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Review

Plant NB-LRR Immune Receptors: From Recognition to Transcriptional Reprogramming

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Both plants and animals contain nucleotide-binding domain and leucine-rich repeat (NB-LRR)-type immune receptors that function during defense against pathogens. Unlike animal NB-LRRs that recognize general pathogen or microbe-associated molecular patterns (PAMPs or MAMPs), plant NB-LRR immune receptors have evolved the ability to specifically recognize a wide range of effector proteins from different pathogens. Recent research has revealed that plant NB-LRRs are incredibly adaptive in their ways of pathogen recognition and defense initiation. This review focuses on the remarkable variety of functions, recognition mechanisms, subcellular localizations, and host factors associated with plant NB-LRR immune receptors.

Introduction

Plants have developed sophisticated mechanisms of perceiving pathogen ingress and, if needed, producing a highly localized and specific response resulting in visible, localized programmed cell death (PCD) called the hypersensitive response (HR) (reviewed by Heath, 2000). Once initiated, HR is highly effective at halting pathogenesis at the site of invasion. Unlike mammals, which rely on specialized, circulating defense cells, each plant cell has the ability to recognize nonself molecules. Plant cells synthesize a large number of surveillance-type immune receptors that function to detect the presence of pathogens and relay the message of invasion.

Intracellular immune receptors encoded by *R* (resistance) genes perceive specific virulence proteins called pathogen effectors. The majority of *R* genes encode immune receptors containing a nucleotide-binding and a leucine-rich repeat domain (NB-LRR). These domains are also present in mammalian NOD-like immune receptors (Ausubel, 2005). Plant NB-LRR proteins contain a variable amino terminus. The largest group of NB-LRRs contains a Toll interleukin 1 receptor homology (TIR) protein-protein interaction domain at the amino terminus. Interestingly, TIR-NB-LRRs receptors share remarkable structural and functional similarities to the TOLL immune receptor in *Drosophila* and Toll-like receptors (TLR) in mammals. Rather than resulting from common ancestry, the similarity between insect, mammalian, and plant NB-LRRs is thought to be an excellent example of convergent evolution (Ausubel, 2005). Plants and animals recruited TIR, NB, and LRR domains independently in response to similar selective pressures. Plants, however, possess a far greater number and diversity of NB-LRR proteins than animals. Some of this diversity lies in the fact that a second common class of NB-LRR proteins exists with a coiled-coil (CC) protein-protein interaction domain (CC-NB-LRR) in place of the TIR domain at the N terminus. These proteins, known as CC-NB-LRRs, make up the second most common class of NB-LRRs in *Arabidopsis thaliana* (Meyers et al., 2003). The rice (*Oryza sativa*) genome lacks TIR-NB-LRRs entirely and, in their place, contains 261 NB-LRRs with N-terminal domains of unknown function (X-NB-LRR), further emphasizing the complexity of NB-LRR evolution and function in plants (Monosi et al., 2004). It is clear, however, that the NB-LRR domains in combination with CC and TIR protein-protein interaction domains provide remarkable adaptability and flexibility during pathogen recognition and subsequent defense activation. In this review, we discuss the fascinating variety of recognition mechanisms, subcellular localizations, and host factors associated with plant innate immune receptors.

Two Modes of Pathogen Recognition by NB-LRR Immune Receptors

Unlike mammalian TLRs and NLRs (NOD-like receptors), which recognize generic MAMPs (microbe-associated molecular patterns), plant NB-LRR immune receptors recognize specific pathogen-encoded effector proteins. Over the past decade, a tour de force of research has uncovered extraordinary diversity in the way NB-LRRs recognize pathogens (Figure 1). This has revealed two distinct mechanisms of molecular recognition of pathogen effectors by NB-LRRs and a unique mechanism for non-NB-LRRs.

Direct Interactions

The simplest form of recognition occurs via direct physical association of the pathogen effector with the R immune receptor, similar to a ligand binding to its receptor (Figure 1A). The first example of this mode was shown between the Pita CC-NB-LRR immune receptor in rice and the AVR-Pita effector from the fungus *Magnaporthe grisea* (Jia et al., 2000). The LRR domain of Pita directly interacts with the AVR-Pita effector, and a single amino acid substitution in the LRR can abolish this interaction, resulting in loss of resistance (Bryan et al., 2000). Another example of recognition by direct association was discovered between the *Arabidopsis* RRS1-R immune receptor with the bacterial wilt PopP2 effector (Deslandes et al., 2003).

Perhaps the best example of direct recognition originates from the research on flax multigenic loci (*K*, *L*, *M*, *N*, and *P*) whose products recognize about 30 effector proteins from the flax rust fungus (reviewed by Ellis et al., 2007). The polymorphic *L* locus encodes a TIR-NB-LRR gene with 13 allelic variants (*L*, *L1*-*L11*, and *LH*). *L5*, *L6*, and *L7* are alleles that show differing resistance specificities to corresponding fungal effectors encoded at the *AvrL567* multigenic locus of related genes (Ellis et al., 1999; Dodds et al., 2004). Yeast two-hybrid analysis

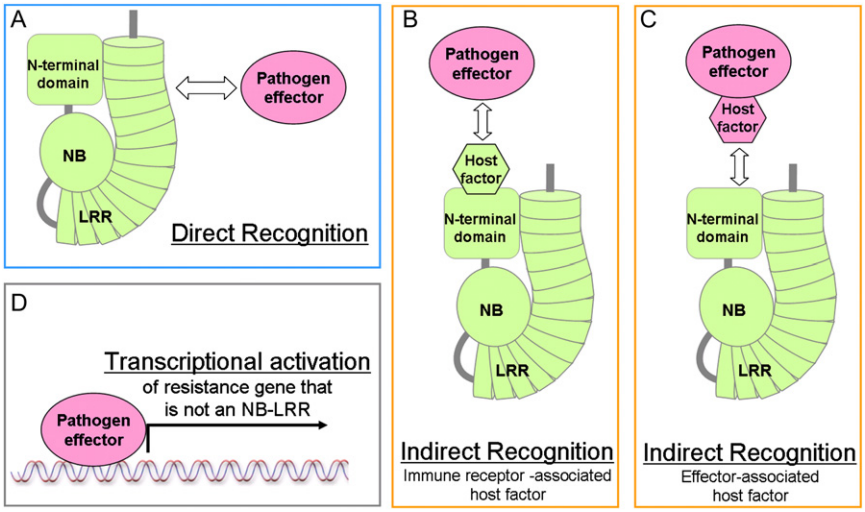

Figure 1. The Three Modes of Pathogen Recognition

(A) Pathogen recognition can occur if NB-LRR immune receptors (green) directly bind pathogen effectors (pink). Alternatively, NB-LRRs can indirectly recognize pathogens through the N-terminal domain (CC or TIR) using an intermediary host factor. The host factor (also referred to as guardee) can be constitutively associated with the immune receptor (B) or it may first associate with the pathogen effector (C) and then is subsequently recognized by the immune receptor. The third type of recognition occurs when a pathogen effector mimics a transcription factor and directly induces the expression of a non-NB-LRR resistance protein (D).

showed a correlation between the direct physical interaction of *AvrL567* effectors with their cognate L immune receptor and the activation of resistance (Dodds et al., 2006). Further sequence analysis, X-ray crystallography, and targeted mutagenesis identified key amino acids that can alter local surface properties of effectors, which in turn determine recognition by the corresponding NB-LRR immune receptor (Wang et al., 2007). These detailed studies convincingly demonstrate that direct interactions can drive recognition specificity, suggesting that, in this case, physical association and recognition of pathogen effectors by an NB-LRR occur as a one-step process (Figure 2, steps 1 and 2). In the future, the dynamics of these direct interactions should be examined in vivo.

Sequence analysis and domain swap experiments with L immune receptors have shown that the LRR domain is the major determinant for effector specificity (Ellis et al., 1999; Dodds et al., 2001). Comparative protein interaction models hint at the curved beta-sheet of the LRR of L proteins as the possible docking site for the AvrL567 effectors (Wang et al., 2007). In addition to the LRR domain, the TIR domain can determine the specificity of rust strains recognized by L6 but not L7 immune receptors. This specificity is conferred by an unlinked *inhibitor of avirulence* gene (*I*) and not differences in AvrL567 effectors (Ellis et al., 2007). The I factor may function within the pathogen by modifying AvrL567 effectors proteins or their secretion into the host (Lawrence et al., 2007). Alternatively, the I factor may be secreted into the host to disturb the association of the TIR domain with an unknown protein required for the activation of defense signaling or disrupt intramolecular interactions with the NB or LRR domains. This would be an excellent example of the uncoupling of pathogen effector association and specific activation of defense (Figure 2, step 1 and 2).

Indirect Interactions

R immune receptors can also recognize pathogen effectors in a more mechanistically complex, indirect way. Many effector proteins alter or modify certain host proteins during pathogen infection. The "Guard Hypothesis" suggests that R proteins monitor these host target proteins and activate defenses if they are perturbed (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). Two variations of this model now exist. The R immune receptor can be constitutively bound to its guardee host factor (Figure 1B), or alternatively, the R immune receptor may bind to its guardee only after the guardee is bound by the pathogen effector (Figure 1C).

The most extensively studied host target (guardee) protein is *Arabidopsis* RIN4 (RPM1 interacting protein 4), which constitutively associates with the CC-NB-LRR immune receptors RPM1 and RPS2. Three structurally unrelated *Pseudomonas syringae* effector proteins modify RIN4, which leads to activation of RPM1 and RPS2. RPS2 is activated when AvrRpt2, a cysteine protease from *P. syringae*, promotes cleavage of RIN4 (Axtell and Staskawicz, 2003; Mackey et al., 2003; Kim et al., 2005a). Structure-function analysis of the RIN4-RPS2 association suggests that RIN4 keeps RPS2 in an inactive state until it is cleaved (Day et al., 2005). RIN4 associates with two other *P. syringae* effectors, AvrRpm1 and AvrB, which promote the phosphorylation of RIN4 that then induces the activation of RPM1 immune response by an unknown mechanism (Mackey et al., 2002).

Recently, the crystal structure of AvrB bound to the C-terminal region of RIN4 was solved (Desveaux et al., 2007). The cocrystallization and targeted mutagenesis revealed crucial structural regions and amino acids at the AvrB-RIN4 interaction interface required by RPM1 to activate defenses. Intriguingly, random mutagenesis of AvrB identified a set of amino acid residues required for the activation of HR both by RPM1 and another immune receptor, Rpg1-b, from soybean, suggesting that the Rpg1-b immune receptor may also guard RIN4 (Ong and Innes, 2006). Phylogenetic analysis suggests that RPM1 and Rpg1-b have independently evolved to recognize AvrB (Ashfield et al., 2004). It is, therefore, tempting to hypothesize that two different species of plants have evolved resistance proteins that identify an effector protein through the same host protein. Functionally, RIN4 acts as a negative regulator of basal immunity, and it is possible that pathogen effectors modify RIN4 to stabilize its ability to suppress host defenses (Kim et al., 2005b; Grant et al., 2006).

Another *Arabidopsis* CC-NB-LRR protein, RPS5, recognizes the *P. syringae* effector protein AvrPphB by indirectly "sensing" its enzymatic activity (Shao et al., 2003). The RPS5 protein maintains extensive intramolecular and interdomain associations that

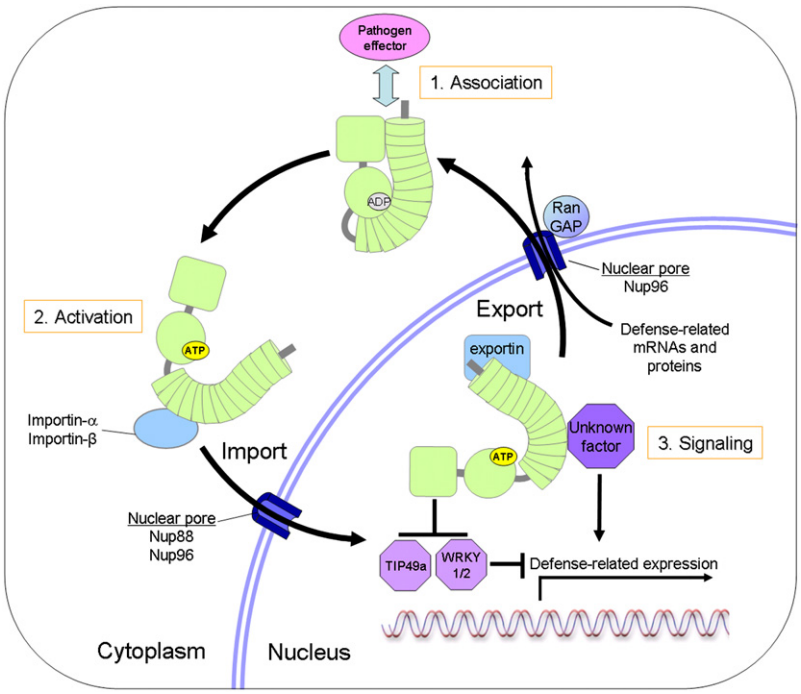

Figure 2. Model for Defense Activation by NB-LRRs

Induction of defense responses by NB-LRRs occurs in three phases. In the first phase, the pathogen effector (pink) will associate with the NB-LRR immune receptor (green). In the second phase, the NB-LRR immune receptor is activated by a conformational change and ATP binding (yellow) to the NB domain. In third phase, activated NB-LRRs function in the nucleus to induce defense-related signaling. This can occur by inhibiting negative regulators of defense (light purple), such as WRKY transcription factors or the TIP49a transcriptional cofactor. Alternatively, an unknown transcription factor (dark purple) may bind to NB-LRRs to positively regulate and induce defense-related expression. The nuclear and cytoplasmic pools of NB-LRRs are most likely maintained by nucleocytoplasmic shuttling. To cross the nuclear pore (dark blue), NB-LRRs with a classical NLS will require importin-α and importin-β for import and exportin for export (light blue). Nup88 and Nup96 are important components of the nuclear pore during innate immunity, and Nup96 may be required for the export of specific defense-related mRNAs or proteins. RanGAP2 (Ran GTPase-activating protein 2) may function during the release of NB-LRRs or defense-related proteins exiting the nucleus during nucleocytoplasmic shuttling. The inactive form of NB-LRRs is found in both the cytoplasm and nucleus in the absence of the pathogen. However, in the presence of the pathogen effector, the activated form of an NB-LRR accumulates in the nucleus to initiate of defense signaling.

help keep RPS5 in a functionally inactive state (Ade et al., 2007). The LRR region appears to be an inhibitory domain, associating with the NB domain when there is no infection (Figure 2, step 1). The CC domain associates with the guardee, protein kinase PBS1 (avrPphB susceptible 1) (Warren et al., 1999; Shao et al., 2003). The current model for RPS5 activation suggests that, during infection, the bacterial AvrPphB effector specifically cleaves PBS1, leading to significant conformational changes in the associated RPS5. The NB domain is thereby relieved from LRR repression, and it is speculated that exchange of ADP for ATP at the NB domain results in an activated, ATP-bound form of RPS5 (Figure 2, step 2). This model is supported by evidence from in vitro studies with the tomato I-2 and Mi-1 immune receptors, which both bind and hydrolyze ATP at their NB domain (Tameling et al., 2002). Mutants that can bind, but not hydrolyze, ATP are constitutively active, suggesting that immune receptors are in their active state when bound to ATP (Tameling et al., 2006). The selective advantage for the pathogen conferred by cleaving PBS1 remains unknown since the exact biological function (or target) of AvrPphB remains to be defined.

The Pto kinase was originally identified in tomato as an immune receptor that provides resistance to *P. syringae* expressing the AvrPto effector (Martin et al., 1993). However, further research suggests that Pto is actually a host factor that mediates the association of AvrPto and the NB-LRR immune receptor Prf in tomato (Mucyn et al., 2006). A structural investigation of AvrPto-Pto binding has revealed a complex engagement between AvrPto, Pto, and Prf (Xing et al., 2007). The inhibition of Pto kinase activity does not trigger defense, but rather, the AvrPto-Pto association is the impetus. Prf and Pto physically interact in vivo and the protein levels of Prf and Pto are reciprocally increased by each other, suggesting that the association may lead to stable accumulation (Mucyn et al., 2006). The Prf-Pto preimmune complex is in an inactive state until the association of AvrPto with Pto disrupts the inhibitory activity of Pto, which subsequently allows Prf to trigger a defense response.

Studies with RIN4, PBS1, and Pto indicate that immune receptors may constitutively bind to their host targets (Mackey et al., 2002; Shao et al., 2003; Mucyn et al., 2006) (Figure 1B). Immune signaling is, thus, activated when the effector protein modifies the bound host factor. Furthermore, studies with RIN4 showed that different R immune receptors can recognize their corresponding pathogen effectors with the same host factors (reviewed by Grant et al., 2006).

A recent report on the indirect association between the TIR-NB-LRR immune receptor N from *Nicotiana glutinosa* and its effector p50 from Tobacco mosaic virus (TMV) suggests that immune receptors are not always bound to their host factors. In this case, N associates with its host factor only when the p50 effector is present (Caplan et al., 2008) (Figure 1C). p50 encodes the helicase domain of the TMV replicase proteins and was shown to indirectly associate with the TIR domain of the N immune receptor using in vivo assays in living tissue (Burch-Smith et al., 2007). Interestingly, the indirect association between N and p50 is mediated by a chloroplastic sulfurtransferase NRIP1 (N receptor interacting protein 1) (Caplan et al., 2008). NRIP1 normally localizes solely to the chloroplasts, but is recruited by the p50 effector to the cytoplasm and nucleus via an unknown mechanism. A preimmune complex containing p50 and NRIP1 is hypothesized to associate with the N immune receptor to ultimately activate defenses (Figure 1C). Interestingly, both the recognizable and the defense-evading forms of p50 can interact with NRIP1 and redirect its localization, suggesting that the indirect association of p50 and N is not sufficient for specific recognition. Therefore, association of an immune receptor with its effector and specific activation can be uncoupled (Figure 2, step 1 and 2). Therefore,

immune receptor activation appears to occur in three distinct phases: association, specific activation, and defense signal initiation (Figure 2, steps 1, 2, and 3, respectively). Although NRIP1 is required for association, it remains unclear if NRIP1 or another host factor is responsible for specific activation. Alternatively, following the indirect association through NRIP1, p50 may associate with the LRR of N to induce activation, since p50 also directly interacts with the LRR of N in yeast two-hybrid assays (Ueda et al., 2006).

Pathogen Recognition through Effector-Mediated Transcriptional Activation of Non-NB-LRR Resistance Proteins

Recent work on two non-NB-LRR immune receptors has discovered a third novel mechanism of pathogen recognition (Figure 1D) that activates the transcription resistance proteins by effector molecules that act as transcription factors. The AvrBs3 effector protein from *Xanthomonas campestris* contains a central repeat region, leucine zipper domains, an NLS (nuclear localization signal), and an acidic activation domain, which makes it similar to eukaryotic transcription factors (Gurlebeck et al., 2006). AvrBs3 localizes to the nucleus and is recognized by the pepper Bs3 resistance gene product (Van den Ackerveken et al., 1996). AvrBs3 directly binds to the promoter of *Bs3* leading to *Bs3* transcript accumulation, eventually resulting in HR (Romer et al., 2007).

An AvrBs3-related protein, AvrXa27, from *Xanthomonas oryzae* is recognized by rice *Xa27*, which encodes a novel protein (Gu et al., 2005). Besides two predicted alpha helixes, the protein's coding sequence does not contain homology to known domains to predict its structure and function. Resistant and susceptible *Xa27* alleles show near identical protein sequences with substantial differences only in their promoter regions. AvrXa27 specifically induces the transcription of the resistant allele of *Xa27*, indicating that the *Xa27* promoter determines its effector specificity. Direct binding of AvrXa27 to the promoter of *Xa27*, however, remains to be demonstrated. Similarly, the *Pantoea agglomerans* HsvG effector recognized in gypsophila and the HsvB effector recognized in beet have putative transcriptional activity in plants (Nissan et al., 2006). Swapping of the transcriptionally active repeats of HsvG and HsvB swaps their specificity, suggesting that these repeats provide recognition specificity. Further studies on the targets of HsvG and HsvB will determine if they transcriptionally activate resistance proteins.

The AvrBs3-Bs3 and AvrXa27-Xa27 interactions are examples of the pathogen-host arms race at the transcriptional level. These effector proteins act as transcription-factor mimics that activate synthesis of desired host proteins by binding to select promoter elements (Kay et al., 2007). Hence, the promoter is functioning as the immune receptor. The hosts, in turn, evolved to exploit this strategy to trigger expression of defense genes. The transcriptional recognition mechanism of Bs3 and Xa27 probably evolved independently because Bs3 was found in dicot plants while Xa27 was found in monocot plants, and their structures are unrelated. It will be interesting to see if NB-LRRs have also evolved a transcription-based recognition mechanism. Logically, plants would induce transcriptional recognition downstream of canonical NB-LRRs, but we should be aware of this possibility as we forge forward.

NB-LRRs Required by Other Immune Receptors

It has recently been discovered that NB-LRRs that are not themselves receptors can act coincidently or downstream of NB-LRR immune receptors during immunity and development. The tomato CC-NB-LRR protein NRC1 is required for HR triggered by the Cf-4, LeEix, Pto, Rx, and Mi immune receptors but is not required by the N immune receptor (Gabriels et al., 2007). However, to provide complete resistance to TMV, the N immune receptor requires a different CC-NB-LRR called N requirement gene 1 (NRG1) from *N. benthamiana* (Peart et al., 2005). It remains unclear if NRG1 is a specific factor required by N or if it is a signaling component of multiple immune receptors. It is possible that a number of key CC-NB-LRRs function downstream during the activation of defense. NB-LRRs can also work in concert with each other to provide immunity. For example, *Arabidopsis* RPP2A and RPP2B are TIR-NB-LRRs required to provide resistance to the *Peronospora parasitica* isolate Cala2 (Sinapidou et al., 2004). Neither RPP2A nor RPP2B can provide resistance independently. Therefore, they interact genetically and perhaps physically to induce an immune response.

A surprising role for TIR-NB-LRRs has emerged in the sensing of red light (Faigon-Soverna et al., 2006). A mutant of the *constitutive shade avoidance* (*CSA1*) gene in *Arabidopsis* is defective in red light-induced responses and produces a truncated protein with a structure similar to TIR adapters such as MyD88, which have crucial functions for innate immunity signaling in animals (reviewed by O'Neill and Bowie, 2007). Truncated CSA1 may block downstream signaling by dimerizing with TIR-NB-LRRs such as CSA1, RPS4, and At5g44870, since mutations in all of these genes led to a subtle reduced sensitivity to red light. The association of the truncated form of CSA1 was not examined; however, the N immune receptor can form oligomers through its TIR domain (Mestre and Baulcombe, 2006). Furthermore, N produces an alternative transcript that is predicted to produce a protein containing only TIR-NB domains (Dinesh-Kumar et al., 2000). Similarly, the M flax rust resistance gene and RPS4 contain different splice isoforms with the majority of them having only an intact TIR or TIR-NB domain structure (Zhang and Gassmann, 2003; Schmidt et al., 2007). Interestingly, the *Arabidopsis* genome contains 27 TIR-X and 20 TIR-NBS genes (Meyers et al., 2002). Thus, TIR-NB or TIR-X proteins may function as adapters to TIR-NB-LRR immune receptors. This question requires further attention, as it would lead to the possibility that other aspects of defense signaling are conserved in plants and animals.

NB-LRR Immune Receptors for Other Functions

NB-LRRs may function as general cell death receptors that recognize a variety of pro-cell death signals. During innate immunity, the pathogen recognition leads to HR-PCD, which is correlated with restricting the pathogen to the infection site. There is now evidence that necrotrophic fungal pathogens can manipulate the plant's defensive arsenal of NB-LRRs to promote necrotic PCD required for pathogenesis. In oats, the *Pc-2* gene confers immunity to the biotrophic pathogen, *Puccinia coronata*, but is maliciously used by necrotrophic pathogen, *Cochliobolus victoriae*, to induce necrosis (Meehan and Murphy, 1946). Similarly in *Arabidopsis*, the LOV1 CC-NB-LRR is required for the recognition of victorin toxin and provides susceptibility to

C. victoriae (Lorang et al., 2007). *C. victoriae* uses the victorin toxin to cause HR-like necrosis; however, it is unclear if victorin is recognized by LOV1 or an unknown receptor.

NB-LRRs also function as cell death receptors during hybrid necrosis, which maintains gene flow barriers between both outcrossing and inbreeding plant species (Bomblies et al., 2007). Hybrid necrosis in plants is similar to autoimmunity in mammals. In hindsight, it makes sense that plants co-opted their innate immune system to perform this related function. DM1 is a TIR-NB-LRR that interacts genetically with DM2 to induce autoimmunity in *Arabidopsis*. Although DM2 has not been cloned, researchers have speculated that DM2 is one of two NB-LRRs within the region it was mapped to (Bomblies et al., 2007). In this scenario, DM1 recognizes the unknown death signal encoded by DM2 to induce an autoimmune response. Therefore, NB-LRRs can recognize both external pathogen effector molecules and toxins and possibly an internal unknown autoimmune factor. It will be extremely interesting to investigate whether NB-LRRs are used as death receptors during other biological processes such as PCD during development.

Subcellular Location of NB-LRR Immune Receptors

In mammals, the localization of immune receptors is quite variable, depending on their function. A number of TLRs containing a TIR, LRR, and transmembrane domain localize to the plasma membrane to detect extracellular MAMPs (reviewed by Kawai and Akira, 2006). The majority of NB-LRRs containing a NOD-like domain mainly localize to the cytoplasm to recognize intracellular MAMPs. CIITA is NOD-like NB-LRR containing an activation domain and localizes to the nucleus (reviewed by Kanneganti et al., 2007). Similar to mammalian immune receptors, there is an amazing diversity in the subcellular localization of plant immune receptors. It was previously thought that most NB-LRR proteins were cytoplasmic because they lack canonical signal sequences. In recent years, however, a number of powerful localization prediction tools have been developed. One such program, LocTree, is a hierarchical system that uses support vector machines to predict localization (Nair and Rost, 2005). LocTree was used to predict the localization of the 154 presumed *Arabidopsis* NB-LRR proteins (Meyers et al., 2003). Surprisingly, over 80% were predicted to be nuclear while only 8.4% were predicted to be cytoplasmic. Another 8.4% were predicted to be chloroplastic. Interestingly, many of the cloned immune receptors contain putative nuclear localization signals (Figure 3).

The predicted chloroplastic localization of some NB-LRRs is surprising but warrants further investigation, since the function of the TIR-NB-LRR immune receptor, N, requires the host factor NRIP1, which is localized to the chloroplasts (Caplan et al., 2008). ChloroP was used to find approximately 22 NB-LRRs with putative chloroplast targeting sequences (Emanuelsson et al., 1999). Approximately 90% of these were TIR-NB-LRRs from five different families. In fact, over half of the members of one TIR-NB-LRRs family had predicted chloroplast-targeting sequences detected by both ChloroP and LocTree. It also raises another interesting question—can recognition of pathogens occur in the chloroplasts? Indeed, the majority of secreted proteins from *P. syringae* have a chloroplast-targeting signal (Guttman et al., 2002). For example, the HopI1 effector alters chloroplast ultrastructure and decreases production of the chloroplast-derived defense signal, salicylic acid (Jelenska et al., 2007). Furthermore, the yellow mosaic symptoms of TMV infection are caused by a depletion of photosystem II core complex (Lehto et al., 2003). Hence, it would not be surprising if TIR-NB-LRRs can function in the chloroplasts to recognize perturbations in the host's chloroplastic machinery. It remains to be seen if TIR-NB-LRRs can recognize pathogens within the chloroplasts. Chloroplastic recognition would require retrograde signaling to the nucleus in order to trigger defense. Furthermore, since tobacco NRIP1 specifically interacts with the TIR domain of the N immune receptor, it will be interesting to determine if NRIP1 associates with other chloroplastic TIR-NB-LRRs.

Even though numerous NB-LRR immune receptors have been cloned in the past 15 years, only four NB-LRR immune receptors have been observed to date in intact, living cells. In all of these cases, they were found to be nuclear or nuclear and cytoplasmic (Deslandes et al., 2003; Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). The first indications that NB-LRRs function directly in the nucleus arose with the cloning of the *Arabidopsis* TIR-NB-LRR-WRKY immune receptor RRS1-R (Deslandes et al., 2003). RRS1-R recognizes the PopP2 effector from *Ralstonia solanacearum*. Sequence analysis of RRS1-R revealed classical NLSs and a WRKY domain that is a signature domain found in a class of plant transcription factors instrumental in transcriptional reprogramming during plant immune responses (reviewed by Eulgem and Somssich, 2007). Therefore, RRS1-R must localize to the nucleus for the WRKY domain to function as a transcription factor. RRS1-R labeled with green fluorescent protein (GFP)-transient expression in *Arabidopsis* protoplasts revealed that it is nuclear (Deslandes et al., 2003). Furthermore, detectable RRS1-R protein accumulation was only observed in the presence of its PopP2 pathogen effector, which colocalizes with RRS1-R in the nucleus. This suggests that expression of *RRS1* is either induced by PopP2 or the RRS1-R protein is stabilized by associating with PopP2. When RRS1-R is coexpressed with truncated PopP2 lacking its NLS, RRS1-R and PopP2 localize only to the cytoplasm. This suggests that the NLS of PopP2 drives the nuclear localization of both PopP2 and RRS1-R. This was surprising because RRS1-R contains two putative NLSs (Figure 3). The RRS1-R NLSs may be functional and the interaction with PopP2, rather than PopP2's NLS, may induce the nuclear localization RRS1-R via its own NLSs. Future studies will determine whether the PopP2 NLS or the RRS1-R NLSs drive RRS1-R nuclear localization.

The nuclear localization of RRS1-R was thought to be unique until it was recently discovered that the immune receptors MLA10, MLA1, N, and RPS4 are also localized to the nucleus. Biochemical fractionation experiments revealed that barley MLA1 is predominantly cytoplasmic with approximately 5% of the total MLA1 localizing to the nucleus (Shen et al., 2007). Interestingly, there appears to be an increase in MLA1 in the nucleus, but not in the cytoplasm, during a defense response to *Blumeria graminis* that correlates with its association with a WRKY transcription factor. Transient particle bombardment experiments in barley demonstrated that MLA10 tagged with yellow fluorescent protein (YFP) localized to the cytoplasm and nucleus (Shen et al., 2007). The nuclear localization is required for MLA10-mediated resistance to *B. graminis*. Like MLA1 and

A NB-LRRs with predicted NLS

NB-LRR	NucPred Score	NucPred NLS sequence	Organism	Actual localization
RPP1	0.99	CSKRKATNQDVDSESRKRRKICS SFRRKGID	*Arabidopsis*	
Rpg1-b	0.89	VHSKRKRGSN	Soybean	
Bs2	0.81	KSQKKKARRRFR KSLKRKRYLI	Pepper	
Rpm1	0.77	NYRMKRKRLI	*Arabidopsis*	**Plasma membrane** (Boyes et al., 1998)
N	0.77	YLYTKRKKLHRVKL	*Nicotiana glutinosa*	**Nucleus and cytoplasm** (Burch-Smith et al., 2007)
RRS1-R	0.75	IERRRRLWE VPKKKKKHS	*Arabidopsis*	**Nuclear** (Deslandes et al., 2003)
Pib	0.72	EARRKGCELKRKIR	Rice	
RPS4	0.71	VDGRVNKKKKTRMDNGRPKKKQRSGRD DERKKKWK	*Arabidopsis*	**Nuclear and cytoplasm** (Wirthmuelller et al., 2007)
Bs4	0.71	GEKRKE KLQKKRSR	Tomato	
Dm1	0.70	SLRRKSK	*Arabidopsis*	
RPS2	0.68	RMRRRY	*Arabidopsis*	
RPP13	0.68	SEKRKNKRMRSFLYFGEF ITRKRET	*Arabidopsis*	
HRT	0.62	FPRKRGW	*Arabidopsis*	
RPP8	0.51	FPRKRGW	*Arabidopsis*	
RPS5	0.40	VLRRRKFVL	*Arabidopsis*	
RCY1	0.39	FPRKRGW	*Arabidopsis*	

B NB-LRRs without NLS

NB-LRR	NucPred Score	Organism	Actual localization
Prf	0.80	Tomato	
Pi-ta	0.71	Rice	
LOV1	0.53	*Arabidopsis*	
SNC1	0.49	*Arabidopsis*	
Mla13	0.47	Barley	
Csa1	0.46	*Arabidopsis*	
Mla10	0.45	Barley	**Nucleus and cytoplasm** (Shen et al., 2007)
L6	0.44	Flax	
L7	0.43	Flax	
Rx	0.41	Potato	
Mla6	0.39	Barley	
Mla1	0.38	Barley	**Nucleus and cytoplasm** (Shen et al., 2007)
NRC1	0.33	Tomato	
NRG1	0.33	*Nicotiana Benthamiana*	

Figure 3. Predicted Nuclear Localization Signals in NB-LRRs

(A) NB-LRRs with a NLS that were predicted using the program NucPred (Brameier et al., 2007). Hypothetical NLS sequences are underlined and color-coded (scale below table) to show amino acids that have a negative (blue) and positive (yellow to red) influence on nuclear localization.

(B) NB-LRRs without a predicted NLS. The NucPred score threshold is a measurement of the fraction of proteins that are correctly predicted as nuclear (specificity) versus the fraction of true nuclear proteins predicted (sensitivity) at or below that score. A higher score is more likely to be nuclear than a lower score.

MLA10, the N immune receptor in *N. benthamiana* is both cytoplasmic and nuclear localized and the nuclear pool of N is required for initiating defense (Burch-Smith et al., 2007). Nuclear N is not required, however, for specific recognition of the p50 effector. Lastly, the localization of *Arabidopsis* RPS4 was determined by biochemical fractionation in transgenic *Arabidopsis* lines expressing epitope tagged RPS4 and by confocal microscopy in tobacco leaves transiently expressing YFP-RPS4. RPS4 was detected in both the nucleus and in the soluble cytoplasmic fraction (Wirthmueller et al., 2007). These recent studies suggest that nuclear localization of NB-LRRs may be the rule rather than the exception.

If nuclear localization is becoming the rule, RPM1 appears to be the exception, as biochemical characterization showed that RPM1 is a peripheral plasma membrane protein (Boyes et al., 1998). Indeed, both AvrRpm1 and AvrB, the pathogen effectors that RPM1 recognizes, are localized to the plasma membrane, suggesting that the plasma membrane is the site of recognition (Nimchuk et al., 2000; Mackey et al., 2002; Kim et al., 2005a). However, it is possible that the site of recognition and the site of activation of defense signaling are different. The guardee RIN4, which RPM1 shares with RPS2, also localizes to the plasma membrane after acetylation (Kim et al., 2005a). Cleavage by AvrRpt2 releases RIN4 followed by RPS2 triggered defenses (Takemoto and Jones, 2005). Could the release of one of the cleaved products of RIN4 also release its associated immune receptors from the plasma membrane? Since RPM1 is a peripheral membrane protein, it must be held in place by binding to another protein. This other protein might be RIN4. AvrRpm1- and AvrB-dependent hyperphosphorylation of RIN4 may release RPM1 from the plasma membrane to induce defense signaling. Interestingly, RPM1 possesses a classical NLS (Figure 3). It is possible that RPM1 is released into the cytoplasm following recognition and travels to the nucleus to directly activate defenses. Observing RPM1 in intact, living cells should reveal if RPM1 localization is altered during a defense response.

Nuclear Import and Export during NB-LRR-Mediated Immunity

The discovery that plant immune receptors localize to the nucleus means that immune receptor nuclear import plays a role in defense. All nuclear-localized immune receptors are larger than 40 kDa, suggesting that they require active, facilitated transport (DeYoung and Innes, 2006) (Figure 3). The importance of nuclear

import for defense was shown in a screen for suppressors of the constitutive defense phenotype of the *suppressor of npr1-1* (snc1) mutant (Li et al., 2001). SNC1 from *Arabidopsis* is a TIR-NB-LRR of unknown function. The screen uncovered MOS6 (modifier of snc1), which was identified as an importin-α homolog (Palma et al., 2005). As expected, importin-α homologs localize around the nucleus, probably to nuclear pores within the nuclear envelope (Palukaitis et al., 1992; Hubner et al., 1999; Palma et al., 2005). NB-LRRs such as RPS4 that contain classical NLS (Figure 3) are most likely imported into the nucleus using an importin-α homolog. Importin-α bind to the phenylalanine-glycine (FG) repeats within the nuclear pore to aid in the transport across the nuclear envelope (Beck et al., 2007; Patel et al., 2007).

MOS3 and MOS7, two other modifiers of SNC1, were identified as nucleoporin homologs required for the formation of the nuclear pore (Palukaitis et al., 1992; Wiermer et al., 2007). MOS3 is homologous to the vertebrate Nup96 (nucleoporin 96), which plays a role in mRNA export (Enninga et al., 2002). Although the exact mechanistic function of MOS3 during plant immunity is unknown, it may be similar to the interferon induction by mammalian Nup96 that aids in specific upregulation of factors required for both adaptive and innate immunity (Faria et al., 2006). It will be exciting to determine if MOS3 is upregulated during plant defense and if that subsequently leads to upregulation of a subgroup of proteins that require MOS3-dependent export of their respective mRNA during defense. MOS7 is a homolog to Nup88 (nucleoporin 88) and is required for both basal, systemic acquired, and NB-LRR-mediated resistance (Wiermer et al., 2007). Currently, there is very little published data on MOS7, but its putative role in multiple branches of immunity suggests MOS7 may be required for the transport of many different factors during innate immunity.

In addition to the modifiers of SNC1, biochemical purification of components associated with immune receptors has provided compelling evidence of the nuclear import pathway's role in NB-LRR function. Tandem affinity purification of the CC-NB-LRR immune receptor potato Rx combined with mass spectrometry identified a Ran GTPase-activating protein 2 (RanGAP2) (Tameling and Baulcombe, 2007). RanGAPs stimulate the hydrolysis of RanGTP to RanGDP, releasing importins and exported cargo on the cytoplasmic face of the nuclear envelope, thereby recycling nuclear import machinery. Both potato Rx and GPA2 (an immune receptor for nematodes) participate in a complex with RanGAP2. In both cases the CC domain of the immune receptors was required for the association (Sacco et al., 2007). The CC-NB-LRR immune receptors Bs2 from pepper and HRT from *Arabidopsis*, however, were not shown to associate with RanGAP2. Furthermore, silencing RanGAP2 affected the function of Rx but not the function of the TIR-NB-LRR immune receptor N. It is not likely that RanGAP2 is required for the nuclear localization of NB-LRRs, but it may regulate the nucleo-cytoplasmic shuttling of NB-LRRs or other defense components. Since the observed associations of RanGAPs with NB-LRRs were quite strong, they may co-localize in cells. Plant and animal RanGAPs are cytoplasmic although clearly enriched at the nuclear envelope (Maule et al., 2000; Rose and Meier, 2001). The localization of Rx and GPA2, however, has not been investigated in intact plant tissue.

Direct Role of NB-LRR Immune Receptors in Transcriptional Reprogramming

With evidence of nuclear actions for NB-LRRs mounting, their function in the nucleus emerges as an interesting question. The fusion of transcription factor domains to NB-LRRs, like in RRS1-R, could be a way for NB-LRRs to directly trigger transcriptional reprogramming during defense. In addition to RRS1-R, *Arabidopsis* has a second NB-LRR containing a WRKY domain and a C-terminal kinase domain (Meyers et al., 2003). Furthermore, there are numerous NB-LRRs in rice (japonica group) that contain an N-terminal BEAF and DREF DNA-binding finger (BED) domains (Aravind, 2000).

The MLA and N immune receptors do not have their own transcription factor domains, but instead they directly associate with transcription factors (Shen et al., 2007); T. Burch-Smith, M.P., K. Czymmek, and S.P.D.-K., unpublished data). MLA binds WRKY1 and WRKY2 transcription factors. MLA association with WRKY1 and WRKY2 is induced by the pathogen AVR_{A10} effector accompanied by an increase in the abundance of MLA within the nucleus. The CC domain of MLA10 directly binds WRKY transcription factors while the LRR domain is thought to function during recognition (Shen et al., 2007). In contrast, the TIR domain of N is thought to be required for recognition (Burch-Smith et al., 2007), and the LRR domain binds to squamosa promoter-like (SPL) transcription factors (T. Burch-Smith, M.P., K. Czymmek, and S.P.D.-K., unpublished data). Hence, the roles of the N-terminal domains and the C-terminal LRR domain of MLA10 and N are reversed. The diversity of the protein-protein interaction modules like CC, TIR, and LRR to switch and swap functions further exemplifies the remarkable variety by which NB-LRRs activate defenses.

An insertion in the W-box of the WRKY domain of RRS1-R, also known as sensitive to low humidity 1 (SLH1), constitutively activates defenses similarly to the constitutively active phenotypes of *Arabidopsis* SNC1 and SSI4 (suppressors of *npr1-5*-based salicylic acid (SA) insensitivity 4) (Li et al., 2001; Noutoshi et al., 2005; Shirano et al., 2002). Furthermore, WRKY1 and WRKY2, which associate with MLA10, function as negative regulators of basal defense in barley plants. Similar to MLA, the N-terminal half of RPM1 (CC-NB-LRR) and RPP5 (TIR-NB-LRR) associate with a transcriptional regulator, TIP49a (Holt et al., 2002). TIP49 and TIP48 in animals have been shown to associate with a number of transcription factors including B-catenin, c-Myc, and E2F1 (Bauer et al., 2000; Wood et al., 2000; Dugan et al., 2002). TIP49a may be a common transcriptional cofactor required for the function of the various transcription factors associated with NB-LRRs. In the cases investigated so far, the majority of transcription factors fused to or associated with NB-LRRs function as transcriptional repressors of nonspecific basal immunity.

Conclusions

The similarities between plant NB-LRRs and animal NB-LRRs appear to be largely superficial, while the differences are striking and extensive. The diversity and complexity of recognition mechanisms, associated factors, and activation observed with plant NB-LRRs appear to surpass their animal counterparts. For example, the function of NB-LRRs as immune receptors was originally thought to be a simple receptor-ligand interaction. Indeed, some NB-LRRs directly bind pathogen effectors, but the

majority of NB-LRRs employ indirect recognition or transcriptional recognition of pathogens. Impressively, immune receptors use components originating from the plasma membrane, cytoplasm, chloroplasts, and nucleus during recognition. The TIR, CC, and LRR protein interaction domains have the ability to swap functions, such as recognition and recruitment of transcription factors or other host proteins. There appears to be no restrictions on the type of proteins these domains can recruit to immune receptor complexes. Furthermore, NB-LRRs can function downstream, upstream, or parallel to one another. This level of flexibility and intricacy was difficult to imagine as little as 5 years ago. Progress in the field can only be made with an open mind because it is difficult to speculate what other novel mechanisms of action and functions for NB-LRRs remain to be discovered.

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