CURRENT REVIEW

Legume Nodules: Massive Infection in the Absence of Defense Induction

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Plants of the legume family host massive intracellular bacterial populations in the tissues of specialized organs, the nodules. In these organs, the bacteria, named rhizobia, can fix atmospheric nitrogen and transfer it to the plant. This special metabolic skill provides to the legumes an advantage when they grow on nitrogen-scarce substrates. While packed with rhizobia, the nodule cells remain alive, metabolically active, and do not develop defense reactions. Here, we review our knowledge on the control of plant immunity during the rhizobia-legume symbiosis. We present the results of an evolutionary process that selected both divergence of microbial-associated molecular motifs and active suppressors of immunity on the rhizobial side and, on the legume side, active mechanisms that contribute to suppression of immunity.

LEGUME NODULES: MASSIVELY INVADED ORGANS THAT DO NOT DEVELOP DEFENSE

Rhizobia are often described as soil bacteria able to establish symbiosis with plants of the legume family, during which these bacteria fix atmospheric nitrogen for the plant benefit. However, this definition is biased by the focused attention on the mechanisms governing mutualistic interactions. Many rhizobia trigger nodules but only fix a small amount or no atmospheric nitrogen (Schumpp and Deakin 2010). In such a situation, plants do not benefit from nodulation, but, in all cases, the multiplication of rhizobia in the nodules is spectacular and populations reach densities that are superior to the ones of many very-efficient plant pathogens. The rhizobia that do not fix nitrogen in nodules behave as more- or less-efficient parasitic organisms (Crook et al. 2012; Price et al. 2015; Regus et al. 2017). Interestingly, some rhizobial strains display a parasitic or a mutualistic behavior in a host-dependent way (Crook et al.

2012). Considering similarity between pathogenic and mutualistic bacteria in the way they infect plants, it is not surprising that, during early stages of the infection process of rhizobium-legume interactions, some transient defense-like reactions are observed (Gourion et al. 2015; Kouchi et al. 2004; Libault et al. 2010; Lohar et al. 2006; Lopez-Gomez et al. 2012). However, once the nodules are mature they do not develop common defense reactions, despite the tremendous rhizobial density (Gourion et al. 2015). Such reactions would kill the rhizobia, ending the association and, in the case of mutualistic interactions, avoid the trophic exchanges established between intracellular rhizobia (called bacteroids) and their legume host.

The crucial roles of plant immunity during rhizobia-legumes mutualistic interactions have been recently reviewed (Gourion et al. 2015). Here, we present an update with special emphasis on the late stage of the symbiotic process, i.e., when rhizobia are internalized within the plant cells, and on the potential presence of microbe-associated molecular patterns (MAMPs) of rhizobia that could trigger innate immunity as well as on the mechanisms preventing host defense.

DETECTION OF MAMPS, THE FIRST LAYER OF MICROBIAL PERCEPTION SYSTEMS IN PLANTS

Plant defense against biotic stresses relies on innate immunity that involves different types of perception systems (Jones and Dangl 2006) allowing one or both direct and indirect detection of microorganisms. Plant cells can detect molecular patterns that are widely distributed among (a group of) microbes. Such molecules are referred to as MAMPs or pathogen-associated molecular patterns (PAMPs) and can be recognized by membrane associated receptors, the pattern (or pathogen) recognition receptors (PRRs). PRRs display various domain architectures but they have in common the requirement to act together with a coreceptor (Fig. 1A).

Upon PAMP perception, a signaling cascade involving notable phosphorylation of mitogen-activated kinase proteins (MAPKs) and Ca^{2+} influx in the plant cell is activated (Bigeard et al. 2015). The resulting responses include, notably, the accumulation of callose polymer, the transient production of reactive oxygen species as well as of defense-related proteins. In addition, phytohormones, including ethylene, are produced and stimulate the expression of PRR, thus amplifying the response (Boutrot et al. 2010; Mersmann et al. 2010). Together, these responses result in a form of immunity referred to as PAMP-triggered immunity (PTI). PTI is a relatively weak response that

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is, however, efficient enough to avoid the development of a wide variety of microbes in the plant tissues. With respect to these, PTI contrasts with the intense but very specific responses that are triggered upon recognition of effectors (discussed below).

PRESENCE OR ABSENCE OF PAMPS IN RHIZOBIA

PAMPs are generally described as conserved microbial motifs. Considering this and the density of rhizobia present in the nodule tissues, it is puzzling that PTI is not strongly elicited in the legume nodules. Indeed, the conservation of PAMPs is relative; PAMPs display various taxonomic distributions in the microbial tree of life. For instance, the RaxX sulfated protein recognized by the rice PRR Xa21 seems to be specifically present in bacteria of the genus *Xanthomonas* (Pruitt et al. 2015) and is not present in rhizobia. Similarly but less extreme are the necrosis- and ethylene-inducing proteins (NEP) or NEP-like protein (NLP) that form a large protein family with some members produced by eubacteria, fungi, and oomycetes. Some NLP family members are cytotoxic (Oome and Van Den Ackerveken 2014) and elicit PTI (Böhm et al. 2014; Oome et al. 2014). NLPs are not present in all bacterial strains (Oome and Van Den Ackerveken 2014), and the genetic potential to produce this type of protein is absent in most of the rhizobial clades. More precisely, NLP homologs are present only in a limited number of *Sinorhizobium* (synonym *Ensifer*) strains (including the genome-sequenced strains SM11, AK83, and

WSM419) and are absent in the model strains *Sinorhizobium meliloti* RM41, 1021, and 2011, *S. fredii* HH103 and NGR234, *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium* sp. strains Bta11, ORS278, *Rhizobium etli* CFN42 and Mim1, and *Methylobacterium* sp. strains 2060 and 4-46, as well as the *Mesorhizobium loti* type strain. In the same vein, the PAMP containing protein xanthine/uracile permease, recently identified during an elegant genomic screen (Mott et al. 2016), is present in some but not all rhizobia. These three examples clearly illustrate that not all PAMPs are present in rhizobia. The absence of certain PAMPs could contribute to the prevention of plant defense development during the interactions with the legume hosts (Fig. 1B).

Other PAMPs, like the active elf18 peptide of the elongation factor EF-TU (Kunze et al. 2004) and the csp22 peptide of the cold-shock proteins (Felix and Boller 2003) are present in rhizobia. However, for different reasons, they might not trigger the development of defense reactions during interactions with legumes (discussed below). Among these reasons, it is important to stress here that not all PAMPs are recognized by all plant species. A nonexhaustive list of PAMPs is presented in Table 1, in which their status in rhizobia is summarized.

THE DIVERGENCE OF PAMPS IN RHIZOBIA

For some PAMPs present in rhizobia, divergence has been proposed to explain the lack of PTI during interaction with legumes (Fig. 1C). For example, the peptide flg22 present in the N-terminal region of the bacterial flagellin protein is divergent

Fig. 1. General principle of pathogen-associated molecular pattern (PAMP)-triggered immunity avoidance during rhizobium-legume symbiosis. **A**, The initial step of the PAMP-triggered immunity (PTI). A membrane-located receptor interacts (purple) with both its PAMP ligand (blue) and with its coreceptor (green). The interaction activates a regulatory cascade that elicits the responses leading to PTI. **B**, PTI is not induced as the PAMP is absent from the rhizobial surface. **C**, The divergence of the rhizobial PAMP prevents the activation of PTI (this is the case for flg22, for instance). **D**, The pattern (or pathogen) recognition receptor is not produced by the plant (as it has been suggested for the FLS2 homolog in *Lotus japonicus* nodules). **E**, The activation of PTI is prevented by translocated rhizobial effectors.

in rhizobia and does not elicit the alkalization of cell culture suspensions, a typical feature of PTI, in *Lycopersicon peruvianum* (a tomato sister species), potato, *Arabidopsis*, and tobacco (Felix et al. 1999). These data suggest that modification of PAMPs in rhizobia might account for their capacity to colonize plants without triggering defense. This initial observation of rhizobial flg22 divergence was based on the sequence of one rhizobial flagellin (Felix et al. 1999) and can now be confirmed with many more rhizobial sequences (Fig. 2A). Sequence analysis indicates that four of five residues shown to be important for defense-eliciting activity are not conserved in the rhizobial flg22 peptide.

It is not established if the divergence of rhizobial flg22 to the motif recognized by the PRR FLS2 is an advantage during the colonization of legumes (discussed below). However, FLS2 is conserved in all vascular plants, including nonlegumes (Boller and Felix 2009). This last point is worth noting when considering that rhizobia inhabit rhizosphere of various plant species

including nonlegumes, which they can colonize as endophytes. In this last situation, the selective pressure of the plant innate immunity on the endophytic rhizobial PAMPs (including flagellins) might have predated symbiosis with legumes. With this respect, it is interesting to note that another flagellin region, referred to as FlgII-28, which is present in some bacteria of the *Pseudomonas* genus and which triggers PTI in tomato (Cai et al. 2011), is, like flg22, very divergent in rhizobia (Fliegmann and Felix 2016).

Similar to the divergence observed with the immunogenic epitopes of flagellin, the nlp20 peptides (the active epitope of the NLP proteins [Böhm et al. 2014; Oome et al. 2014]) are divergent in the rhizobial species harboring the corresponding gene. Indeed, these peptides display three additional amino acid residues that increase the distances between residues that are important for the immunogenic property of the peptide (Fig. 2B). In addition, two amino acid residues that are important for the eliciting activity are not conserved in rhizobia (Fig. 2B).

Table 1. Non-exhaustive pathogen-associated molecular patterns (PAMPs) and their status in rhizobia

PAMP	Origin	Perception	Distribution/divergent in rhizobia	Reference
Peptides				
flg22	Flagellin from bacterial flagella	Vascular plants	Divergent	Felix et al. 1999
flgII-28	Flagellin from bacterial flagella		Totally divergent	Cai et al. 2011
csp22	Cold-shock protein	Restricted to Solanaceae	Conserved	Felix and Boller 2003
nlp20	Necrosis- and ethylene-inducing protein-like proteins		Present in a restricted number of strains of the genus <i>Sinorhizobium</i> , in which it is divergent	Oome et al. 2014; Böhm et al. 2014
elf26	Bacterial translation factor EF-TU	Restricted to Solanaceae	The active peptide is present in rhizobia	Kunze et al. 2004
xup25	Bacterial xanthine/uracil permease	<i>Arabidopsis</i>	The protein is present in some but not all rhizobia, in which the peptide is totally divergent	Mott et al. 2016
RaxX21-sY	RaxX, sulfated protein of unknown function	Rice (more?)	Absent	Pruitt et al. 2015
Glycans				
Peptidoglycan	Bacterial cell wall		Present	Gust et al. 2007
Chitin	Fungus cell wall		Present as Nod factors?	
Lipids				
LipidA?	Lipopolysaccharide from gram-bacterial cell wall		Present, strain-specific structures	
Bacterial outer membrane vesicles	Bacterial outer membrane vesicles		Not determined	Bahar et al. 2016
Nucleic acids				
dsRNA			Not determined	Niehl et al. 2016
bRNA	Bacterial RNAs	<i>Arabidopsis</i>	Present	Lee et al. 2016

Fig. 2. Divergence and conservation of selected peptidic pathogen-associated molecular patterns in rhizobia. **A**, The flg22 peptide (Felix et al. 1999). Asterisks (*) indicate residues in the peptide that are critical to elicit plant defenses; weblogos (Crooks et al. 2004) illustrate the divergence of some of these amino acid residues in rhizobia. **B**, The divergence of the nlp20 peptide (Böhm et al. 2014) in rhizobia. Represented in a similar way. **C** and **D**, The conservation of csp22 and of elf26 peptides (Felix and Boller 2003; Kunze et al. 2004), respectively in rhizobia.

Together, this information indicates that the rhizobial nlp20-like peptides are likely not able to trigger PTI.

Peptidoglycan (PG) is another example illustrating that divergence of PAMP in rhizobia might avoid the development of defense reactions during interactions with legumes. PG is a constituent of the bacterial cell wall that provokes PTI in plants (Gust et al. 2007). PG is composed of a sugar backbone of *N*-acetylglucosamine and *N*-acetylmuramic residues alternatively linked in β -(1,4) configuration and of some peptidic chains that reticulate the polymer. PGs are different from one strain to another in their peptidic chain composition and length. It has been suggested, in a *Bradyrhizobium* strain, that alteration of the PG causes defects at different steps of the symbiotic process. Interestingly, those defects are associated with the development of defense reactions, suggesting that only altered rhizobial PG induces defense in legumes (Gully et al. 2016). Noteworthy, up to now, no rhizobial PAMP has been demonstrated to trigger PTI on its legume host and, together, the cited examples suggest that PAMP divergence from the defense-eliciting motifs might be an important feature in the compatibility between rhizobia and their legume and nonlegume hosts.

ABSENCE OF SOME PRRS IN LEGUME GENOMES: A DETERMINANT OF NODULE TOLERANCE TO RHIZOBIA?

PTI has been extensively studied in the nonlegume model *Arabidopsis thaliana*, but perception systems allowing PAMP recognition in these species are not all conserved in plants outside of the Brassicaceae clade. One example is the receptor-like protein 23 (RLP23), which recognizes the nlp20 peptide (Albert et al. 2015) that elicits typical features of PTI on only a restricted set of plant species, including some but not all *Arabidopsis* accessions. In addition to these *Arabidopsis* species, nlp20 is also recognized by lettuce but not by members of family Solanaceae (Albert et al. 2015), parsley, or wheat (Böhm et al. 2014). It is not known whether legumes respond to nlp20, but RLP23 has no clear homolog in the model legume *Medicago truncatula*, for which a genome sequence is available (Young et al. 2011). Although one must be very careful when interpreting this type of sequence-comparative study, especially as a basis to define orthology, this observation suggests inability of *M. truncatula* to perceive the active epitope of NEP (even if it is not excluded that a protein with no or little similarity with RLP23 might play this role in *M. truncatula*). With this respect, the reason for nlp20 divergence in rhizobia is likely not to avoid legume PTI and might rather be important to colonize plants from other phyla.

As with RLP23, phylogenetic studies suggest that LORE1, one of the key receptors responsible for lipopolysaccharide (LPS) perception (Ranf et al. 2015; Shang-Guan et al. 2018), is also restricted to members of Brassicaceae (Ranf et al. 2015), the plant clade of *Arabidopsis thaliana*. The *Arabidopsis thaliana* receptor EFR (Zipfel et al. 2006) perceives elf26, the active epitope of the elongation factor EF-Tu. EFR is believed to be specifically present in Brassicaceae species. Indeed, non-Brassicaceae leaf materials (including some from the legume *Medicago sativa*) do not evolve ethylene (a typical feature of PTI) in response to elf26 (Kunze et al. 2004). Absence of elf26 perception system in *Medicago* explains why elf26 peptide from *S. meliloti* EF-Tu does not compromise the establishment of the symbiotic process, even if it is conserved enough to trigger defense reactions in *Arabidopsis* (Fig. 2D) (Kunze et al. 2004).

To finish, other examples of PRRs specific to a phylogenetic clade are found outside the Brassicaceae taxon. The perception

systems of csp22, the immunogenic epitope of the cold-shock protein, seem to be restricted to family Solanaceae (Saur et al. 2016; Wang et al. 2016b) and are thus, like RLP23 and LORE1, absent from the legume clade. Interestingly, csp22 is strongly conserved in rhizobia (Fig. 2C) and it would likely trigger defense reactions preventing mutualism in case of co-occurrence with its receptor. All these examples illustrate how absence of some PRRs might have contributed during evolution to the emergence of rhizobia-legume symbiosis.

CONTROL OF PRR ACCUMULATION IN THE NODULES?

In most of the studies, responsivity to PAMPs is evaluated with protoplast or leaf material, which might lead to over-interpretations, as the expression of some PRR might be tissue-specific and repressed in the aerial part of the plant or in protoplast. With this respect, it is interesting to note that, according to Roux et al. (2014), in the model legume *M. truncatula*, EFR-like transcripts (48% identity at the amino acid level) are detected in nodules in which they are clearly accumulated as compared with the root samples. These data contrast with some obtained with microarray technology, in which the corresponding gene probeset (Mtr.39099.1.S1_at) displays signals only in roots and not in leaves or nodules (the *Medicago truncatula* Gene Expression Atlas website). The discrepancy between these expression data might result from the different plant culture conditions used in these two studies. Indeed, it is known that the abiotic environment influences the nodule immune status (Berrabah et al. 2014a). Whether this EFR-like protein recognizes the elf26 remains unknown, but this example illustrates the organ-specific expression of a predicted PRR.

The receptor-like kinase FLS2, which perceives flg22 in *Arabidopsis*, was proposed to be present in *Lotus japonicus* (53% identity at the amino acid level) and to be strongly downregulated in mature nodules (Høgslund et al. 2009; Lopez-Gomez et al. 2012). Although it might be noticed here that the *Lotus* homolog was not shown to be functional for flg22 perception, this situation is interesting, as the *Mesorhizobium loti* flg22 is likely divergent enough to not trigger plant defense even in the presence of FLS2. Indeed, purified *Mesorhizobium loti* flagellin does not induce PTI hallmarks on *L. japonicus* seedling (Lopez-Gomez et al. 2012). Furthermore, observations suggest that, in the *Lotus japonicus* nodules, the *Mesorhizobium loti* flagellin biosynthesis is repressed (Tatsukami et al. 2013; Uchiumi et al. 2004).

Downregulation of PRR expression during rhizobium-legume interactions might contribute to the establishment and to the persistence of the symbiotic process (Fig. 1D). However, this hypothesis remains to be clarified as, up to now, no PRR able to recognize rhizobial PAMP has been experimentally characterized in legumes. An alternative explanation might be that nodules in a natural environment host nonrhizobial bacteria (discussed below) that might have some benefit and should not be recognized as pathogens.

In addition to the absence of some PRR genes in the legume genomes or to the suppression of PRR expression in the nodules, a tight control of PRR localization or their specific degradation at the symbiosome membrane are open hypotheses. If true, these hypotheses might also explain the absence of plant defense reactions in the symbiotic organ. Indeed, PRR content may vary between peribacteroid and plant cell membranes. Interestingly nodules of the *symCRK* mutant of *Medicago truncatula*, which develop defenses at the level of rhizobia-infected cells, strongly accumulate various leucine-rich repeat receptor-like kinases, a protein family in which PRRs are well-represented (Berrabah et al. 2018).

PAMPS PRESENT IN RHIZOBIA?

To our knowledge, no PAMP isolated from rhizobia has been shown to activate defense reactions in its legume host. However, some PAMPs are essential constituents of bacteria, such as the LPS and the PG present in the cell envelope. Those last PAMPs diverge from strain to strain. However, the conservation is likely higher for other PAMPs. For example, RNAs isolated from *Pseudomonas syringae* are able to trigger defense reactions and PTI features in *Arabidopsis* (Lee et al. 2016). Whether this defense-eliciting property is present in the rhizobial equivalent molecules in general and, more specifically, on their legume host remains unknown.

The moderate and transitory defense-like reactions observed during the very first stages of the symbiotic process suggest that rhizobial PAMPs can trigger defense reactions in legumes (Jones et al. 2008; Libault et al. 2010; Kouchi et al. 2004; Lohar et al. 2006; Lopez-Gomez et al. 2012). However, such patterns remain to be identified.

COUNTERING THE PTI: A ROLE FOR BACTERIAL SECRETED PROTEINS AND FOR NODULATION FACTORS

Some pathogens are able to interfere with plant cell signaling and to impair PTI. For instance, some bacterial exopolysaccharides (EPS) prevent or reduce PTI by chelating Ca^{2+} , an essential component of PTI signaling (Aslam et al. 2008). Other molecules responsible for PTI inhibition, referred to as effectors, are delivered into the plant cell cytoplasm by translocation machinery. In the case of bacteria, the best-known machinery is the type three secretion system (T3SS). When the effector inhibits PTI, the plant is susceptible to the pathogen, a situation referred to as effector-triggered susceptibility (ETS) (Jones and Dangl 2006). In some special cases, the plants are able to perceive the effectors and develop an immune response that shares some similarities with PTI but that is more intense and faster, leading to the so-called effector-triggered immunity (ETI) (Jones and Dangl 2006). ETI is characterized by the hypersensitive response associated with programmed cell death. In the next paragraphs, we will discuss some equivalent mechanisms and factors found in rhizobia that suggest that the conceptual framework theorized for plant-pathogen interactions (Fei et al. 2016; Jones and Dangl 2006; Keller et al. 2016) can be directly transposed to the rhizobia-legume symbiosis.

Genetic analyses indicate that similar bacterial factors are involved in the suppression of PTI in pathogenic bacteria and in rhizobia. For instance, EPS of both bacterial types are able to inhibit the plant defense and it seems that this suppression is important at various steps of the interactions (Aslam et al. 2008; Berrabah et al. 2015; Jones et al. 2008). Also, in some cases, T3SS have been shown to be important for successful rhizobia-legume interactions. Never-the-less, this assumption should be nuanced; in some other cases, T3SS also restrict the host range of the rhizobia, likely because of an ETI-like mechanism. The importance of the rhizobial protein secretion system for the interactions with legumes has been recently extensively discussed (Gourion et al. 2015; Nelson and Sadowsky 2015; Staehelin and Krishnan 2015) and, here, we will only focus attention on general features and on recent advances.

Such recent advances have been described in two papers in which Jiménez-Guerrero et al. (2015a and b) reported that *S. fredii* T3SS suppresses the early defense response to effectively nodulate soybean. Furthermore, the authors showed that NopC, a rhizobium-specific effector has a positive effect on *Glycine max* and *Vigna unguiculata* nodulation. This effect suggests a role for ETS in these symbioses (Fig. 1D and E).

Interestingly, references to ETI or, more generally, to modification of host specificity mediated by T3SS are present in related rhizobia-legume literature, while rhizobial PAMPs have, so far, not been identified as active on their legume host. This distinction might be due to the subcellular location of the two types of molecules and of their cognate receptors. Indeed, one can imagine that PRRs are not present or not active at the peribacteroid membrane and that, on the other hand, the resistance proteins perceiving effectors are present in the plant cytoplasm.

The T3SS is present in some but not in all rhizobia. It is described in members of the *Bradyrhizobium* genus as well as in the *Sinorhizobium fredii* species (the model strains being NGR234 and HH103). Remarkably, these two groups of rhizobia have relatively large host spectra. The fact that T3SS is absent in most sequenced strains of the other rhizobial group does not necessarily indicate that bacteria of these clades are not able to inject one or more effectors. Indeed, effectors can be translocated by the T4SS, which is efficient for protein translocation in the *Mesorhizobium* genus and was recently proposed to be effective in some *Sinorhizobium* strains (Hubber et al. 2004; Nelson et al. 2017; Sugawara et al. 2013). It is also not excluded that other mechanisms allow protein translocation.

Interestingly the T3SS known to be involved in the suppression of PTI in pathogenic bacteria can compensate for the lack of nodulation factor (NF) in *Bradyrhizobium elkani* for nodulation of soybean (Okazaki et al. 2013). Similarly, nodulation is T3SS-dependent in some but not all species of the legume genus *Aeschynomene*, for which nodulation does not depend on NFs (Giraud et al. 2007; Okazaki et al. 2016). Other remarkable data suggest that NFs have a suppressive effect on PTI in a nonsymbiotic context (Liang et al. 2013). On *M. truncatula*, NFs were shown to have either an inducer or, on the contrary, a repressor effect on the expression of various defense-related genes (Jardinaud et al. 2016). Thus, the precise role of NFs on the plant immune status during rhizobia-legume interactions remains to be clarified.

Beyond the role of NF, these illustrations indicate that, like pathogenic bacteria, rhizobia can interfere with the plant PTI signaling.

RESISTANCE GENES AND OTHER PLANT FACTORS RESTRICTING HOST RANGE DURING RHIZOBIA-LEGUME INTERACTIONS

As mentioned above, rhizobial type three effectors can modulate host ranges. In some cases, the reason might be a key role in ETS. In other situations, the effectors might restrict the host range because they trigger ETI. For example, in soybean, the *Rj2* gene restricts nodulation with specific strains of *B. japonicum* and *S. fredii*. *Rj2* encodes a protein harboring a typical structure of plant resistance proteins, i.e., the three domains toll/interleukin-1 receptor, nucleotide-binding site, and leucine-rich repeat. Allelic variation of the *Rj2* gene modifies the host spectrum of the soybean (Yang et al. 2010). The description of this gene suggests the existence of a recognized effector.

In soybean, the *Rj4* gene restricts nodulation by many *B. elkani* strains. *Rj4* encodes a thaumatin-like protein (a member of the pathogenesis-related protein family 5) (Hayashi et al. 2014; Tang et al. 2016). On the bacterial side of this interaction, Okazaki and colleagues (2009) have shown that mutation in the T3SS overcomes nodulation restriction on *Rj4*. *B. elkani* genes potentially involved in incompatibility have been identified during a genetic screen (Faruque et al. 2015) and the candidate genes are not exclusively effectors or

components of the T3SS. In strain Is-34 of *B. japonicum*, which is also incompatible with *Rj4*, a putative effector has been identified (Tsurumaru et al. 2015). Deletion of the corresponding gene confers the ability to nodulate Rj4 soybean (Tsurumaru et al. 2015).

In a *rj4/rj4* line during incompatible interactions, the infection process is early aborted. Indeed, root hair curling and infection threads are not observed in such a situation. Also, the defense-associated molecules, salicylic acid (SA), and hydrogen peroxide are accumulated in roots, whereas they do not during the interaction with a T3SS-defective mutant (Yasuda et al. 2016). These observations indicate that, although not obviously dependent on a typical resistance protein, ETI restricts nodulation in this system and inhibits the normal plant response to Nod factor.

BEYOND THE SECRETED EFFECTORS: OTHER RHIZOBIAL FACTORS POTENTIALLY PREVENTING THE PLANT DEFENSE

In plants, virtually all phytohormones impact immunity. Three of them, ethylene, jasmonic acid, and SA, together with PAD4, which is important for SA accumulation under certain circumstances and has an SA-independent immune function, play a key role in PTI and ETI (Tsuda et al. 2009). Those three hormones have a negative effect on the early stages of the development of nodules, as discussed by Gourion et al. (2015). Other hormones related to plant development like cytokinins, auxins, and abscisic acid may also participate to the immune response during symbiosis (Boivin et al. 2016; Nadzieja et al. 2018; Pieterse et al. 2012). Although the potential contribution of rhizobia in hormonal signaling is not clear, it has been proposed that the rhizobial ability to degrade the ethylene precursor promotes the nodulation process in pea (Ma et al. 2003). Such interference with ethylene signaling might also occur during the later stage of symbiosis. Indeed, in *Mesorhizobium loti*, the expression of the *acdS* gene, encoding an enzyme degrading the ethylene precursor, depends on NifA2 (Nukui et al. 2006), a regulator essential for nitrogen fixation, which is specifically expressed when rhizobia are within the plant cells. In addition, rhizobia-mediated interference with the hormone signaling pathways that are associated with plant defenses cannot be excluded. For instance, production of bioactive cytokinins, known to interfere with plant defenses (Laffont et al. 2015), seems to be a general feature of rhizobia (Kisiala et al. 2013; Podlešáková et al. 2013). Such potential interferences are difficult to interpret, as these hormones are not only connected with defense but also with developmental processes.

Other bacterial factors are involved in the control of innate immunity. Two bacterial signaling pathways have been identified in the soybean symbiont *Bradyrhizobium diazoefficiens* (previously *B. japonicum*) as required to prevent the development of defense reactions. The general stress-response signaling cascade that involves the response regulators PhyR and the sigma factor EcfG is required to prevent the development of nodule necrosis (Gourion et al. 2009). In addition, the key regulator of nitrogenase production, the NifA protein, is also required in *B. diazoefficiens* to prevent nodule necrosis as well as the accumulation of defense compounds (Fischer et al. 1986; Lardi et al. 2016). Interestingly, the lack of nitrogen fixation is clearly not the cause of the intense necrosis observed in the nodules triggered by the *B. diazoefficiens* *nifA* mutant. Indeed, the *B. diazoefficiens* mutant *nifH*, which does not produce nitrogenase, does not trigger necrotic nodules. Supporting this idea, the defense-related compound resveratrol, is specifically accumulated in the *nifA*-triggered nodules but not in the *nifH*

ones (Lardi et al. 2016). The NifA target contributing to the suppression of immunity in the soybean nodules remains to be identified. Interestingly, in contrast to the soybean-*Bradyrhizobium* system, NifA is dispensable in *S. meliloti* to prevent intense defense reactions in *Medicago* nodules (Berrabah et al. 2015). Also, a role in the suppression of immunity in plants of the genus *Medicago* has been proposed for *S. meliloti* LPS (Ferguson et al. 2005; Scheidle et al. 2005).

INEFFICIENT BACTERIODS DO NOT INDUCE PLANT DEFENSE

As mentioned in the introduction, some rhizobia-legume interactions result in the development of nonfixing nodules. In this case, rhizobia can be considered as parasites inducing a niche on their host. Such behavior, per se, does not trigger the development of intense defense. Indeed, rhizobial *nifH* mutants unable to produce the nitrogenase complex elicit nodules that are not necrotic, do not accumulate phytoalexins, and in which the expression of defense-related genes is not induced. This has been documented in soybean and in *M. truncatula* inoculated, respectively, with *B. diazoefficiens* and *S. meliloti* *nifH* mutants (Berrabah et al. 2015).

Also, early death of the bacteroid within *M. truncatula* cells does not trigger the development of defense, as illustrated with the *nifH*, the *nifA*, and the *bacA* mutants of *S. meliloti*. *NifA* encodes a regulatory protein essential for the production of the nitrogenase complex (Dixon and Kahn 2004), and *bacA* is essential for the survival of *S. meliloti* in the symbiotic cells (Haag et al. 2011) and, thus, also for nitrogen fixation (Glazebrook et al. 1993). Nodules triggered by these mutants do not display necrosis or induction of defense-related genes (Berrabah et al. 2014a, 2015). Interestingly, in soybean, despite the lack of nitrogen fixation, the plant continues to provide photosynthetates to the nodules induced by the Fix-negative mutant (Lardi et al. 2016). Together, these results clearly indicate that inability to fix nitrogen does not induce typical plant defense reactions.

PLANT GENES PREVENTING DEFENSE REACTIONS IN NODULES

In recent years, plant genes involved in the control of plant defense specifically during the symbiosis have been identified in *M. truncatula*. The corresponding mutants display features of defense reactions in nodules once the rhizobia have been internalized in the plant cells (Berrabah et al. 2014a and b, 2015; Bourcy et al. 2013; Sinharoy et al. 2013; Wang et al. 2016a). The process was shown to act sequentially (Berrabah et al. 2015) and to involve at least four *Medicago* genes, *DNF2*, *SymCRK*, *RSD*, and *NAD1*. The functions of *DNF2* and *SymCRK* remains unknown, the first protein presenting similarity with phosphatidyl inositol phospholipases C (Bourcy et al. 2013) that cleaves one or both phospholipids and glycosylphosphatidylinositol (GPI)-anchored proteins. It is interesting to note that some PRRs are anchored to the membrane by a GPI. Also, mutations in *DNF2* trigger a conditional phenotype and *dnf2* mutant is able to fix nitrogen upon special culture conditions (Berrabah et al. 2014a). *SymCRK* is specifically expressed in nodule cells hosting rhizobia and encodes a receptor-like kinase harboring a non-arginine-aspartate (non-RD) kinase motif typical of PRRs (Berrabah et al. 2014b; Dardick and Ronald 2006; Dardick et al. 2012). Recent data indicate that ethylene signaling contributes to the defense phenotype of the *dnf2* and the *symCRK* mutants (Berrabah et al. 2018). The precise role of *SymCRK* remains unknown; however, the presence of the non-RD motif suggests that *SymCRK* might interfere with PRR

coreceptors. The RSD gene encodes a transcriptional regulator that has been proposed to act through the inhibition of vesicle-associated membrane protein 721A expression and by modifying vesicle trafficking (Sinharoy et al. 2013). RSD and SymCRK act later than DNF2 during the symbiotic process (Berrabah et al. 2015). The temporal action of NAD1 has not been determined, so far, even though the phenotype of the corresponding mutant harbors strong similarity with *dnf2* (Domonkos et al. 2017), which leads us to presume that it acts before RSD and SymCRK. The molecular role of NAD1 remains unknown, the predicted protein is rather small, harbors two transmembrane helices, and localizes to the endoplasmic reticulum of symbiotic cells (Wang et al. 2016a).

DNF2, *SymCRK*, *RSD*, and *NAD1* were identified in *M. truncatula*, a plant species that triggers terminal bacteroid differentiation (TBD) (Alunni and Gourion 2016). This plant-triggered phenomenon results in the loss of bacteroid capacity to resume growth and is associated with genome endoreduplication, cell elongation, and increased membrane permeability (Alunni and Gourion 2016; Mergaert et al. 2006). The consequences of bacteroid membrane permeability on the potential leakage of intracellular or periplasmic material, potentially containing PAMPs, was not estimated. However, terminally differentiated bacteroids do not seem to trigger defense response, perhaps because of a form of control involving *DNF2*, *NAD1*, *RSD*, and *SymCRK*. Mutants altered in these genes display an incomplete bacteroid differentiation process associated with plant defense and the early death of intracellular rhizobia. Such a differentiation defect associated with development of defense was also reported in the DD-Cpase1 mutant of photosynthetic bradyrhizobia nodulating another plant inducing TBD (Gully et al. 2016). On the contrary, the phenotype of nodules induced by the *S. meliloti bacA* mutant (impaired in the bacteroid differentiation process and triggering an early senescence of the nodule) indicates that the absence of differentiation is not, per se, triggering defense in the nodules (Berrabah et al. 2014a).

Interestingly, *M. truncatula* can be nodulated by symbionts lacking both the T3SS and the T4SS. Whether or not *DNF2*, *NAD1*, *RSD*, and *SymCRK* compensate the inability of the rhizobial microsymbiont to suppress immunity remains to be determined.

Recently, in *Lotus japonicus*, a legume species that does not trigger TBD, the *APN1* gene was shown to prevent the development of defenses after bacteria internalization within the plant cells in a strain-specific way. The gene encodes for an aspartate peptidase displaying a signal peptide, suggesting a possible targeting to the symbiosome (Yamaya-Ito et al. 2018).

The mechanisms controlling immunity in the nodules and involving *DNF2*, *SymCRK*, *RSD*, *NAD1*, and *APN1* remain to be characterized.

NODULE SENESCENCE AND DEFENSE

Nodule senescence is developed under various conditions including stresses and metabolic imbalance, encountered, notably, when the nitrogen source is becoming available to the plant (Puppo et al. 2005). Nodules of the *dnf2*, *nad1*, *rsd*, *symCRK*, and *apn1* mutants display early senescence features in addition to the defense-like reactions they develop (Berrabah et al. 2014b; Bourcy et al. 2013; Sinharoy et al. 2013; Wang et al. 2016a; Yamaya-Ito et al. 2018). Whether innate immunity and PTI is reactivated in senescent nodules, as proposed by Puppo et al. (2005), remains unknown.

The potential contribution of defense in the nodule senescence process is not now clear. Interestingly, nodule senescence is activated by nitric oxide (Cam et al. 2012) and involves cysteine proteinases (Pierre et al. 2014), both being important

during some plant responses to pathogens (Gourion et al. 2015). The type III effector NopL of *Sinorhizobium fredii* NGR234 is a MAPK substrate (Ge et al. 2016) and the phosphorylatable sites of NopL are important to prevent senescence of the bean nodules (Ge et al. 2016). This observation also links nodule senescence to plant immunity.

ONE MORE LEVEL OF COMPLEXITY: NODULES HOST ENDOPHYTES

The control of immunity in the symbiotic organ can even be more complex than expected if we consider that, in fields, legume nodules are not only hosting rhizobia but also non-rhizobial endophytes (De Meyer et al. 2015; Leite et al. 2017; Velázquez et al. 2013; Zgadzaj et al. 2015). The studies by De Meyer et al. (2015) and Leite et al. (2017), for example, indicate that, in some plants, there are more nonrhizobial endophytes in the symbiotic organ than rhizobia and that there is some form of specificity of the nodule microbiome. This suggests one or both a selection by the plant and a compatibility with the rhizobial partner. It is, thus, possible to hypothesize that immunity plays a role in the recognition of these non-rhizobial endophytes. In any case, some of the peculiarity of the symbiotic immunity described in this review might be, in the natural environment, not only driven by the rhizobial partner but also by the nonrhizobial endophytes.

CONCLUDING REMARKS

The question of the control of immunity during rhizobium-legume symbiosis is particularly intriguing, notably, because of the spectacularly dense microbial populations in the mature nodules. Together, the transitory induced defense during the earliest stages of the symbiosis and the lack of observable defense reactions in mature nodules suggest the suppression or at least the partial suppression of immunity in rhizobia-infected tissues.

Overall, plant defense during rhizobia-legume symbiosis seems to be prevented by mechanisms that are similar to those observed during plant interactions with pathogens, i.e., by PAMP divergence, chelation of Ca²⁺, proteic effectors, and, perhaps, interference with phytohormone pathways (Box 1).

In addition to these mechanisms shared with phytopathogens, plant defenses are also actively repressed by the plants themselves, using symbiosis-specific mechanisms. These processes are not well understood. This immunity shutdown might not be restricted to the symbiosis with rhizobia but be more generally required as, for example, in the case of mutualistic interactions with root endophytes.

The existence of nodules developing defense reactions when induced by some bacterial mutants (or on some plant mutants) indicates that immunity is normally (partially?) suppressed in these organs. If this is the case, legume nodules should display

BOX 1

FACTORS PROPOSED TO LIMIT THE DEVELOPMENT OF PLANT DEFENSE DURING RHIZOBIA-LEGUME INTERACTIONS

- PAMP divergence in rhizobia
- Bacterial factors repressing plant defenses
- PRR absence or silencing in symbiotic tissues
- Active plant repression of defense signaling pathways

altered defense capacity. This remains to be experimentally tested, as a potential impact on plant vulnerability would considerably alter the advantage of legumes to form nodules. In this respect, it is interesting to note that strong selective pressure has been recently proposed to act against nodulation in legumes (Griesmann et al. 2018).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Medicago truncatula Gene Expression Atlas website:
<http://mtgea.noble.org/v3>