Microreview

Perspectives on intracellular perception of plant viruses

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SUMMARY

The intracellular nucleotide-binding domain leucine-rich repeat (NLR) class of immune receptors plays an important role in plant viral defence. Plant NLRs recognize viruses through direct or indirect association of viral proteins, triggering a downstream defence response to prevent viral proliferation and movement within the plant. This review focuses on current knowledge of intracellular perception of viral pathogens, activation of NLRs and the downstream signalling components involved in plant viral defence.

Keywords: Effector-triggered immunity (ETI), Immune receptors, immune signalling, NLR activation, nucleotide-binding domain leucine-rich repeat receptors (NLRs), plant viruses, viral effectors.

INTRODUCTION

Plants have a two-layered innate immune system to defend against pathogen infections (Caplan *et al.*, 2008b; Jones and Dangl, 2006; Macho and Zipfel, 2014). The first layer consists of extracellular recognition of pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin or fungal chitin by cell surface-localized pattern recognition receptors (PRRs), leading to pattern-triggered immunity (PTI) (Macho and Zipfel, 2014). Highly adapted pathogens breach PTI by injecting pathogen-encoded proteins called effectors into plant cells. The second layer of the plant immune system consists of intracellular recognition of pathogen effectors by corresponding nucleotide-binding leucine-rich repeat (NLR) receptors, leading to effector-triggered immunity (ETI) (Caplan *et al.*, 2008b; Jones and Dangl, 2006). Both PTI and ETI culminate in a rapid burst of extracellular reactive oxygen species (ROS), change in ion fluxes, increase in cytosolic calcium, activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CPKs), elevation of phytohormones, and transcriptional and proteome reprogramming (Peng *et al.*, 2018; Yu *et al.*, 2017). Amplitude of these responses during ETI is much higher than in PTI and often leads to induction of hypersensitive response (HR), a type of programmed cell death (PCD), at the site of infection that restricts the pathogen to the sites of localized cell death.

Viruses are intracellular obligate pathogens with RNA or DNA genomes and cause significant crop losses (Anderson *et al.*, 2004). Since viruses encode a minimal number of proteins (approximately 5–10), they hijack host cellular machinery in order to replicate their genome, produce the viral capsid protein for encapsidation of its genome, facilitate cell-to-cell movement and for long distance transport within the plant. Most of our understanding of the plant antiviral immune response against virus infections comes from studies involving host-induced RNA interference (RNAi) and the NLR-mediated ETI response (Caplan *et al.*, 2008b; Guo *et al.*, 2019; Padmanabhan and Dinesh-Kumar, 2014). Another major form of antiviral defence in plants is through recessive resistance, in which the plant fails to produce factors required for the virus to complete its life cycle (Wang and Krishnaswamy, 2012). Since plant viruses are intracellular pathogens, it is widely accepted that plants would not invoke PTI responses against viral infection. However, there has been some recent indirect evidence for PTI-like responses against viral infection (Macho and Lozano-Duran, 2019; Teixeira *et al.*, 2019). In this review, we primarily discuss NLR-mediated intracellular perception of viruses by plants, as well as some of the downstream signalling pathways that lead to the innate immune response against viral pathogens.

Intracellular perception of viruses by NLRs

Intracellular NLRs play an important role in both plants and animals for the perception of pathogens and activation of defence (Caplan *et al.*, 2008b; Jones and Dangl, 2006). In plants, canonical NLRs are subdivided based on the presence of a Toll-Interleukin-1-receptor (TIR) homology domain or coiled-coil (CC) domain at the N-terminus. Unlike mammalian NLRs, which

perceive PAMPs including viral nucleic acid, plant NLRs recognize pathogen effector proteins to activate defence responses that often lead to induction of HR at the site of infection and subsequent containment of the pathogen to the infection site. The first TIR-NLR cloned was the tobacco *N* gene, which confers resistance to *Tobacco mosaic virus* (TMV) (Whitham *et al.*, 1994). Since the cloning of *N*, at least 15 NLRs that confer resistance to various viruses have been cloned or identified, many of which encode CC-NLRs (Gouveia *et al.*, 2016; Padmanabhan and Dinesh-Kumar, 2014).

Despite having cloned many NLRs and their corresponding pathogen effectors, experimental evidence for NLR association (direct or indirect) with effectors has only been demonstrated in a few cases. The tomato Sw-5b, a CC-NLR, confers broad-spectrum resistance to a wide range of agriculturally devastating American-type tospoviruses, including *Tomato spotted wilt virus* (TSWV) (Brommonschenkel *et al.*, 2000). In addition to the canonical CC-NLR domains, Sw-5b contains an extra N-terminal Solanaceae domain (SD) (Chen *et al.*, 2016). Sw-5b recognizes the cell-to-cell movement protein, NSm, of TSWV (Hallwass *et al.*, 2014; Peiro *et al.*, 2014; Zhu *et al.*, 2017) (Fig. 1). Interestingly, a highly conserved 21 amino acid peptide region within NSm (NSm[21]) from American-type tospoviruses is sufficient to induce Sw-5b mediated defences (Zhu *et al.*, 2017). The NB-LRR region of Sw-5b directly interacts with NSm[21] (Zhu *et al.*, 2017). Furthermore, domain swap and mutational analyses indicate that the LRR domain
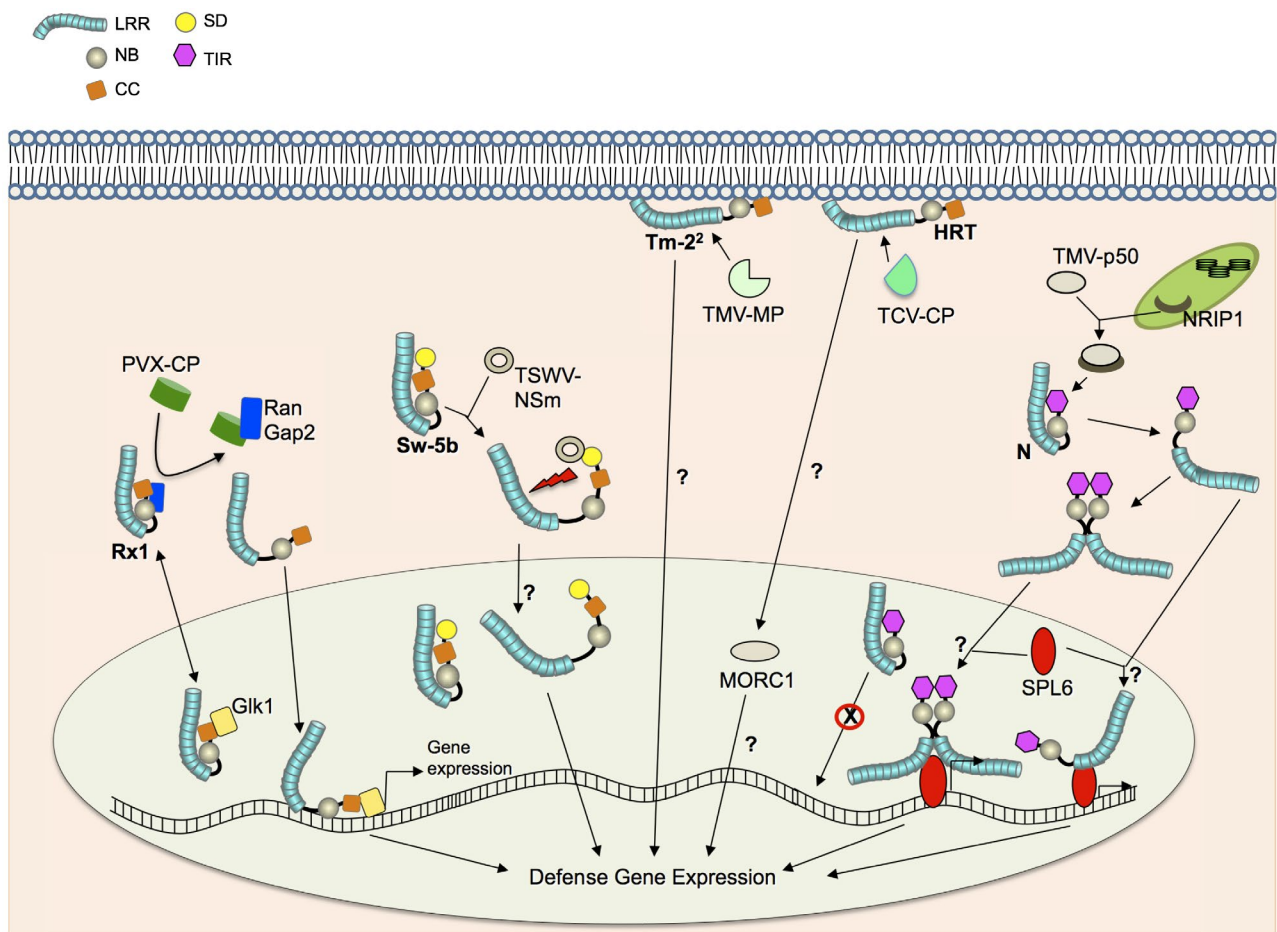

Fig. 1 Recognition of viral pathogens by intracellular NLRs. In the absence of infection, the Rx1 CC-NLR exists in an autoinhibited state stabilized by RanGAP2. Upon recognition of the PVX coat protein (PVX-CP), Rx1 releases its autoinhibition and enters the nucleus, where it associates with the Golden2-like transcription factor NbGlk1 to promote the transcription of defence genes. The SD and NB-LRR domains of the Sw-5b CC-NLR interact with the movement protein NSm of TSWV to relieve auto-inhibition and activate the defence response. Sw-5b is present in the nucleus in the absence of infection but its role in the nucleus is unknown. It is not known if activated Sw-5b moves into the nucleus. TM-2[2] and HRT are membrane-associated CC-NLRs; TM-2[2] recognizes the movement protein of tobamoviruses including TMV (TMV-MP), and HRT recognizes CP of TCV (TCV-CP). HRT and Rx1 (not shown) interact with MORC1, which has an unknown nuclear function. The nucleocytoplasmic TIR-NLR N confers resistance to TMV through recognition of the 50 kDa helicase domain of the TMV replicase (TMV-p50). Recognition of p50 by N requires NRIP1, which relocalizes from the chloroplast to the cytoplasm during infection. Following p50 recognition, N undergoes self-association; it also associates with the transcription factor SPL6 in the nucleus to promote transcription of defence genes. It is currently unknown which activated form (monomeric or self-associated N) associates with SPL6 in the nucleus.

of Sw-5b is involved in the recognition of NSm[21]. Therefore, Sw-5b recognizes a small PAMP-like peptide within a pathogen-encoded effector to activate broad-spectrum resistance, analogous to the mechanism of PRR-mediated recognition of PAMPs. In addition to the NB-LRR region, the N-terminal SD also interacts with NSm[21] (Li *et al.*, 2019). Interestingly, the SD domain plays an important role in enhancing the ability of NB-LRR region to recognize low levels of NSm or NSm[21] and to induce robust HR (Li *et al.*, 2019) (Fig. 1). These findings indicate that Sw-5b uses a novel two-step recognition mechanism involving the SD and LRR domains to perceive TSWV and to activate a robust defence response.

N, a TIR-NLR that confers broad-spectrum resistance to all strains of tobamoviruses except the Ob strain of TMV, recognizes the 50 kDa helicase domain of the TMV replicase (p50) through its TIR domain (Burch-Smith *et al.*, 2007; Caplan *et al.*, 2008a); this recognition is mediated by an intermediate protein, N receptor interacting protein 1 (NRIP1) (Caplan *et al.*, 2008a) (Fig. 1). NRIP1 is a sulfurtransferase and is localized to the chloroplast in the absence of infection. Upon TMV infection, NRIP1 relocalizes to the cytoplasm and forms a complex with p50. N then associates with the NRIP1-p50 complex in the cytoplasm to activate defence (Fig. 1). The LRR domain of N has also been shown to interact with p50 in yeast two-hybrid and *in vitro* pull-down assays (Ueda *et al.*, 2006). Despite the lack of *in vivo* evidence for an LRR–p50 interaction, it is possible that N uses a two-step recognition mechanism similar to that of Sw-5b involving both the TIR and LRR domains to perceive and respond to TMV.

Similar to N, tomato Tm-2[2], a CC-NLR, confers resistance to some strains of tobamoviruses, including TMV. Tm-2[2] recognizes the movement protein (MP) from TMV but not p50 (Fig. 1). Furthermore, Tm-2[2] associates with MP at the plasma membrane (Chen *et al.*, 2017) and recognition of MP by Tm-2[2] may involve an intermediate MP Interacting Protein 1 (NbMIP1) (Du *et al.*, 2013). However, evidence for the existence of Tm-2[2], MP and NbMIP1 in a complex is currently lacking.

Unlike N, Sw-5b, Tm-2[2] and other NLRs that recognize virus-encoded effectors, Vat (Virus aphid transmission), a CC-NLR from melon, activates defence against aphids, which in turn prevents the transmission of *Cucumber mosaic virus* (CMV) and several potyviruses (Dogimont *et al.*, 2014). The virus resistance induced by Vat is only observed during aphid transmission. This suggests that activation of Vat is linked to the recognition of effector(s) released by aphids during their initial feeding. Consistent with this, Vat fails to induce a defence response when viruses are introduced mechanically.

Viral NLR activation

As reported for N, the association between NLRs and their corresponding effector is not sufficient to activate NLR and to induce defence (Padmanabhan *et al.*, 2013). N not only associates with p50 from defence-eliciting TMV-U1 but also with p50 from non-defence-eliciting TMV-Ob (Padmanabhan *et al.*, 2013). However, only N associated with p50-U1 will activate the defence response. Furthermore, an ATP-binding site mutation in N protein that fails to initiate defence signalling can also interact with p50-U1. Similar to N, SW-5b is able to associate with non-eliciting NSm[21] mutants, but fails to induce HR (Zhu *et al.*, 2017). Therefore, pathogen recognition and subsequent defence activation are two independent processes in NLR-mediated defence.

Early studies of Rx1, a CC-NLR, which recognizes the CP of *Potato virus X* (PVX), indicated that intramolecular interactions between the CC domain and NB or LRR domains retain NLRs in an auto-inhibited state (Fig. 1) (Moffett *et al.*, 2002; Rairdan *et al.*, 2008; Slootweg *et al.*, 2013). These interactions are disrupted upon effector association. The exchange of ADP for ATP at the NB domain is then predicted to activate NLRs. Compared to Rx1, the SD and CC domains of Sw-5b additively retain Sw-5b in an auto-inhibited state (Chen *et al.*, 2016). Consistent with the intramolecular domain interaction model, mutations in NB or LRR domains of NLRs that disrupt these interactions have been shown to activate NLRs in the absence of their cognate effectors (Rairdan and Moffett, 2006; Zhu *et al.*, 2017). In Sw-5b, residue R927 in the LRR domain is critical for auto-inhibition; association with NSm[21] modulates R927 and releases the interaction between the NB and LRR domains (Zhu *et al.*, 2017). Compared to Rx1 and Sw-5b, intramolecular interactions have not been observed for N (Mestre and Baulcombe, 2006). However, activation of N requires self-association/dimerization after p50 recognition (Fig. 1), which is dependent on a functional NB domain. Interestingly, a mutation in the TIR domain that fails to induce HR can still undergo self-association (Mestre and Baulcombe, 2006), suggesting that self-association is not sufficient to activate downstream defence. Therefore, understanding steps downstream of N self-association should provide mechanistic insights into how viral NLRs function to induce defence.

In contrast to the Rx1 and Sw-5b studies described above, a recently reported cryo-EM structure of ZAR1, a CC-NLR, suggests that NLR activation may not involve major disruption of intramolecular interaction (Wang *et al.*, 2019b). ZAR1 together with RKS1 kinase recognizes the uridylyltransferase effector AvrAC from *Xanthomonas campestris* pv. *campestris* (Wang *et al.*, 2015). AvrAc-uridylated PBL2 kinase (PBL2[UMP]) associates with the ZAR1-RKS1 complex through interaction with RKS1 (Wang *et al.*, 2015). The structure of the ZAR1-RKS1 complex showed that ADP binding stabilizes the intramolecular interaction within ZAR1 domains and keeps ZAR1 in an inactive monomeric state. The structure of ZAR1-RKS1-PBL2[UMP] indicated that RKS1 binding to PBL2[UMP] results in a slight conformational change in only the NB domain of ZAR1, indicating that PBL2[UMP] binding to RKS1 releases bound ADP from ZAR1. However, the ZAR1-RKS1-PBL2[UMP] complex is also monomeric and the conformation of this complex is similar to that of the inactive ZAR1-RKS1 complex. Therefore,

contrary to previously proposed models, activation of ZAR1 does not involve major disruption of intramolecular interactions between N-terminal domains and the C-terminal LRR. Furthermore, ADP release is not sufficient to activate ZAR1. ZAR1-RKS1-PBL2[UMP] forms a higher-order oligomeric complex in the presence of ATP or dATP, indicating that this resistome complex is the active form (Wang *et al.*, 2019a). This higher-order complex forms a pentameric wheel involving all domains of ZAR1 (Wang *et al.*, 2019a). Based on the structure-guided mutagenesis experiments, Wang *et al.* suggest that the funnel-shaped pentameric complex of ZAR1 associates with the plasma membrane to cause HR (Wang *et al.*, 2019a). Further biochemical and structural analyses of other plant NLRs should shed light on whether the ZAR1 model is universal in the activation of NLRs and defence.

Viral NLR signalling

Activated NLRs use downstream signalling components to initiate immune responses. ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) act as a shared, central signalling node for TIR-NLRs including *N*-mediated resistance to TMV (Cui *et al.*, 2015). EDS1 resides in the cytoplasmic and nuclear pools, and interacts dynamically with two regulators of defence, PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101 (SAG101). While EDS1-SAG101 complexes are formed exclusively in the nucleus, EDS1-PAD4 complexes are found in both the cytoplasm and the nucleus, and EDS1 homodimers are strictly cytoplasmic (reviewed in Cui *et al.* (2015)). Nuclear EDS1 is required for immune receptor-mediated induction of transcriptional reprogramming and HR (Cui *et al.*, 2015). EDS1 and SAG101 are also required by HRT, a CC-NLR that recognizes the CP of *Turnip crinkle virus* (TCV-CP) (Zhu *et al.*, 2011).

N TIR-NLR-mediated resistance to TMV requires NRG1 (N Required Gene 1), a CC-NLR (Peart *et al.*, 2005). Based on this, it was proposed that NLRs, which recognize pathogens, recruit other NLRs for downstream signalling (Peart *et al.*, 2005). Consistent with this earlier observation, recent studies reported that other pathogen-recognizing NLRs (called sensor NLRs) also require other NLRs or a network of NLRs (called helper NLRs) for immune signalling (Wu *et al.*, 2017). Sw-5b and Rx1 require NRC2/3/4 NLRs for the induction of TSWV-NSm and PVX-CP-mediated HR-PCD, respectively (Li *et al.*, 2019; Wu *et al.*, 2017). Recently, EDS1 has been shown to associate with NRG1 (Qi *et al.*, 2018). Since silencing of NRG1 does not affect N self-association/dimerization (Peart *et al.*, 2005), the NRG1-EDS1 complex must function downstream of the dimerization step.

Evidence suggests that some NLRs may function as transcriptional co-regulators to control transcriptional reprogramming during defence through direct association with transcription factors (Cui *et al.*, 2015; Padmanabhan and

Dinesh-Kumar, 2010, 2014). N associates with the Squamosa Promoter Binding Protein Like 6 (SPL6) in the nucleus only in the presence of defence-eliciting p50-U1 but not in the presence of non-defence-eliciting p50-Ob, indicating that N-SPL6 association is dependent on the N activation step (Fig. 1) (Padmanabhan *et al.*, 2013). However, it is currently unknown if activated monomeric N from the cytoplasm moves to the nucleus and associates with SPL6 or if activated dimeric N from the cytoplasm moves to the nucleus and associates with SPL6 (Fig. 1). Rx1 associates with Ran GTPase-activating protein 2 (RanGAP2), which is required for defence against PVX (Sacco *et al.*, 2007; Tameling and Baulcombe, 2007) as well as the nucleocytoplasmic partitioning of Rx1 (Tameling *et al.*, 2010) (Fig. 1). Rx1 has been shown to bind DNA following activation with PVX-CP effector (Fenyk *et al.*, 2015). Furthermore, Rx1 interacts with a Golden2-like transcription factor, NbGlk1, that binds to DNA when Rx1 is activated by PVX-CP (Townsend *et al.*, 2018) (Fig. 1). Sw-5b is also a nucleocytoplasmic protein (De Oliveira *et al.*, 2016) (Fig. 1); however, its role in the nucleus remains unknown.

A GHKL (gyrase, heat-shock protein 90, histidine kinase, MutL)-type ATPase, Microchidia 1 [MORC1; formerly called Compromised Recognition of TCV (CRT1)], that interacts with many CC-NLRs, including viral NLRs such as HRT, Rx1 and RCY1 (which confers resistance to *Cucumber mosaic virus*), has also been shown to have a nuclear role (Fig. 1) (Kang *et al.*, 2008, 2010, 2012). The interaction between NLRs and MORC1 is disrupted when NLRs are activated (Kang *et al.*, 2008, 2010). Recent evidence suggests that MORC1 binds nucleic acid and possesses endonuclease activity *in vitro* and activation of immune receptors increases MORC1 levels in the nucleus (Kang *et al.*, 2012). Considering MORC family members participate in transcriptional gene silencing (TGS) and interact with the components of RNA-dependent DNA methylation (RDDM) and chromatin remodelling complex, it has been proposed that MORC family members may regulate transcriptional reprogramming during immunity (see review in Koch *et al.* (2017)). Many questions remain with MORC function. How does endosome-localized MORC1 interact with plasma membrane- and nucleocytoplasmic-localized NLRs? What processes are involved in the disruption of MORC1 interaction with NLRs post-activation? What processes enhance accumulation of MORC in the nucleus during defence? What is the role of MORC-mediated chromatin remodelling during immunity?

CONCLUSIONS AND PERSPECTIVES

In this review, we have highlighted some of the recent advances in understanding viral recognition, signalling intermediates, and the mechanisms of NLR regulation and activation. We have also discussed emerging evidence for the possible

involvement of cell-surface receptors in the perception of viruses or viral nucleic acids. However, further studies are needed to establish the existence of "viral PTI" in plants. It has been 25 years since the first plant NLRs were cloned. Despite this, our understanding of how NLRs recognize pathogens and activate defence is far from complete. The recent reporting of the first plant NLR structure has provided insights into the intramolecular interaction and nucleotide-binding state of ZAR1 (Wang *et al.*, 2019b). To understand whether the model of ZAR1 activation and HR induction (Wang *et al.*, 2019a) is universal to all NLRs will require further biochemical and structural analyses of other NLRs. It will be interesting to see how nucleocytoplasmic NLRs such as N and Rx1, which interact with transcription factors, are activated following effector recognition compared to plasma membrane-localized ZAR1. Furthermore, structural comparison between TIR-NLRs and CC-NLRs as additional structural information becomes available for plant NLRs should provide insights into how these two classes of NLRs function. Further structural studies will also provide information on how NLRs are recognizing effectors. Do effectors form a complex with their cognate NLRs? Or, as in the case of PBL2^{UMP}, do NLRs recognize effector-modified ligands? Considering CP and MP do not possess any known enzymatic activity and helicase activity of TMV-p50 is not required to activate N, how would these effectors modify and recruit a decoy like PBL2? Based on the ZAR1 resistome structure, Wang *et al.* (2019a) suggested that helper CC-NLRs like NRG1 and NRC4 might form higher-order pentamer structures like ZAR1 to induce HR during N- or Rx1-mediated immunity, respectively. Considering NRC4 and NRG1 are cytoplasmic proteins, it is unclear how these proteins would be recruited to the plasma membrane to execute cell death. Regardless, the knowledge we have gained over the last few years has provided the framework for future studies to understand the precise molecular and biochemical function of NLRs in antiviral immunity and immunity in general.

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