

# NLR singletons, pairs, and networks: evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of plants

Hiroaki Adachi, Lida Derevnina and Sophien Kamoun



NLRs are modular plant and animal proteins that are intracellular sensors of pathogen-associated molecules. Upon pathogen perception, NLRs trigger a potent broad-spectrum immune reaction known as the hypersensitive response. An emerging paradigm is that plant NLR immune receptors form networks with varying degrees of complexity. NLRs may have evolved from multifunctional singleton receptors, which combine pathogen detection (sensor activity) and immune signalling (helper or executor activity) into a single protein, to functionally specialized interconnected receptor pairs and networks. In this article, we highlight some of the recent advances in plant NLR biology by discussing models of NLR evolution, NLR complex formation, and how NLR (mis)regulation modulates immunity and autoimmunity. Multidisciplinary approaches are required to dissect the evolution, assembly, and regulation of the immune receptor circuitry of plants. With the new conceptual framework provided by the elucidation of the structure and activation mechanism of a plant NLR resistosome, this field is entering an exciting era of research.

## Address

The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Norwich, NR4 7UH, UK

Corresponding author: Kamoun, Sophien ([sophien.kamoun@tsl.ac.uk](mailto:sophien.kamoun@tsl.ac.uk))

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## Introduction

Plants have a multi-layered immune system activated by receptor proteins that detect pathogen molecules and trigger an immune response. Nucleotide-binding domain and leucine-rich repeat (NLR) proteins make up one of the largest and widespread family of immune receptors. NLRs function inside plant cells by monitoring the activity of pathogen secreted molecules known as ‘effectors’, which have evolved to facilitate pathogen

spread and infection by modulating host processes. The recognition of pathogen effectors by host NLRs leads to NLR-triggered immunity (also known as effector-triggered immunity), a robust immune response that is often accompanied by localized cell death in the host known as the hypersensitive response (HR) [1,2]. In addition to plants, NLR-mediated immunity is an important component of the immune system of animals, and possibly fungi [3]. Unlike plants, animal NLRs tend to respond to pathogen molecular patterns rather than the rapidly evolving effectors [3,4]. Whether plant and animal NLRs share a common evolutionary origin has been under debate. Urbach and Ausubel argued that the occurrence of NLRs across diverse eukaryotes is a result of convergent evolution [5]. However, the recent elucidation by cryo-electron microscopy (cryo-EM) of the structure of a plant NLR oligomer revealed a wheel-like pentameric resistosome with remarkable similarity to metazoan inflammasomes and apoptosomes [6\*\*], indicating that these divergent NLRs probably evolved from a common ancestor.

NLRs show a multi-domain structure and have a conserved nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain. In addition to the NB and LRR domains, most NLRs have characteristic N-terminal domains that are likely involved in immune activation [7]. Considering N-terminal domain features, plant NLRs can be broadly categorized into three subgroups, toll and interleukin-1 receptor (TIR)-type NLR (TIR-NLR), coiled-coil (CC)-type NLR (CC-NLR), and RPW8-like CC-type NLR (RPW8-NLR) [8]. Recognition of effectors by plant NLRs follows multiple mechanistic models; ‘Direct’, ‘Guard’, ‘Decoy’, and ‘Integrated-decoy’ (reviewed by Refs. [9] and [10]). The ‘Integrated-decoy’ model was revealed from functional analyses of unusual domains in several NLRs. These NLRs are referred to as ‘NLR-ID’ based on their additional accessory domains called ‘integrated domains (IDs)’ [11]. The IDs have a pivotal role in effector recognition and are proposed to have evolved by integration of effector host targets into the classical NLR domain architecture.

Recent progress in understanding plant immunity revealed that immune receptors, including NLRs, can form networks of varying complexity that translate pathogen detection into activation of hypersensitive cell death and immunity [12,13]. However, despite notable advances in NLR biology, including the recent

elucidation of the structure and activation mechanism of the resistosome [6\*\*,14\*\*], how NLRs and their networks are regulated at multiple levels remains largely unknown. In this review, we highlight some of the recent advances in plant NLR biology by discussing models of NLR evolution and functional specialization, NLR complex formation, and how NLR regulation modulates immunity and autoimmunity.

### NLR evolution: from singleton to pairs to network

The conceptual basis of host–pathogen interactions was first described by Harold Henry Flor in his gene-for-gene model. Flor's model postulated that inheritance of resistance and parasitism is determined by matching single genes in plants and pathogens [15]. Fifty years after Flor proposed his gene for gene model, the first *R*-gene in plants were cloned [10]. To date dozens of *R* genes have been cloned and remarkably the majority of these *R* genes encode NLR proteins. True to Flor's model, some NLRs indeed function as a single genetic unit for sensing and signalling, referred to here as 'singleton NLR' (Figure 1a, Table 1). These NLRs sense effectors either directly or indirectly and trigger the so-called hypersensitive immune response in the host. MILDEW LOCUS A (MLA) protein family, found broadly across different barley accessions, is a well-studied NLR locus required for isolate-specific resistance to powdery mildew fungi [16]. MLA directly recognizes matching avirulence effector, AVRa, and triggers cell death in the heterologous *Nicotiana benthamiana* system, suggesting that it probably behaves both as a sensor and as a signal inducer [17]. Other likely singleton NLRs that could sense effectors and independently trigger hypersensitive cell death in heterologous plant systems include Sr50, a rye ortholog of MLA, L6, a flax NLR, and some Arabidopsis NLRs such as RESISTANCE TO PERONOSPORA PARASITICA 13 (RPP13), RESISTANCE TO PSEUDOMONAS SYRINGAE5 (RPS5) and HOPZ-ACTIVATED RESISTANCE1 (ZAR1) [18–24].

Recent findings revealed that the functional principles of NLR-mediated immunity are more complex than previously anticipated with many NLRs requiring other NLR proteins to function [25,26] (Figure 1a, Table 2). Some NLRs work in pairs, in which a sensor NLR, specialized to recognize the pathogen, is coupled with a helper (also known as executor) NLR that is involved in initiating immune signalling [27]. In Arabidopsis and rice, genetically linked NLR pairs, 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 together. In these pairs, RRS1, RGA5, and Pik-1 are typical NLR-IDs, possessing additional domains for effector recognition, WRKY domain in RRS1, and

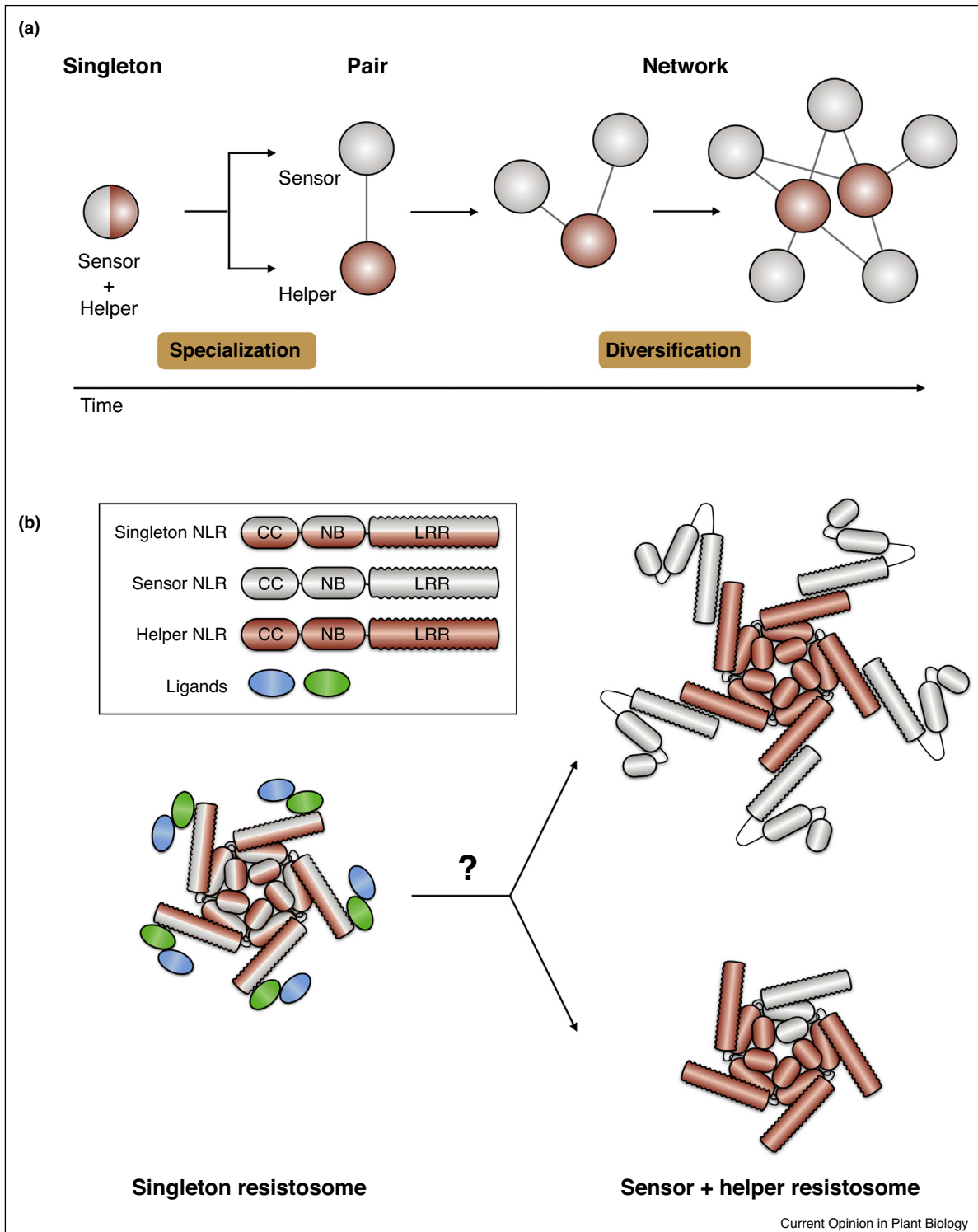
HEAVY METAL-ASSOCIATED (HMA) domain in both RGA5 and Pik-1 and require RPS4, RGA4, and Pik-2, respectively, to trigger immune signalling [28–34].

We propose that functional specialization has been a critical event in NLR evolution that enhances NLR evolvability, that is their capacity to keep up with rapidly evolving pathogens without being constrained by signalling activity (Figure 1a). This view is supported by the emerging paradigm that many NLRs are interconnected not only in one-to-one relationship, but also through a complex network architecture [12]. Recently, a major clade of NLRs in Solanaceae plant species was shown to form an intricate immunoreceptor network. In this network, multiple helper NLRs, known as NLR-REQUIRED FOR CELL DEATH (NRC), are required by a large number of sensor NLRs that mediate resistance against diverse pathogens, such as viruses, bacteria, oomycetes, nematodes, and insects [35\*\*]. Unlike typical paired NLRs, NRCs are not always clustered with sensor NLRs in plant genomes even though they are evolutionarily related [35\*\*]. The current evolutionary model for the NRC network is that it has evolved from a few genetically linked NLRs, as in the Caryophyllales, through massive expansion of sensors and a relatively limited expansion of helpers, possibly to maintain robustness of the network against rapidly evolving pathogens. As shown in Table 2, other NLR networks defined by the ubiquitous helper NLRs ADR1 and NRG1 have also been reported across several plant families.

### NLR protein complexes: assembling the immunoreceptor circuitry

How do plant NLRs activate immunity after effector recognition? A common model is that plant NLRs, maintained in an inactive form through intramolecular associations, self-associate through their N-terminal domains to trigger cell death and initiate immune signalling (Table 1) [36,37]. The TIR domain of a flax TIR-NLR L6, and Arabidopsis TIR-NLRs RPS4 and SUPPRESSOR OF *npr1-1* CONSTITUTIVE 1 (SNC1) self-associate and is sufficient for HR cell death induction *in planta* [38,39]. Site-directed mutagenesis of the self-association interfaces disrupted signalling activity of the TIR domains and full-length TIR-NLRs [39]. The CC domain of CC-NLRs is also thought to mediate self-association to activate immunity. The CC domains of MLA10 and the wheat and rye orthologs, Sr33 and Sr50, can self-associate independently and trigger cell death *in planta*. Mutations in these CC domains abolished their self-association and induction of HR activity [40–42]. However, the greatest insight into CC domain function emerged from two recent ground-breaking studies on the structure and function of the ZAR1 CC-NLR [6\*\*,14\*\*]. Activation by the *Xanthomonas campestris* effector AvrAC triggers a series of molecular events through which an inactive monomer of ZAR1 forms a pentameric wheel-like

Figure 1



Evolution of NLR networks.

**(a)** NLR functions can be separated into pathogen detection termed ‘sensor function’ and signalling or ‘helper (executor) function’. In this model, singleton NLRs have both activities ‘sensor + helper’. We propose that throughout evolution, some singleton NLRs have duplicated and functionally specialized into ‘sensors’ and ‘helpers’ forming connections that range from pairs to complex networks. **(b)** Possible resistosome configurations of sensor/helper NLR hetero-complexes. One hypothesis is that the sensor NLRs engage into the resistosome as functional equivalents of RLCKs in the ZAR1 resistosome. Another possibility is that sensor NLRs are recruited as one of the wheel spokes in a hetero-oligomeric resistosome comprises sensors and helper NLRs, as reported for the mammalian NAIP/NLRC4 inflammasome.

**Table 1****Examples of potential singleton NLRs**

Name	NLR type	Host organism	HR in heterologous plant	Effector	Host target	Self-association	References
MLA10	CC-NLR	Barley	Yes	AVRa10		Yes	[17,40]
Sr50	CC-NLR	Rye	Yes	AvrSr50		Yes	[18,41,42]
RPP13	CC-NLR	Arabidopsis	Yes	ATR13		NR	[21]
RPS5	CC-NLR	Arabidopsis	Yes	AvrPphB	PBS1	Yes	[22,23]
ZAR1				HopZ1	ZED1		
	CC-NLR	Arabidopsis	Yes	AvrAC	RKS1/PBL2	Yes	[6**,14**,24,83–85]
				HopF2a	ZRK3		
L6	TIR-NLR	Flax	Yes	AvrL567		Yes	[19,20,38]

**Table 2****Examples of NLR pairs and networks**

Sensor	NLR type	Host organism	Helper	NLR type	Host organism	Biochemical connection	References
RGA5	CC-NLR	Rice	RGA4	CC-NLR	Rice	Yes	[32]
Pik-1	CC-NLR	Rice	Pik-2	CC-NLR	Rice	NR	[33]
PigmS	CC-NLR	Rice	PigmR	CC-NLR	Rice	Yes	[70*]
RRS1	TIR-NLR	Arabidopsis	RPS4	TIR-NLR	Arabidopsis	Yes	[39,48,86]
CHS1	TIR-NLR	Arabidopsis	SOC3	TIR-NLR	Arabidopsis	Yes	[87]
CHS3	TIR-NLR	Arabidopsis	CSA1	TIR-NLR	Arabidopsis	NR	[88]
Rx2	CC-NLR	Solanaceae spp.	ADR1	RPW8-NLR	Solanaceae spp.	NR	[50]
RPS2	CC-NLR		ADR1	RPW8-NLR			
RPP2	TIR-NLR		ADR1-L1	RPW8-NLR			
RPP4	TIR-NLR		ADR1-L2	RPW8-NLR			
SNC1	TIR-NLR	Arabidopsis			Arabidopsis	NR	[49**,51,52**]
CHS1 (/SOC3)	TIR-NLR						
RRS1 (/RPS4)	TIR-NLR						
Rx2	CC-NLR						
N	TIR-NLR	Solanaceae spp.	NRG1	RPW8-NLR	Solanaceae spp.	NR	[50,53*,54]
Roq1	TIR-NLR						
RPM1	CC-NLR						
RPS2	CC-NLR						
RPP1	TIR-NLR						
RPP2	TIR-NLR						
RPP4	TIR-NLR	Arabidopsis	NRG1	RPW8-NLR	Arabidopsis	NR	[52**,54,55**]
WRR4	TIR-NLR						
CHS1 (/SOC3)	TIR-NLR						
CHS3 (/CSA1)	TIR-NLR						
RRS1 (/RPS4)	TIR-NLR						
Bs2	CC-NLR		NRC2	CC-NLR			
Prf	CC-NLR		NRC3	CC-NLR			
R8	CC-NLR	Solanaceae spp.			Solanaceae spp.	NR	[35**]
Rx	CC-NLR						
Sw5b	CC-NLR						
Bs2	CC-NLR						
CNL_11990	CC-NLR						
Mi-1.2	CC-NLR						
R1	CC-NLR						
R8	CC-NLR	Solanaceae spp.	NRC4	CC-NLR	Solanaceae spp.	NR	[35**]
Rpi-blb2	CC-NLR						
Rx	CC-NLR						
Sw5b	CC-NLR						

complex termed a ‘ZAR1 resistosome’ (Figure 1b) [6\*\*,14\*\*]. Remarkably, a stunning conformational switch—the death switch—releases the very terminal  $\alpha 1$  helices of the CC domain, from being buried in the inactive ZAR1 to projecting out of the ZAR1 resistosome plane, to form a funnel-like structure. Structure-function studies revealed that the  $\alpha 1$  funnel is required for cell

death activity possibly by inserting into the plasma membrane and forming a toxic pore [6\*\*]. The structure of the activated ZAR1 resistosome takes us a leap forward in our understanding of CC-NLR activation with an emerging model of the CC domain directly exerting HR cell death through membrane perturbations without relying on downstream signalling pathway.

Not all CC domains appear to function as signalling domains. For instance, the CC domain of Arabidopsis CC-NLR RESISTANCE TO PSEUDOMONAS MACULICOLA 1 (RPM1) self-associates but does not trigger an immune response *in planta* [43]. Also, the CC domain of potato Rx does not self-associate and fails to trigger HR [44,45]. We propose three possibilities to explain these inconsistencies. First, key residues or regions may be missing in the CC domains tested in self-association or functional assays, because different studies have used different domain boundaries and one amino acid difference of CC domain length can affect cell death inducing activity [40–42,46\*\*]. Second, other NLR domains (NB or LRR) could be involved in signalling along with the CC domain by supporting the overall protein complex integrity or by mediating subcellular localization. A recent finding demonstrated that a functional p-loop in the RPM1 NB domain is important for binding to the interactor RPM1-INTERACTING PROTEIN 4 (RIN4) and for proper subcellular localization [43]. Third, some CC domains may be non-functional relying on the CC domains of their helper NLRs for function [6\*\*,47]. Thus, expression of CC domain truncations may be inadequately paired or may even lack their partners in heterologous expression systems. Therefore, going forward it is important to consider NLR activities in the context of intra-molecular and inter-molecular connections between different NLR domains and appreciate that different CC domains may have distinct activities and functions.

Biochemical studies have revealed that several paired NLRs physically interact with each other for optimal function (Table 2). Arabidopsis RRS1/RPS4 and rice RGA5/RGA4 form homo-complexes and hetero-complexes through their N-terminal domains [32,48]. The interaction interface of the TIR domains of RRS1 and RPS4 is required for the immunoreceptor complex to respond to the pathogen effectors, AvrRps4 and PopP2 [48]. Recent large-scale interactome analyses revealed that N-terminal CC domains of Arabidopsis CC-NLR and RPW8-NLR (defined as ‘extended CC domains’) are capable of forming highly redundant interaction networks suggestive of a high frequency of heteromeric interactions [46\*\*]. This biochemical interaction network is consistent with the emerging view that NLRs can form complex receptor network [12]. In Arabidopsis, the ACTIVATED DISEASE RESISTANCE 1 (ADR1) family (ADR1, ADR1-L1, ADR1-L2), members of the RPW8-NLR clade, are required by CC-NLRs (RPS2 and Rx2) and TIR-NLRs [RESISTANCE TO PERONOSPORA PARASITICA 2 (RPP2), RPP4, SNC1, RRS1, and CHILLING SENSITIVE 1 (CHS1)/SUPPRESSORS OF *chs1-2* 3 (SOC3)] for immune response [49\*\*,50,51,52\*\*]. Another RPW8-NLR, known as N REQUIREMENT GENE 1 (NRG1), is necessary for full function of TIR-NLRs

[N, RECOGNITION OF XOPQ 1 (Roq1), WHITE RUST RESISTANCE 4A (WRR4A), WRR4B, RPP1, RPP2, RPP4, RRS1/RPS4, RRS1B/RPS4B, CHS1/SOC3, and CHS3/CONSTITUTIVE SHADE-AVOIDANCE 1 (CSA1)] and CC-NLRs (Rx2, RPS2, and RPM1) [50,52\*\*,53\*,54,55\*\*]. One hypothesis is that RPW8-NLR family members may have evolved as helper NLRs that co-operate with diverse sensor NLRs, including not only CC-NLRs but also TIR-NLRs. In the future, it would be interesting to match the genetic evidence for NLR networks with biochemical models and determine how NLR hetero-complexes are configured especially in light of the ZAR1 resistance structural model (Figure 1b).

### NLR (mis)regulation: modulating immunity and autoimmunity

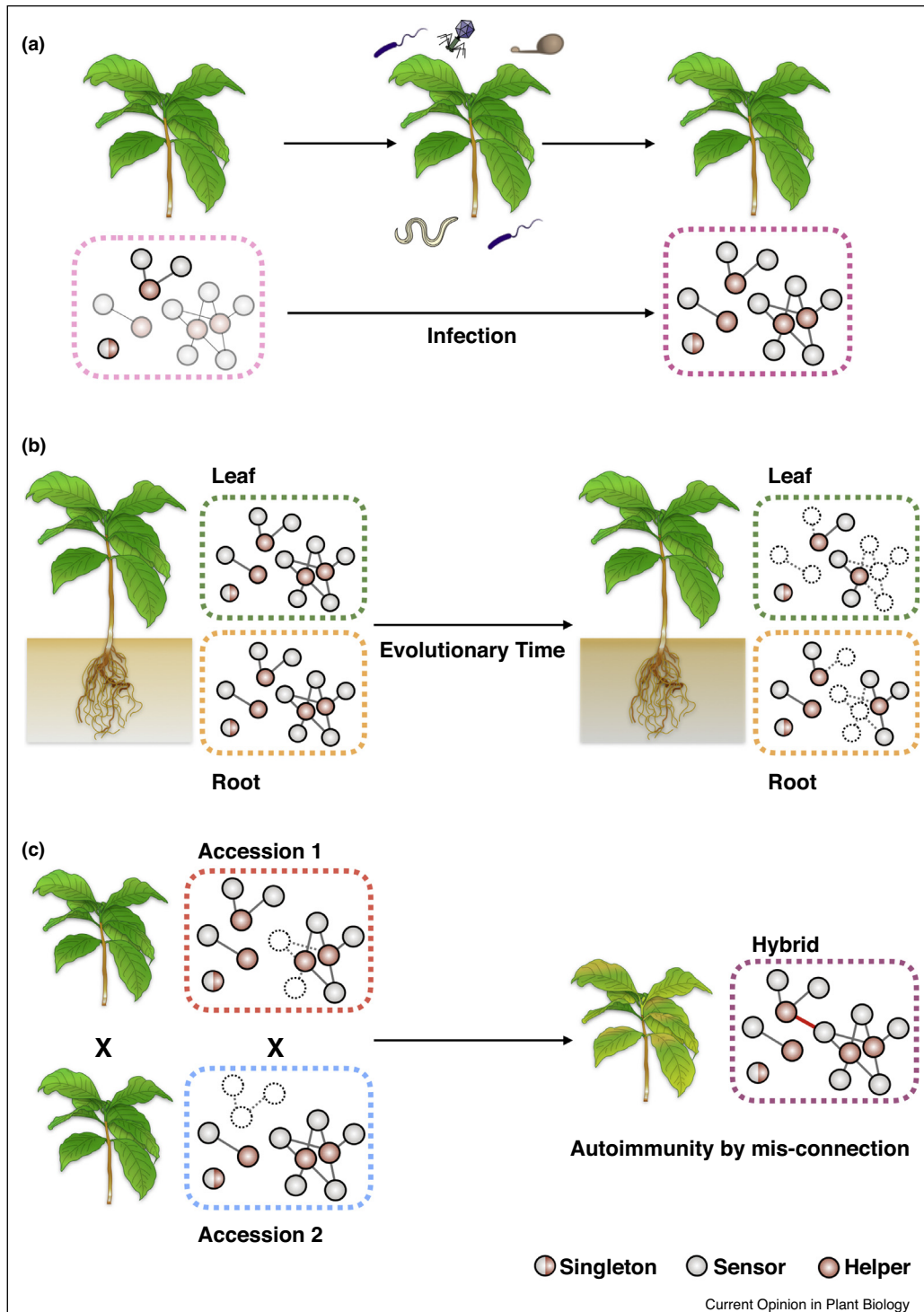
NLR networks are regulated at different levels in plants. Plant NLRs are known to undergo trade-offs between disease resistance and growth and abiotic stress [56,57]. Mis-regulated NLRs trigger physiological phenotypes in plants including growth suppression known as the auto-immune phenotype [58–60]. In their resting state, NLRs are thought to be kept under check through chaperone complexes. For example, SKP1-CULLIN1-F-box (SCF) and other chaperone complexes control NLR protein levels and conformation in the absence of pathogen activation [61,62].

Plant NLRs can also be tightly regulated at the transcriptional level [63]. Expression levels of some *NLR* genes are upregulated in response to flg22, a bacterial microbe-associated molecular pattern (MAMP), or pathogen-related treatment [64,65], suggesting that basal expression level of NLRs is kept low but is amplified in the cells attacked by plant pathogens (Figure 2a). MicroRNA-mediated gene silencing has been implicated in dynamic regulation of NLR transcripts [66]. Post-transcriptional NLR regulation by microRNAs such as miR482 superfamily is conserved in several plant species [67,68\*]. The miR482 level decreases in response to virus and bacterial pathogen infection, resulting in increased transcript levels of targeted NLRs [67]. Thus, the microRNA-mediated silencing machinery may contribute to suppress excessive accumulation of NLR transcripts and minimize fitness cost to the plant.

Interestingly, recent meta-analyses of *NLR* gene expression profiles revealed that plant species have distinct tissue-specific expression patterns of *NLR* genes [69\*]. Transcriptome analyses across nine plant species revealed that monocot and Fabaceae plants preferentially express *NLR* genes in roots, but Brassicaceae plants, including Arabidopsis, show relatively high NLR expression in shoots [69\*]. These findings are consistent with the view that plants may have evolved tissue-specific NLR networks in a species-dependent manner, possibly to



Figure 2



Examples of NLR network regulation and mis-regulation.

**(a)** Dynamic expression of *NLR* genes during pathogen infection results in a different network architecture throughout the host–pathogen interaction. **(b)** Tissue-specific *NLR* networks may have evolved to adapt to tissue-specific pathogen infections (e.g. nematodes in roots) and reduce the risk of autoimmunity. **(c)** Genetic crosses between distinct plant genotypes often cause incorrect connections between nodes in the *NLR* network, resulting in *NLR* mis-regulation and autoimmunity.

match the distinct pathogen attacks of different organs and tissues (Figure 2b). One model of tissue-specific NLR expression is epigenetic regulation of *NLR* promoters. A recent study focusing on the rice NLR *Pigm* cluster indicated that methylation levels of a transposable element in the *Pigm* promoter matched tissue-specific expression in pollen and leaves [70]. However, the regulatory mechanisms of *NLR* gene expression and their functional relevance to disease resistance remain largely unknown.

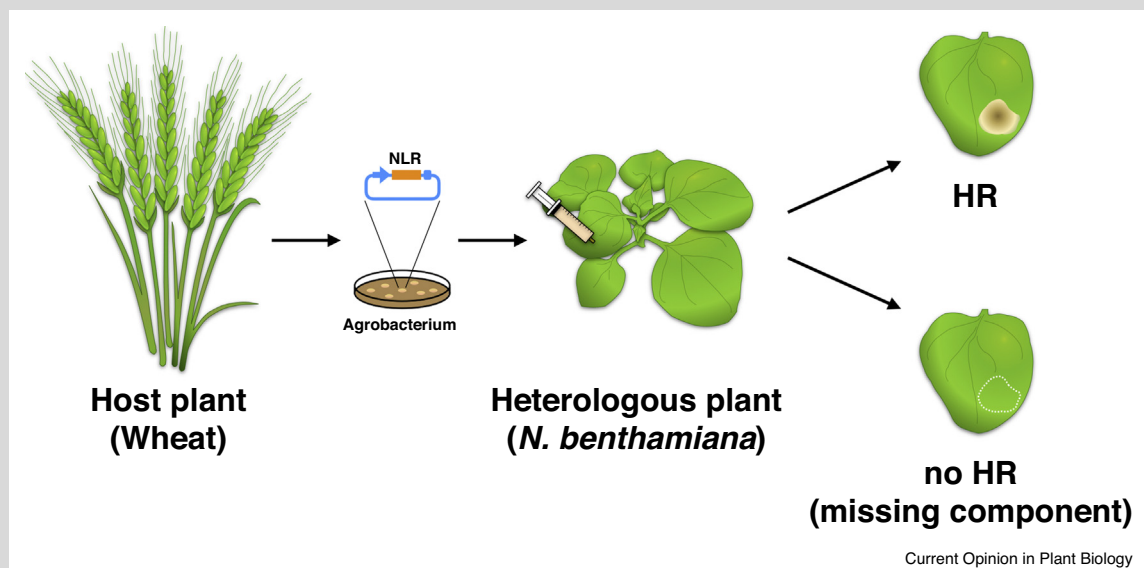
Intra-molecular and inter-molecular interactions form another layer of regulation of plant NLR activity. A widely cited model of intramolecular regulation is that the NB and LRR domains bind each other to suppress NLR activity in the absence of the pathogen [23,71,72]. Mutations in the NB or LRR domains of plant NLRs often confer auto-activity [73,74]. Consistently, the cryo-EM structure of ZAR1–RKS1 revealed that the LRR domain maintains ZAR1 in an inactive monomeric and ADP-bound state [14]. Intermolecular regulation

between plant NLRs are known for NLR pairs. In some paired NLRs, such as RRS1/RPS4, RGA5/RGA4 and *PigmS/PigmR*, ectopic expression of helper NLRs results in auto-activity, which is suppressed by co-expressing the matching sensor NLRs [32,70,75]. In contrast, in the *Pik-1/Pik-2* pair, helper NLR *Pik-2* does not show autoimmunity in an ectopic expression system, and both NLRs are required to trigger an immune response in response to the matching effector [33,76]. Therefore, interconnections between sensor and helper NLRs are presumably regulated through different mechanisms depending on the paired genes. In line with this model, autoimmune responses are often observed at the F1 generation when wild *Arabidopsis* accessions are crossed—a phenomenon known as ‘hybrid necrosis’ [59]. QTL mapping revealed that hybrid necrosis can be correlated with genetically unlinked *NLR* gene loci, for instance, between DANGEROUS MIX 1 (DM1) from *Arabidopsis* accession Uk-3 and DM2 from Uk-1 [59,77,78]. Interestingly, the auto-immune phenotype was triggered by hetero-complex of NLR proteins from the unlinked DM1 (DM1<sup>Uk-3</sup>)

#### Box 1 How to define singleton NLRs?

Given our relatively limited mechanistic understanding of NLR activities, it is not straightforward to determine whether a given NLR protein functions as a singleton or whether it requires other NLRs. NLRs with integrated domains (NLR-IDs) are thought to be exclusively involved in pathogen sensing and those characterized so far have turned out to be associated with a paired NLR helper (aka executor) [27]. Classic NLRs, such as Rx, Bs2, Mi, Rpi-blb2, R1, and others, were first cloned as *R* genes segregating for resistance against particular pathogens but turned out to require NLRs of the NRC helper class [35]. Typically, such NLRs cannot be transferred to taxonomically distinct plant families in the absence of their NLR mates [89]. This phenomenon quipped ‘restricted taxonomic functionality’ by Tai *et al.* [90] may not apply to singleton NLRs, which tend to confer hypersensitivity when expressed alone in heterologous plants (Table 1). In contrast, the monocot CC-NLRs MLA10 and Sr50 can trigger HR cell death when co-expressed with matching effectors in *Nicotiana benthamiana* and their CC domains confer HR cell death when transiently expressed in this heterologous plant [17,18,41,42]. Although this test is not definitive and may depend on the degree of conservation of any endogenous helper NLRs (e.g. NRC and NRG1), it remains a first step to sort out NLRs into separate functional categories and dissect the NLRome network architecture of a given plant species (Figure 1).

Figure 1



Work flow to validate NLR function for hypersensitive response (HR) cell death in heterologous plant system.

and DM2d (DM2<sup>Uk-1</sup>) alleles [79\*]. Thus, mis-regulated interconnections between genetically unlinked plant NLRs may rewire the immunoreceptor network resulting in auto-activation of the NLR complex (Figure 2c).

### Outlook: unravelling the genetic, biochemical and mechanistic links that define NLR immunoreceptor networks

In this review, we highlight some of the recent findings on NLR biology by covering advances in NLR evolution, biochemical functions and genetic regulation. The emerging view is that NLRs form receptor networks that include not only singletons and pairs but also more complex connections that vary in their specificity and redundancy. It is likely that classic NLRs, such as MLA10, RPS5, ZAR1 and L6, function as singletons that do not require other NLRs to sense pathogens and activate immunity (Table 1, Box 1). In the ZAR1 resistosome, ZAR1 clearly functions as a singleton as it forms wheel-like pentamer in 1:1 relationship with its RLCK ligands RKS1/PBL2<sup>UMP</sup> and may trigger cell death directly through the funnel-shaped  $\alpha$ 1 helices of the CC domain [6\*\*]. However, other NLRs can be paired or networked and probably form various hetero-complexes to sense and respond to invading pathogens and pests (Table 2). Here, we propose an evolutionary model, in which NLR singletons independently gave rise to functionally specialized NLRs that are connected in various ways (Figure 1a). Understanding the genetic, biochemical and mechanistic links between NLR network nodes should reveal a complex and dynamic architecture that would help to organize the hugely expanded NLRome of plants.

One pending question is the degree to which the meta-zoan inflammasome and apoptosome models apply to paired plant NLRs. In mammalian systems, NLR family CARD domain-containing 4 (NLRC4) forms a wheel-like hetero-oligomer complex with the sensors NLR family apoptosis inhibitory proteins (NAIPs), to detect diverse ligands and recruit and activate Caspase-1 protease [80–82]. Whether or not sensor and helper NLRs form hetero-oligomer complexes similar to NLRC4/NAIPs is currently unknown (Figure 1b). A deeper mechanistic understanding of NLR networks is required and the recent resolution of the structure of the ZAR1 resistosome by Wang *et al.* [6\*\*] should pave the way to address this question.

Many other important questions remain unanswered. What is the molecular basis of the evolutionary transition from singletons to NLR pairs and networks, and how does this impact NLR protein functions and activities? What are the regulatory mechanisms of plant NLRs, and how do they differ between singleton and paired NLRs? How do sensor and helper NLRs function together, and do they form dynamic higher level resistosome-type complexes? What are the determinants of sensor/helper

specificity in paired and network forming NLRs? Multi-disciplinary approaches are required to dissect the evolution, assembly and regulation of the immune receptor circuitry of plants. But there is a reason for optimism especially given the new conceptual framework provided by the elucidation of the structure and activation mechanism of the ZAR1 resistosome.

### Conflict of interest statement

LD and SK have filed a patent application based on NLR networks. SK receives industry funding on NLR biology.

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