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Activation and Regulation of NLR Immune Receptor Networks

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Plants have many types of immune receptors that recognize diverse pathogen molecules and activate the innate immune system. The intracellular immune receptor family of nucleotide-binding domain leucine-rich repeat-containing proteins (NLRs) perceives translocated pathogen effector proteins and executes a robust immune response, including programmed cell death. Many plant NLRs have functionally specialized to sense pathogen effectors (sensor NLRs) or to execute immune signaling (helper NLRs). Sub-functionalized NLRs form a network-type receptor system known as the NLR network. In this review, we highlight the concept of NLR networks, discussing how they are formed, activated and regulated. Two main types of NLR networks have been described in plants: the ACTIVATED DISEASE RESISTANCE 1/N REQUIREMENT GENE 1 network and the NLR-REQUIRED FOR CELL DEATH network. In both networks, multiple helper NLRs function as signaling hubs for sensor NLRs and cell-surface-localized immune receptors. Additionally, the networks are regulated at the transcriptional and posttranscriptional levels, and are also modulated by other host proteins to ensure proper network activation and prevent autoimmunity. Plant pathogens in turn have converged on suppressing NLR networks, thereby facilitating infection and disease. Understanding the NLR immune system at the network level could inform future breeding programs by highlighting the appropriate genetic combinations of immunoreceptors to use while avoiding deleterious autoimmunity and suppression by pathogens.

Keywords: Autoimmunity • Host–pathogen interaction • Immune receptor network • NLR integrated domain • Nucleotide-binding domain and leucine-rich-repeat-containing protein • Pathogen effector

Introduction

Plants have an effective innate immune system, which can be activated by several types of immune receptors upon recognition of diverse pathogen molecules. These immune receptors are located either at the cell surface or in the nucleocytoplasm of plant cells (Lu and Tsuda 2021). Cell-surface receptors can recognize pathogen-associated molecular patterns or pathogen-secreted proteins, known as effectors, in the extracellular apoplastic space. In addition to secreting pathogen effectors into the apoplast, many plant pathogens also translocate effectors into the host nucleocytoplasm, thereby altering host physiological processes and facilitating infection. In response, plants have evolved a diverse repertoire of intracellular immune receptors, predominantly belonging to the nucleotide-binding domain and leucine-rich repeat-containing protein (NLR) family, to recognize these translocated pathogen effectors (Jones et al. 2016). Upon recognition of their cognate ligands by either cell-surface or NLR receptors, both classes of immune receptors activate common signaling pathways to trigger defense responses (Lu and Tsuda 2021). The activation of some cell-surface receptors and most NLRs results in the induction of a localized programmed-cell-death response, known as the hypersensitive response (HR), in the infected cells. This cellular suicide machinery is thought to restrict the spread of pathogens from the infection site to neighboring cells (Balint-Kurti 2019). In this manner, plants can fight off invading pathogens and prevent disease while maintaining their health at the tissue level.

Our understanding of how NLRs are activated and induce downstream signaling and immunity has expanded tremendously in recent years. NLR immune receptors are important components of the innate immunity of plants and animals (Jones et al. 2016). Plant NLRs share a multidomain architecture, characterized by a central nucleotide-binding domain shared with APAF-1, various R proteins and CED-4 (NB-ARC) domain, and a C-terminal leucine-rich repeat (LRR) domain (Kourelis et al. 2021b). The C-terminal LRR domain is typically involved in effector recognition, while the NB-ARC domain mediates the intramolecular activation of the NLR protein presumably by exchanging ADP for ATP in the nucleotide-binding pocket. In addition to the conserved NB-ARC and LRR domains, most plant NLRs have a variable N-terminal domain, which can be used to broadly classify these proteins into four

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subgroups: Toll/Interleukin-1 Receptor (TIR)-type NLRs (TIR-NLR or TNL), coiled-coil (CC)-type NLRs (CC-NLR or CNL), RESISTANCE TO POWDERY MILDEW 8 (RPW8)-type CC-NLRs (CC_R-NLR or RNL) and the more recently described G10-type CC-NLRs (CC_{G10}-NLR) (Lee et al. 2021). The N-terminal domains, TIR, CC, CC_R and CC_{G10}, are generally thought of as the signaling domain that executes downstream immune responses upon ligand recognition.

Effector recognition by plant NLRs can be direct or indirect. The structural elucidation of both the direct and the indirect recognition mechanisms has benefited from developments in biophysics and cryo-electron microscopy (cryo-EM). Both the *Arabidopsis thaliana* TIR-NLR RECOGNITION OF PERONOSPORA PARASITICA 1 (RPP1) and the *Nicotiana benthamiana* TIR-NLR RECOGNITION OF XOPQ 1 (Roq1) are activated upon the direct binding of their cognate effector ligands (Ma et al. 2020, Martin et al. 2020). The RPP1 and Roq1 structures reveal that effector binding is mediated by two distinct interaction surfaces: the LRR and a post-LRR C-terminal jelly roll and Ig-like (C-JID) domain (Ma et al. 2020, Martin et al. 2020). Similar to direct effector binding by TIR-NLRs, the wheat (*Triticum monococcum*) CC-NLR Sr35 also directly binds its cognate effector AvrSr35, which is mediated by the LRR domain (Förderer et al. 2022). By contrast, the structure of the Arabidopsis CC-NLR HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) reveals how indirect recognition can function (Wang et al. 2019b, 2019a). ZAR1 indirectly recognizes multiple bacterial effectors through host receptor-like cytoplasmic kinases (RLCKs). One such partner RLCK is RESISTANCE-RELATED KINASE 1 (RKS1), which interacts with the ZAR1 LRR domain and recruits RLCK AVRPPHB SUSCEPTIBLE 1 (PBS1)-LIKE PROTEIN 2 (PBL2) upon its uridylylation by the *Xanthomonas campestris* pv. *campestris* (*Xcc*) effector AvrAC (Wang et al. 2015). Finally, as an additional mode of recognition, the 'integrated-decoy' model was proposed based on functional analyses of several unusual NLRs that carry noncanonical integrated domains (IDs) required for effector perception (Césari et al. 2014a). These NLRs are referred to as 'NLR-IDs'. These receptors use IDs as a bait to directly or indirectly recognize effectors. Given that these effectors appear to exert their virulence activity by targeting host proteins containing the same domains as the IDs, NLR-IDs may represent fusions of effector target genes with NLR genes (Białas et al. 2017).

Upon effector recognition, activated NLRs form a high-order 'resistosome' complex. The first example of a resistosome was revealed by elucidating the structure of the Arabidopsis CC-NLR ZAR1 (Wang et al. 2019a). The ZAR1–RKS1 complex associates with uridylylated PBL2 ($PBL2^{UMP}$, a form of PBL2 modulated by the *Xcc* effector AvrAC), inducing a conformational change in monomeric ZAR1, leading to the replacement of ADP by ATP or deoxyadenosine triphosphate (dATP) in the ZAR1 NB-ARC domain in vitro (Wang et al. 2019b, 2019a). This ADP–ATP switch is required for the oligomerization of activated ZAR1 monomers into a pentameric resistosome structure. In the activated ZAR1–RKS1–$PBL2^{UMP}$ resistosome structure, the first N-terminal α helix (α1 helix) of the CC domain of the ZAR1 monomers is exposed and forms a funnel-shaped structure (Wang et al. 2019a). The exposed funnel on the ZAR1 resistosome is thought to insert into the plasma membrane to form a pore (Wang et al. 2019a). As such, the ZAR1 resistosome functions as a calcium ion (Ca^{2+}) channel on the plasma membrane, which induces Ca^{2+} influx and subsequent hypersensitive cell death (Bi et al. 2021). Supporting this model, substitutions in the ZAR1 α1 helix, impairing Ca^{2+} influx, lead to the loss of the hypersensitive cell-death response and immunity to *Xcc*, although they do not affect the formation of the ZAR1 resistosome *in vivo* (Wang et al. 2019a, Hu et al. 2020, Bi et al. 2021). Additionally, the CC-NLR Sr35 also forms a similar pentameric resistosome structure which also acts as a Ca^{2+} channel (Förderer et al. 2022).

The cryo-EM structures of activated RPP1 and Roq1 reveal two examples of tetrameric resistosomes formed by TIR-NLRs (Ma et al. 2020, Martin et al. 2020). The RPP1 resistosome is bound by ADP, although it might be that the switch of ADP for ATP is crucial for oligomerization (Ma et al. 2020). Activated RPP1 and Roq1 oligomerize and their N-terminal TIR domains form two active centers for NAD^+ cleaving activity (Ma et al. 2020, Martin et al. 2020). The enzymatic activity of these TIR domains results in the release of a variant of cyclic-ADP-ribose (v-cADPR), and this enzymatic activity is required for the induction of hypersensitive cell death (Horsefield et al. 2019, Wan et al. 2019). In addition, plant TIR proteins appear to form a distinct structure in which the TIR domain displays 2′,3′-cAMP/cGMP synthetase activity via the hydrolysis of RNA/DNA, and this catalytic activity is also required for the induction of hypersensitive cell death (Yu et al. 2022). The N-terminal domains on the NLR resistosomes therefore directly mediate immune responses in distinct ways.

Thirty years of research on cloning plant disease resistance (*R*) genes has led to the identification of hundreds of *R* genes, which generally encode plant immune receptors (Kourelis and van der Hoorn 2018, Ngou et al. 2022a). Furthermore, as discussed above, the remarkable recent progress in plant NLR structural biology has dramatically advanced our understanding of how plant NLRs function at the molecular level. However, beyond the function of individual NLRs, a picture is now emerging in which intricate receptor network systems require multiple NLRs to function together to recognize diverse pathogen effectors and trigger immune signaling (Ngou et al. 2022b). In this review, we discuss our current understanding of how NLR immune receptor networks form, become activated and are regulated in plant immunity.

NLR Networks Consist of Sensors and Helpers

NLR networks comprise sensor and helper NLRs

The conceptual basis of plant–microbe interactions was initially defined by the influential gene-for-gene model proposed by the

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plant pathologist Harold Flor (Flor 1971). In this model, an *R* gene from the host plant evolves alongside a specific avirulence (*AVR*) gene from the pathogen. On a biochemical level, the gene-for-gene model dictates that plant NLR immune receptors (encoded by *R* genes) can recognize pathogen effector ligands, either directly or indirectly, and trigger an immune response as a single NLR unit. This means that plant NLRs are receptors that have both sensing and signaling functions and are therefore referred to as 'singleton NLRs' (Adachi et al. 2019b). The best described singleton NLR is ZAR1, since its sensing and signaling functions are described at the structural level. ZAR1 both recognizes its cognate effectors and executes immune signaling through homo-oligomerization and complex formation without relying on other NLRs (Wang et al. 2019b, 2019a).

In addition to the singleton NLRs, it is now clear that many plant NLRs have functionally specialized to either sense pathogen effectors (sensor NLRs) or execute immune signaling (helper NLRs, also known as executor NLRs). Sensor NLRs can recognize pathogen effectors either directly or indirectly by recognizing the modification of host target proteins, but they require helper NLRs to induce downstream immune signaling. Sensor and helper NLRs often work in pairs; for instance, the rice (*Oryza sativa*) CC-NLRs 'Sasanishiki' *RESISTANCE GENE ANALOG 5* (*SasRGA5*) and *PYRICULARIA ORYZAE RESISTANCE K-1* (*Pik-1*) CC-NLRs are sensor NLRs that are genetically linked to the CC-NLR genes, *SasRGA4* and *Pik-2*, respectively (Ashikawa et al. 2008, Okuyama et al. 2011). RGA4 and Pik-2 function as helper NLRs that form a heterocomplex with the corresponding sensor NLRs to trigger immune signaling (Césari et al. 2014b, Zdrzałek et al. 2020).

In other cases, however, helper NLRs can function as signaling nodes for multiple sensor NLRs (Adachi et al. 2019b). In this receptor system, many NLRs form a complex network architecture—an NLR network—beyond the one-to-one relationship of NLR pairs. As discussed in Adachi and Kamoun (2022), the potential benefits of NLR networks are evolvability and redundancy. For example, functional specialization of NLR receptors into sensors and helpers may allow sensor NLRs to diversify by diversifying selection, accumulating mutations or acquiring novel domain to recognize fast-evolving pathogen effectors. Helper NLRs instead maintain the ability to induce immune responses as signaling hubs, experiencing limited expansion and purifying selection. Redundancy in helper NLRs can allow the immune system to be more resilient from the suppression of central signaling nodes by pathogen effectors. The diversification and resilience in NLR receptor networks are distinct properties from other plant signaling networks and have presumably occurred in evolutionary arms races with fast-evolving pathogen effectors which are not only perceived as signal inputs but also evolved to act as suppressors of these NLR networks (Adachi and Kamoun, 2022, Katagiri 2018). Hereafter, we review examples of NLR immune receptor networks.

The NRC network is an expanded helper/sensor clade in the Asterids

The NLR network model was first proposed upon the realization that a phylogenetic subclade of NLRs in the Solanaceae act as helper NLRs, which are differentially and redundantly required for the function of sensor NLRs (Wu et al. 2017). In this network, CC-NLRs known as NLR-REQUIRED FOR CELL DEATH (NRC) proteins function as helper NLRs for multiple sensor NLRs to mediate immune responses (**Fig. 1**); therefore, this immune receptor network is referred to as the NRC network. In the solanaceous model plant *N. benthamiana*, *NRC2*, *NRC3* and *NRC4* act redundantly and with different specificities as helper NLRs for many sensor NLRs (Wu et al. 2017, Lin et al. 2021, Witek et al. 2021); for example, the sensor NLR Rpi-blb2 specifically activates hypersensitive cell death and disease resistance to the oomycete potato blight pathogen *Phytophthora infestans* through *NRC4*, while the sensor NLR Prf-mediated response is dependent on *NRC2* and *NRC3* (Wu et al. 2016, 2017). All three helper NLRs redundantly contribute to sensor NLR Rx-mediated immunity against *potato virus X* (PVX) (Wu et al. 2017).

Although NRC paralogs and NRC-dependent sensor NLRs are genetically unlinked and dispersed throughout the genomes of solanaceous plant species, they form a phylogenetically well-supported clade (NRC-helper clade) (Wu et al. 2017). Interestingly, the NRC-helper clade phylogenetically clusters with a hugely expanded CC-NLR clade (NRC-sensor clade), which includes many sensor NLRs encoded by *R* genes from different solanaceous plant species. This phylogenetic relationship between helper and sensor NLRs in the NRC network indicates a common origin. Indeed, outside of the Asterid lineages, sugar beet (*Beta vulgaris*)—belonging to the Caryophyllales—has one NRC helper and two NRC sensors, which are genetically linked (Wu et al. 2017). The NRC network components therefore presumably emerged as a sensor–helper gene cluster about 100 million years ago, before the Asterids and Caryophyllales linages split. Subsequently, this sensor–helper gene cluster massively expanded into the current NRC network through gene duplication and diversification in the Solanaceae and several other Asterids; in some species, as much as 50% of all NLRs belong to this superclade of NRCs and their *R* sensors.

How do helper NRCs activate immune signaling? One clue is provided by the fact that the first 29 amino acids of NRC4 are sufficient to trigger an HR (Adachi et al. 2019a). Notably, the N-termini of helper NRCs show a high sequence similarity to the N-terminal α1 helix of ZAR1, which forms the funnel and creates a pore at the plasma membrane for Ca^{2+} influx. The consensus sequence motif for this N-terminus—MADAxVSFxVxKLxxLLxxEx—is called the 'MADA motif'. The MADA motif is present in about 20% of all CC-NLRs across flowering plant species, but it has degenerated in sensor CC-NLRs (Adachi et al. 2019a). The MADA motif of NRC4 can be functionally replaced by the N-terminal sequence of multiple other MADA-type CC-NLRs from both dicots and monocots (Adachi et al. 2019a). As in ZAR1, mutations of some

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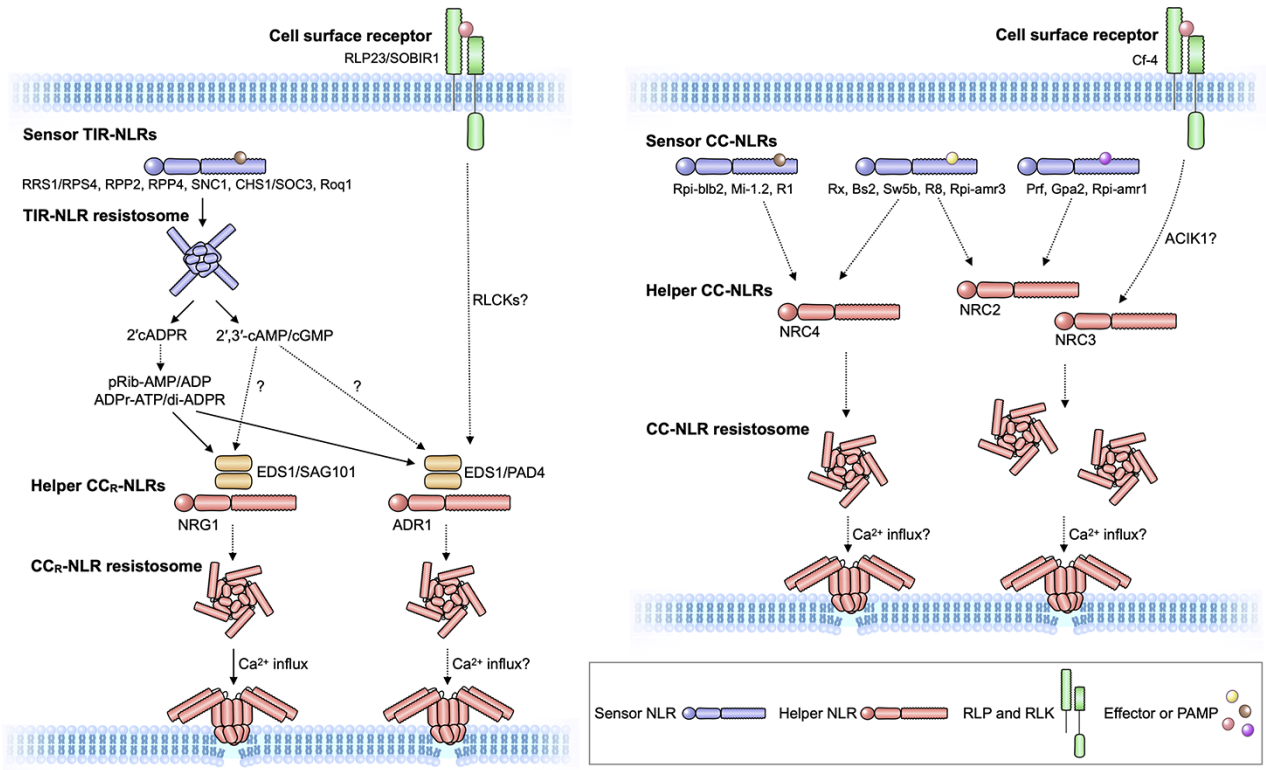

Fig. 1 Activation of NLR immune receptor networks. Pathogen ligand perception by cell-surface receptors and sensor NLRs leads to the activation of signaling pathways through helper NLRs. In the ADR1/NRG1 network (left), the EDS1–PAD4–ADR1 and EDS1–SAG101–NRG1 modules function downstream of the TIR-NLRs and some cell-surface RLP/RLKs. The activated TIR-NLR resistosome has enzymatic activity to produce v-cADPR and 2′,3′-cAMP/cGMP, which likely activate the helper CC_R-NLRs through EDS1–PAD4 and EDS1–SAG101. Upon activation, ADR1 and NRG1 form a high-order complex (CC_R-NLR resistosome), acting as a Ca^{2+} channel to induce immunity and hypersensitive cell death. In the NRC network (right), NRC helper subfamily members function downstream of phylogenetically linked sensor CC-NLRs and the cell-surface RLP(s). NRC2, NRC3 and NRC4 are functionally validated helpers for resistance-gene-encoded sensors. The effector recognition by sensor NLRs results in NRC homo-oligomerization by an activation-and-release mechanism. The resulting homo-oligomerized NRC complex may function as a CC-NLR resistosome, inducing a Ca^{2+} influx resulting in immunity and hypersensitive cell death. Solid lines indicate validated molecular mechanisms, while dashed lines indicate hypothetical models requiring further mechanistic elucidation.

hydrophobic residues in the NRC MADA motif ($NRC2^{L17E}$, $NRC3^{L21E}$, $NRC4^{L9E}$, $NRC4^{L13E}$, $NRC4^{L17E}$ and $NRC4^{L9E/V10E/L14E}$) impair cell-death activity (Adachi et al. 2019a, Kourelis et al. 2021a). Unlike in ZAR1, however, the E11A mutation in the MADA motif of NRC4 does not lead to loss of the hypersensitive cell-death response (Adachi et al. 2019a). Additionally, similar mutations of charged residues in the predicted α1 helix of Sr35 also do not abolish hypersensitive cell-death induction, while mutating hydrophobic residues ($Sr35^{L15E/L19E}$) does result in the loss of hypersensitive cell-death induction (Förderer et al. 2022). Therefore, hydrophobic residues in the ZAR1 MADA/α1 helix are likely involved in pore formation, while the negatively charged residue E11 could be essential for ZAR1 Ca^{2+} channel activity, but not necessarily for other CC-NLRs (Adachi et al. 2019a, Wang et al. 2019a, Hu et al. 2020, Bi et al. 2021, Förderer et al. 2022). The helper NRCs may therefore function like ZAR1, forming a resistosome upon activation with an N-terminal funnel structure to make a pore on the plasma membrane (**Fig. 1**); however, it remains unknown whether, like ZAR1, helper NRCs function as Ca^{2+} channels.

The ADR1/NRG1 network mediates TIR-NLR signaling

Another well-characterized NLR network is formed by the N REQUIREMENT GENE 1 (NRG1) and ACTIVATED DISEASE RESISTANCE 1 (ADR1) subfamilies of CC_R-type helper NLRs (**Fig. 1**). CC_R-NLRs are required as helper NLRs for TIR-NLR-mediated immunity. Since the CC_R-NLR subfamily is found in the genomes of most flowering plant species and is smaller than the CC-NLR and TIR-NLR subfamilies, the CC_R-NLRs are considered to be conserved throughout angiosperm evolution as helper NLRs for sensor NLRs (Shao et al. 2016, Baggs et al. 2020, Liu et al. 2021). Interestingly, the copy-number variation of the TIR-NLR and CC_R-NLR genes (primarily *NRG1*) is tightly correlated, reflecting an evolutionary association among

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these NLR subfamilies (Liu et al. 2021). The TIR-NLR and NRG1 CC_R-NLR subfamily lineages have been lost in the monocots, although most monocot species do possess ADR1 subfamily CC_R-NLRs. This suggests that ADR1 is a helper subfamily not only for TIR-NLRs, but also for other types of sensors.

Arabidopsis possesses five full-length CC_R-NLR helpers, two NRG1 paralogs (*NRG1.1* and *NRG1.2*, also known as *NRG1A* and *NRG1B*, respectively) and three ADR1 paralogs (*ADR1*, *ADR1-L1* and *ADR1-L2*). Saile et al. (2020) recently characterized the genetic requirement for *ADR1* and *NRG1* in immunity by comparing the phenotypes of the *adr1 adr1-L1 adr1-L2* triple mutant, *nrg1.1 nrg1.2* double mutant and *nrg1.1 nrg1.2 adr1 adr1-L1 adr1-L2* '*helperless*' pentuple mutant lacking all full-length CC_R-NLR helpers. This comparison revealed that Arabidopsis *ADR1* genes are required for full resistance mediated by the TIR-NLRs RESISTANT TO RALSTONIA SOLANACEARUM 1 (RRS1)/RESISTANT TO P. SYRINGAE 4 (RPS4), RPP2 and RPP4 (Saile et al. 2020). *NRG1* genes can partially substitute for *ADR1* function in resistance mediated by RRS1/RPS4 and RPP2; hence, the *helperless* mutant has a more susceptible phenotype than the *adr1* triple mutant (Saile et al. 2020). In addition, the RRS1/RPS4-triggered hypersensitive cell-death response requires only NRG1s (Saile et al. 2020). This reveals an unequal genetic redundancy between the *ADR1* and *NRG1* genes for TIR-NLR-mediated resistance and hypersensitive cell death. The CC-NLRs do not appear to require *ADR1* or *NRG1* for the activation of hypersensitive cell death and immunity, as the singleton CC-NLRs ZAR1 and RESISTANCE TO P. SYRINGAE PV MACULICOLA 1 (RPM1) do not require *ADR1* or *NRG1* (Saile et al. 2020).

An elicitor-independent autoactive mutant of NRG1.1, $NRG1.1^{D485V}$, forms higher-order complexes and associates with the plasma membrane when it is expressed in *N. benthamiana*, while wild-type NRG1.1 does not (Jacob et al. 2021). Furthermore, ADR1s can form self-associated complexes and localize to the plasma membrane through the interaction of their N-terminal CC_R domain and the anionic lipid phosphatidylinositol-4-phosphate of the plant plasma membrane (Saile et al. 2021). Interestingly, the N-terminal CC_R domain of NRG1.1 is composed of a four-helical bundle structure like the ZAR1 CC domain (Jacob et al. 2021), which implies that helper NRG1 and ADR1 form a ZAR1-type resistosome to execute immune signaling. Although the N-terminus of NRG1 and ADR1 do not share a similar sequence pattern to the ZAR1 MADA/α1 helix, their N-termini carry negatively charged residues similar to ZAR1, which are required for Ca^{2+} influx and the initiation of cell death (Jacob et al. 2021, Sun et al. 2021). The activated helper NRG1 and ADR1 may, therefore, function like the ZAR1 resistosome on the plasma membrane, mediating the hypersensitive cell-death response by Ca^{2+} influx (**Fig. 1**).

Activation of NLR Immune Receptor Networks

Sensor NLRs activate helper NLRs

In the ADR1/NRG1 network, sensor TIR-NLRs form an enzymatically active resistosome upon effector recognition, and this enzymatic activity is required to induce the hypersensitive cell-death response and immunity (**Fig. 1**). In addition to this catalytic activity, the hypersensitive cell-death response triggered by TIR-NLRs depends on lipase-like proteins belonging to the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) family (Gantner et al. 2019, Lapin et al. 2019). This family contains homologs of EDS1, SENESCENCE-ASSOCIATED GENE 101 (SAG101) and PHYTOALEXIN-DEFICIENT 4 (PAD4) (Lapin et al. 2020). EDS1 forms mutually exclusive dimers with either SAG101 or PAD4 (Wagner et al. 2013), which are required for NRG1- or ADR1-mediated immunity, respectively (Sun et al. 2021) (**Fig. 1**). EDS1–SAG101–NRG1 and EDS1–PAD4–ADR1 physically associate during some stages of the immune signal transduction, but the hypersensitive cell death mediated by autoactive mutants of NRG1 does not require *EDS1* (Jacob et al. 2021, Sun et al. 2021).

Because *EDS1* is not required for the production of v-cADPR mediated by TIR proteins in planta (Wan et al. 2019), it was proposed that v-cADPR may activate the EDS1–SAG101–NRG1 and/or EDS1–PAD4–ADR1 modules, in turn inducing the formation of the NRG1 and ADR1 resistosomes, which function as Ca^{2+}-permeable nonselective cation channels (**Fig. 1**) (Saur et al. 2021). TIR-NLRs thus indirectly induce a similar immune response to the CC-NLRs. The likely structure of plant TIR domain that produced v-cADPR was recently identified as 2′cADPR (independently identified and named 1′–2′ glycocyclic ADPR) (Leavitt et al. 2022, Manik et al. 2022). 2′cADPR may serve as an intermediate in the synthesis of novel nucleosides associated with plant immunity (Manik et al. 2022). Indeed, Huang et al. (2022) show that TIR-NLRs catalyze the production of 2′-(5′′-phosphoribosyl)-5′-adenosine mono-/di-phosphate (pRib-AMP/ADP) and that EDS1-PAD4 act as a receptor complex for pRib-AMP/ADP. Binding of pRib-ADP to EDS1-PAD4 results in a conformational change in the PAD4 C-terminal domain, thereby promoting interaction with ADR1s (Huang et al. 2022). pRib-AMP can be directly derived from 2′cADPR by cleavage of its pyrophosphate bond (Manik et al. 2022). EDS1–SAG101, instead, acts as a receptor complex for other TIR-catalyzed second messengers, ADP-ribosylated ATP (ADPr-ATP) and ADPr-ADPR (di-ADPR), that induce EDS1–SAG101 association with NRG1.1 but not with ADR1-L1 (Jia et al. 2022). These second messenger interactions with the EDS1–PAD4 and EDS1–SAG101 complexes then likely promote ADR1 and NRG1 resistosome formation and Ca^{2+} channel activity. Finally, aside from their NAD^+ cleaving activity, the TIR domain of some TIR-NLRs displays 2′,3′-cAMP/cGMP synthetase activity via the hydrolysis of RNA/DNA (Yu et al. 2022). While this activity is also required for the induction of hypersensitive cell death, it is unclear which pathways these signaling molecules activate.

The mechanisms by which sensor NLRs activate helper NLRs in the NRC networks are the subject of ongoing investigation. A primary technical barrier for in planta biochemical and cell biological analyses of activated NRC network components is the cell-death response elicited upon their activation. Contreras et al. (2022) and Ahn et al. (2022) took advantage of an NRC2

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MADA motif mutant (NRC2$^{L9E/L13E/L17E}$) to abolish the cell-death response without affecting resistosome assembly and plasma membrane localization, similar to what was previously shown for the MADA-type CC-NLRs ZAR1, NRC4 and Sr35 (Hu et al. 2020, Duggan et al. 2021, Förderer et al. 2022). NRC2 oligomerization is induced upon effector recognition by sensor NLRs Rx, Bs2, Rpi-amr1 or Rpi-amr3, resulting in molecular complexes in the ~720–1,048 kDa range (Ahn et al. 2022, Contreras et al. 2022). Interestingly, the activated sensor NLRs do not appear to be incorporated in the NRC2 higher-order complex (Ahn et al. 2022, Contreras et al. 2022). Instead, the activated sensor NLRs are proposed to trigger homo-oligomerization of helper NRCs by an activation-and-release mechanism (**Fig. 1**) (Ahn et al. 2022, Contreras et al. 2022). This activation also results in a subcellular relocalization of helper NRCs. The helper NRC4, for example, localizes to the plasma membrane around the *P. infestans* invasion site where the effectors are secreted, and activation of NRC4 by the sensor Rpi-blb2, either by *P. infestans* secreting the Rpi-blb2 ligand AVR-blb2 or by co-expression of AVR-blb2, results in a punctate distribution of NRC4 (Duggan et al. 2021). Similarly, the activation of NRC2 by the sensor Rx, either upon PVX infection or by co-expressing the PVX coat protein ligand of Rx, results in the subcellular relocalization of NRC2 to plasma-membrane-localized puncta (Contreras et al. 2022). In contrast, upon activation, the sensor NLR Rx does not form plasma-membrane-associated puncta and remains cytosolic, further supporting an activation-and-release mechanism for NRC activation (Contreras et al. 2022). This activation-and-release mechanism is distinct from the activation mechanism of the mammalian paired NLRs NLR neuronal apoptosis inhibitory protein/nucleotide-binding oligomerization domain (NOD)-like receptor containing a caspase activating and recruitment domain 4, which form a heterocomplex upon ligand perception (Zhang et al. 2015, Tenthorey et al. 2017). The exact mechanism by which sensor NLRs trigger oligomerization of helper NRCs and whether this involves a transient interaction state or other components are currently not known.

Autoactive sensor NLR mutants require helper NLRs in immune signaling

In addition to effector recognition, amino acid insertions or substitutions in NLR proteins often result in autoimmunity. In Arabidopsis, some alleles of TIR-NLRs, such as *suppressor of npr1-1, constitutive 1* (*snc1*), *chilling-sensitive mutant 1* (*chs1*) and *chs3-2D*, result in an autoimmune phenotype (Zhang et al. 2003, Bi et al. 2011, Wang et al. 2013). These alleles encode TIR-NLR proteins with gain-of-function mutations, and the autoimmunity is dependent on *EDS1* (Zhang et al. 2003, Wang et al. 2013) and *NRG1* and *ADR1* with different strengths (Castel et al. 2019, Wu et al. 2019).

Furthermore, substitutions in a conserved motif in the NB-ARC domain typically containing the amino acids MHD (MHD motif) are commonly used to generate autoactive versions of NLRs. The MHD motif is located in a position binding to ADP in the ZAR1 structure, suggesting that the MHD motif–ADP interaction may have a role in intramolecular regulation of NLR proteins (Wang et al. 2019a). The first example of such a MHD autoactive mutant was identified through the random mutagenesis of the NRC sensor Rx (Bendahmane et al. 2002). The MHD mutant Rx^{D460V} can induce the autoimmune cell-death response in the absence of the cognate pathogen ligand (Bendahmane et al. 2002). Similarly, MHD mutants of helper NLRs, such as NRC1^{D481V}, NRC2^{H480R}, NRC3^{D480V}, NRC4^{D478V}, NRG1.1^{D485V} and ADR1-L2^{D484V}, are also autoactive (Gabriëls et al. 2007, Roberts et al. 2013, Derevnina et al. 2021, Jacob et al. 2021). The autoactive NRG1.1^{D485V} mutant forms a high-order complex *in vivo* (Jacob et al. 2021), indicating that autoactive mutants could be used to analyze the biochemical and biophysical properties of sensor and helper NLRs in their activated state. Finally, autoactive sensor NLR mutants are useful tools for dissecting the NLR network specificity in the absence of a known pathogen ligand, as this autoactivity requires helper NLRs (Derevnina et al. 2021).

Cell-surface receptors can signal through NLR networks

Finally, it is becoming increasingly clear that helper NLRs are also required for signaling mediated by cell-surface receptors. Cell-surface receptors are typically divided into two categories: the receptor-like kinases (RKs, also known as RLKs) and the receptor-like proteins (RPs, also known as RLPs) (Saijo et al. 2018). Upon ligand recognition, many LRR-RKs involved in immunity hetero-oligomerize with the LRR-RK co-receptor SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3 (SERK3, also known as BRI1-associated kinase 1 [BAK1]). Similarly, most LRR-RPs constitutively interact with the LRR-RK SUPPRESSOR OF BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) 1 (SOBIR1) and hetero-oligomerize with SERK3 upon ligand binding. This in turn activates downstream RLCKs, which together relay the immune response.

In Arabidopsis, the EDS1–PAD4–ADR1 and, to a lesser extent, EDS1–SAG101–NRG1 modules are genetically required for a subset of the immune responses triggered by LRR-RPs and LRR-RKs (Pruitt et al. 2021, Tian et al. 2021) (**Fig. 1**). For example, the LRR-RP RECEPTOR-LIKE PROTEIN 23 (RLP23)-mediated immunity is dependent on SOBIR1, the RLCK-VII subfamily protein PBS1-LIKE 31 (PBL31), and the EDS1–PAD4–ADR1 module (Pruitt et al. 2021). Protein–protein interaction analyses suggest that a cell-surface receptor complex including SOBIR1 and PBL31 associates with the EDS1–PAD4–ADR1 module in a ligand-independent manner (Pruitt et al. 2021); therefore, upon ligand perception, LRR-RPs and LRR-RKs converge to activate NLR networks for a subset of their immune functions, possibly by protein-kinase-mediated phosphorylation.

In addition to CC_R-type helper NLRs, a helper NLR in the NRC network is also involved in cell-surface-receptor-mediated immune responses (**Fig. 1**). In virus-induced gene silencing experiments, the helper NRC1 was previously implicated as a

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key component in the cell death mediated by the LRR-RPs Cf-4 (Gabriëls et al. 2006), LeEIX2 (Gabriëls et al. 2007) and Ve1 (Fradin et al. 2009). Recently, the precise contribution of helper NRCs to the HR mediated by Cf-4 has been validated using *N. benthamiana* CRISPR mutants of various NRCs (Kourelis et al. 2021a). This showed that the Cf-4-mediated hypersensitive cell death in response to the recognition of the *Cladosporium fulvum* (syn. *Passalora fulva*) effector Avr4 is lost in a *nrc2/3* CRISPR line, which could be restored by expressing *NRC3* (Kourelis et al. 2021a). Furthermore, a functional MADA motif in NRC3 is required for the hypersensitive cell-death response (Kourelis et al. 2021a). This implies the function of a signaling pathway downstream of the cell-surface receptor Cf-4, which activates NRC3 to trigger hypersensitive cell death, presumably through a ZAR1-resistosome-type mechanism. The RLCK-VII member Avr9/Cf-9 induced kinase 1 (ACIK1) was identified as a downstream component in Cf-4- and Cf-9-mediated hypersensitive cell death (Rowland et al. 2005). Although the exact signaling components and the molecular mechanism by which the LRR-RP signals are transduced into the NRC network are currently unknown, the phosphorylation of helper NLRs by cell-surface receptor complexes such as RLCKs might be a key of helper activation. Indeed, the function of the TIR-NLR RRS1 is regulated by the phosphorylation of its C terminus by unknown protein kinase(s) (Guo et al. 2020).

NLR Networks are Regulated at Multiple Levels

Transcriptional and posttranscriptional regulation of NLR networks

Plant NLRs are regulated at the transcriptional, posttranscriptional and posttranslational levels to prevent the autoimmune fitness costs associated with the inappropriate activation of immune signaling. Our current understanding is that many NLR genes are expressed at a low basal level but are amplified upon the activation of immunity. For example, at the transcriptional level, many plant NLRs are subject to the premature termination of transcription mediated by the RNA-binding protein FPA, thereby regulating NLR protein levels (Parker et al. 2021) (**Fig. 2**).

The activation of cell-surface receptors leads to the transcriptional upregulation of immune-related genes, including the NLRs, which is required for the induction of NLR-mediated hypersensitive cell death and immunity (Ngou et al. 2021, Yuan et al. 2021). Notably, the activation of the TIR-NLR pair RRS1/RPS4 by AvrRps4 (Ngou et al. 2021) and the CC_{G10}-NLRs RPS2 and RPS5 by AvrRpt2 (Ngou et al. 2021, Yuan et al. 2021) and AvrPphB (Ngou et al. 2021) requires the cell-surface-receptor-mediated potentiation of signaling to trigger hypersensitive cell death.

At the posttranscriptional level, microRNA-mediated gene silencing has been shown to regulate NLR genes in NLR immune receptor networks (**Fig. 2**); for example, miR-n033 regulates a large number of CC-NLR genes in Solanaceae species (Seo et al. 2018). Most of the miR-n033 targets belong to the NRC sensor superfamily, including the *R* genes *Rpi-blb2*, *Mi-1.2* and *Hero*. Additionally, homologs of the Solanaceous NRC-sensor *R* genes *Rx1*, *R2* and *R1* are targeted by the microRNAs stu-miR6024, stu-miR482d and nta-miR6025a, respectively (Li et al. 2012). In addition to the NRC network, the ADR1/NRG1 network is also regulated by microRNAs; for example, in *N. benthamiana*, nta-miR6019 and nta-miR6020 lead to the cleavage of transcripts from the TIR-NLR *R* gene *N*, which encodes a sensor NLR in the ADR1/NRG1 network (Li et al. 2012). In addition to the sensor NLRs, transcripts of the LRR-RP genes are also regulated by microRNAs. In tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*), sly-miR6022, sly-miR6023 and miR-n026 target LRR-RPs belonging to the *Homologs of C. fulvum resistance 9* (*Hcr9*) clade, which are homologs of the *Cf-9R* gene (Li et al. 2012, Seo et al. 2018). MicroRNAs presumably regulate the transcript levels of diverse sensor NLRs and RPs in NLR networks, thereby preventing autoimmunity.

Non-NLR host proteins modulate NLR networks

In addition to transcriptional and posttranscriptional regulation, plant NLR networks are regulated at the posttranslational level. One such mechanism is the ubiquitin–proteasome degradation pathway (**Fig. 2**). In the ADR1/NRG1 network, the Arabidopsis TIR-NLR SNC1 and the related proteins SIDEKICK SNC1 1/2/3 (SIKIC1/2/3) are targeted for protein degradation by SKP1–CULLIN1–F-box (SCF) E3 complex and the RING-type E3 ligases MUTANT and SNC1-ENHANCING 1/2 (Cheng et al. 2011, Dong et al. 2018). A recent study identified the novel E3 ligases *SNC1*-INFLUENCING PLANT E3 LIGASE REVERSE 1/2, which broadly regulate protein levels of sensor NLRs in Arabidopsis (Wu et al. 2020). In *N. benthamiana*, the putative E3 ubiquitin ligase UBR7 modulates the protein levels of the TIR-NLR N (Zhang et al. 2019).

In addition to the ubiquitin–proteasome-mediated degradation of NLR proteins, other host components can negatively regulate NLR-network-mediated immunity; for instance, RPS2-mediated hypersensitive cell death is suppressed by salicylic acid (SA) treatment (Zavaliev et al. 2020). SA is a plant hormone that induces systemic acquired resistance through the master regulator NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1). Interestingly, the suppression of hypersensitive cell death is dependent on NPR1 and is not limited to RPS2 but also to the TIR-NLRs RPS4 and RPP1 (Zavaliev et al. 2020). Spatiotemporal analyses of phytohormone responses during RPS2-mediated immunity reveal that the SA-signaling pathway is activated in the areas surrounding cells displaying hypersensitive cell death (Betsuyaku et al. 2018, Zavaliev et al. 2020). These findings suggest that the NPR1 pathway may regulate the ADR1/NRG1-network-mediated hypersensitive cell-death response and contribute to the survival of cells adjacent to the pathogen infection site.

The ADR1/NRG1 network and NRC network are modulated by NRG1C and NRCX, respectively

There are also cases where NLR proteins regulate NLR networks in plants (**Fig. 2**); for example, in the ADR1/NRG1 network, the

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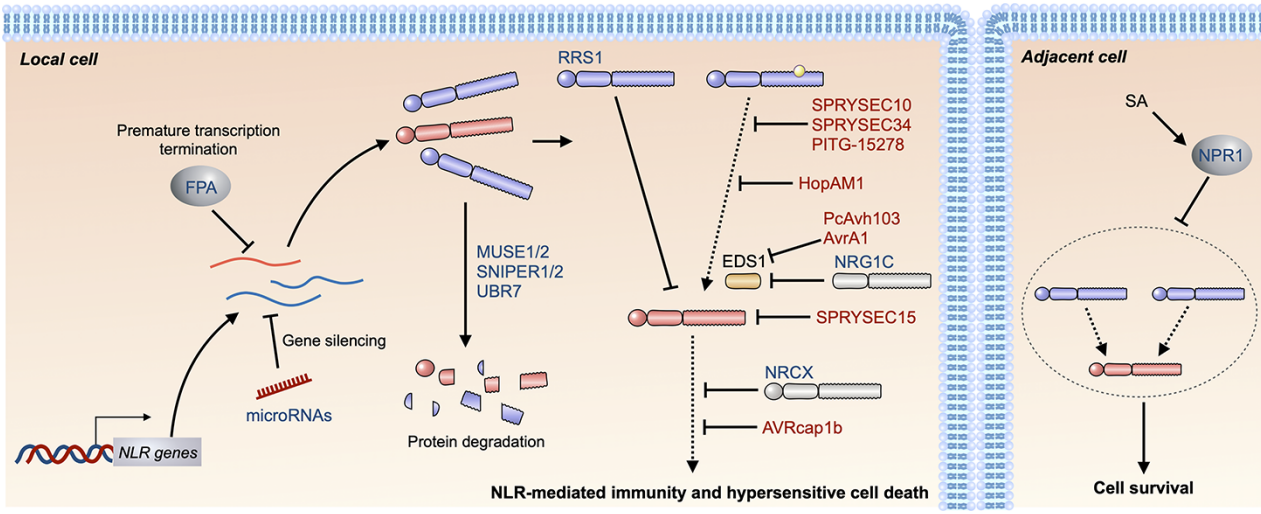

Fig. 2 Negative regulation of NLR immune receptor networks. NLR network components are tightly regulated at multiple levels. NLR transcripts are regulated by host premature transcription termination and microRNA-mediated silencing machineries. NLR networks are modulated by other host proteins, such as E3 ligases and NPR1, and by NLRs such as RRS1, NRG1C and NRCX, which likely suppress the inappropriate activation of the networks. Plant pathogens have evolved effectors to target NLR networks or other key host proteins.

Arabidopsis TIR-NLR RRS1 associates with its genetically linked NLR partner RPS4, thereby negatively regulating its autoactivity (Williams et al. 2014). The overexpression of *RPS4* results in the constitutive activation of immunity in tobacco and Arabidopsis, which is suppressed in the presence of RRS1 (Huh et al. 2017). In contrast to this one-to-one regulation, Wu et al. (2022) recently showed that NRG1C negatively regulates TIR-NLR-mediated immunity and autoimmunity of its paralog NRG1.1. NRG1C is a member of the CC_R-NLR family, forming a gene cluster with helper NRG1.1 and NRG1.2; however, unlike NRG1.1 and NRG1.2, NRG1C lacks the N-terminal CC_R domain and has a severely truncated NB-ARC domain, suggesting that NRG1C has lost its signaling activity and the capacity to induce hypersensitive cell death. Protein–protein interaction analyses indicate that the negative regulation by NRG1C likely occurs through its interference with the EDS1–SAG101 complex rather than an interaction with its helper NLR NRG1.1 (Wu et al. 2022).

Similarly, the NRC network is also regulated by other NLRs; for example, in *N. benthamiana*, systemic gene silencing of *NRCX* markedly impairs plant growth, resulting in a dwarf phenotype (Adachi et al. 2021). Although NRCX is a member of the helper NRC family with a CC-NLR domain architecture, it lacks certain canonical features of helper NRCs, such as a functional N-terminal MADA motif and the capacity to trigger autoimmunity. The alteration of *NRCX* expression modulates the hypersensitive cell death mediated by NRC2 and NRC3 but not by NRC4 (Adachi et al. 2021), although the molecular mechanism underpinning the NRCX antagonism remains unknown. An emerging picture is that NRG1C and NRCX are atypical homologs of helper NLRs, which lost their cell death executor activity and instead evolved to modulate the signaling hubs of NLR networks.

Pathogen effectors have evolved to suppress NLR networks

Because helper NLRs in NLR networks are central hubs in mediating immune responses, diverse plant pathogens have evolved effectors to suppress them and thereby establish infection (**Fig. 2**). Derevnina et al. (2021) conducted a screen using effector libraries from pathogens of solanaceous plant species and identified five effectors suppressing hypersensitive cell death induced by NRC network components in *N. benthamiana*. Three of these effectors, SPRYSEC10 and SPRYSEC34 from the potato cyst nematode *Globodera rostochiensis* and PITG-15278 from the oomycete *P. infestans*, suppress the hypersensitive cell-death response mediated by the sensor NLR Rpi-blb2 (Derevnina et al. 2021). By contrast, two other effectors, *G. rostochiensis* SPRYSEC15 and *P. infestans* AVRcap1b, suppress the helper NRCs NRC2 and NRC3, thereby preventing the recognition of effectors mediated by NRC2/3-dependent sensor NLRs (Derevnina et al. 2021). Interestingly, SPRYSEC15 directly binds to the NB-ARC domain of NRC2 and NRC3, suggesting that this direct association interferes with the helper NLR function. AVRcap1b, however, appears to indirectly suppress NRC2- and NRC3-mediated hypersensitive cell death by binding to the host protein Target of Myb 1-like protein 9a (TOL9a). The suppression of NRC2 and NRC3 by AVRcap1b is compromised when *TOL9a* expression is silenced by RNA interference.

In addition to the suppression of the NRC network, pathogens have evolved effectors to suppress NRG1 and ADR1 subfamily CC_R-type helper NLRs; for example, a *Phytophthora capsici* effector PcAvh103 associates with the lipase domain of EDS1 and promotes the dissociation of the EDS1–PAD4 interaction (Li et al. 2020), thereby disrupting the function of the EDS1–PAD4–ADR1 network. Similarly, the AvrA1 effector from the bacterial pathogen *P. syringae* interacts with the soybean

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(*Glycine max*) homologs of EDS1 and requires these proteins to exert its virulence function (Wang et al. 2014). Finally, the *P. syringae* effector HopAM1 is a TIR-domain-containing effector which can suppress cell-surface-mediated signaling and TIR-NLR-mediated signaling (Eastman et al. 2022, Manik et al. 2022). HopAM1 serves to produce a distinct version of v-cADPR recently identified as 3′cADPR (Manik et al. 2022). It appears that 3′cADPR and its derivatives can manipulate ADR1/NRG1 network signaling (Manik et al. 2022). This host NAD^+ manipulation could be a conserved virulence mechanism of *P. syringae*, considering that 93% of the primary phylogroup *P. syringae* strains have at least one NADase effector (Hulin and Ma 2022). Taken together, these findings indicate that plant pathogens have evolved effectors targeting NLR networks at multiple levels, thereby enabling them to establish infection and cause disease in the host.

Future Perspectives

In this review, we highlight major advances in our understanding of NLR biology and NLR immune receptor networks in plants. Recent discoveries of NLR protein structures have provided mechanistic insights into how plant NLRs are activated and initiate downstream signaling; however, there are many unanswered questions about NLR function in NLR networks. Two main types of NLR networks have been described thus far: (i) CC_R-NLRs acting as helper NLRs downstream of TIR-NLRs and (ii) the NRC network of phylogenetically related CC-NLRs, which diversified into helper and sensor NLRs in Asterid species. In addition, it is now evident that both types of networks are also activated during the activation of some cell-surface receptors. How are helper NLRs activated by sensor NLRs and cell-surface receptors? What is the determinant of the sensor NLR/helper NLR or cell-surface receptor/helper NLR connections?

In addition to the activation of helper NLRs, how are genetically scattered NLR components coordinately regulated at the transcriptional level? How do host modulators appropriately regulate massively expanded NLR components to maintain homeostasis in NLR receptor networks? How are NLR networks activated and regulated at the single-cell level during pathogen infection? Further studies combining molecular evolution, biochemistry, biophysics and cell biology approaches are required to fully understand the activation and regulation of network-forming NLRs.

Most molecular breeding programs incorporate *R*-gene-encoded sensor NLRs to generate disease-resistant crops. Given that a large number of sensor NLRs can function together with one or more helper NLRs, incorporating knowledge of NLR networks in future breeding programs could ensure that proper combinations of sensor/helper and cell-surface receptor/helper NLRs are achieved. A further understanding of how NLR network homeostasis is maintained will provide new insights into breeding disease-resistant crops without the potential fitness costs and yield loss. Furthermore, given that plant pathogens have evolved effectors to target NLR networks and overcome host immunity, the molecular engineering of NLRs and cell-surface receptors would make the NLR receptor network system more resilient by avoiding suppression by effectors. In conclusion, understanding NLR networks at multiple levels is required to inform future plant breeding programs.

Data Availability

No new datasets were generated or analyzed in this study.

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