NLR receptor networks in plants

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Abstract

To fight off diverse pathogens and pests, the plant immune system must recognize these invaders; however, as plant immune receptors evolve to recognize a pathogen, the pathogen often evolves to escape this recognition. Plant–pathogen co-evolution has led to the vast expansion of a family of intracellular immune receptors—nucleotide-binding domain and leucine-rich repeat proteins (NLRs). When an NLR receptor recognizes a pathogen ligand, it activates immune signaling and thus initiates defense responses. However, in contrast to the model of NLRs acting individually to activate resistance, an emerging paradigm holds that plants have complex receptor networks where the large repertoire of functionally specialized NLRs operate together to act against an equally large repertoire of rapidly evolving pathogen effectors. In this article, we highlight key aspects of immune receptor networks in plant NLR biology and discuss NLR network architecture, the advantages of this receptor network system, and the evolution of the NLR network in asterid plants.

INTRODUCTION

One dogma in plant pathology is that most plants are resistant to most plant pathogens. Disease is the exception, not the rule, and plants use their effective and complex immune system to fight off most pathogens. In the first layer of this immune system, immune receptors act as part of the surveillance system that detects pathogens [1]. One class of plant immune receptors is the intracellular nucleotide-binding domain and leucine-rich repeat containing (NLR) family, which perceives molecules—known as effectors—derived from varied pathogenic fungi, oomycetes, bacteria, nematodes, and aphids [2, 3]. When an NLR detects a pathogen effector, the receptor activates the immune system with a multitude of defense responses, often resulting in programmed cell death at the infection site [4]. This localized cell death reaction is known as the hypersensitive response and limits the spread of pathogens by killing the infected cells before the pathogen reaches neighboring cells.

NLRs are multi-domain proteins that generally harbor a central NB-ARC (nucleotide-binding domain shared with APAF-1, various R proteins, and CED-4) domain and a C-terminal LRR (leucine-rich repeat) domain [5]. The LRR domain recognizes the effector and the NB-ARC domain mediates conformational changes of the entire NLR protein by exchanging an ADP molecule to ATP at the nucleotide binding pocket [6, 7]. In addition to NB-ARC and LRR domains, most plant NLRs have a variable domain at their N termini that defines which sub-class they belong to. For example, CC-NLR proteins have an N-terminal coiled-coil (CC) domain and TIR-NLRs have a Toll/Interleukin-1 Receptor homology (TIR) domain [8]. Although CC and TIR domains are structurally different, both domains are known to execute immune signaling.

Since the cloning of the first NLR genes in the early 90s, over 400 experimentally validated NLR genes have been identified in 30 genera of flowering plants [5]. This collection of validated NLRs—the RefPlantNLR dataset—illustrates the diversity of NLR genes across plant species. Indeed, NLRs are the most diverse gene family in flowering plants, as many plant species have large (>100 genes) and diverse repertoires of NLRs in their genomes [5, 9, 10]. NLRs typically exhibit hallmarks of rapid evolution even at the intraspecific level [11-13]. Although many immune receptor genes have been identified for over 20 years, the complete picture of how diverse plant NLRs are and how they activate immune responses remained in the dark for a long time.

The advent of new technologies in biophysics and cryo-electron microscopy has revealed the structures of activated NLR oligomers, called ‘resistosomes’. The first example of a resistosome structure was revealed by characterization of HOPZ-ACTIVATED RESISTANCE 1 (ZAR1), a prototypical ancient CC-NLR conserved across flowering plant species [6, 14, 15]. ZAR1 partners recognize pathogen effectors; these partner proteins are receptor-like cytoplasmic kinases that trigger a conformational change in monomeric ZAR1 [6]. ZAR1 then assembles into a pentameric resistosome whose CC domains form a funnel-shaped structure due to rotation of its N-terminal α helices upon ATP hydrolysis [14]. The ZAR1 resistosome is thought to evoke local cell death by translocating to the plasma membrane where it inserts itself and functions as a calcium (Ca^{2+}) channel [14, 16]. Other recent studies described two examples of tetrameric resistosomes formed by the TIR-NLRs RECOGNITION OF PERONOSPORA PARASITICA 1 (RPP1) and RECOGNITION OF XOPQ 1 (ROQ1) [17, 18]. The N-terminal TIR domain is activated through oligomerization and acquires NAD^+ cleaving activity [17, 18]. This enzymatic activity of TIR domains is required for executing hypersensitive cell death [19, 20]. Therefore, NLR resistosomes induce immune responses in different ways depending on their NLR class.

Recent progress in defining NLR structures has dramatically advanced our understanding of how plant NLRs function at a molecular level. Notably, these structural insights support the extremely influential gene-for-gene model proposed by the plant pathologist Harold Flor [21]. In the gene-for-gene model, a resistance gene from the host plant forms a unique pair with an avirulence gene from the pathogen. To this day, the gene-for-gene model is often simplified to the biochemical equation that one plant NLR immune receptor (encoded by the resistance gene) recognizes one pathogen effector ligand. In agreement with the gene-for-gene model, ZAR1 functions as a single biochemical unit that reacts to the presence of its cognate effector and forms a homo-NLR oligomer complex to execute immune signaling. However, beyond this one-to-one relationship, an emerging paradigm is that plants have very complex receptor networks composed of multiple NLRs to confer some advantage in recognizing fast-evolving pathogen effectors to trigger immune signaling [22]. Here, we review some key aspects of NLR receptor networks that have emerged from plant NLR biology.

Sensor NLRs and helper NLRs

Researchers have now classified many plant NLRs as sensor NLRs or helper NLRs (also known as executor NLRs) based on their functional roles [22, 23]. Sensor NLRs recognize pathogen effectors directly or sense the modification of host target proteins by pathogens; helper NLRs induce the downstream immune responses. These two types of NLRs even sometimes work in pairs. For instance, in Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*), NLRs encoded by genetically linked genes, RESISTANCE TO RALSTONIA SOLANACEARUM 1 (RRS1)/RESISTANCE TO PSEUDOMONAS SYRINGAE 4 (RPS4), RESISTANCE GENE ANALOG 5 (RGA5)/RGA4, and PYRICULARIA ORYZAE RESISTANCE K-1 (Pik-1)/Pik-2, function as dedicated pairs, whereby a sensor NLR requires a helper NLR partner to trigger immune responses [24-30]. Interestingly, the sensor NLRs have acquired new domains that act as baits or decoys for detecting effectors [31].

In other cases, individual sensor NLRs function together with multiple helper NLRs [32, 33]. For example, a major clade of CC-NLRs in Solanaceous plants forms a complex network architecture, in which multiple helper NLRs, known as the REQUIRED FOR CELL DEATH (NRC) subfamily of NLR proteins, are required to activate immune responses after pathogen perception by the upstream sensor NLR(s) (Figure 1A) [34]. Similarly, members from the two RESISTANCE TO POWDERY MILDEW8 (RPW8)-like CC-NLR subfamilies, N REQUIREMENT GENE 1 (NRG1) and ACTIVATED DISEASE RESISTANCE 1 (ADR1), contribute as helper NLR nodes of TIR-NLRs and a subset of CC-NLRs across several plant species [35-38].

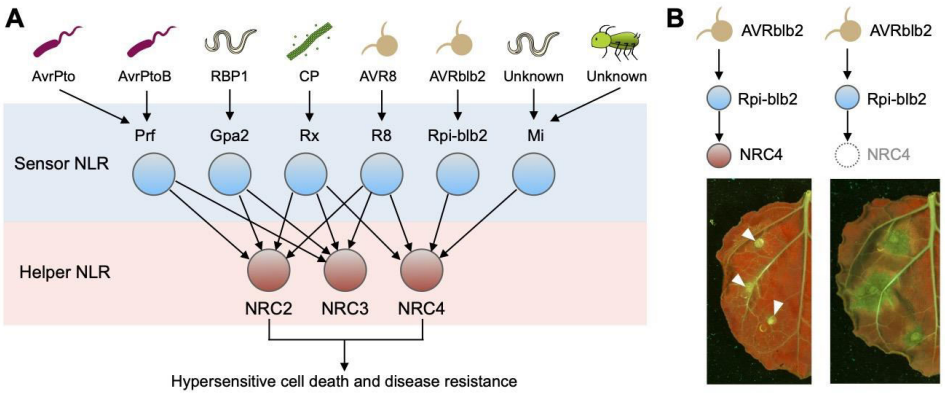

Figure 1. The NRC network mediates immunity to diverse plant pathogens.

(A) Sensor NLRs confer disease resistance to diverse pathogens including bacteria, oomycetes, virus, nematodes and aphids through helper NLRs. Helper NLRs specifically or redundantly function with multiple sensor NLRs. **(B)** The combination of the sensor Rpi-blb2 and the helper NRC4 induces a hypersensitive response upon infection with *Phytophthora infestans* carrying the effector AVRblb2. Arrowheads indicate the hypersensitive response in a *Nicotiana benthamiana* leaf, which suppresses the spread of the *P. infestans* infection. Right panel: NRC4 is essential for Rpi-blb2-mediated mitigation of pathogen infection.

The NRC network

The NRC network provides a model for plant NLR networks. The cloning and initial characterization of NRC network components spanned many years of classical genetics work on disease resistance. In this network, sensor NLRs mediate resistance against diverse pathogens and pests. The sensors illustrated in Figure 1A are mostly encoded by *Resistance* (*R*) genes. For example, *R* gene *Rpi-blb2* from the wild potato species (*Solanum bulbocastanum*) confers specific resistance to the oomycete pathogen *Phytophthora infestans* carrying the effector (avirulence gene) *AVRblb2* [39]. Other well-characterized *R* genes include *Prf* from a wild relative of cultivated tomato (*Solanum pimpinellifolium*) against the bacterial pathogen *Pseudomonas syringae* and potato (*S. tuberosum* ssp. *andigena*) *Rx* against *Potato virus X* [40-42].

To activate defense responses, individual R protein sensors within the NRC network require one or more NRC proteins, which are themselves also typical NLRs [34]. In the simplified model illustrated in Figure 1A, NRC2, NRC3 and NRC4 are helper NLRs for many sensor NLRs with different specificity and redundancy. For example, Rpi-blb2 specifically activates immunity through NRC4, but not NRC2 and NRC3, while all three helper NLRs redundantly contribute to Rx-mediated immunity. The complex redundancy between helper NLRs may help explain why their identification took much longer than that of the more specific sensor NLRs. Indeed, it would have been challenging to unravel

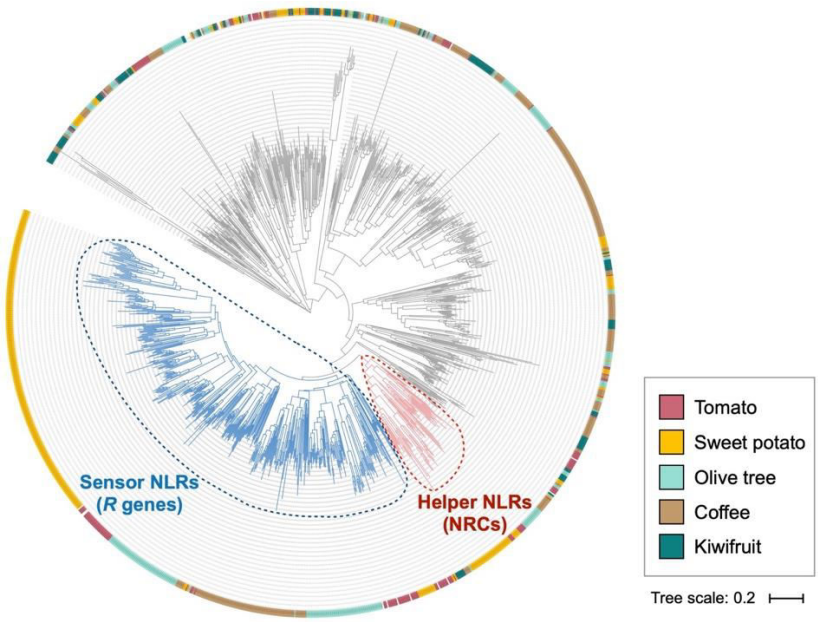

Figure 2. The NRC network in asterid plant species.

Phylogenetic tree showing the relationship between NLR proteins identified from tomato, sweet potato, olive tree, coffee and kiwifruit. The sensor and helper clades within the NRC superclade are highlighted in blue and red, respectively. The color codes in the outer circle denote the plant species.

the function of three redundant genes by classical genetics studies. Among the redundant NRCs, the exclusive association between NRC4 and Rpi-blb2 was instrumental in deciphering NLR networks. Wu et al. [34] identified the NRC network through genetic analyses of Rpi-blb2-mediated immunity that revealed NRC4 as a component downstream of Rpi-blb2 (Figure 1B). The overexpression of *Rpi-blb2* in *Nicotiana benthamiana* conferred resistance against *P. infestans* by inducing a hypersensitive response and silencing of the helper *NRC4* abolished resistance.

Identifying the connection between Rpi-blb2 and NRC4 enabled to use comparative genomics and evolutionary analyses to explore the NLR network. In a phylogenetic tree of all NLR proteins, the helper NRCs form a tight and well-supported sister clade next to an expanded clade that includes many sensor R proteins from different plant species (Figure 2). This network of related proteins is massively expanded in Solanaceae and several other asterids—in some species, as much as fifty percent of all NLRs belong to this superclade of NRCs and their R sensors [34]. The expansion of the NRC network occurred about 100 million years ago before most asterid species diverged. This NRC superclade likely evolved from an ancestral gene pair consisting of one sensor and one helper NLR gene before massive gene duplication and expansion. The sensor NLRs diversified to detect various types of pathogens such as bacteria, viruses, oomycetes, aphids, and nematodes, while helper NLRs underwent limited expansion and remained constrained by some redundant roles.

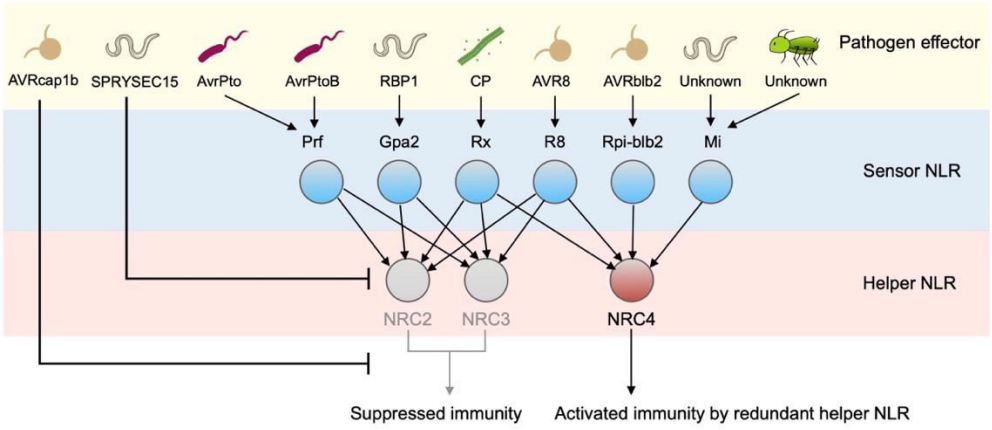

Figure 3. Redundancy in the Solanaceae NLR network avoids immune suppression by diverse pathogen effectors.

The *Phytophthora infestans* and *Globodera rostochiensis* effectors AVRcap1b and SPRYSEC15 target distinct signaling components of the NRC network. SPRYSEC15 directly binds NRC2 and NRC3 to suppress their function. AVRcap1b suppresses NRC2- and NRC3-mediated immune responses by associating with other host proteins. When the NRC2 and NRC3 pathways are suppressed by effectors, the helper NLR NRC4 can substitute as a redundant node for several sensor NLRs.

Redundant receptor networks in plant immunity ensure robustness

Signaling convergence in the NRC network makes helper NRCs obvious targets for pathogens, as the suppression of helper nodes will impair the immune responses initiated by many sensor NLRs. Indeed, a screen for effectors from various solanaceous pathogens identified five effectors that suppress the hypersensitive response induced by the NRC-dependent sensor NLRs, *Prf* and *Rpi-blb2* in *N. benthamiana*. Two of these effectors, SPRYSEC15 from the potato cyst nematode *Globodera rostochiensis*, and AVRcap1b from the oomycete *Phytophthora infestans*, blocked the cell death response mediated by NRC2 and NRC3, but did not affect NRC4 activity (Figure 3) [43]. Therefore, pathogen effectors exhibit some specificity in suppressing helper NLR nodes in the NRC network and redundancy in these core immune elements may help plants evade the suppression of their immune systems by pathogen effectors.

Pathogen effectors suppress the NRC-mediated immune response in different ways (Figure 3). For example, the cyst nematode effector SPRYSEC15 directly binds to NRC2 and NRC3 but does not show a strong affinity for NRC4 [43]. A set of protein-protein interaction analyses such as *in planta* co-immunoprecipitation, yeast two-hybrid assays, and *in vitro* gel filtration assays determined that the central NB-ARC domain of NRCs is a SPRYSEC15 target site. This direct association is thought to interfere with the function of the helper NLR. The *P. infestans* effector AVRcap1b indirectly

suppresses the NRC response by binding to another host protein [43]. Indeed, AVRcap1b interacted with a single host target in yeast-two-hybrid and co-immunoprecipitation assays: Target of Myb 1-like protein 9a (TOL9a), which is generally involved in endosome or vesicle trafficking. In agreement with this result, the suppression of NRC2- and NRC3-mediated immunity by AVRcap1b was compromised when *TOL9a* was silenced by RNA interference [43]. Although role of the TOL9a in NRC-dependent immunity remains unknown, TOL9a may act downstream of activated helper NLRs. Taken together, these observations show that pathogens have evolved to target the NRC network at multiple levels to circumvent plant immunity and enable their infection of the host.

The emerging model is that co-evolution between effectors and NRCs drove the emergence of multiple NRCs to evade suppression by pathogen effectors, while retaining helper function with different sensor NLRs. In such a model, the suppression of one helper node by a pathogen can be, at least partially, compensated for by another node in executing the immune responses. This model would offer one possible explanation for the observed network complexity of the plant NLR immune system.

Redundant receptor networks in plant immunity allow for evolvability

A second potential benefit of the NLR network system is evolvability—the ability to generate phenotypic variation. We hypothesize that a complex immune system such as that of plants composed of functionally specialized receptors can acquire variation in the encoding receptor genes against fast-evolving pathogen effectors. The function of a typical receptor has been uncoupled into two distinct NLR proteins in the NLR network: a sensor NLR for pathogen detection and a helper NLR to activate immune signaling. While helper NLRs must maintain their ability to mediate immune responses, sensor NLRs can be more flexible and prone to diversification such as accumulating new mutations or even gain an entirely new domain to detect effectors.

Indeed, the sheer number of sensor NLRs in the solanaceous NRC network is much greater than the limited number of helper NLRs (Figure 2). Based on phylogenomics analyses, helper NRCs are more highly conserved than sensor NLRs across the Solanaceae [34, 44]. In addition, unlike helper NRCs, about half of all sensor NLRs carry additional N-terminal extension domains prior to their CC domains [45, 46]. Some of these N-terminal extension contribute to the direct detection of pathogen effectors as baits, but they are not directly involved in activating immune responses [47, 48]. This integration of novel domains in sensor NLRs may be a consequence of their relaxed selective pressure, as they rely on their helper NLR partners to execute the immune responses. Overall, the network organization of the immune system has allowed plants to keep up with diverse pathogens that are continuously changing to evade the plant immune system.

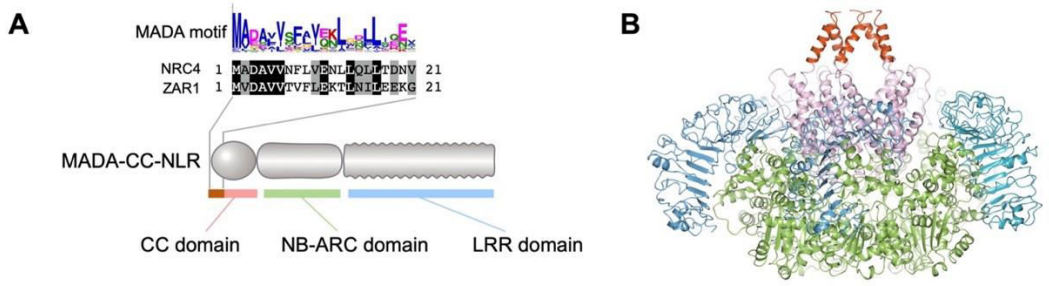

Figure 4. ZAR1 and NRC4 share the N-terminal MADA motif/α helix on the resistosome.

(A) Schematic diagram of a prototypical CC-NLR with the conserved MADA motif (shown above as a protein logo and alignment). The MADA motif is located in the N terminus of about 20% of CC-NLRs, including NRC4 and ZAR1, across flowering plant species. **(B)** A structure model of the resistosome highlighting the position of the MADA motif in orange. The MADA motif forms the funnel-like structure of the resistosome.

Evolution of NLRs and their networks

We have developed an evolutionary model of plant NLRs and their underlying networks. The sensor and helper NLRs presumably emerged through asymmetric evolution from a multifunctional ancestor receptor that possessed the ability to recognize effectors and the ability to trigger immune signaling. This sub-functionalization resulted in the current pairs of NLRs that participate in the immune system and have assembled into networks of specialized NLR proteins.

There is a key molecular signature suggesting the transition from multifunctional singletons into paired and networked NLR receptors. Using helper NRC4 as a template, a transposon mutagenesis screen that introduced stop codons at random positions along NRC4 revealed that the first 29 amino acids of NRC4 are sufficient to trigger a hypersensitive response [45]. Notably, the N terminus of helper NRCs shows high sequence similarity to the N-terminal α helix of ZAR1, which comes together to form the funnel in the resistosome and creates a pore at the plasma membrane (Figure 4). This region codes for a consensus sequence motif, designated the 'MADA motif', with the sequence signature (MADAxVSFxVxKLxxLLxxEx). The MADA motif is present in about one-fifth of all CC-type NLRs across flowering plant species [45]. Mutations in the MADA motif impair the cell death activity of ZAR1 and helper NRCs [14, 16, 45, 49]. In addition, the MADA motif of NRC4 can be functionally replaced by the N-terminal sequence of multiple MADA-type CC-NLRs from both dicots and monocots [45]. These findings suggest that the MADA sequence signature may have emerged early in the evolution of CC-NLRs and has been functionally conserved in a substantial fraction of CC-NLRs across distantly related plant species.

Notably, the MADA-type sequence is only detected in helper NLRs, but not in sensor NLRs among

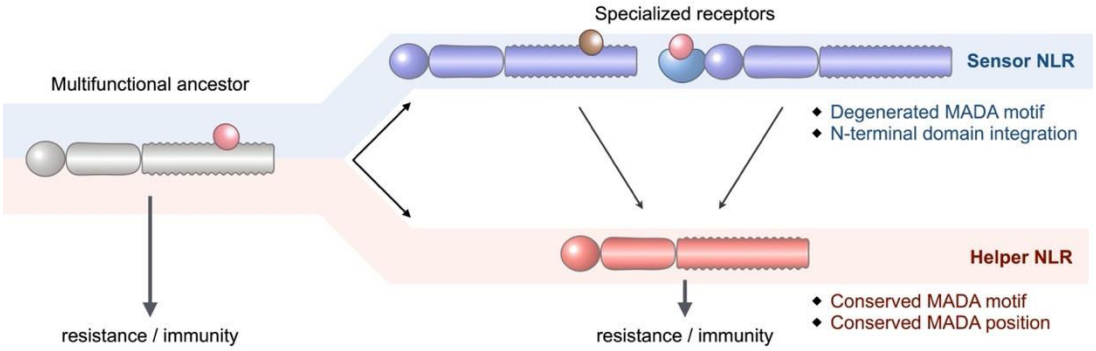

Figure 5. Evolution of CC-NLRs from a multifunctional receptor to networks.

The N-terminal MADA motif emerged early during CC-NLR evolution and remained constrained over time from the multifunctional ancestor into specialized helper NLRs in pairs and networks. By contrast, the MADA motif likely degenerated in sensor NLRs that rely on helper NLRs to execute the immune responses. Several sensor NLRs have acquired an additional N-terminal extension domain for effector recognition.

the NRC network [45]. As mentioned above, about half of all sensors have an N-terminal insertion prior to the CC domain. Given that many of these N-terminal insertions are quite large (spanning several hundred amino acids), the model offered by the ZAR1 resistosome cannot be applied for these sensor NLRs. Thus, we think that sensor NLRs have diversified by losing their MADA motifs, and often dedicating their N termini for effector recognition (Figure 5). This diversification of sensor NLRs occured because the sensors rely on the helpers for executing the immune response.

We propose that the evolutionary model of the NLR network follows a “use it or lose it” principle (Figure 5). As sensor NLRs have relegated the signaling function to their helper NLRs, the MADA sequence in sensor NLRs has degenerated over time to become nonfunctional, reflecting their functional specialization toward pure sensors. However, the helpers retained the MADA sequence over long evolutionary times. In addition, the MADA sequence has retained its conserved position at the N terminus, which may be critical to function when using the resistosome as a model. This separation of labor between sensor and helper NLRs presumably allows sensors to diversify through co-evolution with pathogen effectors by acquiring new domains and mutations. The distinct evolutionary paths that have led to the functional specialization into either sensing or helper activity make this network a robust and evolvable immune system.

Summary Points

- Plants have complex immune receptor networks where functionally specialized sensor and helper NLRs function together against diverse plant pathogens.
- Redundancy in the NLR receptor network can allow the immune system to be more resilient in terms of dealing with perturbations from the environment.
- Asymmetric evolution from a multifunctional ancestor to functionally specialized NLRs enables rapid evolution and diversification of NLR immune system.

Author contributions

Conceptualization: H.A., S.K.; Figure preparation: H.A.; Writing initial draft: H.A., S.K.; Editing: H.A., S.K.

Declaration of interests

S.K. receive funding from industry and has filed patents on NLR biology.

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