could also be involved in the regulation of Atpep-induced $Ca^{2+}$ influx. However, many PRRs including AtFLS2 and OsCERK1 do not have a cytosolic GC domain similar to that in AtPEPR1, implying that $Ca^{2+}$ mobilization mechanisms may differ between different PRRs.

In addition to CNGC channels, plant glutamate receptor-like channels that are permeable to $Ca^{2+}$ have also been implicated in mediating PAMP-induced $Ca^{2+}$ influx [3]. In the opposite direction, energy-dependent active transport of $Ca^{2+}$ out of the cytosol by P-type $Ca^{2+}$-ATPases and/or CAX antiporters is required to return $[Ca^{2+}]_{cyt}$ to resting [3]. The multiple regulatory properties of $Ca^{2+}$ channels, transporters, and pumps, and their distinct subcellular locations, provide great flexibility for dynamic modulation of $Ca^{2+}$ oscillations. A future challenge is to decipher how $Ca^{2+}$ influx and efflux processes are coordinated to generate specific $Ca^{2+}$ signatures triggered by PAMPs/pathogens.

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## Spotlight

# $NAD^+$ Cleavage: TIR Domain-Containing Resistance Proteins in Action

Ming Chang,[1,2] Michael Clinton,[2] Fengquan Liu,[1,*] and Zheng Qing Fu[2,*]

Plant resistance proteins play a key role in detecting pathogen infection and activating plant defense. Two recent papers by Horsefield *et al.* and Wan *et al.* revealed that the TIR domain in resistance (R) proteins functions as an $NAD^+$-cleaving enzyme. This enzymatic activity is induced by pathogen recognition and is indispensable for the R protein-dependent cell death response.

To defend themselves against infection by pathogens, plants have established an elegant multilayered immune system [1]. During millions of years of coevolution with pathogens, plants have developed pattern recognition receptors (PRRs) as a part of this immune system. PRRs detect conserved molecules in plant pathogens, termed pathogen-associated molecular patterns (PAMPs), that activate PAMP-triggered immunity (PTI). To cause disease, plant pathogens have acquired the ability to deliver effectors into host cells. In susceptible plants, these effectors promote pathogen virulence by altering plant physiology to suppress PTI, resulting in effector-triggered susceptibility (ETS). In resistant plants, the effectors or their activities can be directly or indirectly detected by R proteins. This phenomenon is termed effector-triggered immunity (ETI) [2]. In contrast to PTI, which is relatively mild, ETI is usually intense. It involves amplification of PTI defense pathways and is often associated with rapid localized cell death at the infection site, termed the hypersensitive response (HR).

Most R proteins in plants belong to the nucleotide-binding leucine-rich repeat (NB–LRR) receptors which contain an additional N-terminal Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) domain [2]. The TIR–NB–LRR class of R proteins require downstream EDS1 for activation of plant defense, whereas in CC–NB–LRR R protein-mediated immune response NDR1 plays a crucial role [3]. Since the first report of an

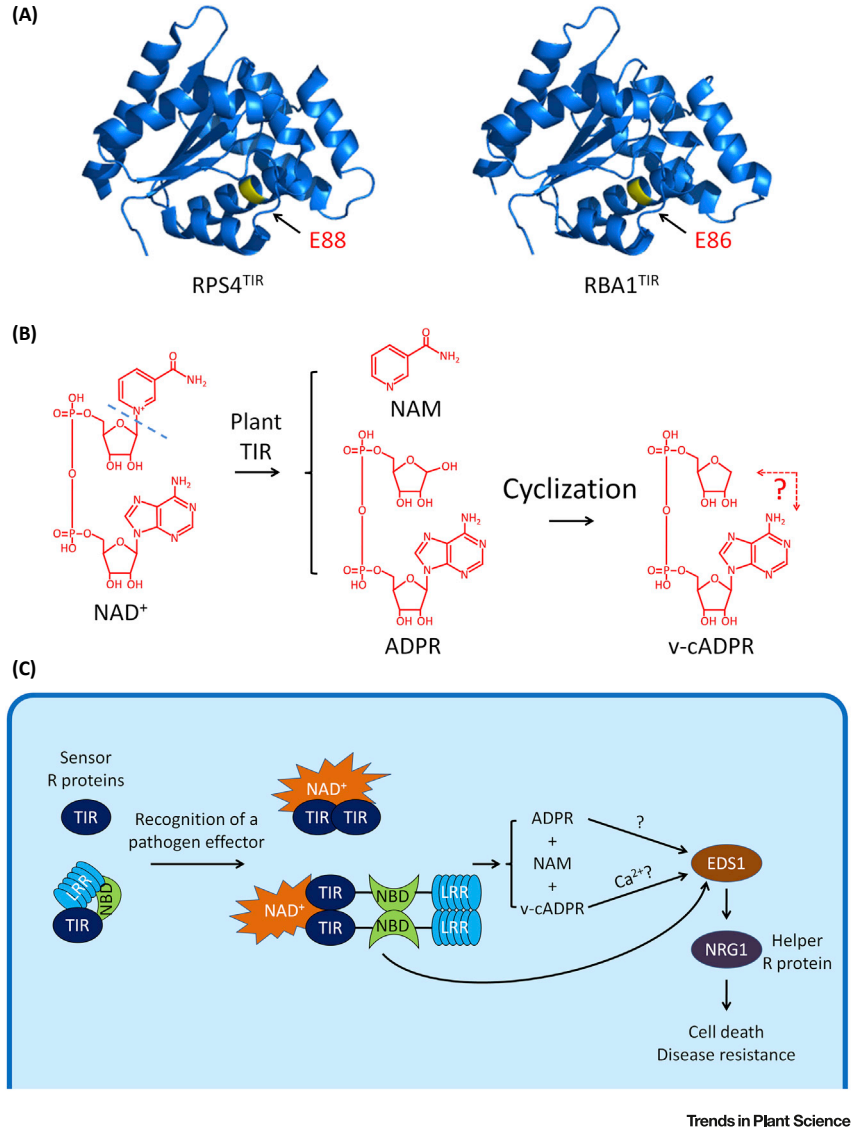


**Figure 1. Structures and Enzymatic Activity of Plant Toll/Interleukin-1 Receptor (TIR) Domains, and a Proposed Model for the Function of Plant TIR Domains in Effector-Triggered Immunity.**
(A) Crystal structures of the TIR domain of RPS4 (PDB i4C6T) and RBA1 (modeling with the structure of $RPS4^{TIR}$). The catalytic glutamic acid residues of $RPS4^{TIR}$ (E88) and $RBA1^{TIR}$ (E86), which are required for the $NAD^+$ cleavage activity, are shown in yellow. RPS4 and RBA1 are typical examples of TIR–NBD–LRR and TIR domain only resistance (R) proteins, respectively. (B) The plant TIR domain in R proteins functions as an $NAD^+$ cleavage enzyme to degrade $NAD^+$ into nicotinamide (NAM), ADP-ribose (ADPR), and variant cyclic ADP-ribose (v-cADPR). The cleavage site in $NAD^+$ is marked with a blue broken line. The v-cADPR with an unknown cyclizing bond (indicated with red broken arrows) is a special product of $NAD^+$ cleavage by plant TIR domains. (C) A proposed model for the function of plant TIR domains in effector-triggered immunity. Upon recognition of their cognate effectors, the $NAD^+$ cleavage activity of TIR domains in TIR only (top) and TIR–NBD–LRR R proteins (bottom) is induced, degrading $NAD^+$ into NAM, ADPR, and v-cADPR. It is hypothesized that ADPR and v-cADPR then transfer immune signals to the downstream signaling proteins EDS1 and NRG1 (a helper R protein) via an unknown mechanism, and finally induce cell death to resist pathogen growth. As an alternative signaling pathway, TIR–NBD–LRR R proteins have been shown to associate with EDS1 to mediate downstream immune signaling. Abbreviations: NBD, nucleotide-binding domain; LRR, leucine-rich repeat.

NB–LRR R protein in 1994 [4], little progress has been made towards understanding the exact biochemical function of R proteins.

Recently, an R protein which recognizes the type III effector HopBA1 was identified in *Arabidopsis thaliana* [5] (Figure 1A). Surprisingly, this TIR-only R protein, RBA1, does not have canonical NB–LRR domains. RBA1 in the presence of HopBA1 is sufficient to trigger a cell death response, suggesting that TIR domains in other TIR–NB–LRR R proteins play a key role in R protein-mediated plant defense. TIR domains are found in plant TIR–NB–LRR and TIR-only receptors, as well as in animal Toll-like receptors [6]. Therefore, it is a common interest in both plant and animal science to determine how the TIR domain triggers an immune response.

On 23 August 2019, two papers regarding TIR domain-containing R proteins were published back-to-back in *Science* and presented a new paradigm for TIR domain function [7,8]. Both papers revealed that the TIR domain in several R proteins functions as an $NAD^+$-cleaving enzyme (Figure 1B). This enzymatic activity can be induced by the recognition of a pathogen effector and is required for these R proteins to trigger cell death [8].

An earlier study found that loss of SARM1 (sterile alpha and Toll/interleukin-1 receptor motif-containing 1) blocks axonal degeneration for weeks after nerve damage, demonstrating that SARM1 functions as a key executioner of axon degeneration after injury [9]. Axonal injury induces $NAD^+$ depletion, and this requires SARM1 both *in vitro* and *in vivo*. More excitingly, dimerization of the TIR domain in SARM1 is sufficient to induce axonal

degeneration in the absence of injury through severe depletion of axonal $NAD^+$. Biochemical studies have shown that purified SARM1–TIR proteins from mammalian and bacterial cells, as well as from cell-free *in vitro* translation systems, have NADase activity, cleaving $NAD^+$ to produce ADP-ribose (ADPR), cyclic ADP-ribose (cADPR), and nicotinamide (NAM) [9]. This NADase activity is required for full-length SARM1 to induce axonal degeneration post-injury. Consistent with these discoveries, it was demonstrated that the TIR domains of prokaryotic proteins also possess $NAD^+$ cleavage activity and require a conserved glutamic acid for their catalytic functions [10].

In an effort to study the mechanistic basis for its $NAD^+$ cleavage activity, Horsefield *et al.* crystallized the TIR domain of human SARM1 [7]. They determined that the crystal structure of the TIR domain of human SARM1 shows more similarity to the plant TIR domain than to the bacterial TIR domain (Figure 1A). The purified TIR domains of the R proteins L6 from flax and RUN1 from grapevine are capable of cleaving $NAD^+$ and $NADP^+$ at high concentrations. Moreover, mutation of the catalytic glutamic acid abolishes their $NAD^+$ cleavage activities. Consistent with these data, the TIR-only plant immune receptors RBA1 and BdTIR (from the model monocot plant *Brachypodium distachyon*), when expressed in *Escherichia coli*, degrade $NAD^+$ and $NADP^+$, a function that is also dependent on the putative catalytic glutamic acid (Figure 1A,B). When the plant TIR proteins RBA1 and BdTIR were transiently expressed in *Nicotiana benthamiana* plants, or delivered into *Arabidopsis* plants via *Pseudomonas fluorescens*, a special variant of cyclic ADP-ribose (v-cADPR) was detected as a cleavage product [8] (Figure 1B). The accumulation of v-cADPR is dependent on the putative catalytic glutamic acid (Figure 1A). This v-cADPR wsa identified as a biomarker for plant TIRs because v-cADPR is not a cleavage product of human SARM1–TIR.

Through extensive protein sequence analysis, Wan *et al.* found that 131 of 146 *Arabidopsis* TIR domains contain this conserved glutamic acid [8]. Those without the conserved glutamic acid are reminiscent of the cell death-inactive R protein, RRS1. Therefore, the presence of this putative catalytic glutamic acid correlates with the cell death phenotype. Indeed, transient expression of the TIR-only proteins RBA1 and BdTIR, and of the TIR domains of $RPP1_{NdA}$, L6, RUN1, SNC1, and RPS4, all induce cell death in *N. benthamiana*. When the conserved glutamic acid was mutated to alanine in these TIR proteins, the autonomous cell death phenotype of these TIR proteins in *N. benthamiana* plants was abolished, supporting the hypothesis that these TIR proteins rely on their $NAD^+$ cleavage activities to cause cell death (Figure 1C).

It appears that plant TIR proteins require self-association for their ability to induce cell death. Mutations of the AE and DE interfaces, which are common in plant TIR proteins, perturb the self-association of L6. Consequently, the cell death phenotype of the mutated L6 proteins was greatly compromised [7]. In addition, mutation of either the AE or DE self-association interfaces abolishes the NADase activities of several other plant TIR proteins (RBA1, RPP1, and RPS4) in *E. coli* and the cell death phenotype *in planta* [8]. This indicates that self-association of plant TIRs is required for both $NAD^+$ cleavage activity and the cell death phenotype (Figure 1C).

Even though both RUN1 and $hSARM1^{tSAM–TIR}$ (the tandem SAM and TIR domains of human SARM1 together) cause cell death in plants, RUN1-triggered cell death requires the downstream protein EDS1 (Figure 1C), whereas $hSARM1^{tSAM–TIR}$ can cause HR even in *eds1* mutant plants. This indicates that $hSARM1^{tSAM–TIR}$ induces cell death through a pathway distinct from plant TIRs. It is known that EDS1 physically associates with TIR–NB–LRR R proteins [11,12] (Figure 1C). In addition, a helper R protein, NRG1, plays a crucial role in plant TIR-mediated cell death [8] (Figure 1C). Another major difference is that plant TIRs do not cause $NAD^+$ depletion *in planta*, which is consistent with the weak activity of plant TIRs *in vitro*. Therefore, the cleavage products ADPR and v-cADPR were proposed to be the possible cell death triggers given that cADPR has been shown to cause calcium influx into the cytoplasm and Wan *et al.* did not detect *in planta* $NAD^+$ depletion [8] (Figure 1C).

The two papers highlighted above demonstrate that the TIR domain in R proteins functions as an $NAD^+$-cleaving enzyme to trigger cell death. However, additional work will be necessary to address how the recognition of a pathogen effector induces self-association of TIR domains and TIR domain $NAD^+$ cleavage activity, as well as how the cleavage products of $NAD^+$ or NADP trigger cell death to resist pathogen growth (Figure 1C). Likewise, it is important to investigate the biochemical functions of the CC domain in CC–NB–LRR R proteins and how these proteins induce cell death. Future studies on the molecular functions of EDS1 will undoubtedly improve our understanding of how plants activate ETI to defeat pathogens. Furthermore, our very limited understating of

the exact function of helper R proteins such as NRG1 must be rectified.

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Spotlight

# Crop Genomics Goes Beyond a Single Reference Genome

Yongfu Tao,[1] David R. Jordan,[1] and Emma S. Mace[1,2,@,*]

The inadequacy of a single reference genome to capture the full landscape of genetic diversity within a species constrains exploration of genetic variation for crop improvement. A recent study by Yang *et al.* has demonstrated the value of multiple reference-quality genomes in capturing structural variants and guiding biological discovery.

## A Single Reference Is Insufficient to Fully Capture the Diversity within a Crop Species

Improving crop productivity holds the key to ensuring food security under threats of climate change and human population expansion. Such improvement relies on mining and utilizing genetic diversity within gene pools. The access to a reference genome for most crop species has greatly accelerated genetic and genomic studies to explore genetic variation within crop species and link it with important agronomic traits. However, it is increasingly evident that a single reference genome is insufficient to fully capture the diversity within a crop species, as large amounts of both sequence and genes from a single reference genome have not been found in the genomes of other individuals in the species [1,2]. For example, a comparison of two maize inbred lines (B73 and Mo17) revealed that over 10% of genes did not have homologs in the alternative genome [2]. This discrepancy leads to inefficiency when using a reference genome in the exploration of genetic variation for crop improvement, an approach that frequently results in millions of unmapped reads being excluded from resequencing studies and requires sequencing of targeted regions to find the 'invisible' sequence in map-based cloning studies [3,4].

Reliance on a single reference genome also limits the capacity to identify structural variants (SVs) including insertions, deletions, duplications, inversions, and translocations. SVs are prevalent in crop species and play critical roles in the genetic determination of agronomical traits and genome evolution [5–7]. Although reference genome-based approaches, such as genome resequencing and comparative genomic hybridization (CGH), have been developed to investigate SVs, their power is often limited due to the biases towards detecting variants that are present in the reference genome and the inefficiency of detecting SVs using short-read sequences.

## Multiple Reference-Quality Genomes Facilitate Biological Discovery in Maize

In a recent study, Yang *et al.* demonstrated that the development of multiple reference-quality genomes could facilitate investigation of structural variation and the genetic dissection of important agronomical traits [8]. Using a combination of PacBio long-read sequencing, Illumina short-read sequencing, a BioNano optical map, and 10x Genomics Chromium