Plant NB-LRR proteins: tightly regulated sensors in a complex manner

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Abstract

As plants are sessile, they have evolved hundreds of resistance (R) genes to defend themselves against multiple pathogens. Most of plant R genes encode proteins with the nucleotide-binding and leucine-rich repeat (NB-LRR) domains that interact with pathogen effectors to induce defense responses. Recent findings describing R proteins structures, host interactors and transcriptional and posttranscriptional regulators have broadened our understanding of R gene activity regulation. Genome-wide analyses of NB-LRR genes are useful for identifying host and nonhost R genes and elucidating complex resistance mechanisms. This review provides an overview of the functions of identified NB-LRRs and intra- and intermolecular R gene regulators.

Key words: NB-LRR; resistance genes; effectors; autoimmunity; regulatory mechanism

Introduction

Plants are immobile and are therefore constantly exposed to numerous microbial pathogens. As a result, plants have evolved to defend themselves and have developed an immune system comprising several components including physical barriers, antimicrobial compounds, pattern recognition receptors (PRRs) and R genes [1, 2]. When plants are challenged with pathogens, PRRs on plasma membranes recognize pathogen-associated molecular patterns (PAMPs) and plants trigger a basal resistance response called PAMP-triggered immunity (PTI). To overcome the defense response induced by PTI, microbial pathogens secrete a set of effectors via the type III secretion system (T3SS) for bacteria or haustoria for filamentous pathogens [3, 4]. Effectors modulate plant physiology and modify host proteins to increase pathogen virulence [5]. Plants also have hundreds of R genes that mainly encode nucleotide-binding and leucine-rich repeat (NB-LRR) proteins [6, 7]. The proteins encoded by R genes interact with avirulence (Avr) effector proteins to induce a rapid and strong resistance response called effector-triggered immunity (ETI). An ETI response is typically associated with the hypersensitive response (HR), which is localized programmed cell death to restrict pathogen growth in plant cells [2, 8].

Most plant R genes belong to the NB-LRR superfamily. Depending on the N-terminal domain, plant NB-LRR genes can largely be classified into two groups: Toll/interleukin-1 receptor (TIR)–NB-LRRs (TNLs) and coiled-coil (CC)-NB-LRRs (CNLs) [9, 10]. The N-terminal TIR and CC domains are involved in the formation of homo-dimers required to activate defense signaling [11, 12]. Some TIR domains are sufficient to induce cell death on transient expression [13]. The central NB-ARC domain comprises three subdomains: NB, ARC1 and ARC2. The ARC domain was named based on its presence in APAF-1 (apoptotic protease-activating factor-1), R proteins and CED-4 (Caenorhabditis elegans death-4 protein) [14, 15]. The NB-ARC domain acts as a nucleotide-binding pocket and hydrolyzes ATP to induce conformational changes in R proteins [14, 15]. The NB-ARC domain acts as -nucleotide-binding pocket and hydrolyzes ATP to induce conformational changes in R proteins [14, 15]. The NB-ARC domain acts as
variable sequences than N-terminal or NB-ARC domains [18]. The LRR domain forms horseshoe shape and interacts with NB-ARC domain to maintain the ‘OFF’ state in the absence of pathogen effectors [9, 12, 19]. On pathogen attack, R proteins directly or indirectly interact with effectors and shift into the ‘ON’ state to activate defense signaling.

Since the first NB-LRR-type R gene was identified, advances in genetics, functional genomics and biochemistry have broadened our understanding of the complex regulatory mechanisms that underlie plant NB-LRR function and specificity. This review highlights recent key findings of NB-LRR functions in host and nonhost resistance, as well as plant development. It also summarizes recent developments describing how NB-LRR genes are regulated and modulated.

NB-LRR in host resistance
Flor hypothesized the mode of action of the R-effector interaction and termed it the ‘gene-for-gene hypothesis’ [20]. This hypothesis explains how resistance is triggered by interactions between host resistance gene and cognate avirulence gene of the microbial pathogen. Some evidence supports the hypothesis, including Pt-ta and Rpib1 genes. Pt-ta is a rice resistance protein against Magnaporthe oryzae that directly interacts with AvrPita [21]. A single amino acid change determined susceptible allele of Pt-ta suggesting R-Avr specificity [22]. Furthermore, it is known that Rpib1, a late blight resistance gene of Solanum bulbocastanum, interacts with IPI-O (AvrRbs1) based on yeast two-hybrid screens and co-immunoprecipitation (co-IP) experiments [23].

However, many R genes indirectly recognize effectors. This is called the ‘guard hypothesis’ in which R genes guard host proteins (‘guardee’) modified by pathogen effectors and activate signal transduction pathways [24]. Arabidopsis RIN4 (RPM1-interacting protein 4) is a well-studied guardee protein targeted by multiple effectors. RPM1 and RPS2 sense RIPK-dependent phosphorylation and cleavage of RIN4 by AvrRpm1 or AvrRpt2 effectors of bacterial pathogen Pseudomonas syringae, respectively [25–27] (Table 1). In addition to AvrRpm1, AvrRpt2 and AvrB, HopF2/mop targets RIN4 to promote virulence activity [76]. RIN4 negatively regulates PAMP-induced signaling and interacts with H-ATPase to resist pathogen invasion, which implicates RIN4 as a link between PTI and ETI [77, 78]. BSL1, which encodes putative plant phosphatase, has recently been characterized as a guardee protein against the oomycete pathogen Phytophthora infestans and NB-LRR-type R2 guards BSL1 [58]. R2 and Avr2 interacted with BSL1, and knockdown of BSL1 expression impaired the interaction between R2 and Avr2 effectors.

Genome sequencing of P. syringae revealed that it has 298 putative virulence genes including ~50 TSSS effector genes. It is also known that the P. infestans genome has ~550 RXLR effector genes that could be regarded as putative Avr factors [79, 80]. It seems reasonable that plants have evolved surveillance systems to monitor guardee(s) targeted by multiple effectors rather than individual R–Avr interactions.

NB-LRRs involved in NHR
Identified host R genes have been deployed to improve plant resistance but the breakdown of resistance has often been observed in the field [81]. Conversely, NHR has emerged as a source of durable resistance [82]. NHR is the resistance of an entire plant species to an entire pathogen species [83, 84]. It is known that several components shared with host resistance are involved in NHR [85]. Among them, ETI is considered as one of the core components, and the evolutionary concept of NHR explains that the relative contribution of ETI to NHR increases with decreased evolutionary distance between nonhost and host plants [86].

Until now, only a few NB-LRR-type R genes have been identified as nonhost resistance genes. Rxo1 was isolated from maize, which is a nonhost plant against Xanthomonas oryzae pv. oryzicola (Xoc) that causes bacterial leaf streak disease in rice [87]. Rxo1 recognizes AvrRxo1 of Xoc and confers resistance when it is transferred to susceptible rice [88, 89]. Rxo1 also showed resistance against Burkholderia andropogonis, which cause bacterial stripe on maize. This implies that a single R gene confers resistance to a nonhost pathogen and an unrelated host pathogen. In addition, White rust resistance 4 (WRR4) encodes TNL protein and has been identified from nonhost Arabidopsis ecotypes showing variable degrees of resistance using map-based cloning [90]. Brassica napus, a host plant of white rust (Albugo candida) transformed with WRR4, and transgenic plants showed resistance against A. candida [91]. However, nonhost plants carrying non-functional copy of those R genes still showed resistance against the pathogens [89, 91]. High-throughput screening of P. infestans effectors activity on nonhost pepper plants revealed that multiple effectors induced cell death on pepper and that one or two nonhost genes controlled effector-induced cell death [92]. These results suggest that multiple R genes or host proteins rather than a single R gene recognize multiple effectors, leading to NHR. In the near future, the genome sequencing of nonhost plants and pathogens could provide insight into the mechanisms of NHR.

NB-LRR functions beyond resistance
Several studies indicated that R genes also function as non-immune receptors during signal transduction for plant development. Arabidopsis ADR1 encodes a CNL protein and conveys broad-spectrum disease resistance [93]. Enhanced expression of ADR1 confers drought tolerance, suggesting that there is some overlap between disease resistance and abiotic stress signaling pathways [94]. Arabidopsis chilling-sensitive 2 (chs2) mutant exhibited temperature-sensitive growth defects similar to what is observed during defense responses when grown below 16°C [95]. A gain-of-function of RPP4 mutation has been identified using map-based cloning of chs2 mutant that showed increase of pathogenesis-related (PR) genes expression and accumulation of hydrogen peroxide and salicylic acid (SA) when it was grown at 16°C, which result in chilling sensitivity [95]. Similarly, an Arabidopsis chs3 mutant exhibited arrested growth and chlorosis at 16°C by defense response activation [96]. CHS3 was cloned using map-based cloning and was identified as a TNL-LIM-type R gene that controls the freezing tolerance phenotype. The observed deregulation of R genes in a temperature-dependent manner suggests that R genes mediate temperature sensitivity of plant growth.

R genes also control plant morphology. The mutant of UNI that encodes an NB-LRR-type protein showed early termination of inflorescence stem growth with morphological changes and elevated expression of PR and cytokinin-responsive genes [97]. AtTIP49a identified as a factor associated with TATA-binding protein complex is important for sporophyte and female gametophyte viability and serves as a negative regulator of RPP5- and RPP2-mediated resistance [98]. Taken together, the findings suggest a functional link between plant development and R gene-induced defense responses.
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*ND: Not determined.

1I2 homologues of tomato were targeted.
NB-LRR regulatory mechanisms

Intramolecular interaction

Inaccurate R gene regulation results in growth defects and autoimmunity (constitutive expression of defense responses) [99]. Appropriate activation and tight regulation of R gene function are essential for a balance between physiological benefits and the cost for defense response. The mechanism of R gene activity regulation has been extensively investigated, and several factors to regulate R genes have been characterized. First, R genes are activated in a dosage-dependent manner. SRFR1 (Suppressor of rps4-RLD) encodes a TPR domain-containing protein, and srfr1 mutants exhibited autoimmunity with accumulation of R proteins including SNC1, RPS2 and RPS4, suggesting that SRFR1 negatively regulates the accumulation of R proteins to prevent autoimmunity [99]. As mentioned above, ADR1 was identified from an activation-tagging mutant characterized by SA accumulation and increased PR1 expression. The phenotype of the adr1 mutant was stunted growth and curled leaves. It segregated as a semidominant trait, and homozygous plants showed a more severe phenotype [93]. ADR1 overexpression led to elevated resistance against Peronospora parasitica but also resulted in a fitness penalty. Second, it is also important to maintain the appropriate structure of R proteins because interactions between domains suppress autoimmunity. Domain deletion or amino acid substitution in the major motifs of the NB-ARC domain could trigger conformational changes in R proteins that lead to autoimmunity [100]. The TIR domain of L6 exhibited autoimmunity when overexpressed alone on tobacco, but the NB-ARC domain inhibited this effect by preventing self-association of the TIR domain required for defense signaling [15]. A single amino acid change of the non-conserved region of NLS1 mutant (necrotic leaf sheath 1) conferred severe lesion on leaf sheath in rice [101]. Third, host interactors such as guard proteins can stabilize R proteins and keep them inactive. Ectopic RPS2 expression in Nicotiana benthamiana induced cell death by itself, but no cell death was observed when it was co-expressed with RIN4 [102]. As with RPS2, autoimmune cell death induced by an RPM1 D505V allele carrying a mutation in the MHD motif was suppressed by RIN4 [103].

Intermolecular interactions

Expression levels of R genes also need to be controlled at transcriptional and posttranscriptional levels. Epigenetic factors can regulate R gene expression by chromatin remodeling, which leads to altered level of transcription. Histone lysine methyltransferase SDG8 methylates histone H3 at lysine 36 and is involved in transcriptional activation. Histone modification by SDG8 is required for proper expression of LA25, which encodes a TNL protein similar to RPS4 [104]. In addition to LA25, SDG8 altered the transcript levels of RPM1 and RPS5 but not of RPS2 and RPS4, which suggests that methylation by SDG8 controls the transcription of a subset of R genes.

Small RNAs including microRNA (miRNA) and small interfering RNA (siRNA) function in R gene regulation at the posttranscriptional level. Genome-wide miRNAs analyses revealed that plants have hundreds of miRNAs [383 miRNAs in Medicago truncatula, 203 miRNAs in Glycine max and 177 miRNAs in Capsicum annuum] [105, 106]. Regulation of R gene expressions by miRNAs have been extensively explored in tobacco [28], tomato [107], Arabidopsis [41], legumes [106], cotton [108] and even in woody plants such as apple [109]. Decreased expression of miRNAs on pathogen infection resulted in enhanced expression of targeted NB-LRRs and induced resistance against virus or bacterial pathogens. Two miRNAs of Nicotiana, nta-miR6019 and nta-miR6020, cleaved the N-transcript of a TMV resistance gene, and TMV resistance was attenuated when those miRNAs were overexpressed (Table 1) [28]. Generally, cleavage of target NB-LRR genes by miRNAs triggers the production of secondary siRNAs such as phased 21 nt secondary small RNAs (phased siRNAs) or trans-acting siRNAs (ta-siRNAs) [110]. This phenomenon was verified in tomato, M. truncatula and cotton [98, 107, 108]. Plants target a subset of NB-LRR genes via miRNAs and subsequently regulate a large number/group of NB-LRR genes with high sequence homology through secondary siRNAs.

At the protein level, homo- or hetero-dimerization through N-terminal domain of R proteins regulate the R gene activity. Barley mildew A (MLA) encodes a CNL protein against powdery mildew fungus [111]. CC domain of MLA forms a homo-dimer and the activation-independent MLA self-association is necessary for R gene activity leading to host cell death. Also, the rice R proteins of RGA4 and RGA5, which form hetero-dimerization via CC domains, are important for the resistance to M. oryzae [112]. RGA4 acts as cell death inducer and RGA5, which mediates the direct recognition of AVR-Pia and AVR1-CO39 effectors, regulates the RGA4 activity [112, 113]. In the case of TNL-type R proteins, it was recently revealed that TIR domains of RPS4 and RRS1 interact with each other and the RRS1/RPS4 TIR hetero-dimer suppresses effector-independent RPS4 activation that is induced by homo-dimerization of RPS4 TIR domain [114]. These results suppose that plants adopt other NB-LRR proteins as a regulator to inhibit cell death signaling activated by self-association of R protein.

Regulators of R gene-mediated defense signaling

On recognition with pathogen effectors, R gene-mediated signaling requires several conserved factors including HSP90 (heat-shock protein 90), SGT1 (suppressors of G2 allele of skp1) and RAR1 (required of Mla12 resistance). The HSP90-SGT1-RAR1 chaperone complex targets NB-LRR proteins for stable folding or activation [115]. HSP90 is a conserved molecular chaperone involved in the folding of R proteins to be stabilized [116, 117]. The hsp90 mutant compromised RPM1-, RPS4- and RPP4-mediated resistance [118]. SGT1, a co-chaperone of HSP90, is a component of skp1-Cullin-F-box protein ubiquitin ligases for the negative regulation of R protein accumulation and plays a role in multi-protein networks [119, 120]. SGT1 is associated with host and nonhost resistance and interacts with RAR1 to mediate R gene function [116, 121]. RAR1 encodes the CHORD-containing protein, mediates functions of a subset of R proteins and antagonizes SGT1 to regulate R protein accumulation [115, 116]. SGT1 and RAR1 function additively in RPP4-mediated resistance but the involvement of HSP90, SGT1 and RAR1 in R gene regulation varies depending on the R genes [119, 122].

Two central regulators of R gene-mediated defense activation are EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) and NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE) [123, 124]. Both EDS1 and NDR1 are required for basal resistance, as well as for ETI [125, 126]. EDS1 interacts in vivo with PAD4 and SAG101, which are indispensable for EDS1-dependent resistance [127]. EDS1 is important for the resistance response mediated by TNL-type R proteins. In contrast to EDS1, NDR1 regulates resistance responses mediated by CNL-type R proteins such as RPS2, RPM1 and RPS5 [128]. NDR1 was identified from mutant screens

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showing susceptible responses to *P. syringae* DC3000 carrying AvrB, and the ndr1 mutant was susceptible to several avirulent isolates of *P. parasitica* [123]. NDR1 localized on plasma membrane, and overexpression of NDR1 led to enhanced resistance [129, 130]. Recent studies demonstrated that EDS1 and NDR1 can bind pathogen effectors or R proteins, and perturbation of the interaction affects signaling pathway activation [131–134]. EDS1 forms protein complexes with R proteins and interacts with pathogen effectors [132]. Cognate effectors disrupted R–EDS1 complexes, which suggests that EDS1 behaves as an effector target, and perturbation of EDS1 disrupted EDS1 complexes activates TNL-mediated defense signaling [131]. EDS1 identified from soybean interacted with an AvrA1 effector, which was required for virulence function [134]. Soybean NDR1-like proteins also interacted with *P. syringae* effectors AvrB2 and AvrD1, which correlates with enhanced virulence in NDR1-silenced plants [133]. These results raise the possibility that conserved signaling components that regulate several R gene-mediated responses could have evolved as a guardee on the plant side or, conversely, to be deployed as a virulence target on the pathogen side.

**NB-LRR gene family in plant genomes**

Over the past two decades, sequencing technologies have been rapidly developed and used to assess plant-microbe interactions. Their use enables genome-wide analyses of NB-LRR genes based on the NB-ARC domain [135]. The sequenced genomes of various plant species have revealed hundreds of NB-LRR genes [10]. *Arabidopsis thaliana*, the first plant species sequenced, has 159 NB-LRR genes including 43 CNLs and 83 TNLs [136, 137]. Solanaceae plants carry more than twice the number of NB-LRR genes than *Arabidopsis* and possess more CNLs than TNLs [105, 136, 138, 139]. However, TNLs have not been identified in cereal crop species, which suggests that TNLs have evolved after the divergence of monocots and dicots [10, 137, 140, 141]. NB-LRR genes belong to a rapidly evolving gene family, and their compositions differ among plant species [142, 143]. For instance, 185 NB-LRR genes (21 CNLs and 103 TNLs) were identified in *Arabidopsis lyrata* and more differences in R gene composition have been observed at the genus level. Recently, R gene enrichment and sequencing (RenSeq) method has been established and enables re-annotation of NB-LRR family genes with identification of novel NB-LRR genes [144, 145]. Also, partial type R proteins such as TIR-NB (TN) or TIR-unknown domain (TX) have been identified in plant genomes and the role of those proteins in basal defense has been characterized [146]. *Arabidopsis* ecotype Col-0 has 30 TX genes and 21 TN genes and the overexpression of the genes showed various phenotypes associated with basal defense response such as elevated SA level [146]. Genome-wide analysis of NB-LRR genes may broaden the scope to understand the mechanism of plant resistance.
Future perspectives

Numerous NB-LRR-type R genes have been cloned from a variety of plant species. Most of them have been isolated using map-based cloning approaches, and a few were cloned using transposon tagging or comparative genomics approaches (Table 1). Corresponding avirulence factor and host interactor proteins have been characterized using genomic libraries and interactor screening such as yeast two-hybrid screens or co-IP. With the sequencing technology development, comparative genome-wide analyses of the NB-LRR family within and between species provide new insight into the evolution of the NB-LRR family and serve as a valuable and rapid means to identify R genes through a genome-assisted R gene identification approach (Figure 1). Combining conventional map-based cloning with genome information could shorten the development of trait-linked markers and identify candidate genes [147]. Similarly, core effectors that are found in several isolates could be easily predicted using comparative genomics, and high-throughput screening of effector activity could accelerate R gene identification. Merging biochemical approaches with genomic data could deepen our understanding of R gene regulatory mechanisms.

Key points
- The majority of plant resistance proteins belongs to the NB-LRR superfamily, which directly or indirectly recognizes pathogen effectors through sophisticated mechanisms.
- NB-LRR proteins function as immune receptors in host and nonhost resistance and non-immune receptors in plant development as well.
- The structure, host interactors and transcriptional and posttranscriptional regulators of NB-LRRs elucidate the molecular mechanisms of NB-LRR regulation and specificity.
- Genome-based analyses and high-throughput screening of NB-LRRs and effectors could further enhance the understanding of NB-LRR function in disease resistance.

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