The Role of Plant Innate Immunity in the Legume-Rhizobium Symbiosis

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Abstract

A classic view of the evolution of mutualism is that it derives from a pathogenic relationship that attenuated over time to a situation in which both partners can benefit. If this is the case for rhizobia, then one might uncover features of the symbiosis that reflect this earlier pathogenic state. For example, as with plant pathogens, it is now generally assumed that rhizobia actively suppress the host immune response to allow infection and symbiosis establishment. Likewise, the host has retained mechanisms to control the nutrient supply to the symbionts and the number of nodules so that they do not become too burdensome. The open question is whether such events are strictly ancillary to the central symbiotic nodulation factor signaling pathway or are essential for rhizobial host infection. Subsequent to these early infection events, plant immune responses can also be induced inside nodules and likely play a role in, for example, nodule senescence. Thus, a balanced regulation of innate immunity is likely required throughout rhizobial infection, symbiotic establishment, and maintenance. In this review, we discuss the significance of plant immune responses in the regulation of symbiotic associations with rhizobia, as well as rhizobial evasion of the host immune system.

Contents

INTRODUCTION

Despite the presence of numerous microbes in nature, only a few species can establish compatible interactions with their hosts to cause either pathogenic or mutualistic symbiosis. Indeed, most of the mutualistic interactions between rhizobia and legumes are tightly regulated and exhibit species specificity. An increasing number of studies have demonstrated that plant innate immunity plays a critical role in mediating genotype-specific nodulation (58, 131, 153, 163). This restriction of host range by plant immunity resembles well-documented, race-specific interactions between plants and pathogens. Successful nodule formation requires the exchange of specific chemical signals, which results in specific changes in both host and symbiont that facilitate plant infection and nodule formation (48, 120). Ultimately, the rhizobia take up residence as intracellular symbionts inside infected nodule cells. The intimacy of this interaction and signal exchange initiates key recognition steps by both plant hosts and bacterial symbionts and raises the question of why such an infecting bacterium is not recognized as a foreign invader and dealt with by the host innate immune system.

Nodule formation is generally assumed to begin with the exchange of chemical signals between the rhizosphere-localized rhizobia and the compatible host root. These signals include flavonoids, which are derived from the general phenylpropanoid pathway in the plant host (93). Each rhizobial species is adapted to recognize the repertoire of flavonoids made and secreted by its compatible host. Symbiont recognition of the flavonoids results in induction of the bacterial nodulation genes whose primary role is to synthesize the lipo-chitooligosaccharide (LCO) nodulation (Nod) factor (17, 35, 57) (for ease of reference, **Table 1** lists the abbreviations used in this review). Again, the chemistry of the Nod factor formed is tuned to recognition by the compatible host (52, 97). However, although initial papers describing this type of signal exchange stressed the chemical specificity of these signals, subsequent papers have relaxed this interpretation or—as in the case of

Table 1 Abbreviations used in this review

Abbreviation	Full term or name
BAK	BRI1-ASSOCIATED RECEPTOR KINASE
BIK	BOTRYTIS-INDUCED KINASE
CC	Coiled-coil
CEBiP	Chitin elicitor–binding protein
CERK	CHITIN ELICITOR RECEPTOR KINASE
CO	Chitooligosaccharide
DAMP	Damage-associated molecular pattern
DNF	Defective in nitrogen fixation
DORN	DOES NOT RESPOND TO NUCLEOTIDES
eATP	Extracellular ATP
EDS	ENHANCED DISEASE SUSCEPTIBILITY
EF-Tu	Elongation factor Tu
EFR	EF-Tu RECEPTOR
EPR	Exopolysaccharide receptor
ETI	Effector-triggered immunity
FLS	FLAGELLIN-SENSING
IRLC	Inverted-repeat-lacking clade
LCO	Lipo-chitooligosaccharide
LRR	Leucine-rich repeat
LYK	LYSIN MOTIF RECEPTOR-LIKE KINASE
LYM	LYSM-CONTAINING RECEPTOR PROTEIN
LYP	LysM-containing protein
LysM	Lysin motif
MAMP	Microbe-associated molecular pattern
MTI	MAMP-triggered immunity
NAD	NODULES WITH ACTIVATED DEFENSE
NBS	Nucleotide-binding site
NCR	Nodule cysteine-rich
NFR	Nod factor receptor
NLR	NOD-like receptor
Nod	Nodulation
NOD	Nucleotide-binding oligomerization domain
Nop	Nodulation outer protein
PAMP	Pathogen-associated molecular pattern
PBS	AVRPPHB SUSCEPTIBLE
PR	Pathogenesis-Related
PRR	Pattern recognition receptor
RBOH	Respiratory burst oxidase homolog
RIN	RPM1-INTERACTING PROTEIN
RLK	Receptor-like kinase
ROS	Reactive oxygen species

(*Continued*)

Table 1 (*Continued*)

Abbreviation	Full term or name
RPM	RESISTANCE TO *P. SYRINGAE* PV. *MACULICOLA*
RPS	RESISTANT TO *P. SYRINGAE*
RRS	RESISTANT TO *RALSTONIA SOLANACEARUM*
RSD	REGULATOR OF SYMBIOSOME DIFFERENTIATION
SUMO	Small ubiquitin-like modifier
SymCRK	CYSTEINE-RICH RECEPTOR-LIKE KINASE
TAL	Transcription activator–like
TIR	Toll/interleukin-1 receptor
Xop	*Xanthomonas* outer protein

Nod factor sulfation in the *Sinorhizobium meliloti*–alfalfa interaction, for example—have failed to find a mechanistic explanation now that the molecular details of Nod factor receptor function are known (52, 82). Hence, one could argue that a more nuanced mechanism may explain the observed host specificity and that the plant immune system may play a significant role in this interaction. The suggestion is that mechanisms controlling host range in plant pathogens may be a significant component of what ultimately regulates legume-rhizobium specificity.

One can assume that successful establishment of the legume-rhizobium symbiosis depends on how the bacteria adapt themselves to the special conditions on and in legume roots. Nodule development begins after the rhizobia attach to the root hairs. Compatibility is then demonstrated by root hair curling; infection thread formation, by which the rhizobia gain entry to the root cortex; cortical cell division and differentiation, leading to the formation of the nodule primordium; and, eventually, release of the rhizobia from the infection thread into the infected cells of the nodule. The nodule is a true organ that shows tissue differentiation; for example, infected cells that house the bacteroids (the symbiotic form of the bacteria) are found adjacent to the infected cells that function in nitrogen assimilation.

In a mature nodule, the bacteroids are confined within a membrane-bound vesicle called the symbiosome (130). The number of such symbiosomes, the shape and development of the nodule, nodule metabolism, and ultimately nodule senescence are tightly controlled by the host plant, although they can be perturbed by specific bacterial mutations (e.g., those inducing early senescence). Publications that appeared decades ago had already pointed to a connection between plant immunity and nodule senescence—for example, by showing that phytoalexins are induced when soybean nodules senesce (79).

INNATE IMMUNITY TRIGGERED BY MICROBE-ASSOCIATED MOLECULAR PATTERNS

Microbe-Associated Molecular Patterns and Pattern Recognition Receptors

Microbe-associated molecular patterns (MAMPs), also termed pathogen-associated molecular patterns (PAMPs), are conserved motifs present on essential components of a pathogen that plants can recognize, inducing innate immunity pathways (7). This type of immunity is termed MAMP-triggered immunity (MTI). [The term MAMP was suggested as an alternative to PAMP because nonpathogens have the same conserved motifs (4), and is used here because of the consideration of plant interactions with nonpathogenic rhizobia.] In the past decade, impressive research

progress has been made regarding MAMP recognition and MTI, starting from the chemical identification of a variety of MAMPs (102, 177). The best-characterized MAMPs include bacterial flagellin (or the active-epitope flagellin-based 22-amino-acid peptide, flg22), elongation factor Tu (EF-Tu) (or the active-epitope EF-Tu-based 18-amino-acid peptide, elf18), sulfated RaxX, bacterial lipopolysaccharides and peptidoglycans, and fungal chitooligosaccharides (COs) (11, 102, 128).

Perhaps surprisingly, relatively little has been done to explore rhizobia for possible MAMPs. However, rhizobial flagellin appears to lack the flg22 epitope required for FLAGELLIN-SENSING 2 (FLS2)–mediated MAMP activity, which may be one means by which the bacteria avoid host detection as a pathogen (99). In contrast to pathogens, the rhizobial MAMPs identified so far (including flagellin, lipopolysaccharides, and peptidoglycans) do not trigger MTI in their hosts (99, 151). There are some reports of uncharacterized MAMP activity derived from rhizobia (99), and even Nod factors show some weak MAMP activity (33).

Plants sense MAMPs through membrane-localized receptors (40, 177). These pattern recognition receptors (PRRs) directly bind their elicitors, resulting in rapid changes in the cell (102). PRRs are either receptor-like kinases (RLKs), which have an extracellular domain for ligand binding and an intracellular kinase domain for signal transduction, or receptor-like proteins, which lack a significant intracellular domain. Receptor-like proteins are required to form a hetero-receptor complex with RLKs or with receptor-like cytoplasmic kinases to transduce the signal to downstream target proteins. The flagellin receptor FLS2 has a leucine-rich repeat (LRR) extracellular domain and an active intracellular kinase domain (55). Apart from a diverged intracellular domain, it resembles the mammalian Toll-like receptors that recognize MAMPs and trigger immune responses to invading pathogens (56). FLS2 senses bacterial flagellin with its coreceptor, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (21, 55, 68). Both kinases are transphosphorylated, enabling downstream signaling mediated by BOTRYTIS-INDUCED KINASE 1 (BIK1) (101, 172), mitogen-activated protein kinases (3), or calcium-dependent protein kinases (14).

A receptor named EF-Tu RECEPTOR (EFR), which is also an LRR-RLK that requires BAK1, recognizes the MAMP EF-Tu (134, 178). By contrast, fungus-derived COs and the structurally similar peptidoglycans are recognized by the lysin motif (LysM) PRRs (69). In the case of *Arabidopsis*, the CO receptor is most likely a heterotetramer composed of the LysM proteins *Arabidopsis thaliana* CHITIN ELICITOR RECEPTOR KINASE 1 (AtCERK1) and *Arabidopsis thaliana* LYSIN MOTIF RECEPTOR-LIKE KINASE 5 (AtLYK5) (18). This is of particular interest because the legume LCO receptor is also composed of two LysM proteins, Nod factor receptor 1 (NFR1) and NFR5 (using the *Lotus japonicus* nomenclature) (106, 127). In both of these situations, one protein (CERK1 or NFR1) has an active intracellular kinase domain, whereas the second protein (LYK5 or NFR5) has an inactive intracellular kinase domain (18, 105). Hence, although both proteins appear to be necessary to recognize the ligand, signaling might occur only through the protein with an active kinase.

Chitin-Triggered Immunity in Plants

Chitin, the major component of fungal cell walls, is a long-chain polymer of β1–4-linked *N*-acetylglucosamine. Plants secrete chitinases that can degrade this polymer into shorter-chain soluble COs (64). Studies in a variety of plants have shown that hexameric to octameric COs (i.e., those with a degree of polymerization ≥6) are potent MAMP elicitors of the plant immune response, whereas shorter-chain COs elicit at most a weak response (33, 63, 90, 171). It is now clear from studies in rice, *Arabidopsis*, and other plants that a key receptor for long-chain COs is

a protein complex formed by CERK1 and its homologs. Initially, binding of COs to AtCERK1 was thought to induce homodimerization, allowing binding of the long-chain COs across the two subunits and inducing the activity of the intracellular kinase domain (95, 113, 160). However, in *Arabidopsis*, Cao et al. (18) demonstrated that this homodimerization requires AtLYK5, which appears to have a higher affinity for COs. The model is that COs bind to AtLYK5, which exists in the cell as a preformed homodimer, leading to its association with AtCERK1 and inducing its dimerization, which ultimately activates the kinase domain. Thus, a heterotetramer of AtCERK1 and AtLYK5 appears to be the most likely quaternary structure for the MAMP CO receptor (18).

The situation in rice may be somewhat similar in that OsCERK1 does not appear to bind chitin and instead interacts with the *Oryza sativa* chitin elicitor–binding protein (OsCEBiP), which has a high affinity for COs (77, 138). One long-chain CO is sandwiched between two monomers of OsCEBiP, which has only an extracellular lysin domain (65). It is not clear whether an OsCERK1-OsCEBiP heterotetramer forms, but signaling does occur through OsCERK1, because OsCEBiP lacks an intracellular kinase domain (65, 138). Although *Arabidopsis* has three CEBiP-like proteins, they are not required for chitin-triggered innate immunity (139, 159), but two of these proteins do play a role in peptidoglycan recognition, again in association with AtCERK1 [*Oryza sativa* LysM-containing protein 4 (OsLYP4) and OsLYP6 in rice, and *Arabidopsis thaliana* LYSM-CONTAINING RECEPTOR PROTEIN 1 (AtLYM1) and AtLYM3 in *Arabidopsis*] (92, 165). Another of these CEBiP-like proteins, AtLYM2, functions independently of AtCERK1 and mediates chitin-induced suppression of intracellular flux through plasmodesmata (44). This may have some relevance to nodulation because such movement could play a significant role in nodule formation and function. However, such a role for a LysM protein in nodulation has not been reported.

The above discussion of MAMP CO recognition is relevant to this review because NFRs are also composed of two LysM RLKs. Indeed, Liang et al. (90) proposed that CO recognition, which appears to be widely conserved among plants, including primitive plants, is likely the evolutionary progenitor of LCO recognition, which is confined primarily to legumes.

Nod Factor Signaling

Nodulation can sometimes occur in the absence of Nod factor recognition (see the section titled Characterized Effectors in Rhizobia and Nod Factor–Independent Nodulation). However, in the best-studied examples, Nod factors are essential for the initiation of symbiosis with legumes. The core structure of the Nod factors is identical to that of chitin, but with a degree of polymerization of approximately 3–5. Nod factors are LCOs because they are modified with a fatty acyl side chain and, in most cases, a variety of other substituents (sulfate, acetyl, fucosyl, carbomyl, etc.), depending on the rhizobial species (29). These substitutions and the nature of the fatty acyl side chain appear to contribute to host specificity, although the mechanism is unclear (45, 124). For example, the original idea was that the chemistry of the Nod factor determines its binding to the NFRs (i.e., a lock-and-key model). However, although more recent studies have supported some specificity of NFR binding to the LCO, they cannot be strictly interpreted within this model. For example, no NFR has been found in alfalfa that shows selectivity toward a sulfated LCO, although such sulfation appears to be essential to support rhizobial infection (60). Findings from other studies have also run counter to the lock-and-key model. For example, some pea cultivars require an acetylated LCO, but this requirement can be removed by fucosylation at the same location (100).

Based on the available genome sequences, legumes appear to contain more LYK-encoding genes than nonleguminous plants do. For example, the *L. japonicus* genome contains 17

LYK-encoding genes (96), whereas the *Arabidopsis* genome contains only 5 (175, 176). As mentioned above, plant LYK proteins can be divided into two major clades, one that has kinase activity (e.g., NFR1 and CERK1) and one that lacks kinase activity because of key missing amino acids in the conserved kinase domain (e.g., NFR5 and LYK5) (175, 176). The phylogeny of LysM proteins supports the notion that Nod factor recognition evolved from the more widespread CO recognition system. This idea is also supported by more recent reports suggesting that CERK1 and NFR proteins can serve a dual function in either symbiosis or pathogen response (114, 174). For example, a microarray study showed that long-chain CO treatment induces symbiosis gene expression in *L. japonicus* (118). COs and LCOs are also produced by mycorrhizal fungi and are apparently essential for the establishment of this plant symbiosis (146).

In rice, OsCERK1, identified through its role in pathogen recognition, also plays the key role of recognizing LCOs to support mycorrhizal infection (114, 174). Consistent with this functional and evolutionary connection between CO and LCO recognition, expression of a chimeric receptor of both LjNFR1-AtCERK1 and LjNFR5-AtCERK1 in *atcerk1-2* mutant plants resulted in an LCO-triggered immune response in *Arabidopsis*, whereas expression of chimeric receptors of both OsCERK1-LjNFR1 and OsCEBiP-LjNFR5 in lotus *nfr1-1* and *nfr5-2* mutant plants resulted in CO-induced symbiotic responses (164). This significant overlap in the function of the CO and LCO receptors also brings into question whether NFR recognition of LCO *stricto sensu* is sufficient to explain to a significant extent the host specificity exhibited in the legume-rhizobium interaction.

Historically, the prevailing notion was that the LCOs are developmental signals that evolved to promote symbiotic development in either rhizobia or mycorrhizae. Therefore, it was surprising to find that adding either LCOs or short-chain COs to *Arabidopsis* seedlings resulted in a significant suppression of the immune response to MAMP elicitation (89). The suppressive effect of COs or short-chain COs on plant immunity appeared to be conserved across a wide variety of plant species, including both dicots and monocots. Although short-chain COs act similarly to LCOs, the concentration of short-chain COs needed for this effect was ten times that found for LCOs, suggesting some specificity for LCOs. Interestingly, in soybean, suppression of innate immunity directed by COs or LCOs was independent of NFR1 and NFR5. However, AtLYK3, an active-kinase LYK, is necessary for the suppressive effect of COs or LCOs in *Arabidopsis* (89). Consistent with this, *Atlyk3* mutant plants were more resistant to the pathogens *Botrytis cinerea* and *Pectobacterium carotovorum* than wild-type *Arabidopsis* plants were (122).

A study by Liang et al. (89) suggested that the mechanism of this suppressive effect of COs and LCOs involves a reduction in the concentration of PRR proteins (as exemplified by FLS2) at the plasma membrane (**Figure 1**). The authors postulated that further degradation of chitin from long-chain COs, which elicit defense, to short-chain COs, which suppress defense, could be an avoidance mechanism that pathogens use to suppress immunity. They also suggested that the ability of LCOs to suppress immunity may have emerged during a pathogenic stage of plant-rhizobium evolution, before this molecule took on its role of inducing the plant development and differentiation processes necessary for nodulation. This is speculation but is consistent with the more recent finding that Nod factor–independent nodulation requires type III secretion effectors, which are also known to suppress immunity (121) (see the section titled Characterized Effectors in Rhizobia and Nod Factor–Independent Nodulation).

Microbe-Associated Molecular Patterns from Rhizobia

Although plant defense pathways have not been well studied during nodulation, the data suggest that they are initially activated during the rhizobial infection process (**Figure 1**). For example,

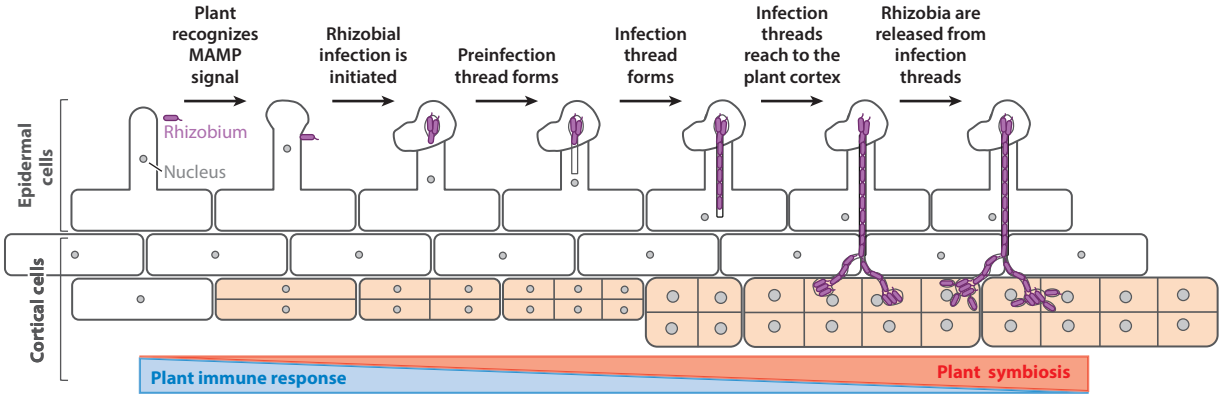

Figure 1

The balance between immune response and symbiosis during nodule development. Rhizobial MAMPs activate a transient immune response (*blue*) in plants termed MTI. Rhizobia then escape MTI by producing Nod factors to suppress MTI. To successfully invade legume roots, rhizobia secrete effectors to modulate host immunity, allowing the establishment of symbiosis (*red*). Abbreviations: MAMP, microbe-associated molecular pattern; MTI, MAMP-triggered immunity; Nod, nodulation.

the expression of a large number of plant immunity-related genes was induced within 12 h of *Bradyrhizobium japonicum* inoculation in soybean, but the expression levels gradually diminished to background levels within 24 h of inoculation (91). *L. japonicus* and *Medicago truncatula* exhibited similar changes, with expression of defense-related genes induced shortly after treatment with rhizobia and then reduced to resting levels after the establishment of symbiosis (76, 143). Indeed, treatment of *L. japonicus* plants with a cell suspension derived from its compatible symbiont, *Mesorhizobium loti*, activated defense responses similar to those that occur following treatment with flg22 (99). For example, such treatment caused an increase in ethylene production and mitogen-activated protein kinase phosphorylation, consistent with the notion that MTI is triggered by a component of the rhizobial culture (99). Therefore, rhizobia do have the ability to induce MTI, and the data suggest that such a response does occur early in the infection process (**Figure 1**). The assumption is that this response is actively suppressed by the compatible symbiont through action of the Nod factor, likely in conjunction with other mechanisms. For example, earlier studies showed that the levels of salicylic acid were significantly elevated in legume roots inoculated with rhizobia that were unable to produce LCOs, but not in roots inoculated with an LCO-producing wild-type strain (109). Salicylic acid is a well-known secondary signal involved in plant disease resistance and strongly inhibits nodulation (143).

The limited searches for active MAMPs in rhizobia have shown that the common MAMPs (**Table 2**)—including flagellin, lipopolysaccharides, exopolysaccharides, β-glucans, and K-antigen-type polysaccharides—appear to lack MAMP activity (58). A recent study found that a peptidoglycan-modifying enzyme in *Bradyrhizobium* strains is required for bacteroid differentiation in *Aeschynomene* species, suggesting that peptidoglycans also play a role in symbiosis (62). Several polysaccharide types are required for successful rhizobial infection (154), indicating that recognition mechanisms for these components likely do exist in legumes. Recent studies have shed some light on the role of exopolysaccharides in the nodulation process. *S. meliloti* and *Rhizobium leguminosarum* mutants defective in exopolysaccharide biosynthesis are blocked early in nodulation, at the stage of infection thread initiation and elongation (142). Kawaharada et al. (81) identified a LYK receptor in *L. japonicus*, exopolysaccharide receptor 3 (EPR3), as the receptor that mediates the recognition of exopolysaccharides during the rhizobial infection process.

Table 2 Rhizobial symbiotic signals and plant proteins/mutants involved in regulating immunity and symbiosis

Rhizobial signal	Plant protein/mutant	Symbiotic response	Immune response	Reference(s)
Nod factor	AtLYK3	Initiation of symbiosis signaling	Suppression of MTI	89
EPS	EPR3 (AtLYK3 homolog)	Rhizobial entry	Successful infection, which may involve suppression of MTI	81
LPS		Establishment of successful symbiosis	Structural modification so that LPSs are not detected by LPS receptors	128
NopL		Suppression of premature nodule senescence	Inhibition of immune response	51
NopM		Promotion of nodule initiation	Suppression of MTI	167
NopP		Inhibition of symbiosis	Upregulation of *PR1* gene expression	141
NopT		Promotion of symbiosis	Induction of cell death	30
	CERK1	Establishment of successful symbiosis	Production of chitin receptors, which induces MTI	114, 174
	RBOH	Rhizobial infection and nodule development	ROS production	2
	NAD1	Failure of bacteroid differentiation	Defense-like responses and necrosis	160
	DNF2	Failure of bacteroid differentiation	Defense-like responses and premature nodule senescence	15
	SymCRK	Nonfunctional nodules and failure of bacteroid differentiation	Defense-like responses and premature nodule senescence	9
	RSD	Incomplete symbiosome and bacteroid differentiation	Production of brown pigment in mutants, indicating an immune response	140
	NCR169	Early senescence and failure of bacteroid differentiation	Polyphenol accumulation and inhibition of bacterial growth	71
	NCR211	Failure of bacteroid differentiation	Polyphenol accumulation and inhibition of bacterial growth	84
	Rj2 (Rfg1)	Genotype-specific nodulation	Expression of resistance proteins that control soybean genotypic nodulation via ETI	168
	Rj4	Genotype-specific nodulation	Expression of PR5-related proteins, which determine genotype-specific nodulation via ETI	126
	Medicago NF0438 mutant	Impairment of rhizobial release from infection threads to epidermal cells	Accumulation of brown pigment	125
	Medicago NF0359 mutant	Uncontrolled cell division in the nodules and absence of root hair curling	Accumulation of brown pigment	125
	Medicago NF2853 mutant	Absence of root hair deformation	Accumulation of brown pigment	125
	Medicago NF1859 mutant	Failure of rhizobial entry into the epidermis	Accumulation of brown pigment in infection sites	125

(*Continued*)

Table 2 (*Continued*)

Rhizobial signal	Plant protein/mutant	Symbiotic response	Immune response	Reference(s)
	Medicago NF0673 mutant	Failure of rhizobial entry into the epidermis	Accumulation of brown pigment in infection sites	125
	Medicago NF2811 mutant	Failure of rhizobial entry into the epidermis	Accumulation of brown pigment in infection sites	125
	Medicago NF5654 mutant	Excessive development of vascular tissue and uncontrolled cortical cell division	Accumulation of brown pigment	125

Abbreviations: AtLYK3, *Arabidopsis thaliana* LYSIN MOTIF RECEPTOR-LIKE KINASE 5; CERK1, CHITIN ELICITOR RECEPTOR KINASE 1; DNF2, defective in nitrogen fixation 2; EPR3, exopolysaccharide receptor 3; EPS, exopolysaccharide; ETI, effector-triggered immunity; LPS, lipopolysaccharide; MTI, microbe-associated molecular pattern (MAMP)–triggered immunity; NAD1, NODULES WITH ACTIVATED DEFENSE 1; NCR, nodule cysteine-rich; Nod, nodulation; Nop, nodulation outer protein; PR, Pathogenesis-Related; RBOH, respiratory burst oxidase homolog; ROS, reactive oxygen species; RSD, REGULATOR OF SYMBIOSOME DIFFERENTIATION; SymCRK, CYSTEINE-RICH RECEPTOR-LIKE KINASE.

Phylogenetic analysis showed that EPR3 is the closest homolog of *Arabidopsis* LYK3, which was previously implicated in the ability of LCOs to suppress defense responses (89). It is therefore possible, although untested, that EPR3 also mediates immune suppression by exopolysaccharides.

Extracellular ATP: A Central Molecule Involved in Plant-Microbe Interactions

In addition to MAMPs, another class of molecules involved in plant biotic stress resistance are the damage-associated molecular patterns (DAMPs). DAMPs are molecules derived from endogenous host substances that are released following physical damage and whose recognition leads to defense responses designed to protect and heal (19). A well-studied DAMP in animals, including mammals, is extracellular ATP (eATP), which is released following tissue damage and aids in the induction of inflammation and other defense and healing responses (145). Targeting of human purine signaling pathways involved in eATP recognition, which is mediated by G protein–coupled receptors, represents a multibillion-dollar pharmaceutical industry [e.g., Plavix, which targets the P2Y12 purine receptor involved in clotting (70)].

Several early reports indicated that eATP is present in plants and acts as a DAMP in addition to playing a general signaling role in plants (147). Some of these studies suggested a possible role for purine signaling in the nodulation process. An early indication of this was the finding that the ecto-apyrase GS52 is induced early during the soybean nodulation process (59, 148). An apyrase is an enzyme that hydrolyzes ATP but with no obvious capture of the released energy; an ecto-apyrase is an apyrase in which the catalytic domain is located extracellularly, suggesting action on an extracellular substrate (e.g., eATP). Indeed, ectopic expression of the soybean ecto-apyrase GS52 enhanced rhizobial infection and nodulation in *L. japonicus*, and inhibition of ecto-apyrase activity by treatment with antibody or by gene silencing resulted in a reduction in nodulation (59). Finally, treatment of soybean roots with ADP but not ATP increased nodule numbers after inoculation by the compatible symbiont *B. japonicum* (59). The authors proposed a model in which homeostatic regulation of the concentration of eATP mediated by the action of the ecto-apyrase is critical to successful nodulation. Kim et al. (86) visualized the presence of eATP at the tips of legume root hairs using a cellulose binding domain–luciferase chimeric protein and found that elicitation of MAMPs (e.g., COs) significantly increased eATP levels.

The canonical G protein–coupled purine receptors in animals are absent from plants. However, Choi et al. (23) showed that a lectin RLK, DOES NOT RESPOND TO NUCLEOTIDES 1 (DORN1), is essential for eATP recognition in *Arabidopsis*. Radiolabeled ATP directly binds to the lectin domain of DORN1 with an affinity and specificity that are consistent with the physiological action of eATP. Elucidation of this receptor should allow more detailed, mechanistic studies of a possible role for eATP in the nodulation process.

Reactive Oxygen Species Signaling in Symbiosis

Production of reactive oxygen species (ROS) is a major physiological response to a variety of stresses, especially pathogens (135). For example, a strong ROS burst is usually observed within 30 min of MAMP treatment. The key enzymes involved in ROS production are the NADPH oxidases known as respiratory burst oxidase homologs (RBOHs). Indeed, *Arabidopsis* plants without RBOHD and RBOHF activity are completely impaired in MAMP-triggered ROS production and are more susceptible to pathogen infection (152). Santos et al. (132) reported that alfalfa responds to wild-type *S. meliloti* with transient ROS production, perhaps reflecting initial recognition as a pathogen.

Other evidence also supports a role for ROS and RBOHs in rhizobial infection, nodule development, and even senescence (115). For example, treatment with diphenylene iodonium, a specific inhibitor of RBOHs, prevents root hair curling and rhizobial infection (28, 123). Consistent with these findings, RNA-interference silencing of *PvRBOHB* prevented infection thread formation and nodulation in *Phaseolus vulgaris*, and ectopic expression of *PvRBOHB* promoted infection thread formation but not progression (2, 116). These results suggest that the legume host may have a mechanism for homeostatic control of ROS levels during the nodulation process. This control would be necessary because although ROS is a key element of plant disease resistance, it is also involved in cell growth (36). In *M. truncatula*, *MtRBOHB*, *MtRBOHD*, and *MtRBOHF* are highly expressed in different zones of the nodules, suggesting that ROS production is likely important throughout the nodulation process (108).

EFFECTOR-TRIGGERED IMMUNITY

In addition to MTI at the cell surface, the plant innate immune system recognizes intracellular proteins that are specifically secreted into the host cell by plant pathogens. In the case of bacterial pathogens, transfer of proteins from the bacterial to the plant cytoplasm mostly occurs directly via a type III secretion system, a needle-like structure that is evolutionarily related to the apparatus that assembles flagella (26). The secreted proteins are termed effector proteins, and the defense responses they induce are termed effector-triggered immunity (ETI) (27, 75).

Much of what is known about ETI comes from studies of interactions between plants and pathogens, which in turn led to an appreciation of effectors as pathogen-deployed virulence factors. The suite of effectors secreted by a given pathogen is a major factor determining whether the interaction with a host plant will be successful for the pathogen. Phytopathogenic bacteria invade plant tissues through openings such as wounds, stomata, and hydathodes. Not all effectors are proteinaceous: Some strains of the well-studied gram-negative bacterium *Pseudomonas syringae* exude the toxin coronatine, which opens stomata and is a molecular mimic of jasmonic acid–isoleucine, interfering with immunity by downregulating the salicylic acid immune signaling pathway against biotrophic pathogens (80, 155). Although symbiotic rhizobia invade roots mainly through root hairs, they also use effectors to combat host immunity (51, 121, 167). The number of effectors secreted varies widely across microbes. For example, the bacterial pathogen *P. syringae* secretes

approximately 30 effectors into host cells (26), whereas soybean oomycete and fungal pathogens secrete several hundred (78).

Plant-encoded resistance gene products detect effectors secreted from potential pathogens, leading to a robust immune response (40, 75). Several interactions between effectors and resistance proteins have been identified, and genome sequencing has identified additional putative effectors. Host modes of recognition of pathogen effectors are of further interest because they can suggest how effectors manipulate the host cell, potentially leading to novel ways of engineering host resistance in agronomic crops.

The ETI response often, but not always, culminates in a hypersensitive response, a programmed cell death response that kills cells to halt biotrophic pathogens (22). Importantly, the hypersensitive response is not an effective resistance mechanism against necrotrophic pathogens that feed on dead tissue (111). Because a constitutively heightened immune response can be detrimental to plant health and reproduction, evolution has selected for specific resistance responses to be initiated only when the plant detects a pathogen. In response to ETI, pathogens can lose the effector that enables the plant to detect them, mutate the effector to maintain virulence while losing host resistance recognition specificities, or acquire new effectors through horizontal gene transfer or evolution to overcome the host immune response.

Effector-Triggered Immunity in Plant Pathogen Responses

Plant resistance proteins share homology with the nucleotide-binding oligomerization domain (NOD)–LRR proteins in mammals. These NOD-like receptor (NLR) proteins are further divided into two subclasses: those with a coiled-coil (CC) domain at the N terminus [the CC–nucleotide-binding site (NBS)–LRRs] and those with a Toll/interleukin-1 receptor (TIR) motif at the N terminus (the TIR-NBS-LRRs), which share sequence similarities with the Toll-like receptors in animals (110). Resistance proteins are usually highly specific in the effectors they recognize, an observation that was generalized by Flor (46) in his gene-for-gene hypothesis. Perhaps the simplest way for this resistance specificity to occur is by the resistance protein directly binding the pathogen effector protein. One of the first defined examples of this was in rice, in which the resistance protein Pi-ta confers resistance to the rice blast fungus *Magnaporthe grisea* by directly binding the AvrPita protein (74). Another well-studied example is from flax, in which effector products of the polymorphic *AvrL567* genes from the flax rust fungus *Melampsora lini* directly bind to and are recognized by the flax NLR proteins encoded by the *L* alleles *L5*, *L6*, and *L7* (39).

Resistance protein signaling can also be activated indirectly. The guard model, for example, postulates that several resistance proteins do not detect the direct action of effectors, but rather sense effector action through protein intermediates within the resistance protein complex (32). An example of this is when an effector targets a host protein that is guarded by the resistance protein: The resistance protein senses the modification of the guarded host protein by the effector and activates immune response signaling.

One well-studied example of the guard model in *Arabidopsis* is AvrRpm1/AvrB-triggered immunity through RESISTANCE TO *P. SYRINGAE* PV. *MACULICOLA* 1 (RPM1), a membrane-localized resistance protein (104). AvrRpm1 induces phosphorylation of RPM1-INTERACTING PROTEIN 4 (RIN4), another plasma membrane–localized protein that serves as a negative regulator of defense in the absence of ETI. In the case of AvrB, the effector causes the RPM1-interacting protein kinase to phosphorylate RIN4 (94), which activates RPM1 and triggers ETI. Another resistance protein, RESISTANT TO *P. SYRINGAE* 2 (RPS2), also functions through RIN4; in this interaction, AvrRpt2 cleaves RIN4, which leads to the activation of RPS2 (5, 103).

In some cases, resistance proteins guard host proteins that have evolved structural similarities to effector targets in the cell. Effectors targeting host proteins are trapped by the decoy protein, activating defense. An example of this model is the targeting and cleavage of the *Arabidopsis* serine/threonine protein kinase AVRPPHB SUSCEPTIBLE 1 (PBS1) by the membrane-localized *P. syringae* effector AvrPphB, which activates the resistance protein RPS5 (1, 137). Kim et al. (85) recently showed that this activation of RPS5 can be used to engineer novel host resistance against cross-kingdom pathogens. The fact that *pbs1* mutants have no detrimental host phenotypes in the absence of AvrPphB makes PBS1 a good candidate to manipulate for engineered resistance. Alterations to the cleavage site of PBS1 by inserting five or seven amino acids can activate RPS5; this NLR activation results from a conformational change in PBS1 independent of proteolytic cleavage (38). Modifying the PBS1 bait to respond to different pathogen effectors widens the specificity of the NLR to a multitude of effectors from different pathogens, hypothetically leading to novel engineered disease resistance in crops (85).

Another example of the decoy model is the recently characterized RPS4–RESISTANT TO *RALSTONIA SOLANACEARUM* 1 (RRS1) pair, which functions in triggering ETI through an integrated decoy within RRS1. RPS4 and RRS1 are resistance proteins with the structure of TIR-NBS-LRRs, the class of resistance proteins that additionally requires the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) protein to function (37, 50). Additionally, RRS1 codes for a WRKY transcription factor domain at its C terminus. The effector PopP2 from *Ralstonia solanacearum* is secreted into host cells, where it serves as an acetyltransferase, acetylating host proteins to the benefit of the pathogen (88, 133, 150). Among the targets for acetylation are WRKY transcription factors at residues critical for DNA binding, and PopP2 acetylates corresponding residues in the integrated WRKY domain (the decoy WRKY) of RRS1. This modification of RRS1 activates RPS4, which subsequently works against the pathogen. RRS1 is encoded in the genome in a head-to-head orientation with *RPS4*.

RPS4 and RRS1 thus work as a protein pair to trigger resistance (12, 119). The current model suggests that RRS1 keeps RPS4 in an inactive "off" state, and following effector action on RRS1, RPS4 can be switched to an active "on" state, leading to a robust immune response. The effector proteins that target host WRKY transcription factors important to the defense response are intercepted by the decoy WRKY domain in RRS1, leading to activation of RPS4 and subsequent downstream ETI-triggered responses. In essence, the guard model helps to explain how a relatively small number of resistance proteins can detect numerous pathogen effectors (31, 49).

Characterized Roles of Pathogenic Effectors

Effectors have numerous roles, including reprogramming host cells in order to suppress MTI and convert host cells into a nutrient source for the pathogen (168). Suppressing MTI allows the pathogen to successfully colonize the host. As described above, both pathogenic and beneficial microbes contain highly conserved MAMPs, suggesting that suppression of MTI is critical to both mutualism and pathogenesis. Bacterial effector proteins have evolved to function in eukaryotic plant cells, and studies in the past few years have elucidated the biochemical functions of specific effectors.

The transcription activator–like (TAL) effectors from various *Xanthomonas* species function as transcription factors, binding to the promoter regions of genes and regulating their transcription to the benefit of the pathogen (13). Some TAL effectors bind to promoters of SWEET sugar transporters in rice and cassava, an example of plant pathogens hijacking host machinery to give the pathogen a steady nutrient source (20, 25). A few resistance genes directly bind TAL effectors in their promoter regions and initiate a defense response mediated by the encoded downstream

resistance protein. One example of these so-called executor genes is pepper *Bs3*, which recognizes AvrBs3 from *Xanthomonas campestris* pv. *vesicatoria* (129, 157). Executor genes trap the TAL effectors that specifically bind DNA sequences in their promoter regions, which could lead to the synthetic cloning of executors against identified TAL effectors (173). TAL effectors have also been used as genome editing tools. By fusing a TAL DNA-binding domain to a cleavage domain of a restriction enzyme, researchers can cut specific parts of the genome to silence a gene or introduce a new DNA fragment into the genome (24). Alternatively, fusion of a TAL DNA-binding domain to a transcriptional activator or repressor can alter transcription of a targeted gene (117).

Other effectors that interfere with transcription include the above-mentioned acetyltransferase PopP2. PopP2 acetylates host WRKY proteins, presumably to inhibit their binding to host target promoters and thereby promote the virulence of the pathogen on susceptible plants. PopP2 can dislodge a subset of WRKY transcription factors from host DNA, strongly suggesting that it directly interferes with host transcription (88).

Additionally, the *X. campestris* pv. *vesicatoria* effector *Xanthomonas* outer protein D (XopD) functions as a small ubiquitin-like modifier (SUMO) protease that desumoylates the ethylene response factor SIERF4 in tomato, suppressing ethylene production, which is required for immunity to *X. campestris* pv. *vesicatoria* (83). Importantly, the symbiotic bacterium *Bradyrhizobium elkanii* has a XopD homolog with putative ETI function in soybean, further supporting the hypothesis that effectors secreted from symbiotic bacteria function in suppression of host MTI (43).

Suppressing MTI is a critical first step for a successful pathogen or symbiont. Distinct effector proteins from pathogenic bacteria directly target the PRR MAMP receptors, contributing to the virulence of the pathogen. An example is the *P. syringae* effector AvrPto, which targets and binds to FLS2 and BAK1 (a signaling partner common to several PRRs), thereby disrupting MTI (136, 166), whereas AvrPtoB targets AtCERK1 (53). AvrPto can betray the pathogen on resistant tomatoes expressing the Pto-encoding gene, leading to ETI and unsuccessful colonization. The major implication of MTI for symbiosis is that beneficial microbes also contain these highly conserved patterns, which may be detrimental to successful interaction. However, similarly to how effector proteins secreted from phytopathogens can suppress MTI responses, current data suggest that effectors from beneficial microbes can also suppress these responses, providing a favorable environment for symbiosis (58). Conversely, ETI could be detrimental to successful symbiosis: Certain soybean cultivars with the *Rj4* gene might recognize the above-mentioned rhizobial effector with homology to XopD, triggering ETI and halting symbiosis (170).

Characterized Effectors in Rhizobia and Nod Factor–Independent Nodulation

The intimacy of the legume-rhizobium symbiosis suggests the need for detailed and constant communication between the two partners to promote the infection process, allow development, and maintain the symbiosis. In addition to flavonoids, MAMPs, and LCO signals, rhizobia can produce and inject effector proteins into the plant cytosol via type III, type IV, or type VI secretion systems (120). The effectors presumably act similarly to those of plant pathogens to promote the infection process. In a few cases, resistance proteins can prevent nodulation in specific strains, presumably mediated by effector recognition (170) (**Figure 2**).

The well-documented symbiosis pathway between legumes and rhizobia starts with flavonoid-induced synthesis of Nod factors in rhizobia. However, the symbiosis between photosynthetic *Bradyrhizobium* species and some *Aeschynomene* species is independent of Nod factor signaling (54). Interestingly, Nod factor–independent symbiosis is initiated through crack entry (i.e., infection through an opening in the epidermis, such as at the emergence of a lateral root) and not through root hair infection. The presence of a Nod factor–independent pathway was also supported by

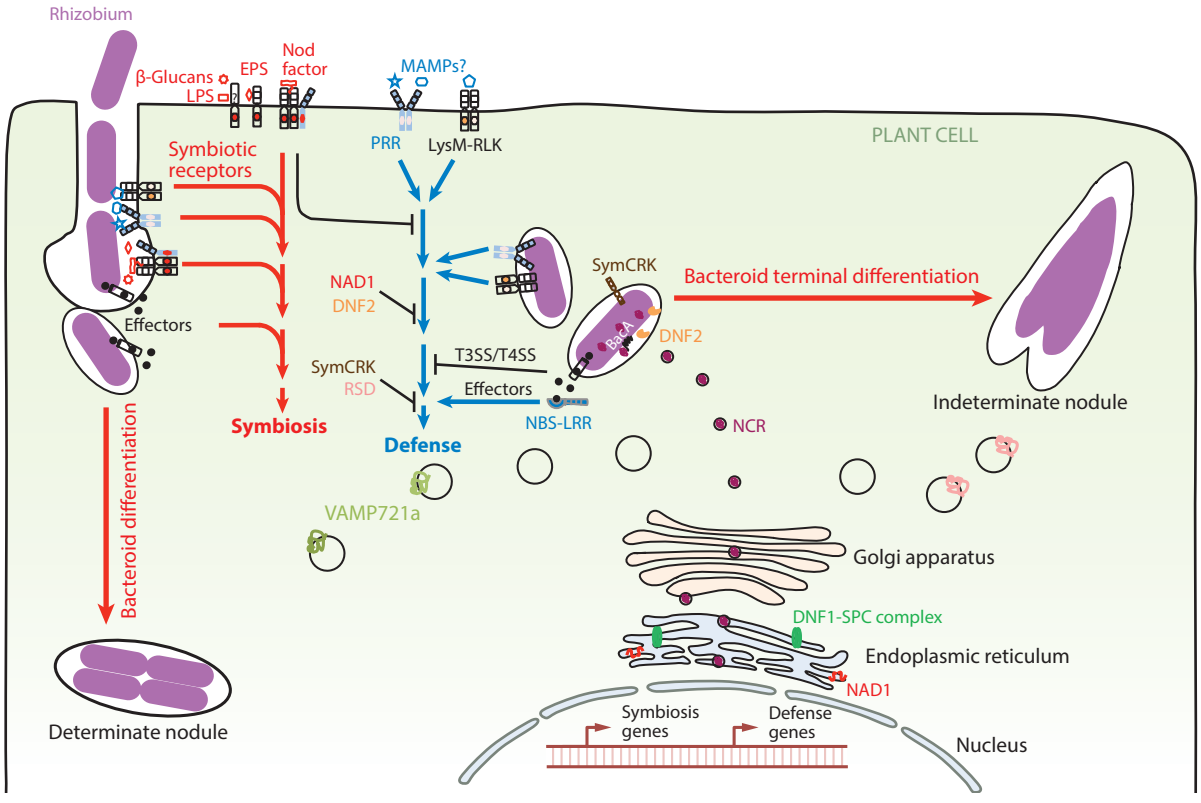

Figure 2

Overall defense response involved in symbiosis. Host PRRs recognize MAMPs from rhizobia, triggering transient immune responses in plants. NFR1 and NFR5 from legumes can detect Nod factors to initiate symbiosis signaling. During rhizobial colonization, effector proteins are secreted through the T3SS and T4SS by rhizobia to suppress or block host immunity, allowing the establishment of symbiosis. DNF2, NAD1, RSD, and SymCRK from plants are involved in suppressing host immunity, which seems to specifically inhibit terminal bacteroid differentiation. Abbreviations: DNF, defective in nitrogen fixation; EPS, exopolysaccharide; LPS, lipopolysaccharide; LRR, leucine-rich repeat; LysM, lysin motif; MAMP, microbe-associated molecular pattern; NAD1, NODULES WITH ACTIVATED DEFENSE 1; NBS, nucleotide-binding site; NCR, nodule cysteine-rich; NFR, Nod factor receptor; Nod, nodulation; PRR, pattern recognition receptor; RLK, receptor-like kinase; RSD, REGULATOR OF SYMBIOSOME DIFFERENTIATION; SPC, signal peptidase complex; SymCRK, CYSTEINE-RICH RECEPTOR-LIKE KINASE; T3SS, type III secretion system; T4SS, type IV secretion system; VAMP721a, VESICLE-ASSOCIATED MEMBRANE PROTEIN 721a.

the finding that *M. loti nod* mutants can form a symbiosis with lotus plants defective in Nod factor recognition, albeit at a very low efficiency; this infection appears to be mediated via crack entry (107).

The studies mentioned above did not directly examine the role of rhizobial effector proteins. More recently, however, Okazaki et al. (121) showed that a *B. elkanii* strain that lacks the canonical *nodABC* genes required for Nod factor synthesis is able to nodulate soybean independently of Nod factors through a mechanism that requires the type III protein secretion system. This exciting finding points to a separate, Nod factor–independent system to support rhizobial infection and nodule formation that involves a mechanism that utilizes components usually associated with ETI. This is perhaps the best evidence that the mechanisms by which plants and pathogens interact are not just ancillary to legume nodulation but may represent an ancestral link to a previous pathogenic

lifestyle that has now evolved to support nodulation in the absence of Nod factor signaling. One can further conjecture that the high efficiency of Nod factor–mediated nodulation obscures this alternative pathway except in some unique cases.

Similarly to LCO production, effector protein expression in rhizobia is induced in the presence of host-produced flavonoids, consistent with these effectors playing a specific and important role in the nodulation process (34). Effectors can be secreted through either the type III secretion system (as in *Bradyrhizobium* species) or the type IV secretion system (as in *M. loti*). Approximately ten effector proteins have clearly been identified in rhizobia (144), but more almost certainly exist, given the diversity of rhizobia and the repertoire of effectors produced by plant pathogens (**Table 2**). In the cases that have been studied in detail, rhizobial effectors appear to promote successful legume infection, similarly to the plant pathogen examples. For example, studies of the *Sinorhizobium fredii* effector proteins nodulation outer protein L (NopL) and NopM demonstrated their involvement in the inhibition of plant immunity through misregulation of the host mitogen-activated protein kinase pathway and inhibition of ROS production in plants, respectively (51, 167). Another effector protein, NopT, induces immune responses in plants (30), suggesting the presence of a cognate resistance protein. The same is likely true for the rhizobial effector NopP, because an *S. fredii nopP* mutant showed enhanced nodule formation and lower *Pathogenesis-Related 1* (*PR1*) gene expression when inoculated onto soybean (98, 141).

In addition to modulating host immunity, specific rhizobial effectors affect host range, reminiscent of the pathogen race specificity determined by interactions between effectors and resistance proteins. For example, the effector NolX from *S. fredii* USDA257 is involved in host range determination and plays a role in the early stages of nodule development (87). Rather than studying specific effectors, some studies have used bacterial mutants defective in the type III secretion system, effectively blocking all effectors; in these cases, such mutations broadened the host range to include previously incompatible hosts. The function of effectors in regulating host range is always linked with host resistance proteins or defense-related proteins (43, 169) (see the section titled Effector-Triggered Immunity in the Legume-Rhizobium Symbiosis), indicating the critical role of ETI in the legume-rhizobium symbiosis. Similarly to pathogens, significant conservation in the suite of effectors rhizobia produce is not apparent, and related strains can have distinct sets of effectors (6, 144, 158). This suggests that negative selection may have led to the loss of effectors so as not to inhibit the nodulation process or, perhaps, to better regulate host range in order to increase the efficiency of compatible interactions.

Effector-Triggered Immunity in the Legume-Rhizobium Symbiosis

The cloning of the soybean *Rj2/Rfg1* locus, which encodes a TIR-NBS-LRR-type disease resistance protein (169), clearly demonstrated that ETI can determine genotype-specific nodulation. In soybean, for example, the Williams 82 variety expressing *Rfg1* restricts nodulation with specific strains of *S. fredii* USDA257 but not with a type III secretion system mutant, suggesting a typical effector–resistance protein recognition mechanism. The specific effector acting on Rfg1 remains to be identified.

Rj4 is a dominant gene in soybean that restricts nodulation by many strains of *B. elkanii*, a common species in soils in the southern United States that exhibits low nitrogen-fixation efficiency. Some *B. elkanii* strains can also produce rhizobitoxine, which can be detrimental to plant growth (41). The negative effects of these strains are perhaps best demonstrated by the fact that soybean breeders, without directly selecting for this trait, have incorporated the *Rj4* alleles into most of the elite varieties grown in the southern United States (126). The *Rj4* locus was recently cloned and shown to encode a thaumatin-like protein from the PR5 family (67, 149). How a

thaumatin-like protein would affect the nodulation process is unknown. Incompatible interactions determined by Rj2/Rfg1 are blocked very early and do not allow infection thread formation to begin (169). Interestingly, Rj2/Rfg1- and Rj4-mediated nodulation restriction requires bacterial type III effectors (43, 169), indicating the critical role of ETI in this process.

Although nodulation restriction encoded by the *Rj2/Rfg1* and *Rj4* loci likely respond to rhizobial effectors, the soybean recessive loci *rj1* and *rj5/rj6*, which also restrict nodulation, correspond to GmNFR1α and GmNFR5α (72, 73), respectively, indicating the critical role of Nod factors in mediating genotype-specific nodulation. The protein encoded by the soybean *Rj3* locus, which also restricts nodulation by *B. elkanii* strains, remains to be identified (66).

IMMUNITY IN TERMINAL BACTEROID DIFFERENTIATION

On the basis of their morphology, nodules can be defined as either determinate or indeterminate (16). In both cases, they can be further defined by the presence of peripheral (as opposed to central) vascular tissue. Determinate nodules lack a persistent meristem, and after initial cell division, they grow primarily through cellular expansion. Indeterminate nodules, by contrast, are defined by their persistent meristem and concomitant terminal differentiation of the bacteroids.

Interestingly, this bacteroid terminal differentiation is generally restricted to legumes from the small inverted-repeat-lacking clade (IRLC), which lack a generally conserved DNA fragment in their chloroplast genomes. Bacteroids in non-IRLC legumes, such as soybean, maintain their normal bacterial size and genome content; terminally differentiated bacteroids in IRLC legumes, by contrast, are usually significantly larger and misformed relative to their free-living state. Direct evidence that these differences are controlled by plants came from a study that examined rhizobia able to infect both determinate and indeterminate hosts; the authors found large, terminally differentiated rhizobial cells only in the indeterminate hosts (47) (**Figure 2**).

Nodule Cysteine-Rich Peptides in Terminal Bacteroid Differentiation

A key difference between indeterminate nodules formed on IRLC legumes is the large abundance and diversity of cysteine-rich proteins, termed nodule cysteine-rich (NCR) peptides (112) (**Figure 2**). For example, the *M. truncatula* genome is predicted to encode more than 600 unique NCR peptides (112). The presence of the NCR proteins appears to be responsible for terminal differentiation of the bacteroids in IRLC legumes as well as their unique shape (156, 162). Indeed, previous work showed that plant peptides control terminal bacteroid differentiation, whereas these proteins seem not to be involved in determinate nodule development. A mutation in a subunit of the signal peptidase complex defective in nitrogen fixation 1 (DNF1) blocked bacteroid and symbiosome development and ectopic expression of NCR035. Challenging rhizobia with NCR035 induced terminal bacteroid differentiation in *dnf1* mutant plants (156, 162). In addition, recently published data have confirmed the critical role of NCRs in mediating terminal bacteroid differentiation: *M. truncatula* plants with mutations in the *DNF4* and *DNF7* genes, which encode NCR211 and NCR169, respectively, showed severe defects in nodule development (71, 84) (**Figure 2**).

One surprising finding was that, despite the large redundancy of NCR peptides, mutations in individual NCR-encoding genes can exhibit distinct effects. For example, although *dnf4* and *dnf7* mutant plants have similar phenotypes, the proteins encoded by the *DNF4* and *DNF7* genes have different functions: NCR211 does not rescue the defects of *dnf7* mutant plants, and NCR169 does not rescue the defects of *dnf4* mutant plants. The proposed role of NCR211 is to protect differentiating bacteroids from degeneration, whereas NCR169 and NCR247 regulate bacteroid

differentiation (71, 84). Because of the strong antimicrobial activity of NCR peptides, the rhizobia use different strategies to survive in the nodule environment, where these peptides are abundant. For example, the rhizobial BacA transporter appears to function in exporting excess peptide from the bacteroids to maintain homeostasis and bacteroid viability (61) (**Figure 2**).

Consistent with their antimicrobial activity in vitro, NCR peptides are similar in sequence to defensin-like antimicrobial peptides, which disrupt membrane permeability and/or inhibit cell division. The NCR247 peptide interacts with several bacterial proteins (42), such as the cell division protein FtsZ and the cell cycle transcriptional regulators GcrA and CtrA, to inhibit both protein synthesis and septal ring formation during cell division (10). The NCR211 peptide also inhibits the growth of free-living rhizobia (84). In addition to the role of NCR peptides in terminal bacteroid differentiation, their antimicrobial activity suggests that they play a role in controlling the number of bacteria inside each nodule. The fact that exogenous addition of NCR peptide to *dnf* mutant plants restores terminal bacteroid differentiation suggests that an unknown receptor or sensor protein may mediate this response (**Figure 2**).

Host Immunity Involving Terminal Bacteroid Differentiation

Several *M. truncatula* genes have been cloned and found to be essential for bacteroid survival in planta, perhaps by repressing defense responses (**Table 2**). *M. truncatula* plants with mutations in the *DNF2*, *CYSTEINE-RICH RECEPTOR-LIKE KINASE* (*SymCRK*), *REGULATOR OF SYMBIOSOME DIFFERENTIATION* (*RSD*), or *NODULES WITH ACTIVATED DEFENSE 1* (*NAD1*) genes exhibit necrosis and an apparent strong defense response, as demonstrated, for example, by the accumulation of phenolic compounds and induction of defense-related genes (9, 15, 140, 161) (**Figure 2**). Consistent with these findings, the nodules from *dnf2*, *symcrk*, and *rsd* mutant plants, but not those of *nad1* mutant plants, showed an early senescence phenotype (9, 15), and the degree of bacterial colonization of the nodules differed significantly from the wild type. *nad1* mutant plants exhibited the strongest defense response, with very few rhizobia detected in the nodules, whereas *symcrk* and *rsd* mutant plants showed a higher degree of colonization (161).

The proteins encoded by these genes appear in different cellular locations. For example, RSD appears to be involved in protein-directed immune responses in vesicles (140). NAD1 is predicted to have three transmembrane domains and localizes to the endoplasmic reticulum, perhaps playing a role in protein maturation (161). SymCRK is a transmembrane-localized kinase (9). Evidence from genetic and molecular analyses suggests that DNF2, bacterial BacA, SymCRK, and RSD work successively to regulate bacterial internalization and persistence during nodule development. A proposed model implicates these proteins in a multilayer defense response important for nodule development and persistence (9, 10, 140). In this model, DNF2 functions as a suppressor of immunity involved in initiating rhizobial internalization, rhizobial BacA works as an antagonistic factor for NCR peptides in direct dialogue between rhizobia and plant cells (15), and SymCRK and RSD suppress immune responses triggered during the massive colonization of the plant cell and subsequent bacteroid differentiation (9, 140) (**Figure 2**).

In addition, several *M. truncatula* mutant plants with defects in nodule development have enhanced immune responses, such as accumulation of pigment at infection sites (125) (**Table 2**). One intriguing finding was that the nodule-defective phenotype of *dnf2* mutant plants depended on the plant growth medium: Plants grown on regular agar formed defective nodules, whereas those grown on the purer phytogel showed no defect (8). The authors interpreted these results to suggest that the agar likely had an elicitor that triggered defense responses in *dnf2* mutant plants and that this elicitor was not present in the phytogel.

CONCLUSIONS

The evolutionary arms race between plant hosts and pathogens is a crucial environmental factor that affects plant growth and reproduction. The arms, in this case, are molecules that are recognized in various ways by either the plant or the pathogen. Evidence suggests that many of these pathogenic systems are active during the legume-rhizobium symbiosis and are either actively suppressed by the action of the symbiont or used in a beneficial way to promote infection and/or nodule development. We favor the hypothesis that the interaction between plants and rhizobia began as a pathogenic association and remnants of this lifestyle persist—providing a mechanism for more primitive forms of plant infection and remaining essential for nodulation, but obscured by the efficiency of Nod factor–associated mechanisms.

This hypothesis seems fully consistent with current knowledge. Under this scenario, the pathogenic features of the progenitor rhizobia were attenuated over time and became neofunctionalized to support an endosymbiotic lifestyle. Although adoption of the model plants *L. japonicas* and *M. truncatula* has greatly advanced understanding of the legume-rhizobium symbiosis, nodule formation in these IRLC legumes likely represents the most highly evolved and specialized version of this process. The overwhelming focus on these systems has therefore led to an underappreciation of the full diversity of rhizobial infection mechanisms, many of which are significantly more primitive and appear to exploit functions normally associated with interactions between plants and pathogens. The most obvious example is the Nod factor–independent crack-entry nodulation, which requires an active type III secretion system (121). However, much more research is needed before we will fully understand the role of plant immunity and the rhizobial response.

SUMMARY POINTS

1. Rhizobial microbe-associated molecular patterns are critical for establishment of symbiosis.
2. Nod factors play roles in both symbiosis and suppression of the plant immune response.
3. Rhizobial effectors regulate the host immune response.
4. Plant immunity is involved in regulating the rhizobial host range.
5. Host immunity is involved in terminal bacteroid differentiation.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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