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

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NLRs are modular plant and animal proteins that are intracellular sensors of pathogen-associated molecules that trigger a potent broad-spectrum immune reaction known as the hypersensitive response. An emerging paradigm is that plant NLRs form immune signalling 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.

Introduction

Plants have a multi-layered immune system activated by receptor proteins that detect pathogen molecules and trigger immune signalling. 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. A subset of pathogen effectors, however, are detected by host NLRs leading to NLR-triggered immunity (also known as effector-triggered immunity), a robust immune response that is often accompanied by localised cell death in the host known as the hypersensitive response (HR) (Dodds and Rathjen, 2010; Win et al., 2012). Like plants, animals also possess an arsenal of NLRs that tend to respond to pathogen molecular patterns (Jones et al., 2016). The occurrence of NLRs across diverse eukaryotes indicates that NLR-mediated immunity has evolved as an important component of the immune system.

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 signalling (Bentham et al., 2017). Based on N-terminal domain features, plant NLRs can be broadly categorized into three subgroups, toll and interleukin-1 receptor (TIR)-type NLR (TNL), RPW8-like CC-type NLR (RNL) and coiled-coil (CC)-type NLR (CNL) (Shao et al., 2016). Recognition of effectors by plant NLRs follows multiple mechanistic models; “Direct”, “Guard”, “Decoy” and “Integrated-decoy” (reviewed by Cesari, 2018; Kourelis and van der Hoorn, 2018). 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)" (Baggs et al., 2017). 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 immune signalling (Wu et al., 2018). However, despite notable advances in understanding how plant NLRs detect pathogen effectors, other aspects of NLR biology, such as mechanisms of NLR activation and how these activities are regulated at multiple levels, remain 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 developed 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 (Flor, 1971). Fifty years after Flor proposed his gene for gene model, the first *R*-gene in plants were cloned (Kourelis and van der Hoorn, 2018). 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 1**, **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 (Maekawa et al., 2018). MLA recognizes matching avirulence effector, AVRa, in the heterologous Arabidopsis system, suggesting that it probably behaves both as a sensor and as a signal inducer (Lu et al., 2016). Other likely singleton NLRs that sense and trigger an immune response in heterologous plant systems include Sr50, a rye ortholog of MLA, L6, a flax NLR, RESISTANCE TO PSEUDOMONAS SYRINGAE5 (RPS5) and HOPZ-ACTIVATED RESISTANCE1 (ZAR1), Arabidopsis NLRs (Ade et al., 2007; Qi et al., 2012; Ravensdale et al., 2012; Bernoux et al., 2016; Baudin et al., 2017; Chen et al., 2017).

Recent findings revealed that the functional principles of NLR-mediated immunity are more complex than anticipated by the gene for gene model. Many NLRs require other NLR proteins to function (Gabriëls et al., 2007; Roberts et al., 2013) (**Figure 1**, **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 (Cesari et al., 2014). 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, they possess 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,

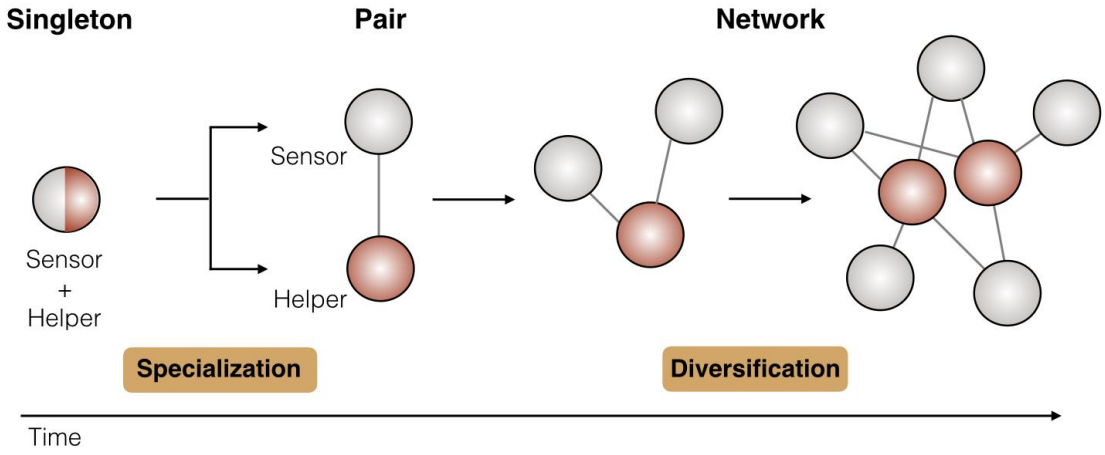


Figure 1. Evolution of NLR networks. NLR functions can be separated into sensing termed "sensor function" and signalling termed "helper function". In this model, singleton NLR has both functions "sensor + helper". We propose that throughout evolution, some singleton NLRs have duplicated and specialized into "sensors" and "helpers" forming connections that range from pairs to complex networks.

respectively, to trigger immune signalling (Narusaka et al., 2009; Cesari et al., 2013; Césari et al., 2014; Le Roux et al., 2015; Maqbool et al., 2015; Sarris et al., 2015; De la Concepcion et al., 2018).

We propose that functional specialization is a critical event in NLR evolution (**Figure 1**) and may enhance the NLR evolvability, i.e. its ability to keep up with rapidly evolving pathogens. 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 (Wu et al., 2018). 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 huge variety of sensor NLRs that mediate resistance against diverse pathogens, such as viruses, bacteria, oomycetes, nematodes and insects (Wu et al., 2017). Unlike typical paired NLRs, NRCs are not genetically linked to the sensor NLRs even though they are evolutionarily related (Wu et al., 2017). The current evolutionary model of 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 somewhat 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 initiate immune signalling after effector recognition? A common model is that plant NLRs self-associate for signal transduction through their N-terminal domains (**Table 1**) (Ve et al., 2015; Bentham et al., 2018). The TIR domain of a flax TNL L6, and Arabidopsis TNLs RPS4 and SUPPRESSOR OF *npr1-1* CONSTITUTIVE 1 (SNC1) self-associate and is sufficient for immune signalling *in planta* (Bernoux et al., 2011; Zhang et al., 2017). Site-directed

Table 1. Examples of potential singleton NLRs

Name	NLR type	Host organism	HR in heterologous plant	Effector	Host target	Self-association	References
MLA10	CNL	Barley	Yes	AVRa10		Yes	Ridout et al. 2006 Maekawa et al. 2011
Sr50	CNL	Rye	Yes	AvrSr50		Yes	Casey et al. 2016 Cesari et al. 2016 Chen et al. 2017
RPS5	CNL	Arabidopsis	Yes	AvrPphB	PBS1	Yes	Ade et al. 2007 Qi et al. 2012
ZAR1	CNL	Arabidopsis	Yes	HopZ1 AvrAC HopF2a	ZED1 RKS1/PBL2 ZRK3	Yes	Wang et al. 2015 Lewis et al. 2013 Baudin et al. 2017 Seto et al. 2017
L6	TNL	Flax	Yes	AvrL567		Yes	Ravensdale et al. 2012 Bernoux et al. 2011 Bernoux et al. 2016

mutagenesis of the self-association interfaces disrupted signalling activity of the TIR domains and full-length TNLs (Zhang et al., 2017). The CC domain of CNLs is also likely to mediate self-association to activate immunity. The CC domains of MLA10 and the wheat and rye orthologs, Sr33 and Sr50, can self-associate by themselves and trigger cell death *in planta*. Mutations in these CC domains abolished their self-association and signalling activity (Maekawa et al., 2011; Casey et al., 2016; Cesari et al., 2016). Arabidopsis ZAR1 CC domain can also initiate immune signalling probably through self-association (Baudin et al., 2017). However, some results are inconsistent with the general model of CC domain function as a signalling domain. For instance, the CC domain of Arabidopsis CNL RESISTANCE TO PSEUDOMONAS MACULICOLA 1 (RPM1) self-associates but does not trigger immune response *in planta* (El Kasmi et al., 2017). Also, the CC domain of potato Rx does not show self-association and signalling activity (Rairdan et al., 2008; Hao et al., 2013). However, given that Rx is dependent on the helper NLRs NRC2, NRC3 or NRC4 (Wu et al., 2017), its CC domain may have lost the capacity to signal by itself (see below).

We propose three possibilities to explain inconsistent cases in CC domain function. First, it can be difficult to confidently predict the CC domain given low sequence similarity and absence of consistent motifs (Bentham et al., 2018). Key residues or regions may therefore be missing in the CC domains tested in self-association or signalling studies, additionally, different studies have used different domain boundaries (Maekawa et al., 2011; Casey et al., 2016; Cesari et al., 2016). Second, other NLR domains (NB or LRR) can be involved in signalling along with the CC domain by supporting accessibility of signalling components or modulating 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 (El Kasmi et al., 2017). Third, non-functional CC domains may require the other NLRs for signalling, which can be inadequately paired or even absent in the heterologous expression system used in functional assays. Therefore, going forward it is important to consider NLR signalling in the context of intra- and inter-molecular connections between different NLR domains and appreciate that different CC domains may have distinct activities and functions.

Biochemical studies revealed that several paired NLRs physically interact with each other for their proper function (**Table 2**). Arabidopsis RRS1/RPS4 and rice RGA5/RGA4, form homo- and hetero-complex through their N-terminal domains (Césari et al., 2014; Williams et al., 2014).

Table 2. Examples of NLR pairs and networks

Sensor	NLR type	Host organism	Helper	NLR type	Host organism	Biochemical connection	References
RGA5	CNL	Rice	RGA4	CNL	Rice	Yes	Césari et al. 2014
Pik-1	CNL	Rice	Pik-2	CNL	Rice	NR	Maqbool et al. 2015
PigmS	CNL	Rice	PigmR	CNL	Rice	Yes	Deng et al. 2017
RRS1	TNL	Arabidopsis	RPS4	TNL	Arabidopsis	Yes	Williams et al. 2014 Narusaka et al. 2016 Zhang et al. 2017
CHS1	TNL	Arabidopsis	SOC3	TNL	Arabidopsis	Yes	Zhang et al. 2017
CHS3	TNL	Arabidopsis	CSA1	TNL	Arabidopsis	NR	Xu et al. 2015
Rx2	CNL	Solanaceae spp.	ADR1	RNL	Solanaceae spp.	NR	Collier et al. 2011
RPS2 RPP2 RPP4 SNC1 CHS1 (/SOC3) RRS1 (/RPS4)	CNL TNL TNL TNL TNL TNL	Arabidopsis	ADR1 ADR1-L1 ADR1-L2	RNL RNL RNL	Arabidopsis	NR	Bonardi et al. 2011 Dong et al. 2016 Wu et al. 2019
Rx2 N Roq1	CNL TNL TNL	Solanaceae spp.	NRG1	RNL	Solanaceae spp.	NR	Peart et al. 2005 Collier et al. 2011 Qi et al. 2018
RPM1 RPS2 RPP1 RPP2 RPP4 WRR4 CHS1 (/SOC3) CHS3 (/CSA1) RRS1 (/RPS4)	CNL CNL TNL TNL TNL TNL TNL TNL TNL	Arabidopsis	NRG1	RNL	Arabidopsis	NR	Qi et al. 2018 Castel et al. 2019 Wu et al. 2019
Bs2 Prf R8 Rx Sw5b	CNL CNL CNL CNL CNL	Solanaceae spp.	NRC2 NRC3	CNL CNL	Solanaceae spp.	NR	Wu et al. 2017
Bs2 CNL_11990 Mi-1.2 R1 R8 Rpi-blb2 Rx Sw5b	CNL CNL CNL CNL CNL CNL CNL CNL	Solanaceae spp.	NRC4	CNL	Solanaceae spp.	NR	Wu et al. 2017

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 (Williams et al., 2014). Recent large-scale interactome analyses revealed that N-terminal CC domains of Arabidopsis CNL and RNL (defined as “extended CC domains”) are capable of forming highly redundant interaction networks suggestive of a high frequency of heteromeric interactions (Wróblewski et al., 2018). This biochemical interaction network is consistent with the emerging view that NLRs can form complex receptor network (Wu et al., 2018). In Arabidopsis, the ACTIVATED DISEASE RESISTANCE 1 (ADR1) family (ADR1, ADR1-L1, ADR1-L2), members of the RNL clade, are required for immune signalling mediated by CNLs (RPS2 and Rx2) and TNLs [RESISTANCE TO PERONOSPORA PARASITICA 2 (RPP2), RPP4, SNC1, RRS1 and CHILLING SENSITIVE 1 (CHS1)/SUPPRESSORS OF *chs1-2* 3 (SOC3)] (Bonardi et al., 2011; Collier et al., 2011; Dong et al., 2016; Wu et al., 2019). Another RNL, known as N REQUIREMENT GENE 1 (NRG1), is necessary for full function of TNLs [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 CNLs (Rx2, RPS2 and RPM1) (Peart et al., 2005; Collier et al., 2011; Qi et al., 2018; Castel et al., 2019; Wu et al.,

2019). One theory is that RNL family members may have evolved as helper NLRs that co-operate with diverse sensor NLRs, including not only CNLs but also TNLs. In future, it would be fascinating to match the biochemical and genetic evidence for NLR networks and determine which types of NLR complexes lead to effective immune signalling.

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 (Karasov et al., 2017; Ariga et al., 2017). Mis-regulated NLRs trigger physiological phenotypes in plants including growth suppression known as the auto-immune phenotype (Shirano et al., 2002; Bomblies et al., 2007; Palma et al., 2010). 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 (Cheng et al., 2011; Kadota and Shirasu, 2012).

Plant NLRs can also be tightly regulated at the transcriptional level (Lai and Eulgem, 2018). Expression levels of some *NLR* genes are up-regulated in response to flg22, a bacterial microbe-associated molecular pattern (MAMP), or pathogen-related treatment (Mohr et al., 2010; Yu et al., 2013), suggesting that basal expression level of NLRs is kept low but is amplified in the cells attacked by plant pathogens (**Figure 2a**). Recently, small RNA-mediated gene silencing has been implicated in dynamic regulation of NLR transcripts (Park and Shin, 2015). Post-transcriptional NLR regulation initiated by microRNA superfamilies is conserved in several plant species (Shivaprasad et al., 2012; Seo et al., 2018). Plant microRNA levels decrease in response to virus and bacterial pathogen infection, resulting in increased transcript levels of targeted NLRs (Shivaprasad et al., 2012). Thus, the small RNA-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 (Munch et al., 2017). 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 (Munch et al., 2017). These findings are consistent with the view that plants may have evolved tissue-specific NLR networks in a species-dependent manner, possibly to 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 (Deng et al., 2017). However, the regulatory mechanisms of *NLR* gene expression and their functional relevance to disease resistance remain largely unknown.

Intra- 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 (Qi et al., 2012; Slootweg et al.,

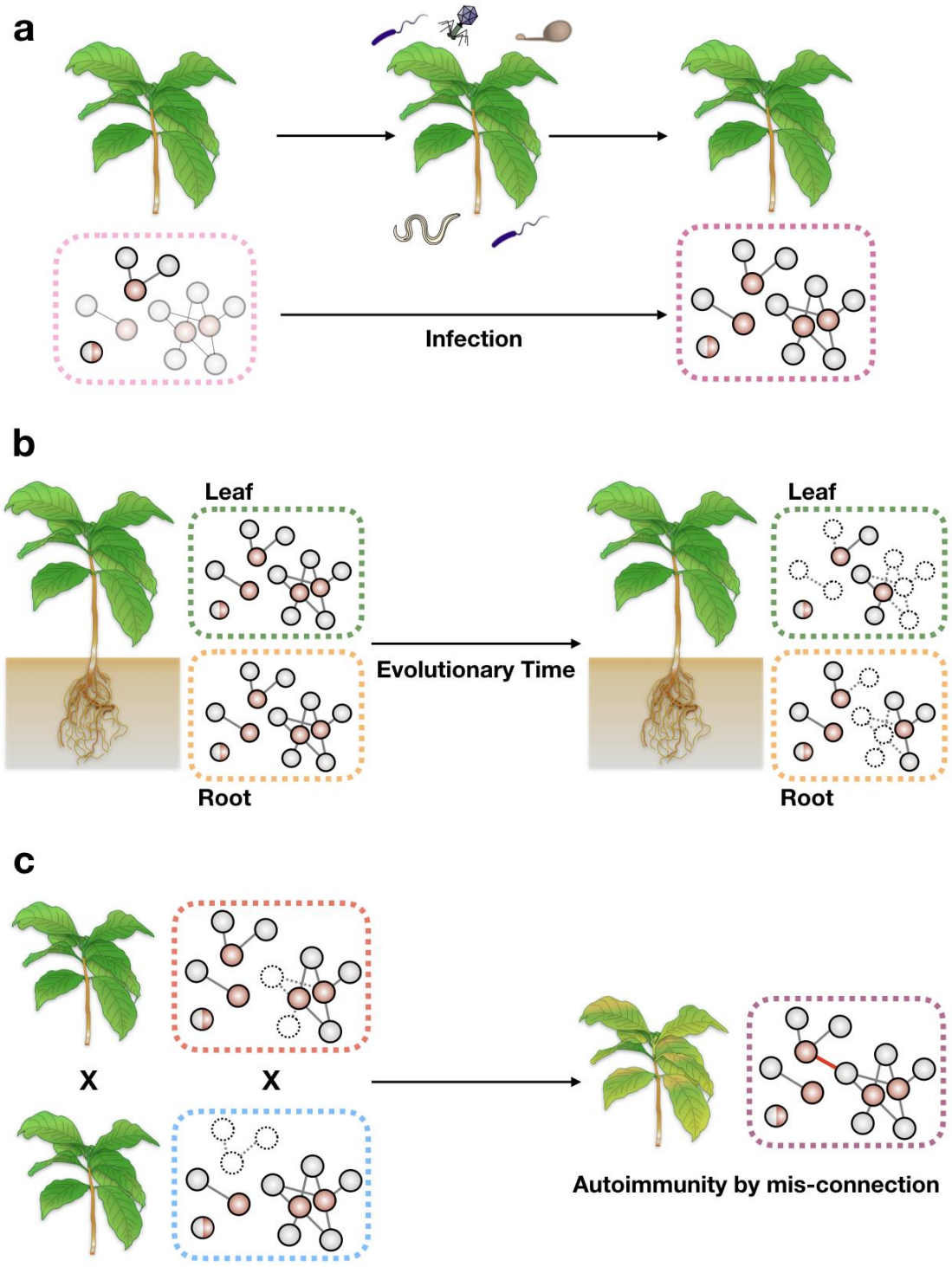


Figure 2. Examples of NLR network regulation and mis-regulation. (a) Dynamic expression of *NLR* genes during pathogen infection results in a different network architecture throughout the host-pathogen interaction. **(b)** Tissue-specific NLR networks may have evolved to adapt to tissue-specific pathogen infections (e.g. nematodes in roots) and reduce the risk of autoimmunity. **(c)** NLR mis-wiring triggers autoimmunity. Genetic crosses between distinct plant genotypes often results in autoimmunity possibly via incorrect connections between nodes in the NLR network.

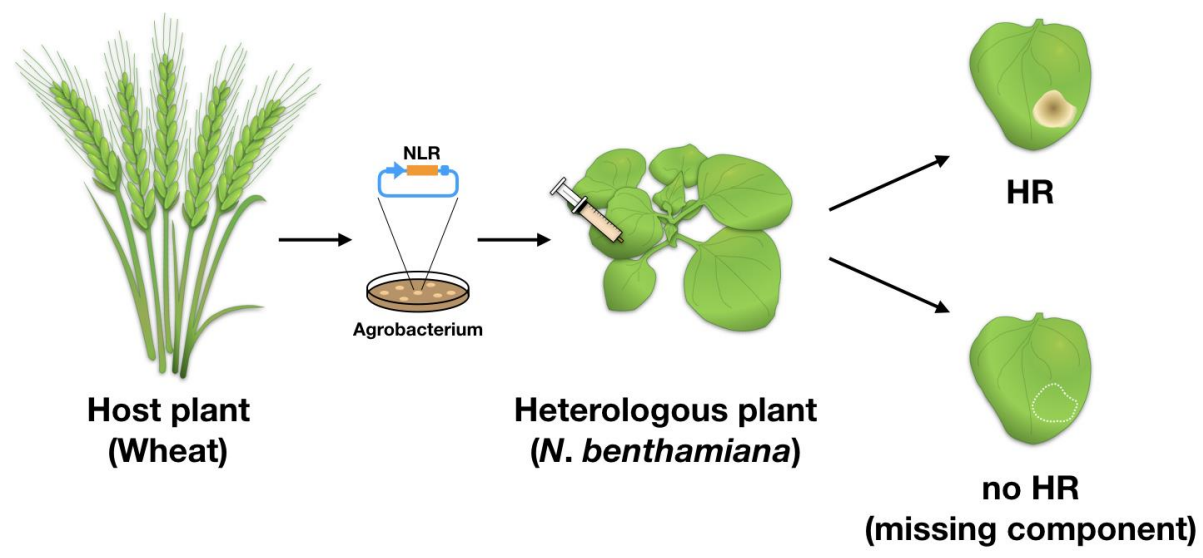
2013; Schreiber et al., 2016). Consistently, mutations in the NB or LRR domains often confer auto-activity to plant NLRs (Bendahmane et al., 2002; Tameling et al., 2006 Intermolecular regulation between plant NLRs are known for NLR pairs. In some paired NLRs, such as RRS1/RPS4, RGA5/RGA4 and PigmS/PigmR, ectopic expression of helper NLRs results in auto-activity, which is suppressed by co-expressing the matching sensor NLRs (Césari et al., 2014; Deng et al., 2017; Huh et al., 2017). 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 (Zhai et al., 2011; Maqbool et al., 2015). 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" (Bomblies et al., 2007). 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 (Bomblies et al., 2007; Chae et al., 2014; Atanasov et al., 2018). Interestingly, the auto-immune phenotype was triggered by hetero-complex of NLR proteins from the unlinked DM1 ($DM1^{Uk-3}$) and DM2d ($DM2^{Uk-1}$) alleles (Tran et al., 2017).]. 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 just singletons and pairs but also more complex connections that vary in their specificity and redundancy. NLRs appear to exhibit a diversity of connections within these networks. 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**). The modern view is that other NLRs do cooperate 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 1**). Understanding the genetic, biochemical and mechanistic links between NLR networks nodes should reveal a complex and dynamic architecture that would help to organize the hugely expanded plant NLRome. However, our knowledge remains limited and fragmented, notably about the biochemical connections between NLRs. In mammalian systems, fundamental advances through biophysical and biochemical studies revealed that NLRs form a sensing and signalling multi-protein complex dubbed "the inflammasome". For example, NLR family CARD domain-containing 4 (NLRC4) forms a hetero-oligomer complex with the sensors NLR family apoptosis inhibitory proteins (NAIPs), to detect diverse ligands and recruit and activate Caspase-1 protease (Zhao et al., 2011; Tenthorey et al., 2014; Tenthorey et al., 2017). Such level of mechanistic knowledge is still lacking for plant NLRs and is required for our understanding of NLR networks to leap forward. Despite undeniable advances in understanding plant NLR detection of pathogen effectors [e.g. Maqbool et al., 2015; De la Concepcion et al., 2018], many key questions remain unanswered. How did NLR pairs and network evolve from singletons? How do

Box 1. How to define singleton NLRs?

Given our overall poor mechanistic understanding of NLR sensing and signalling, it is not straightforward to determine whether a given NLR protein functions as a singleton or whether it requires other NLRs. The converse question can more easily be answered. NLRs with integrated domains (NLR-IDs) are thought be exclusively involved in pathogen sensing and have so far always been associated with a paired helper (or executor) NLR (Cesari et al., 2014). Classic NLRs, such as Rx, Bs2, Mi, Rpi-blb2, R1 and others, were first cloned as segregating *R* genes against multiple pathogens but turned out to require NLRs of the NRC helper class (Wu et al., 2017). Typically, such NLRs cannot be transferred to taxonomically distinct plant families in the absence of their NLR mates (Narusaka et al., 2013). This phenomenon quipped "restricted taxonomic functionality" by Brian Staskawicz (Tai et al., 1999) may not apply to singleton NLRs, which tend to confer hypersensitivity when expressed alone in heterologous plants (**Table 1**). For example, the monocot NLRs MLA10 and Sr50 can confer hypersensitive cell death when transiently expressed in *Nicotiana benthamiana*. Although this test is not definitive and may depend on the degree of conservation of a potential guardee or decoy protein (e.g. RPS5 and PBS1), it remains a necessary assay to sort out NLRs into functional categories.



singleton activities differ from paired NLRs? 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 higher level complexes? What are the determinants of sensor/helper specificity in paired and network forming NLRs? Multidisciplinary approaches are required to dissect the evolution, assembly and regulation of the immune receptor circuitry of plants.

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