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Forum

A TIReless battle: TIR domains in plant–pathogen interactions

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Toll/interleukin-1 receptor (TIR) domain-containing proteins are conserved across kingdoms, and their mechanistic understanding holds promise for basic plant biology and agriculture. Here, we discuss the novel enzymatic TIR domain functions of nucleotide-binding leucine-rich repeat receptors (NLRs) in cell death, and posit how TIR domain-containing effectors mechanistically subvert host immune systems.

TIRed to death in ETI

Plant NLR biology research has grown over the past 5 years, with numerous exciting discoveries furthering our understanding of both coiled-coil NLRs (CNLs) and TIR-NLRs (TNLs), including their mechanistic regulation via 'resistosome' complexes [1–3]. Comparative evolutionary analyses of species-wide full NLR repertoires, also termed NLRomes, uncovered the existence of an intricate immune receptor network, which is punctuated by sensor and helper/executor NLRs that recognize pathogen effectors and transduce immune signals, respectively [4]. This leads to the activation of effector-triggered immunity (ETI), which is often associated with cell death or the hypersensitive response (HR; Figure 1). Since NLR signaling crosstalks with pattern recognition receptors (PRRs), which perceive pathogenic or host patterns and activate pattern-triggered immunity (PTI), ETI is largely considered a reamplified and accelerated form of PTI [5,6] (Figure 1). While a plethora of studies underscore the above-described advances in NLR biology, our focal point here are the emerging reports on enzymatic functions of TIR domains in HR [3,7–9].

Several TIR-only proteins or TNLs, including response to HopBA1 (RBA1), recognition of *Peronospora parasitica* 1 (RPP1), flax immune receptor L7 (L7), and resistant to *Pseudomonas syringae* 4 (RPS4), were shown to hydrolyze NAD in its oxidized forms (NAD^+ and $NADP^+$; Figure 1) [3,7–9]. This TIR-mediated NAD^+ hydrolase (NADase) activity depends upon a putatively catalytic glutamate (Glu) residue and is essential for the activation of HR. The presence of this conserved Glu in ~90% of plant TIR proteins suggests that NADase activity is a widespread phenomenon in the activation of immunocompetent NLR complexes [3,8]. Although this landmark discovery is a new milestone in our understanding of TNL-mediated cell death, it also raises new questions. For instance, monocots lack canonical TNLs and have TIR-only proteins, such as *Brachypodium distachyon* TIR (*Bd*TIR) [3]. Akin to a dicot TIR-only RBA1, *Bd*TIR was shown to exhibit NADase-dependent cell death, which requires a downstream signaling hub, enhanced disease susceptibility 1 (EDS1). While RBA1 co-immunoprecipitated with the *P. syringae* effector HopBA1, exactly how monocot TIRs can recognize effectors remains an area of future study. Do these monocot TIR-only proteins act as sensor NLRs, or do they function in conjunction with other NLRs to recognize effectors? How do they crosstalk with PTI signaling? Regardless of the direct or indirect mechanisms of effector detection, both TNLs and TIR-only proteins form oligomeric protein complexes, although the size of such higher-order structures can differ, and their significance is not yet fully understood [3,8,9] (Figure 1). As such, TIR–TIR heterodimerization for RRS1/RPS4 (a genomically paired TNL) was shown to function as an effector recognition complex [10]. The existence of such TIR–TIR heterodimers, if detected for nonpaired TNLs or TIR-only proteins, could sanction synergistic or antagonistic effects for not only effector detection, but also downstream signal transduction. Does such TIR–TIR heterodimerization increase effector recognition specificities and also influence different activation potencies of NLRs?

Another knowledge gap that needs to be filled is how activated TIRs relay immunostimulatory signals to helper NLRs. Activated disease resistance 1 (ADR1) and N requirement gene 1 (NRG1) belong to the resistance to powdery mildew 8 (RPW8) class of 'helper' NLRs (or RNLs). Upon effector recognition, ADR1 and NRG1 associate with EDS1 lipase family members and form two distinct interaction modules [ADR1-EDS1-phytoalexin-deficient 4 (ADR1-EDS-PAD4) and NRG1-EDS1-senescence-associated gene 101 (NRG1-EDS1-SAG101)] to restrict pathogen growth and HR, respectively [10] (Figure 1). It remains unclear how activated monocot TIR-only proteins, including *Bd*TIR, trigger HR since monocots lack SAG101 and NRG1. Can the observed *Bd*TIR-mediated HR be a consequence of dicot TIR downstream machinery? Do TIR-only proteins in monocots participate in disease resistance but not in HR? Perhaps the answer to linking TIR-only proteins and TNL immunomodulatory signal(s) to RNLs questions lies in TIR-mediated NADase activity, NAD^+ consumption, or NAD^+ hydrolysis products, including nicotinamide (NAM), adenosine diphosphoribose (ADPR), and cyclic ADPR variant (v-cADPR). NAD^+ is a major cofactor in redox reactions in all domains of life [11] (Figure 1). Importantly, the NADase activity of all tested plant TIRs does not consume cellular NAD^+ stores [3,8,9]. However, it does not preclude that other activated plant TIRs reduce

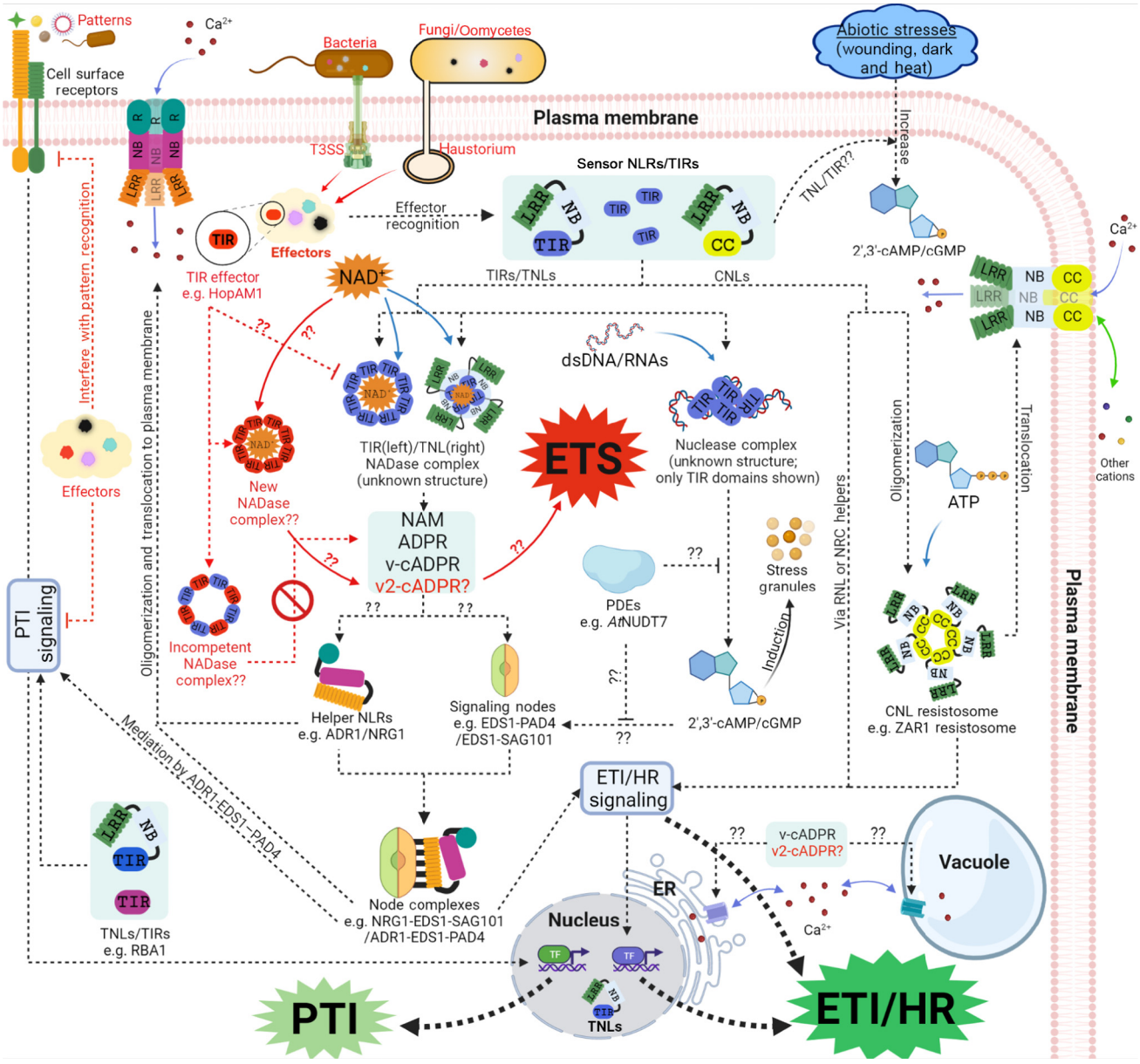

Figure 1. The Toll/interleukin-1 receptor (TIR) domain-mediated pathogen–plant tug-of-war. The recognition of pathogen patterns via pattern recognition receptors (PRRs) localized to the plasma membrane (surface-localized receptors) and pattern-triggered immunity (PTI) signaling are illustrated. To suppress PTI signaling, the delivery of virulence factors (effectors) of a bacterium and fungi/oomycetes into the cytoplasm is demonstrated. In this tug-of-war, nucleotide-binding domain leucine-rich repeat (NLRs) plant sensors, including TIRs, TIR-nucleotide-binding leucine-rich repeat receptors (NLRs) (TNLs), and coiled-coil NLRs (CNLs) are shown to recognize pathogen effectors. CNLs either assemble into higher-order complexes embedded in the plasma membrane and are involved in cation transport or undergo conformational changes directly to deliver signals via resistance to powdery mildew 8 (RPW8) class of 'helper' NLRs (or RNLs) or NLR required for cell death (NRCs) helpers, both activating downstream effector-triggered immunity (ETI) and hypersensitive response (HR) signaling pathways. Dinucleotide hydrolase (NADase) and nuclease functions of the octameric TIRs or tetrameric TNLs are indicated. Intracellular NAD^+ is cleaved by active NADase-TIR complexes, generating nicotinamide (NAM), adenosine diphosphoribose (ADPR), and cyclic ADPR variant (v-cADPR). Processing of cytoplasmic double-strand (ds)DNA and RNAs by TIR complexes to produce 2'3'-cAMP/cGMP is shown. Involvement of enhanced disease susceptibility 1 (EDS1) signaling and suppression by 2'3'-cAMP/cGMP phosphodiesterases (PDEs) are indicated. The enzymatic activities of TIRs potentially activate cytoplasmic NLRs [such as N requirement gene 1 (NRG1) and activated disease resistance 1 (ADR1)] as well as EDS1 signaling nodes [such as EDS1-phytoalexin-deficient 4 (EDS-PAD4) and EDS1-senescence-associated gene 101 (EDS1-SAG101)], followed

(Figure legend continued at the bottom of the next page.)

intracellular NAD^+ concentrations, since the TIR domain of human sterile alpha and TIR motif containing 1 (SARM1) or HopAM1 (a bacterial effector; discussed below) depletes NAD^+ when overexpressed in *Nicotiana benthamiana* and NAD is postulated to be a pathogen target [3,8,12,13]. Intriguingly, NAD derivatives, including cADPR, enhance the conductance of Ca^{2+} channels located in the plasma membrane, the endoplasmic reticulum (ER) membrane, and the tonoplast [11]. Since ADR1 and NRG1 RNLs form Ca^{2+} influx channels [2], it needs to be tested whether such NAD derivatives coordinate with RNL 'resistosome' complexes and directly regulate cytoplasmic Ca^{2+} influx to trigger disease resistance and HR (Box 1; Figure 1). Since ER and vacuole are rich sources of Ca^{2+} [11], such yet-to-be-discovered ER- and tonoplast-resident Ca^{2+} channels likely participate in TIR-mediated HR (Figure 1).

Box 1. What's sTIRring in the future?

How do NAD^+ hydrolysis kinetic properties vary for TIR domains of TIR-only proteins, TNLs, and effectors?

How do TIR domain-containing effectors mechanistically interfere with the activation and signaling of TIR-only proteins/TNLs to establish ETS?

How do different variants of cADPR (v-cADPR and v2-cADPR) modulate the conductance of Ca^{2+} channels or have they evolved new cellular roles?

What distinct characteristics of nucleic acids define the substrate specificities of TIR proteins/receptors?

How do NAD^+ products and 2′,3′-cNMPs crosstalk, and how do they activate diverse RNL/EDS1-member complexes to activate ETI and PTI? How do pathogen effectors (TIRs and non-TIRs) interfere with these activities to establish ETS? *At*NUDT7 and the oomycete effector Avr3b act as 2′,3′-cAMP/cGMP phosphodiesterases and inhibit TIR-mediated HR. Given that *At*NUDT6/7 are major signaling hubs, what effectors target them to interfere with TNL-mediated ETI?

NAD^+ homeostasis is essential for the cellular redox state, circadian clock, and energy metabolism. Is NAD^+ depletion or perturbed NAD^+-cADPR homeostasis a mechanism for ETS?

While their roles are not fully understood, 2′,3′-cAMP/cGMP are shown to induce stress granules (SGs). What RNAs and protein components are sequestered in SGs, and how do they have a role in ETI/HR?

An enTIRely new idea: triple enzymatic functions

A recent breakthrough study demonstrating TIR domains of TIR-only proteins and TNLs functioning as nuclease and 2′,3′-cAMP/cGMP synthetases solved another important piece of the puzzle in TNL-mediated HR [7]. Intriguingly, the substitution of a critical and highly conserved cysteine in the TIR domain of plant $L7^{TIR}$ (Cys^{132}), $L6^{TIR}$, and $RPV1^{TIR}$, as well as bacterial *Acinetobacter baumannii* TIR (*Ab*TIR), did not abolish NADase and nuclease activities but compromised TIR-mediated activation of HR [7]. Subsequently, the authors tested a hypothesis that TIR-mediated degradation products of nucleic acids might be essential for HR activation. Using liquid chromatography–mass spectrometry (LC-MS) followed by multiple reaction monitoring and high-resolution MS analyses, they discovered the presence of 2′,3′-cAMP and other 2′,3′-cNMPs species [7] (Figure 1), which became the focal point of their study. Furthermore, they showed that all tested TIR domain-containing proteins/receptors with intact conserved Cys displayed TIR-mediated nuclease and 2′,3′-cAMP/cGMP synthetase activities. A comprehensive genomic survey among TIR-only proteins or TNLs for the presence of conserved Glu and Cys can further delineate their common or distinct enzymatic functionalities in disease resistance and HR.

The unique feature of the TIR domain, harboring both NADase and 2′,3′-cAMP/cGMP synthetase activities, and the close proximity of the catalytic residues mediating these enzymatic functions, prompted Yu *et al.* to explore whether the regulatory mechanisms controlling these distinct enzymatic activities could stem from the assembly of diverse oligomeric structures. In an elegant structural biology experiment using cryo-electron microscopy (cryo-EM), they demonstrated that the double-strand (ds)DNA-sandwiched symmetric tetramer for $L7^{TIR}$ is markedly different from that of asymmetric $L7^{TIR}$, which is required for the NADase activity of the TNL 'resistosomes' [7] (Figure 1). While the presence of these two types of higher-order structure has been unequivocally shown for TIR domains *in vitro*, whether such a structure is sterically possible in full-length TNLs needs to be tested. Moreover, their stoichiometry and mode of activation remain to be studied. Although still a relatively unexplored area, examples of effectors directly/indirectly targeting host nucleic acids exist in both animal and plants models [5]. Whether these TIR-only proteins and TNLs could guard particular species of nucleic acids or their degrading products will be a subject for future investigations, although TIRs

by the formation of node complexes and activation of ETI/HR signaling pathways in different cellular complexes. Moreover, these EDS1 complexes can contribute to PTI signaling. Notably, the activated helper NLRs or EDS1-signaling node complexes followed by the enzymatic activities of TNLs and TIRs may contribute to the assembly of a Ca^{2+} channel in the plasma membrane via oligomerization and translocation. Ca^{2+} transport from the endoplasmic reticulum (ER) and vacuole via cADPR variants is also postulated. TIR domain-containing effector(s) were shown to generate a novel variant of cADPR (v2-cADPR) that interrupts the enzymatic functions exerted by the host TIRs. Host–pathogen TIR–TIR hetero-oligomerization was reported that could form a signal-incompetent NADase complex. Pathogen activities are presented that may contribute to establishing effector-triggered susceptibility (ETS). Created using BioRender (https://biorender.com/).

binding to dsDNA/dsRNA appears to be nonsequence specific. Additional structure–function and mutagenesis analyses demonstrated that dsDNA binding with the TIR domain is essential for 2′,3′-cAMP/cGMP activity and HR, while dispensable for NADase function [7]. Although the authors elucidated the requirement of these enzymatic activities for HR, they further elaborated the crosstalk between NADase and 2′,3′-cAMP/cGMP synthetase using diverse variants of RBA1 in *N. benthamiana*. Whereas mutations that abrogated 2′,3′-cAMP/cGMP synthetase activity did not impact the NADase activity of RBA1, the conserved Glu was strictly required for all enzymatic activities, indicating that the NADase function of TIR is required for nuclease and 2′,3′-cAMP/cGMP synthetase [7]. Taken together, this report has undoubtedly advanced our mechanistic understanding of TNL-mediated cell death, but also opened new avenues of research and invited several follow-up questions (Box 1).

Pathogen TIRe fire

The phenomenon of TIR-domain containing proteins acting as NADases is evolutionarily conserved because bacterial and archaeal TIR domain proteins can efficiently process NAD^+ [3,12,13]. Pathogens use effectors to manipulate host defenses, including ETI, PTI, and cell death, by targeting key signaling hubs and establishing effector-triggered susceptibility (ETS) [14,15]. HopAM1 is one such TIR domain-containing bacterial effector that has NADase activity via its putatively catalytic Glu and produces v2-cADPR, a novel variant of cyclic ADPR, which is required for susceptibility in the arabidopsis (*Arabidopsis thaliana*) Col-0 accession [12] (Figure 1). Unlike *Ab*TIR discussed above, HopAM1 still has a conserved Cys (Cys^{132} of $L7^{TIR}$) and can trigger cell death in yeast, *Nicotiana tabacum,* and *N. benthamiana* [12]. This further substantiates the finding that multiple functions of TIRs are essential to trigger HR [7]. Intriguingly, overexpression of HopAM1 depletes NAD^+ in yeast and *N. benthamiana,* unlike plant TIR-only proteins or TNLs tested so far. However, HopAM1, when delivered through a modified strain of *P. syringae*, was unable to deplete NAD^+ in ETI- and ETS-inducing arabidopsis accessions [12]. Is *in planta* depletion of NAD^+ by certain TIRs an artifact of overexpression, even though NAD^+ is a target of pathogens? It remains a mystery how HopAM1 establishes ETS since v2-cADPR is produced in both HR and susceptible outcomes. Does HopAM1 form NADase-incompetent heterodimers/oligomers with TNLs and, in turn, suppress their downstream nuclease and 2′,3′-cAMP/cGMP activities to subvert ETI (Figure 1)? If so, do HopAM1- versus TNL-mediated cell death differ mechanistically? Do TNLs/NLRs detect HopAM1 or does HopAM1 directly activate signal-competent RNLs/EDS1-member complexes to trigger HR since HopAM1 retains the conserved Cys and possibly exhibits 2′,3′-cAMP/cGMP synthase function? Since the cell death functions of HopAM1 were abolished by mutating three additional conserved phenylalanines ($HopAM1^{F177}$, $HopAM1^{F207}$, and $HopAM1^{F210}$) [12], another key question is what the roles of these additional conserved residues are in NADase or 2′,3′-cAMP/cGMP synthetase activities (Box 1).

In summary, we highlight an ancient but newly discovered scenario in the tug-of-war between plants and their pathogens, and provide food for thought to fuel further discussions for future discoveries (Box 1).

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Declaration of interests

None declared by authors.

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