in Cell Death Pathways. *Science*, **365**, 793-799. https://doi.org/10.1126/science.aax1911