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Review

R PROTEINS AS FUNDAMENTALS OF PLANT INNATE IMMUNITY

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Abstract: Plants are attacked by a wide spectrum of pathogens, being the targets of viruses, bacteria, fungi, protozoa, nematodes and insects. Over the course of their evolution, plants have developed numerous defense mechanisms including the chemical and physical barriers that are constitutive elements of plant cell responses locally and/or systemically. However, the modern approach in plant sciences focuses on the evolution and role of plant protein receptors corresponding to specific pathogen effectors. The recognition of an invader's molecules could be in most cases a prerequisite *sine qua non* for plant survival. Although the predicted three-dimensional structure of plant resistance proteins (R) is based on research on their animal homologs, advanced technologies in molecular biology and bioinformatics tools enable the investigation or prediction of interaction mechanisms for specific receptors with pathogen effectors. Most of the identified R proteins belong to the NBS-LRR family. The presence of other domains (including the TIR domain) apart from NBS and LRR is fundamental for the classification of R proteins into subclasses. Recently discovered additional domains (e.g. WRKY) of R proteins allowed the examination of their localization in plant cells and the role they play in signal transduction during the plant resistance response to biotic stress factors.

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Abbreviations used: CC – coiled-coil domain; CNL – NBS-LRR receptors containing CC domain; HR – hypersensitive response; LRR – leucine-rich repeat; MAMP – microbe-associated molecular pattern; MIMP – microbe-induced molecular pattern; NB – nucleotide binding; NBARC – NB domain with ARC motif; NBS – nucleotide-binding site; PAMP – pathogen-associated molecular pattern; PRR – pattern recognition receptor; TIR – Toll and interleukin receptor; TLR – Toll-like receptor; TNL – NBS-LRR receptor containing TIR domain

This review focuses on the current state of knowledge about the NBS-LRR family of plant R proteins: their structure, function and evolution, and the role they play in plant innate immunity.

Key words: Plant innate immunity, Pathogen recognition, Plant resistance proteins

INTRODUCTION

The ability to detect the presence of pathogens is a prerequisite for plant cells to respond promptly to invading microorganisms. According to the widely accepted current model, pathogen recognition takes place thanks to two relevant groups of host receptors. The first group, called pattern recognition receptors (PRRs), comprises the receptors specializing in the recognition of pathogen-associated molecular patterns (PAMPs, molecules that are evolutionarily conservative in microorganisms and not present in the host cells, such as lipopolysaccharides, murein, flagellin or chitin). PRRs are usually receptor-like proteins or receptor-like kinases (RLPs or RLKs) that are attached to the cell membrane. They resemble animal Toll-like receptors (TLRs) in terms of their structure and function in plant innate immunity. The resistance to pathogens provided by these receptors is called PAMP-triggered immunity (PTI) [1-4].

The other group mainly comprises intracellular receptors called resistance proteins (R proteins). Most of the identified R proteins belong to the NBS-LRR (synonyms: NBARC-LRR, NB-LRR) family, which is a subgroup within the STAND (Signal Transduction ATPases with Numerous Domains) family [5]. These receptors detect pathogen effectors introduced into the host cells in order to suppress PTI. However, certain NBS-LRR proteins are elements of signal transduction pathways involved in disease resistance processes active downstream of the signaling cascades related to the appropriate R proteins or PRR receptors. This is the case of the *Solanum lycopersicum* ART (Avr4-responsive tomato) protein, which is a mediator in a signal transduction pathway during interaction with *Cladosporium fulvum* [6, 7]. Other NBS-LRR proteins play roles which are not associated with resistance. Some, such as *Arabidopsis thaliana* CSA1 protein, are involved in photomorphogenic processes: a mutation in the *CSA1* gene results in the phenotype characteristic for shade-avoidant plants [8]. Another NBS-LRR protein that is involved in phenomena other than resistance is the product of the *UNI* gene. U*ni-1D* mutation causes both the constitutive activation of defense responses, which leads to accumulation of PR (Pathogenesis-Related) proteins, and alterations in morphogenesis due to the accumulation of cytokinins [9]. These findings suggest a deep relationship between plant immunity and other processes on the molecular level.

If a host does not possess appropriate receptors, pathogen effectors (virulence factors) induce the suppression of defense mechanisms, which results in effector-triggered susceptibility (ETS). If a host has suitable receptors, pathogen effectors (which in this case function as avirulence factors in the target cell)

initiate the relevant defense response, generally referred to as effector-triggered immunity (ETI) [10-12]. According to the zig-zag model, the development of an ETI response by the host forms selective pressure, which directs the pathogen to produce new effectors that are unrecognizable by the plant receptors. Consequently, new effectors exert pressure on the host to form new receptors, and this cycle may potentially last *ad infinitum* [10, 13].

MOLECULAR MODELS OF PATHOGEN RECOGNITION

The relationship between corresponding host receptors and pathogen effectors is defined by the gene-for-gene model [14]. It involves the direct effect of a specifically recognized effector on the receptor (Fig. 1A). Although it was possible to identify the receptors involved in such interactions, in most cases, the cooperation of some of the host's additional proteins is necessary to initiate the resistance response. This phenomenon is explained by the so-called guard model, which is an extension of the original gene-for-gene model (Fig. 1B). According to this model, the target protein of the pathogen effector (guardee) is "guarded" by a suitable guard protein, namely an NBS-LRR receptor. Thus, direct detection of the pathogen effector molecules does not occur; what can be observed instead is just their effects reflected by structural and/or functional changes in the host cell [10, 11, 15]. It should be emphasized that in some cases, it seems that a guardee does not play any important role in the absence of the receptor; its interaction with pathogen effectors is not associated with a display of virulence and, consequently, its presence in the host cell does not enhance the pathogen fitness.

In order to explain this phenomenon, a new model of plant-pathogen interaction has recently been proposed. It is a modification of the guard model, and is called the decoy model (Fig. 1C). According to this model, specific proteins that are similar to those targeted by pathogen effectors are generated by the plant in some plant-pathogen interactions. Their only function is to bind these effectors and act as mediators in interactions with R proteins.

Unlike the usual targets of effectors, in this model referred to as operative targets, these decoy proteins do not perform any function in a cell when R proteins are absent, nor do they affect the pathogen virulence. Through functional competition with operative targets in binding with pathogen effectors, decoys can even reduce the pathogen virulence and fitness [16]. Two scenarios of decoy evolution have been proposed. According to one of them, decoys were formed as a result of a modification and loss of the previous function of operative targets. The second scenario assumes that other molecules underwent the process of specific molecular mimicry. Previously, those molecules had not been related to resistance mechanisms, but they might possess some structural resemblance to the operative targets. This structural similarity was subsequently enhanced by natural selection [16].

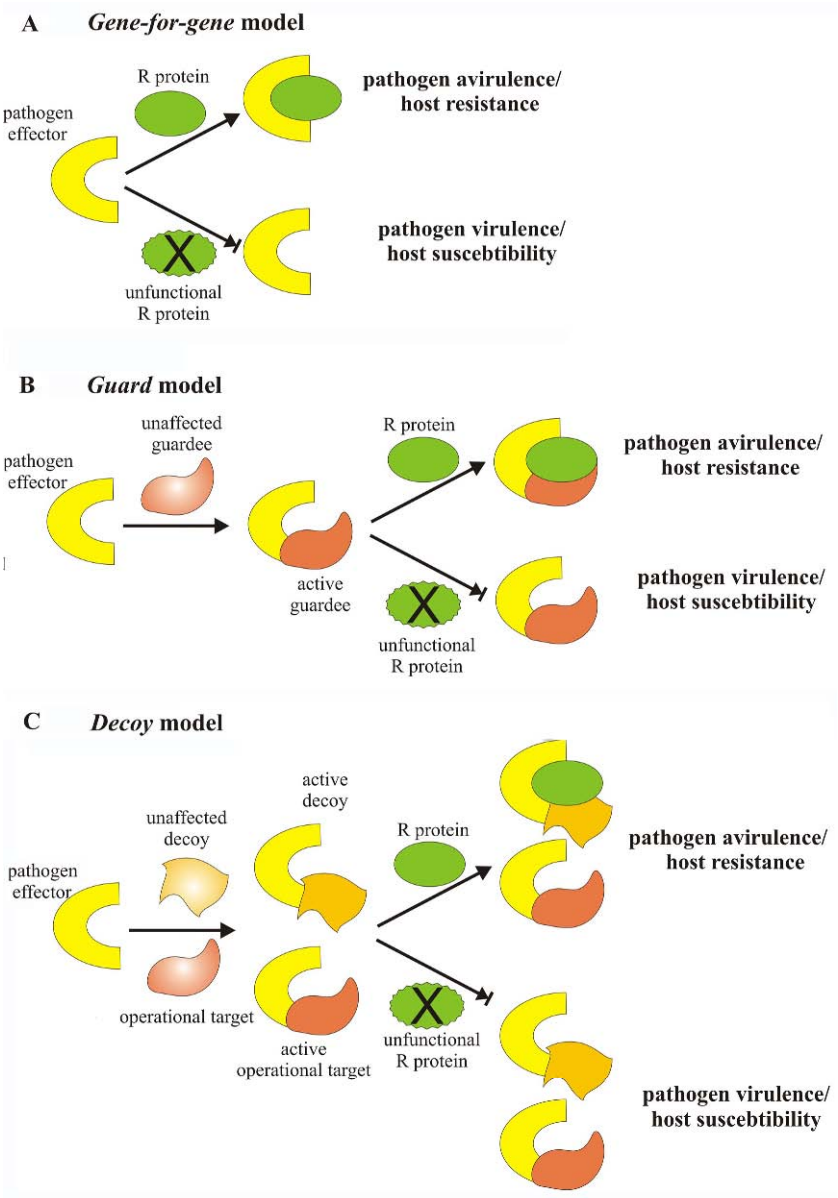

Fig. 1. Models of plant pathogen recognition (drawn up based on [14-16]). Detailed description in the text.

A new concept of receptor and elicitor classification involved in plant innate immunity was recently presented [17]. It was suggested that the molecular factors of microbes recognized by direct interactions with the host receptors should be classified as microbe-associated molecular patterns (MAMPs), even when they are a part of the effectors. At the same time, it was proposed to define the disturbances in the structure or function of host cells recognized by other

receptors as microbe-induced molecular patterns (MIMPs). According to this model, both MAMP and MIMP receptors are classified as R proteins. Moreover, NBS-LRR proteins were previously classified as R proteins, different from PRR. In the new model, some NBS-LRR proteins are classified as PAMP/MAMP receptors that can directly interact with the specific effectors and others as MIMP receptors that detect the interactions between pathogen effectors and their target proteins in the host cell [17-19]. The perception of MAMPs by PRR triggers an immune response within minutes. The types of immune response include an increase in the intracellular Ca^{2+} concentration, which is indispensable for initiating signaling pathways, an oxidative burst, or an induction of mitogen-activated protein kinases (MAPKs) [20-23]. The perception of MAMPs or MIMPs by R protein receptors causes a quantitatively and kinetically different response leading to the hypersensitive response (HR), which is a form of programmed cell death. However, both types of defense response show similar patterns, so it is possible that they involve each other's signaling pathways [23].

THE CLASSIFICATION OF NBS-LRR PROTEINS

The basis for the activity of the NBS-LRR family of plant R proteins is their ability to directly or indirectly recognize MAMPs and MIMPs typical for the particular pathogens prior to initiating the specific resistance response. This is only possible after recruiting a number of domains displaying various functions. Two such domains that are essential for classifying a protein as a member of the NBS-LRR family are the nucleotide-binding domain (NB) and the leucine-rich repeat domain (LRR). They are connected by a homologous region called ARC, which is present in plant R proteins, and in mammal APAF-1 protein and its homolog, CED4 from *Caenorhabditis elegans*. The NB domain combined with an ARC motif is usually referred to as a nucleotide-binding site (NBS) [24].

Further classification of the NBS-LRR proteins depends on the presence of other domains. Two subfamilies of these proteins can be distinguished: TIR-NBS-LRR (TNL) proteins containing the TIR domain, originally identified as an intracellular part of the Toll receptor in *Drosophila* and human interleukin 1 receptor, and non-TIR-NBS-LRR proteins containing other domains, the largest of which is a group of CNL receptors with the coiled-coil (CC) domain (Tab. 1). Such domains are present in various organisms, and they function in oligomerization processes like the TIR domain [25-27]. It should be emphasized that these subfamilies differ not only in terms of the presence or absence of the TIR domain but also in terms of the sequence of the NBS domain [28].

So far, no TNL proteins have been detected in monocotyledonous plants. Although analyzing rice genome sequence databases made it possible to identify several genes encoding proteins that contain the TIR domain, they do not seem to be related to NBS-LRR proteins [29].

Tab. 1. Major classes of plant R proteins.

Domain structure		Example	References
TIR-NBS-LRR			
	TIR-NBS-LRR	N receptor L6 receptor	64 56
	TIR-NBS-LRR-WRKY	RRS1-R receptor	31, 70
	$NBS_{(TIR)}$-LRR	2 Arabidopsis*	30
non-TIR-NBS-LRR			
	CC-NBS-LRR	I-2 RPS5	52 66
	$NBS_{(CC)}$-LRR	4 Arabidopsis*	30
	BED-NBS-LRR	Poptr_1:787192	33
Mixed			
	TIR-CC-NBS-LRR	2 *Populus**	33

*Only the number of the NBS-LRR gene sequences is available.

In dicotyledons, TNL proteins constitute a strongly diversified group in terms of their structure. Analyzing the *A. thaliana* genome made it possible to detect genes containing sequences typical for TNL. However, their structure differs noticeably from the typical TIR-NBS-LRR domain arrangement [30]. For example, the *A. thaliana* RRS1-R protein (defined resistance to *Ralstonia solanacearum*) contains a C-terminal WRKY-type domain [31]. Other identified TNL proteins lack the TIR domain (NBS(TIR)-LRR), and their classification as TNLs is determined by the sequence of an NBS domain [28, 32] or the presence of a C-terminal TIR domain. In the *Populus* genome, sequences encoding proteins which most likely contain a TIR domain at each end were detected. Also, proteins lacking an LRR domain or containing two LRR domains were found in *A. thaliana*, *Populus* and *Oryza sativa* [30, 33].

CC-NBS-LRR-type proteins, the largest group of non-TIR-NBS-LRR proteins, can be found in both dicotyledons and monocotyledons. As with TNL, genome analyses of different plants (*A. thaliana*, *Populus*, *Oryza sativa*) have shown the remarkable structural diversification of this group [34]. In rice, genes encoding proteins with two NBS domains (CC-NBS-NBS-LRR) were identified [35]. On the other hand, in rice, *Arabidopsis* and poplar, genes were found which encode NBS(CC)-LRR proteins lacking a CC domain but showing an NBS domain sequence similar to relevant sequences in the genes of classical CC-NBS-LRR proteins [30, 33]. In the *A. thaliana* and poplar genomes, other proteins showing some similarity to CC-NBS-LRR proteins, including proteins lacking an LRR

domain (CC-NBS) or those exclusively composed of CC or NBS domains were also identified [35].
Apart from CC-NBS-LRR proteins, the non-TIR-NBS-LRR protein family also comprises other smaller families, like the BEAF and DREF proteins containing a zinc-finger DNA-binding domain, and BED-NBS-LRR and BED-NBS and proteins lacking an additional N-terminal domain (NBS(CC/BED)-LRR). In poplar, a group of the so-called mixed proteins containing both TIR and CC domains (TIR-CC-NBS-LRR) was detected [30, 33, 35].
One of the foundations of plant R protein activity is certainly their genomic diversity, which enables the recognition of a diverse array of pathogen effectors. However, in papaya, a small number of genes encoding NBS-LRR proteins were detected. It has been suggested that the diversity of R proteins in papaya is achieved by alternative splicing among other factors; this would compensate for the relatively small genomic diversity of R proteins encoding genes [36].
There are only several member proteins of the NBS-LRR family that have been determined as R proteins. However, there are also atypical R proteins in the plant cell, for example those consisting of kinase domains (Pto) or transmembrane helix domains (Xa13 and MLO). These three proteins respectively take part in innate immunity, fertility and programmed cell death. The functions of the others are still unknown (RPW8) [37].

STRUCTURE AND FUNCTION OF NBS-LRR PROTEINS

The R proteins were identified in several plant species, such as *A. thaliana*, *Populus* or *Medicago trunculata*. The three-dimensional structures of plant resistance proteins are based on research on their animal homologs, but advanced technologies in molecular biology and bioinformatics tools have enabled prediction of the structures and mechanisms of interaction of specific receptors with pathogen effectors. The two main domains of plant R proteins, NBS and LRR, seem to be the most crucial in the pathogen recognition process and the activation of signal transduction in the response to pathogen attack.

The LRR domain

The LRR domain was identified for the first time in the α_2-glycoprotein of human serum [38]. In plants, the first NBS-LRR-encoding genes were cloned in 1994 [39]. The essential structural element of the LRR domain is the tandem repeat of 20-30 amino acids containing a consensus sequence LxxLxLxxNxL, where L is a leucine residue or another aliphatic amino acid, N is asparagin, threonin, serine or cystein, and x is any amino acid [40-42]. A protein with an LRR domain has to contain at least two LRR repeats. The tertiary structure of a single LRR domain is usually a horseshoe-shaped superhelix, and each repeat forms other coils of the superhelix. It is believed that LRR domains constitute a platform for protein-protein interactions [41, 43]. The horseshoe-shaped structure of LRR usually has an inner surface composed of parallel β-strands

containing hydrophobic, aliphatic residues of a consensus sequence, and is believed to be a site of specific interaction with other proteins, which in the case of plant R proteins, provides an essential condition for the specific recognition of elicitors. The outer part of the domain, usually composed of α-helices, is connected with β-strands by β-turns [41].

The involvement of the consensus sequence in interactions with MAMPs or MIMPs can be proved by the differences in the specificity of flax rust (*Melampsora lini*) effector recognition by the P and P2 genes of flax (*Linum usitatissimum*). These genes differ in six amino acids in their β-strands and β-turn motifs [44]. Models of the spatial structure of the complex of the L5 receptor and AvrL567 effector indicating an interaction in the β-strands can provide conclusive evidence to show an active region of the LRR domain [45]. However, it should be emphasized that in animals, other regions of the LRR domain are involved in the protein-protein interaction in receptor proteins involved in immunological processes. An example is the animal TLR2 receptor domain in which the external part of the "horseshoe" (composed of α-helices) interacts with a TLR1 receptor and a bacterial lipopeptide Pam_3CSK_4 [46].

The LRR domain is most frequently attributed the role of an intramolecular regulator that inhibits the receptor activity when an appropriate elicitor is absent. After binding a proper MIMP or MAMP, conformational changes within the LRR domain would take place leading to the dissociation of the LRR domain from the NBS domain and consequently, to the activation of the receptor [47, 48]. Recent data showed that the dissociation of the LRR and NBS domains might not be required for the activation of the receptor [49, 50]. Instead, repetitive rounds of dissociation and re-association could lead to the amplification of the signal originating from elicitor recognition [49].

The NBS domain

The NBS domain was identified in NBS-LRR proteins based on its similarity to the homologous sequences in the animal APAF-1 and CED-4 proteins [51]. This domain is characterized by NTPase activity, and it is suggested to play a crucial role as a molecular switch activating signal transduction. In the signaling pathway, changes in the conformation of the NBS domain occur, caused by reversible nucleotide binding, which leads to the activation/deactivation of the whole receptor [52]. NBS-like domains such as NOD are present in many animal proteins including those involved in immunological processes [53].

Researchers using X-ray crystallography have not yet determined the existence of a three-dimensional structure for the NBS domain for any plant R proteins; however, such a structure was determined for human APAF-1, which makes it possible to speculate about the structure and the function of this domain in plants. The NBS domain of the APAF-1 protein consists of four subdomains: NB, ARC1, ARC2 and ARC-3 [54]. In the plant R proteins, there is no ARC3 domain. Instead, they contain a short linker connecting ARC2 with LRR. ARC1 is composed of a bunch of α-helices and ARC2 of α-helices rolled up in

a winged helix fold. The spatial structure of the NBS domain varies depending on whether it is combined with ATP or ADP. Several conservative motives such as the P loop (Walker A or kinase 1), the RNBS-A, kinase 2 (Walker B), RNBS-B, RNBS-C, GLPL, RNBS-D and MHD motifs can be distinguished in this domain [55]. Research on the tomato mutants *I-2* and *Mi-2* proved that the NBS domain functions by switching R protein from its active to non-active state and showed that it has the capability to bind and hydrolyze ATP. Those mutants capable of an autoactivation of resistance response in the absence of an elicitor and those characterized by an increased susceptibility to pathogens contain several mutations in their NBS domain. It was discovered that mutants showing a disturbance in ATP hydrolysis are characterized by a constitutively active resistance response. Therefore, the binding with ATP rather than ADP seems necessary to activate a receptor. For instance, it was demonstrated that tomato *I-2* mutants in a catalytic region of the NB subdomain display HR even in the absence of an elicitor. At the same time, mutations in the region responsible for nucleotide binding bring about a loss in receptor activity [52]. A similar effect is shown by mutation of the MHD motif at the site of the aspartic acid residue of the flax L6 protein leading to spontaneous necroses. In this case, mutations in the nucleotide-binding regions (P loop) interacting with an elicitor (LRR domain) neutralize the receptor activity [56]. Generally, mutations in the NB and ARC2 subdomains are involved in the mechanism of autoactivation [55]. Similar effects were reported for domain swapping between potato Rx proteins (which recognize the capsid protein of potato virus X) and GPA2 (which recognize the elicitors of pathogenic nematodes) [57, 58]. On the other hand, loss-of-function mutants were identified, defective mainly in the NB and ARC1 subdomains as well as in the ARC2 subdomain [55]. The ARC1 subdomain seems to be involved in binding the LRR domain. However, it is possible that the cooperation of some additional factors is necessary for the process, which can be suggested based on the low stability of ARC1 domain [49].

Other domains

Apart from the domains listed above, there are additional domains, usually located at the amino-terminus, and more rarely at the carboxy-terminus, in most NBS-LRR proteins. The most common ones in this group are the TIR and CC domains [26, 59]. The TIR domain is widely spread among different organisms; for example, it possesses an intracellular domain of animal Toll-like receptors (TLRs). These receptors constitute one of the fundamental elements in animal innate immunity mechanisms. The TIR domain included in TLRs seems to be crucial for interactions with adaptor molecules mediating the initiation of the further steps of signal transduction [60]. Interestingly, many factors interacting with animal TLRs (for example MyD88) also contain the TIR domain. It has been shown that during these interactions, the TIR domains react with each other physically [61]. Furthermore, TIR domains condition the heterodimerization of some animal TLR receptors [62]. An analogous role of the TIR domain has been

proposed for plants. However, no adaptors with which it could interact have thusfar been discovered. The TIR domain in plants is likely to be involved in the recognition of Avr proteins. For instance, there is a proven interaction between the TIR domain of tobacco N protein and the p50 protein of the tobacco mosaic virus, which initiates the hypersensitive response (HR). Importantly, the additional host protein, NRIP1, is indispensable for this interaction [48, 63, 64].
The coiled-coil (CC) domain is composed of two or more α-helices that are usually twisted superhelically around each other in the parallel and antiparallel orientations [25, 65]. The domain is usually attributed an analogous role to that of the TIR domain as a mediator in interactions with other elements of the signaling pathways associated with innate immunity. Research has shown that the conservative motif EDIVD can be identified in the CC domain of all CC-NBS-LRR proteins, except for RPS2, RPS5 and Dm3. Mutations in this motif cause disturbances in the intramolecular interaction with the NBS and LRR domains. The disturbances result in a decreased resistance response to pathogen attack [58]. R proteins with a CC domain that binds target proteins for pathogen effectors are known, e.g. the CC domain of *A. thaliana* RPS5 protein interacts with PBS1, which is a target of AvrPhB, the effector of *Pseudomonas syringae* [66].
Plant WRKY transcription factors identified by the WRKYGQK conservative motif located at the amino-terminus of NBS-LRR, along with a typical domain similar to the zinc finger motif, play a crucial role in regulating the expression of genes involved in plant resistance [67, 68]. Some NBS-LRR proteins have the ability to affect WRKY transcription factors directly. Alleles of barley MLA proteins recognize the corresponding Avr proteins of *Blumeria graminis*, and after recognizing an appropriate elicitor, deactivate HvWRKY1/2 transcription factor, which is a repressor of the resistance genes [69]. There are also NBS-LRR proteins known to contain a domain with the structure of a WRKY transcription factor, which makes it possible to affect gene expression directly. An example of such a protein is the RRS-1R receptor of *A. thaliana*, one of the TIR-NBS-LRR proteins. It recognizes the PopP2 effector of *Ralstonia solanacearum* and contains a C-terminal WRKY domain [70]. The results of an analysis of *A. thaliana* mutants with a change of a single amino acid within the WRKY domain of the TIR-NBS-LRR-WRKY protein, referred to as SLH1, suggest that the WRKY domain may act as an inhibitor of signaling pathways responsible for resistance to a pathogen [71].

THE EVOLUTION OF NBS-LRR PROTEINS

NBS-LRR genes have been identified in most of the major plant groups. It is believed that TNL receptors developed as early as before the separation of the branch leading to bryophytes from the one leading to seed plants. This view is supported by the finding that the PpC gene family in the moss *Physcomitrella patens* displays a significant homology with the NBS region of genes encoding the TIR-NBS-LRR proteins of other plants. The PpC gene family does not show

homology with the other gene families in bryophytes. To identify this homology, sequences of conservative motifs such as the kinase-1 and HD motifs were used; however, instead of TIR domain, a kinase domain was detected at the amino-terminus. It seems that both domain types may play similar roles in signaling pathways [72]. NBS-LRR genes were also found in gymnosperms. As in the case of the homologous sequences used to identify R genes in bryophytes, the PCR technique was applied with primers specific for some conservative motifs occurring in the NBS domain, such as the P loop, kinase-2 and the GLPL motif. Genes from *Pinus monticola* were cloned and compared with the sequences of different R genes in *A. thaliana* and *Brassica*, and were found to display phylogenic links with the TIR-NBS-LRR protein class [73]. Genes cloned from *Pinus lambertiana*, determined as *P1_RGC-CC_1*, showed a significant similarity to the CC-NBS-LRR proteins of *A. thaliana.* A high degree of conservativeness of these genes was discovered, which makes them similar to *ADR1* (Activated Disease Resistance 1) of *A. thaliana*. One of the proposed explanations for this stability assumes that P1_RGC-CC_1 is an example of a guard protein [74].

As mentioned before, no genes coding the TIR-NBS-LRR proteins were identified in monocotyledons, although extensive research was carried out involving database searches and sequence cloning. Since such genes were found in gymnosperms, and they are very common in dicotyledons, it is likely that they occurred in a common ancestor of angiosperms, and then the branch leading to monocotyledons lost the TIR-NBS-LRR genes [29, 75]. The probability of such a scenario is also supported by a similar loss having also been observed in sugar beet (*Beta vulgaris*), a dicotyledon [76]. It remains a mystery, how the loss of a whole gene family could occur in such a relatively short period.

One of the hypotheses assumes that the NBS-LRR genes occurred in the form of several loci before angiosperms came into existence. Then, while some of them were lost in separate phyla, the others underwent a strong diversification. As this phenomenon proceeded independently in unrelated plants (gymnosperms and sugar beet), it is suggested that it could be related to or have been generated by alterations in signaling pathways induced by receptors encoded in individual loci [29, 75, 76]. It was also discovered that the function of the NBS-LRR genes is associated with certain fitness costs which can reduce the adaptation of the plants that possess them. In *A. thaliana*, epistatic effects can result in a necrotic reaction of autoimmune origin. It occurs in the offspring of a plant containing a specific DM1 allele locus (in which a single NBS-LRR gene is located) as well as a specific DM2 allele locus containing sequences typical of the TIR and NBS domains, but having no LRR domain-coding sequences [77].

A classical model of the interaction between plant R proteins and pathogen effectors assumes that the receptors are affected by selective pressure aimed at a stronger interaction with the particular effectors. At the same time, the effectors are influenced by the opposite selective pressure, resulting in the reduction of their interaction with receptors. The consequence of these processes

is the formation of new alleles of effectors and, simultaneously, corresponding new alleles of R proteins. Since the main domain responsible for the recognition of effectors is the LRR domain, the search for symptoms of differentiating selection has focused on it. Research on twelve R genes derived from different plants (e.g. *Zea mays*, *A. thaliana*, *L. usitatissimum*) allowed the detection of traces of the differentiating selection in over 70% of the xxLxLxx motifs present in the LRR domain [78].

Another phenomenon which increases the diversity of R genes is the recombination occurring between their homologous sequences. Multiple sequences of pseudogenes related to genes of R proteins may serve as an additional reservoir of genetic variability. On the other hand, symptoms of the action of the stabilizing selection can often be observed. It results in the establishment of a relatively stable balance of the existing alleles of R genes. This phenomenon seems to be in contradiction to the pressure leading to the generation of new virulence factors in a pathogen population and the corresponding new R genes in a host population [79]. It can be caused either by a favorite influence of individual alleles of R genes on the host fitness in the presence of a pathogen, or by negative influence when a pathogen is absent [80]. A distinct selection of this type has been observed in the locus of the *A. thaliana PRP13* gene, which conditions resistance to *Peronospora parasitica* [81].

THE ROLE OF NBS-LRR PROTEINS IN PLANT INNATE IMMUNITY

Considerable evidence indicates the involvement of the NBS-LRR proteins in plant resistance to many pathogens, particularly the fact that many mapped regions of plant genomes containing different disease resistance genes encode proteins with NBS-LRR domains. By mapping these regions, it was possible to link CNL family genes with the resistance of some cultivars of ginger (*Zingiber officinale*) to *Fusarium oxysporum* [82]. Also, the *A. thaliana* RRS1-R gene, preconditioning resistance to *Ralstonia solanacearum* [70], or the RPP4 gene, responsible for resistance to different strains of *P. parasitica*, were found to belong to the multigenic family of RPP5 receptors of the TNL type [83]. Recent results showed the roles of intracellular interactions between different domains of R proteins and intercellular interactions between Avr and R and other host proteins in the initiation of disease resistance [84].

The NBS-LRR and CC domains of Rx proteins, responsible for the resistance of *Solanaceae* plants to PVX (potato virus X), can interact in the *trans* conformation occurring during the co-expression of their coding sequences. This leads to HR thanks to virus capsid protein detection. It was shown that the integration of the *Hyaloperonospora parasitica* effector gene *ATR13* (*A. thaliana* recognized 13) with the genome of TuMV (Turnip Mosaic Virus) results in the resistance of *A. thaliana* plants to this virus. This is caused by the expression of the bacterial effector inserted into the viral genome in plant cells infected by the virus. As a result, the plant receptor RPP13, which conditions

resistance to *H. parasitica*, becomes an inductor of the resistance response to viral invasion [56, 84]. Interaction between *S. lycopersicum* and *P. syringae* allowed the description of the initiation of the resistance response by NBS-LRR receptors [84]. It was proved that the resistance of *S. lycopersicum* to *P. syringae* results from the presence of Pto kinase and the NBS-LRR protein called Prf. It seems that interactions between AvrPto, Pto and Prf occur according the decoy or guard model. In the first case, Pto is an operative target of the AvrPto effector or a decoy produced by the host to bind pathogen effectors, and Prf is an R protein. According to the guard model, Pto is a target for the AvrPto effector, guarded by Prf. Pto kinase seems to be an inhibitor of Prf activity, and AvrPto probably interacts with Pto, inhibiting its kinase activity through interaction between two specific regions. Such a reaction brings about de-repression of the resistance response initiated by Prf [16, 85, 86]. Resistance initiator proteins are activated by the presence of avirulence factors such as the elements of the signal transduction pathway and signaling networks, and they require the presence of the other host molecules.

R proteins-mediated signaling pathway

Research showed the presence of two partially independent signaling pathways that involve R proteins: the NDR1- and EDS1-dependent pathways (Fig. 2) [87]. EDS1 (Enhanced Disease Susceptibility 1) protein shows homology to eukaryotic lipases and is a mediator in the signaling pathway initiated by most proteins of the TIR-NBS-LRR family, playing a key role in the regulation of the plant response to abiotic and biotic stress [88-90]. Furthermore, EDS1 and PAD4 can regulate the level of salicylate accumulation, and their activity is regulated through FMO1 monooxygenase and NUD7 hydrolase [91]. Null *eds1* mutants of *A. thaliana* are not able to generate the resistance response even if they contain autoactivation mutations within genes encoding TIR-NBS-LRR proteins [90].

The necrotrophic fungus *Botrytis cinerea* can manipulate the plant signal transduction pathway via the activation of the *EDS1* and *SGT1* genes, thus triggering cell death [96]. In *A. thaliana*, SGT1 (the suppressor of the G2 allele of *skp1*) seems to affect the regulation of resistance processes that are initiated both by TIR-NBS-LRR proteins such as RPP2 and RPP4, conditioning resistance to *P. parasitica*, as well as non-TIR-NBS-LRR such as RPP7 and MLA, conditioning the resistance of barley to *Blumeria graminis* [93, 94]. Furthermore, an interaction of SGT1 protein with HSP90 (heat-shock protein 90), which is necessary for the resistance response, was shown. Both interacting proteins might be involved in building a recognition complex that consists of R and RAR1 proteins and recognized effectors [95, 96].

Interestingly, it was recently discovered that in *Arabidopsis*, specific mutations in *HSP90*, referred as *hsp90.2*, suppress the *rar1* mutation and restore the accumulation and function of R proteins [97]. It was observed that SGT1 interacts with another co-chaperone, HSC70, in both the nucleus and cytosol.

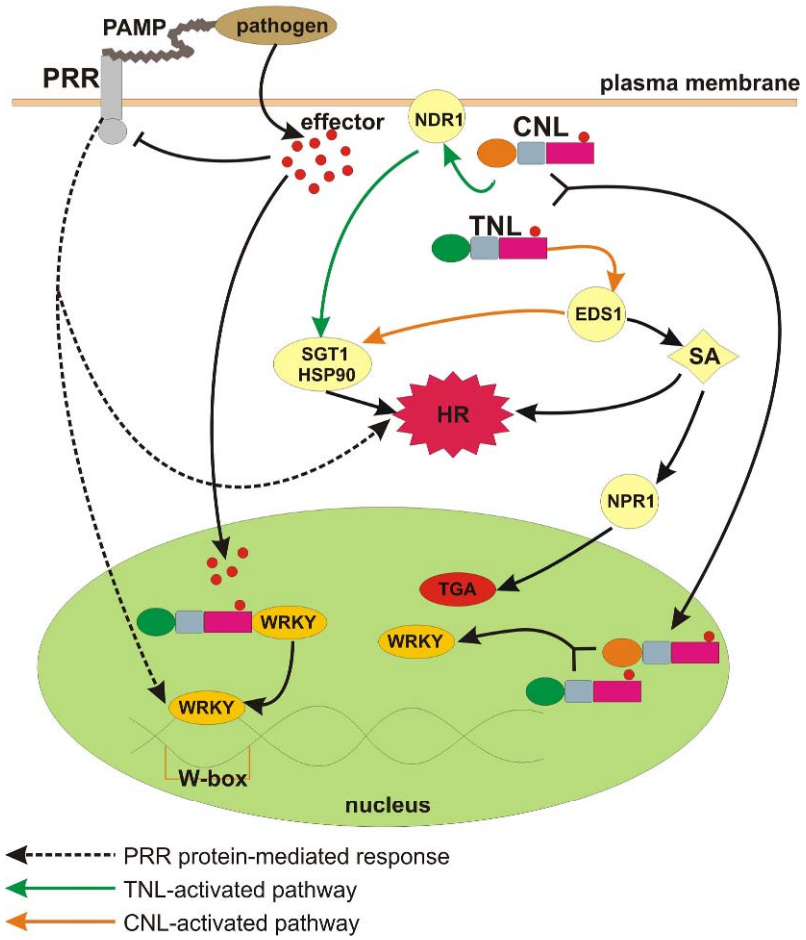

Fig. 2. R protein-mediated signaling pathways in plants (drawn up based on [59, 69, 87, 96]). PTI induced by PAMP recognition through PRR receptors activates signal transduction, thus leading to HR and the expression of resistance-related genes. Pathogens are influenced by the selective pressure as a result of PTI. They develop effectors delivered to the host cell, where they suppress PTI mechanisms. Evolutionarily developed specific effectors function as avirulence factors which initiate the ETI response. The host receptors interacting with pathogen effectors in most cases are NBS-LRR proteins with additional N-terminal domains: TIR or CC. In the response activated via the TNL receptor, EDS1 protein is a downstream mediator, and the CNL-mediated resistance response involves the NDR1 protein pathway.

Abbreviations: CC – coiled-coil; CNL – NBS-LRR receptor containing the CC domain; EDS1 – enhanced disease susceptibility 1; ETI – effector-triggered immunity; HR – hypersensitive response HSP90 – heat-shock protein 90; NBS-LRR – nucleotide-binding site leucine-rich repeats; NDR1 – non-specific disease resistance 1; PAMP – pathogen-associated molecular pattern; PRR – pattern recognition receptor; PTI – PAMP-triggered immunity; SA – salicylic acid; SGT1 – suppressor of the G2 allele of *skp1*; TGA – TGA transcription factors; TIR – Toll and interleukin receptor; TNL – NBS-LRR receptor containing the TIR domain.

Expression of *HSC70* is induced during plant-pathogen interaction, and its overexpression leads to a partial loss of resistance. It has been suggested that SGT1 may function as a bridge between HSP90 and HSP70 chaperones [98]. SGT1 consists of three domains: the TPR (a tetratricopeptide repeat), CS (CHORD-containing protein and SGT1) and SGS (SGT1-specific motif) domains. HSP90 consists of three globular domains including a highly conserved N-terminal ATPase domain (ND), a middle domain (MD) that functions as a platform for client binding, and a C-terminal dimerization domain (CD). Recently, a complete model of the chaperone complex stabilizing NBS-LRR proteins was proposed. It is supposed that it consists of RAR1 interacting via its CHORD domains with the CS domain of SGT1 and the ND domain of HSP90. Additionally, SGT1 interacts via its SGS domain with HSP70 and NBS-LRR proteins. NBS-LRR also interacts with HSP90, but the details of this interaction are unknown. Interestingly, analogous interactions probably exist within mammal chaperone-NLR complexes [99, 100].

The R protein-mediated response is regulated by salicylates, which also influence the activity of the NPR1 protein (non-expressor of PR Genes 1). NPR1 modulates the systemic response, SAR (Systemic Acquired Resistance), through interactions with TGA transcription factors [101]. Most R proteins containing a CC domain initiate a signaling pathway in which the NDR1 (Non-race-specific Disease Resistance 1) protein is a mediator. However, some R proteins (e.g. RPP7, RPP8 and RPP13) act through both EDS1- and NDR1-independent pathways [102]. Although details of the NDR1 mode of action are far from being well known, a few examples of direct interaction of NDR1 with the guardee proteins as well as indirect interactions with R proteins have been discovered. It was found that the cytosolic N-terminal domain of NDR1 interacts with RIN4, a target of the *P. syringae* effector protein AvrRpt2. The guard proteins RPM1 and RPS2 detect RIN4 interaction with the effector, although the two proteins initiate different signaling pathways and require the cooperation with NDR1 that results in resistance response [103, 104].

Until recently, it was thought that the activity of R proteins only exists in the cytosol. However, nuclear localization signal (NLS) motifs in the sequences of several R genes were recently identified, indicating that R proteins can also function in the nucleus [63]. The additional evidence was the discovery of interactions of R proteins with WRKY transcription factors or other proteins with the WRKY-like activity domains [68, 69]. WRKY transcription factors such as CaWRKY in *Capsicum annuum* and WRKY11 and WRKY17 in *A. thaliana* can act as the negative regulators of resistance-related gene expression [105, 106]. Overexpression of CaWRKY1 in transgenic tobacco plants results in increased susceptibility to pathogens, and its silencing in chili pepper shows the opposite effect, causing increased resistance [106]. Different transcription factors play a role as inductors of the resistance response, e.g. WRKY3 and WRKY4 in *A. thaliana*. It seems that overexpression of WRKY4 inhibits the

development of *B. cinerea*. Consequently, *Arabidopsis* mutants with non-active forms of these transcription factors show stronger disease symptoms [107].
In addition to these findings, recent discoveries have shown that some of the NBS-LRR proteins active in antiviral responses may act in cooperation with Argonaute4-like proteins involved in small RNA-mediated transcriptional regulation [108].

CONCLUDING REMARKS

R protein-based resistance of plants to various pathogens is a main area of interest in plant innate immunity. Understanding the evolutionarily developed subtle interactions between plants and pathogens is a necessary starting point to develop a strategy to protect economically important crops. However, only a few such relationships have been successfully deciphered, in spite of the numerous R genes present in plant genomes. Future efforts should focus on identifying new R genes and their corresponding pathogen effectors and modeling these proteins and their interactions. Such research may render it possible to apply this knowledge and improve the resistance of cultivars against various plant pathogens.

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