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The Versatile Roles of Type III Secretion Systems in Rhizobium-Legume Symbioses

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Abstract

To suppress plant immunity and promote the intracellular infection required for fixing nitrogen for the benefit of their legume hosts, many rhizobia use type III secretion systems (T3SSs) that deliver effector proteins (T3Es) inside host cells. As reported for interactions between pathogens and host plants, the immune system of legume hosts and the cocktail of T3Es secreted by rhizobia determine the symbiotic outcome. If they remain undetected, T3Es may reduce plant immunity and thus promote infection of legumes by rhizobia. If one or more of the secreted T3Es are recognized by the cognate plant receptors, defense responses are triggered and rhizobial infection may abort. However, some rhizobial T3Es can also circumvent the need for nodulation (Nod) factors to trigger nodule formation. Here we review the multifaceted roles played by rhizobial T3Es during symbiotic interactions with legumes.

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INTRODUCTION

Rhizobia form a paraphyletic group of alpha- and betaproteobacteria able to activate formation of nitrogen-fixing nodules on the roots of legumes. Once sheltered inside nodule cells, rhizobia provide reduced forms of atmospheric nitrogen (N_2) in exchange for various nutrients (92). Ammonium provided by rhizobia allows legumes to grow in nitrogen-poor soils, thus limiting the need for chemical fertilizers to boost crop yields. Associations between rhizobia and legumes are extremely diverse and occur naturally in many terrestrial ecosystems; as such, they are considered to be major contributors of fixed nitrogen and hence of prime importance for sustainable and environmentally friendly agriculture (30).

Soils are complex ecosystems in which plant roots interact with a broad diversity of microbes, some of which are pathogens or symbionts (73, 89). How legumes secure the entry of symbiotic rhizobia while preventing infections by pathogens remains one of the most burning questions about plant-microbe interactions (42). In general, the symbiotic process begins when rhizobia secrete strain-specific nodulation (Nod) factors (NFs) in response to flavonoids exuded by roots. Perception of NFs by plant receptors activates cellular programs leading to nodule organogenesis and the formation of infection threads that guide infecting bacteria to the emerging nodule primordium (86, 88). When a symbiosis is proficient, each of the infected nodule cells may harbor thousands of nitrogen-fixing bacteroids. How rhizobia can establish such large intracellular communities is intriguing given that plants have evolved potent immune responses to repel microbial infections (6). In plants, recognition of microorganisms is mediated by membrane receptors that detect microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) and that activate a first line of defense called MAMP- or PAMP-triggered immunity (MTI/PTI) (10, 134).

Although no single rhizobium-specific MAMP has been identified as acting on legumes so far, transient defense-like reactions consistent with MTI responses are often observed during the early stages of infection (39, 56, 63, 128). While NFs have been shown to attenuate MTI (28, 61), rhizobia use additional signals and mechanisms to facilitate infection of legume roots. One of the most striking rhizobial strategies to promote legume infection is the use of effector proteins delivered by type 3 secretion systems (T3SSs), which subvert plant immunity in ways similar to mechanisms

of pathogenic bacteria (21). Initially identified in animal- and plant-pathogenic bacteria, T3SSs deliver cocktails of effector proteins (T3Es) inside the cytoplasm of host cells. T3Es interfere with various cellular processes to sabotage immune responses and promote bacterial invasions (15, 24, 31). To defend against these T3SS-mediated attacks, plants have evolved a family of cytoplasmic receptors that, once associated with cognate T3Es, trigger a powerful second line of defense called effector-triggered immunity (ETI) (18). ETI is an extremely potent, hypersensitive response often associated with cell death that halts microbial infection and helps render plants resistant to pathogens (51).

Despite the potential benefits that rhizobia may represent for legumes, a growing body of literature indicates that T3Es can also compromise symbiosis through ETI, as already reported for plant-pathogen interactions (74, 142, 143). This makes rhizobial T3SSs double-edged swords that may promote or prevent symbiosis depending on the host plant (114). Interestingly, recent studies revealed that rhizobial T3SSs can also trigger nodule organogenesis directly, thus bypassing the need for compatible NFs (84, 85, 93, 121). Here, we review the many facets of T3SSs in the context of the beneficial symbioses between rhizobia and legumes.

CHARACTERISTICS SPECIFIC TO RHIZOBIAL T3SSs

T3SS secretory machinery, also called the injectisome, is composed of about 20 different proteins that form a basal body across the bacterial envelope, and an extracellular needle that connects the bacterium to the eukaryotic cell targeted for T3E secretion (**Figure 1**). While the structure of T3SSs of different human pathogens such as *Salmonella*, *Yersinia*, and *Escherichia* species has been extensively studied (14, 24, 83), the corresponding secretion apparatuses of rhizobia remain poorly characterized. Given the high degree of conservation between proteins that form the basal bodies of rhizobia and pathogen T3SSs, the secretion machineries are expected to share similar ultrastructures and functions. However, rhizobial T3SSs also have specific features such as a secretin, which is split in the RhcC1 and RhcC2 components (1).

Like those observed for plant pathogens, the T3SS pili of rhizobia are thin and up to 2-μm-long, flexible extracellular microfilaments (22, 59, 66, 98). In contrast to the 40- to 80-nm needles of T3SSs of animal pathogens, the longer T3SS appendages of rhizobia are possibly adapted to cross plant cell walls and/or result from the absence of molecular rulers, such as the *Yersinia* YscP and *Pseudomonas* PscP proteins, which were shown to control the length of needles (4, 14).

Isolation and partial purification of T3SS appendages showed that pili from *Ensifer* (formerly *Sinorhizobium*) species are made of at least three nodulation outer proteins (Nops) that cross-interact and are specific to rhizobia: NopA, NopB, and NopX (22, 58, 66, 98, 99). It has been suggested that NopA and NopB pilins are part of the extracellular filament, whereas NopX proteins may form translocon structures across the host cell membrane (99). Interestingly, several bradyrhizobia with functional T3SSs, such as the model strain *Bradyrhizobium diazoefficiens* USDA110, were shown to lack a *nopX* homolog (57, 118). Since a recent large-scale comparative analysis of bradyrhizobia genomes showed that strains that lacked *nopX* instead carried T3SS-related *nopE* and *nopH* genes, it is tempting to speculate that products of the two latter genes functionally replace NopX in the translocation of T3Es (122).

As the extracellular appendage of rhizobial T3SSs may come in direct contact with host cells, pilus proteins may act as MAMPs. Indeed, the HrpE pilin of the plant pathogen *Xanthomonas campestris* was shown to elicit MTI (38, 105). The different protein compositions of T3SS pili may therefore correspond to adaptations needed for bacteria to evade plant immune responses or, conversely, to favor recognition by specific hosts (14). Whether T3SS pilins of rhizobia evolved to avoid host immune responses, as already proposed for their flagellins (37, 39, 65), remains to be tested.

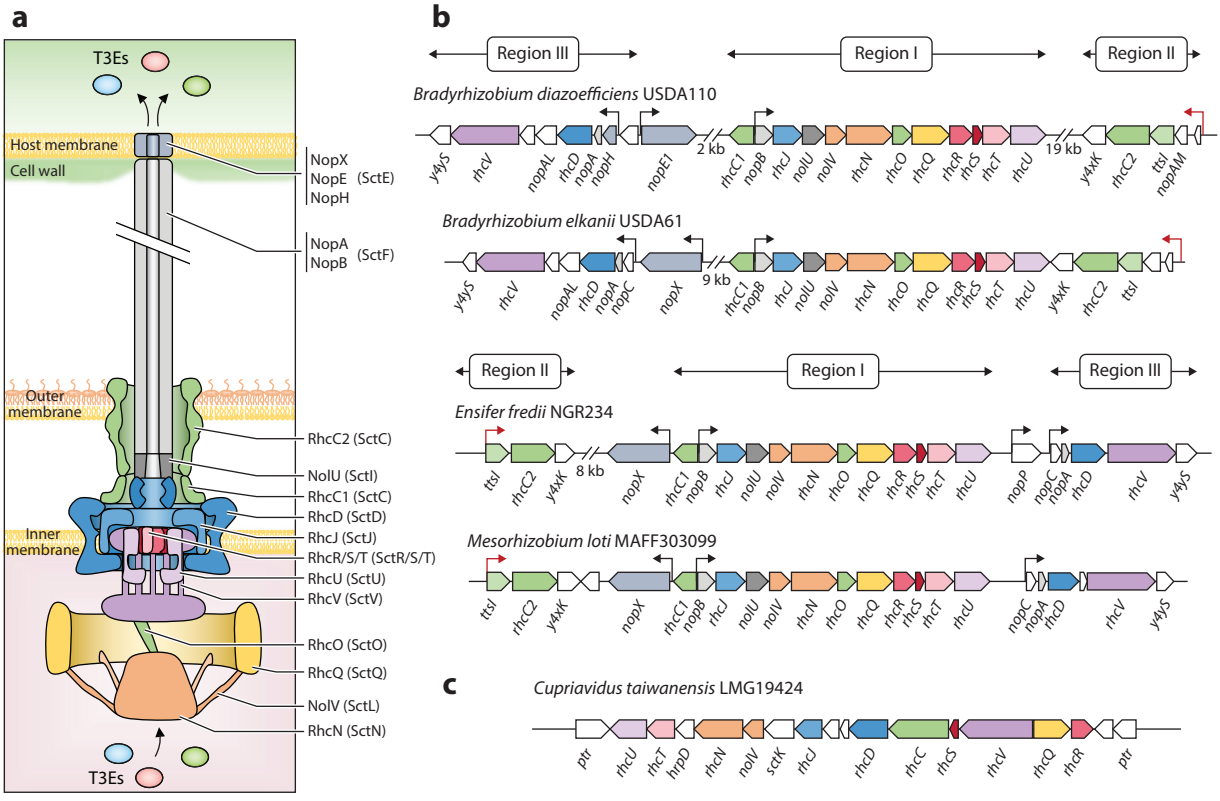


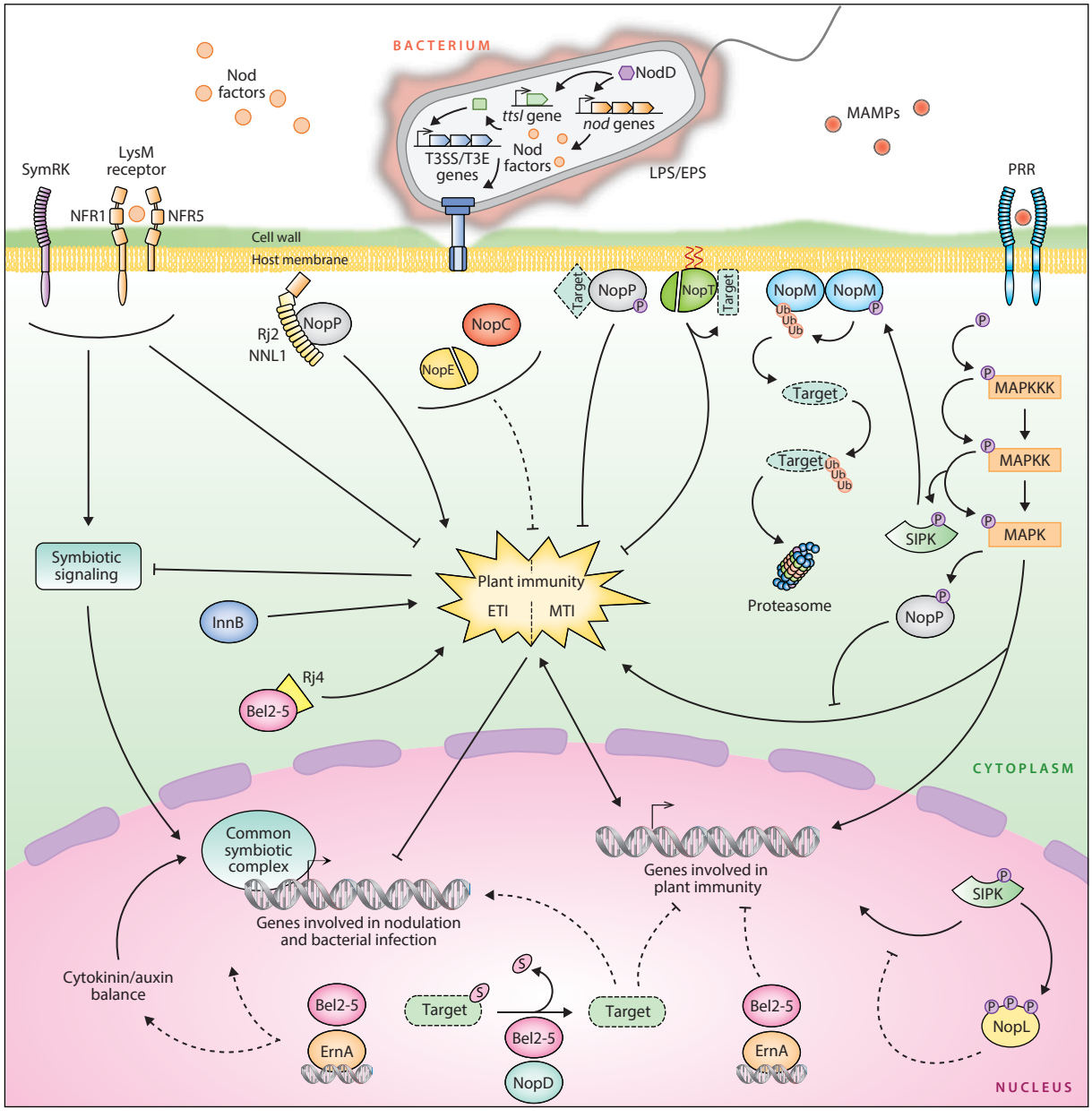
Figure 1

Schematic representation of the type III secretion system (T3SS) machinery and genetic organization of symbiotically active T3SS gene clusters identified in rhizobia. (*a*) Proteins that form the T3SS apparatus are named according to the established rhizobia nomenclature. The corresponding unified Sct (secretion and cellular translocation) names are in parentheses. Panel *a* adapted from References 114 and 130. (*b*) Genetic organization of T3SS clusters identified in various α-rhizobia with three mostly conserved regions (regions I to III) as defined by Tampakaki (118). Genes are represented by arrows outlined in black, oriented according to transcription, and colored according to the colors of the corresponding proteins in panel *a*. White arrows represent genes whose products are type III effectors (T3Es) (e.g., NopP) or of unknown function (e.g., y4yS). Thin black and red arrows indicate promoters with conserved *tts* and *nod* boxes, respectively. (*c*) Atypical T3SS gene cluster identified in the β-rhizobium *Cupriavidus taiwanensis* LMG19424, with its putative transcriptional regulator (*ptr*).

IN RHIZOBIA, T3SSs AND NODULATION GENES ARE COREGULATED

Genes coding for structural components of T3SSs are clustered in rhizobia and are often found close to symbiotic genes like the *nod* genes needed for synthesis of NFs. In α-rhizobia, except for the *rhcC1* secretin and the putative translocators *nopX* and *nopE* monocistronic genes, most T3SS genes are organized in three major genetic units thought to form distinct operons (118, 122) (**Figure 1**). One major operon containing most of the T3SS structural genes is highly conserved, whereas the two remaining operons often display strain-specific differences in gene composition and genetic organization. In β-rhizobia, such as *Cupriavidus taiwanensis* strain LMG19424, genes encoding components of a symbiotically active T3SS are organized in two divergently transcribed units that are more similar in genetic organization to the human opportunistic pathogen *Burkholderia cenocepacia* than they are to the T3SS loci of α-rhizobia (97) (**Figure 1**).

A remarkable feature of α-rhizobial T3SS genes is their coregulation with major *nod* genes, whose transcription is flavonoid- and NodD-dependent. In response to plant-made flavonoids and via binding to specific *nod* box regulatory sequences, transcription regulators of the NodD family activate *nod* genes as well as *ttsI* (57, 129) (**Figure 2**). Once expressed, the transcriptional activator TtsI binds to conserved promoter sequences called *tts* boxes found upstream of genes and operons coding for T3SS components and T3Es (11, 57, 64, 70, 135, 144). Interestingly, TtsI may regulate functions other than those needed for T3SSs, like in *Ensifer fredii* strain NGR234,



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Erratum >

Figure 2 (*Figure appears on preceding page*)

The multifaceted roles of rhizobial T3SSs in legume symbioses. Molecular interplay between factors secreted by rhizobia and some of the plant components involved in perceiving microbes. Plants can detect bacteria via membrane receptors (PPRs) that recognize specific MAMPs and activate a first line of defense (MTI). Concomitantly, in compatible rhizobia, plant flavonoids trigger the synthesis and secretion of NFs and T3Es, via the successive activation of NodD and TtsI transcription regulators. Membrane-bound LysM-RLK receptors (NFR1 and NFR5) associated with SymRK and compatible NFs activate the symbiotic signaling pathway, leading to nodule organogenesis and bacterial infection. T3Es translocated into the host cell cytoplasm by rhizobial T3SSs modulate the symbiotic outcome depending on the host plant. Some T3Es, including NopM, NopP, NopD, NopL, and NopT, interfere with different components of the plant immune systems and thus promote symbiosis by repressing MTI responses. Alternatively, when detected by cytosolic receptors (Rj4, Rj2, GmNNL1), some T3Es (NopP, InnB, Bel2-5) trigger ETI-type responses that block nodulation. Eventually, T3Es such as ErnA and Bel2-5 may directly trigger nodule organogenesis via an unknown mechanism possibly involving binding to nuclear DNA. Note that the figure summarizes a set of different responses mediated by rhizobial T3Es in different plant species. Arrows represent documented interactions. Dashed lines represent hypothetical functions. Abbreviations: EPS, exopolysaccharide; ETI, effector-triggered immunity; LPS, lipopolysaccharide; LysM-RLK, lysin motif receptor-like kinase; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MTI, MAMP-triggered immunity; NF, Nod factor; P, phosphorylated site; PRR, pattern recognition receptor; S, SUMOylation; SIPK, salicylic acid–induced protein kinase; T3E, type III effector; T3SS, type III secretion system; Ub, ubiquitination.

where TtsI also activates loci needed for modifying lipopolysaccharides (LPS) into a symbiotically active form called rhamnan (70, 95). In contrast, activation of T3SS functions in *C. taiwanensis* was reported to be independent of both flavonoids and TtsI, whereas it was triggered by glutamate, like in plant pathogens such as *Ralstonia solanacearum* and *Pseudomonas aeruginosa* (97).

DISTRIBUTION AND EVOLUTION OF T3SSs IN RHIZOBIA

Rhizobia form a large polyphyletic group of hundreds of species distributed in as many as 18 genera of alpha- and betaproteobacteria (20; **https://www.bacterio.net**). The propensity of nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) genes to be carried by genomic islands and plasmids that can be transferred laterally between strains is thought to greatly contribute to the existing diversity of rhizobia (72, 92, 94). Symbiotic tool kits do not necessarily include T3SSs, whose prevalence varies significantly among genera. In the major *Ensifer*, *Mesorhizobium*, and *Rhizobium* genera, co-occurrence of *nod* and T3SS genes is not the rule (115, 125, 133). For example, among 48 *Ensifer* strains analyzed, only a third possessed a T3SS, but all were found to carry type IV secretion systems (T4SSs) (115). Like T3SSs, T4SSs deliver effector molecules to the cytoplasm of host cells (17, 40), and several reports have confirmed that T4SSs of rhizobia also modulate symbiosis positively or negatively, depending on the legume host (21, 45, 77). By contrast, more than 90% of the nodule isolates that belong to the *Bradyrhizobium* genus carry conserved *nod* and T3SS genes that are embedded in symbiotic islands; phylogenetic analyses indicated that these symbiotic genes share a common evolutionary history (122). As several reports have proposed bradyrhizobia to be the ancestors of all rhizobia (46, 87, 133), the transformation of a free-living bacterium into an endosymbiont of legumes may have required both NFs and T3Es to achieve, respectively, nodule formation and subversion of host immunity to secure intracellular infection of nodule cells. If so, during their coevolution with their hosts, rhizobia may also have developed alternatives to T3SSs to cope with plant immunity, for example, by modifying cell surface components (e.g., exopolysaccharides, LPS, or K-antigen polysaccharides) or by using alternative protein secretion systems (34, 39, 47, 78, 92).

Of the seven families of T3SS genetic clusters in gram-negative bacteria that have been cataloged (32, 126), rhizobia primarily use the α-RhcI type to target legume hosts. Some strains possess up to two apparently complete additional atypical T3SS clusters; however, little is known about their function(s) (118, 122). In the *E. fredii* strain NGR234, inactivation of the T3SS-II locus had no measurable effect on symbiosis (103). By contrast, although inactivation of the atypical T3SS of

C. taiwanensis strain LMG19424 had no effect on its primary host, *Mimosa pudica*, it extended the host range of the mutant to include *Leucaena leucocephala* (97). It is possible that these additional atypical T3SSs provide rhizobia with a selective advantage, for example, during colonization of the host rhizosphere and/or competition against other soil microbes or organisms.

NO UNIVERSAL RECIPE FOR COCKTAILS OF RHIZOBIAL EFFECTORS

Approaches based on secretome analysis enabled the first identifications of rhizobial T3Es in *Ensifer* and *Bradyrhizobium* strains (43, 71, 129). Proteins secreted by flavonoid-activated T3SSs were originally called nodulation outer proteins (Nops), to mirror the nomenclature of the *Yersinia* outer proteins (Yops) (69). However, not all Nops should be considered bona fide effectors since NopA, NopB, and NopX are pilus components, and conversely, some of the recently identified T3Es, such as InnB, Bel2-5, and ErnA, were not designated as Nops.

Actually, few of the secreted proteins have been confirmed to possess all the hallmarks of genuine symbiotic T3Es: (*a*) secretion by a T3SS, (*b*) translocation into host cells, and (*c*) modulation of symbiotic interactions with legume hosts. Thus far, only NopC, NopL, and NopP of *E. fredii* strains; NopE1 and NopE2 of *B. diazoefficiens* USDA110; Bel2-5 and InnB of *Bradyrhizobium elkanii* USDA61; and ErnA of *Bradyrhizobium vignae* ORS3257 meet all three criteria for bona fide T3Es (13, 49, 50, 79, 93, 100, 121).

Whereas homology searches for T3SS machinery proteins are simple, identifying the complete set of T3Es (effectome) of a symbiotic strain is more difficult. Genome mining analyses based on combined searches for regulatory *tts* boxes and/or homologies to known T3Es of symbiotic or pathogenic bacteria helped define the putative effectomes of rhizobia (13, 55, 91, 93, 109, 110, 121, 122). Although these *in-silico* searches may occasionally overestimate the number of T3Es secreted by rhizobia (13), comparison of the predicted effectomes revealed interesting properties. The size of T3E repertoires varies considerably between strains, from circa 10 in *Ensifer* strains to more than 50 for some bradyrhizobia such as *B. elkanii* USDA61 (55, 93). Although no core effectome has been identified in rhizobia, NopC, NopM, NopP, and NopT homologs are frequently shared between *Bradyrhizobium* and *Ensifer* strains, suggesting the existence of a possible early effectome core (122). Several of the predicted T3Es, such as ErnA, NopC, NopL, NopP, and NopI, appear to be specific to rhizobia (21, 49, 50, 121). Such differences in the composition of the predicted effectomes indicate high plasticity and rapid evolution of the T3E arsenals in rhizobia, possibly for a better adaptation to specific hosts.

SYMBIOTIC ROLES FOR RHIZOBIAL T3Es

Several studies have highlighted that the deletion of specific T3Es may improve, impair, or have no effect on the symbiotic outcome, depending on host plants (**Figure 3**). For example, compared to the parent *E. fredii* strain NGR234, a *nopT* mutant formed fewer nodules on *Tephrosia vogelii* but nodulated *Crotalaria juncea* better (19, 52), while remaining as proficient as the wild type on *Lablab purpureus* (52). Although listing all of the symbiotic properties of T3E mutants is beyond the scope of this review, it is noteworthy that host-dependent positive or negative effects on symbiosis were also reported when each of the following putative effector genes was mutated: *bel2-5*, *ernA*, *nopAB*, *nopC*, *nopD*, *nopE*, *nopF*, *nopI*, *nopJ*, *nopL*, *nopM*, *nopP* and *innB*, (33, 48–50, 52, 60, 79, 90, 93, 108, 122, 136, 139). A cumulative effect on symbiosis of deleting multiple effectors has been reported in several strains, including in *E. fredii* NGR234, where deletion of both *nopL* and *nopP* reduced *Flemingia congesta* nodulation more than mutation of either of these genes (107). Similarly, in *B. vignae* ORS3257, mutagenesis of both *nopP1* and *nopM1* impacted nodulation of *Aeschynomene indica* and *Vigna mungo* significantly more than single mutations of *nopP1* or *nopM1* (108, 121). It

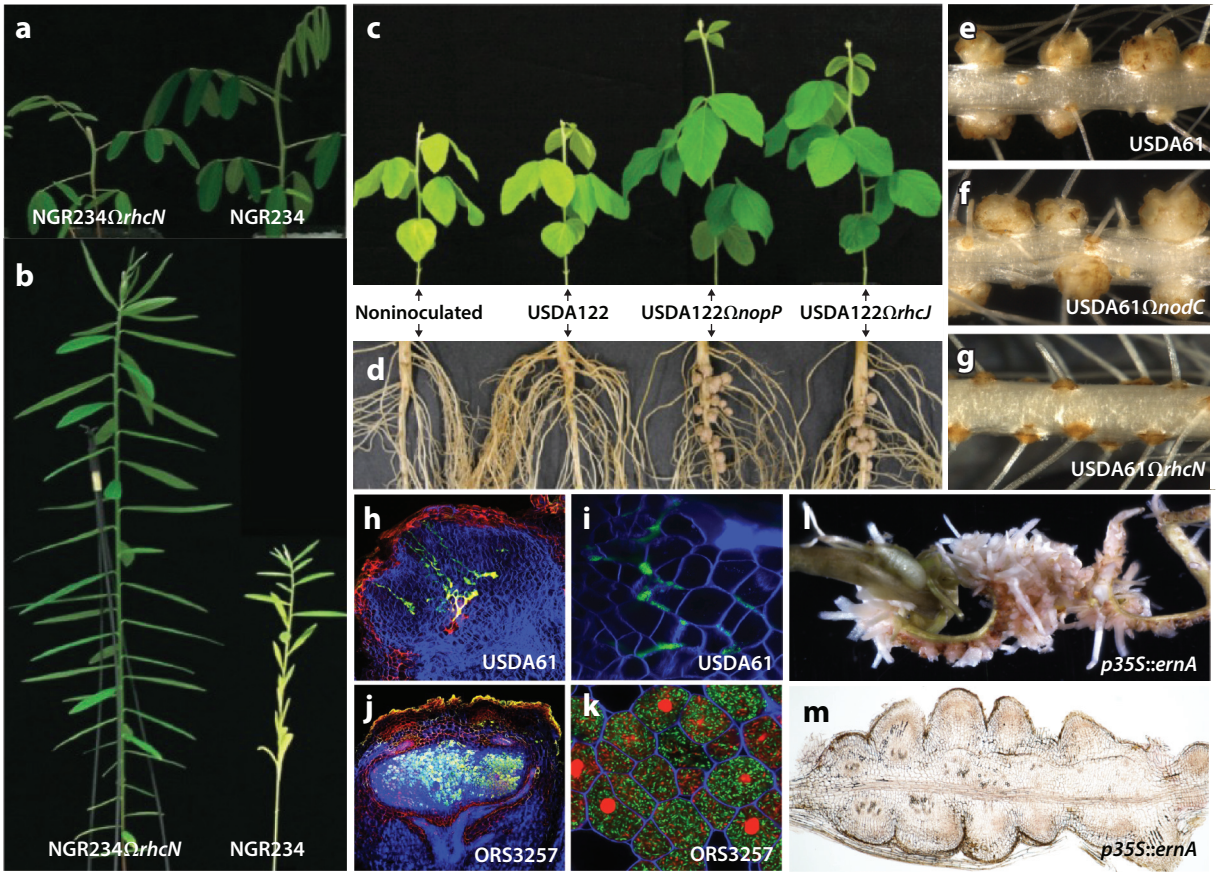

Figure 3

Impacts of T3SSs on rhizobium-legume symbioses. (*a*) In some hosts, a T3SS and its secreted T3Es can promote symbiosis, like that observed during interactions between *Ensifer fredii* NGR234 and *Tephrosia vogelii*, with plants showing reduced growth when inoculated with T3SS-deficient strains (e.g., NGR234Ω*rhcN*). (*b*) Alternatively, T3SSs may completely block symbiotic interactions, as illustrated here in the parent strain *E. fredii* NGR234 in *Crotalaria juncea*. The T3SS-mutant NGR234Ω*rhcN* is fully proficient. (*c*,*d*) One specific effector may suffice to trigger ETI-based incompatibility, as is the case of Rj2-soybean cultivars capable of detecting NopP of *Bradyrhizobium diazoefficiens* USDA122. (*e*–*g*) The T3Es of some rhizobia may also bypass the requirement for NFs to trigger nodulation, as shown in a *nodC* mutant of strain *Bradyrhizobium elkanii* USDA61 (USDA61Ω*nodC*) that is unable to secrete NFs but nonetheless forms nodules on *Aeschynomene indica* and in a similar way to the parent USDA61, while a T3SS mutant (USDA61Ω*rhcN*) fails to nodulate. Depending on the strain, the nodules elicited on *A. indica* via the T3SS are infected (*h*,*i*) intercellularly or (*j*,*k*) intracellularly. (*l*,*m*) The ErnA effector of *Bradyrhizobium vignae* ORS3257 confers the ability to form nodules, as shown by ectopic expression of ErnA in transgenic lines of *A. indica*, which results in formation of nodule-like structures. Abbreviations: ETI, effector-triggered immunity; NF, Nod factor; T3E, type III effector; T3SS, type III secretion system. Panels *a* and *b* adapted from Reference 107; panels *c* and *d* adapted from Reference 116 (CC BY 4.0); panels *e*–*k* adapted from Reference 85; and panels *l* and *m* adapted from Reference 121.

is thus now firmly established that in general, the role of rhizobial T3SSs in symbiosis does not depend on a single effector but rather results from the combined activities of a cocktail of secreted T3Es that can act synergistically, redundantly, and/or antagonistically, depending on the legume host that is challenged (21, 108).

In animal pathogens, effectors are known to be translocated through T3SSs sequentially with chaperone proteins controlling both the order and the time of secretion (14, 127, 138). A study of

the T3SS-mediated interactions between *B. elkanii* USDA61 and several *Lotus* accessions showed that NopF inhibited the infection of *L. japonicus* cultivar Gifu, whereas NopM was responsible for inducing early senescence in nodules of *L. japonicus* cultivar MG-20 (60). Although sequential secretion of T3Es in USDA61 cannot be excluded, no T3SS-dependent chaperone has been identified in rhizobia. Since in vitro, genistein-induced cells of USDA61 secreted NopF and NopM together with other T3Es (60), an alternative hypothesis to sequential secretion of T3Es is that plant cells that are either crossed by infection threads or located inside the nodules differ in susceptibility to T3Es.

FUNCTIONAL CHARACTERIZATION OF T3Es

Like T3Es of pathogenic bacteria, rhizobial T3Es often display a modular architecture with different functional domains and motifs for subcellular localization once T3Es are translocated into host cells (3, 25, 114). Only a few rhizobial effectors have been functionally characterized, mainly using transgenic or transfected nonlegume hosts such as *Nicotiana benthamiana*, *Nicotiana tabacum*, or *Arabidopsis thaliana*, three of the most widely used models for analyzing T3Es. These experimental approaches highlighted functional homologies between T3Es of rhizobia and plant pathogens, in particular for domains needed to subvert the posttranslational modification pathways of the hosts, for example by SUMOylation, ubiquitinylation, or phosphorylation (**Figure 2**).

Targeting the Ubiquitin-SUMO Pathways of Hosts

Ubiquitin and small ubiquitin-like modifier (SUMO) are structurally related small proteins used by eukaryotic cells to specifically modify various substrate proteins. These posttranslational modifications (ubiquitinylation and SUMOylation) can influence several aspects of the protein biology, including stability, activity, location, and interactions with different partners (5, 75, 104). Although SUMO and ubiquitin are both conjugated to target proteins via three-step enzymatic cascades, distinct enzymes are involved in each step: E1 activating enzymes, E2 conjugating enzymes, and E3 ligases. Effectors of the NopM family, found in many rhizobia, carry a C-terminal novel E3 ubiquitin ligase (NEL) domain as well as a conserved N-terminal leucine-rich repeat (LRR) domain that is thought to define substrate specificity (3, 96). When expressed in *N. benthamiana*, NopM of strain NGR234 represses the production of reactive oxygen species induced by flagellin (140). Subsequent in vitro assays showed that NopM forms unanchored polyubiquitin chains and possesses auto-ubiquitination activities, thereby confirming the functionality of its NEL domain (141). Considering that most ubiquitinated proteins are degraded by 26S proteasomes (104), NopM effectors may use the ubiquitin-proteasome pathway to degrade plant proteins possibly involved in defense responses.

Unlike the NopM E3 ubiquitin ligase, other rhizobia effectors are predicted to carry a C-terminal ubiquitin-like protease (ULP)-like domain for deSUMOylation of proteins (93, 139). ULP-like domains are also conserved in several effectors of plant pathogens, including XopD of *Xanthomonas campestris* and PsvA of *Pseudomonas syringae* (54). Interestingly, NopD of *Bradyrhizobium* sp. XS1150 and Bel2-5 of *B. elkanii* USDA61, which both carry a ULP-like domain, share similarity with XopD. NopD and Bel2-5 were both shown to target the plant cell nucleus and, in their corresponding ULP-like domain, to carry the catalytic triad residues H/G/C that are required for activity (44, 53, 54, 93, 139). As NopD of strain XS1150 processed plant SUMO proteins and cleaved SUMO-conjugated substrates, the functionality of its ULP-like domain was confirmed, strengthening the view that NopD homologs act in similar ways to their plant pathogen counterparts by deSUMOylating the targeted plant proteins (139).

Notably, a search for ULP-like and NEL domains in the deduced proteomes of fully sequenced members of the *Bradyrhizobium* genus indicated that NopM and SUMO proteases are abundant in

symbiotic strains with a T3SS and form the two largest families of T3Es (122). Thus, the ubiquitin and SUMO modification pathways could be the plant cellular functions preferentially targeted by T3Es of bradyrhizobia during symbiosis.

Modulation of MAPK Signaling

Perception of MAMPs by plant plasma membrane receptors triggers plant immune responses via activation of mitogen-activated protein kinases (MAPKs) (134). By mimicking substrates for activity of MAPKs, many T3Es of pathogens interfere with the phosphorelays needed for PTI activation (15, 23, 134). In *E. fredii* NGR234, NopL, NopM, and NopP were shown to be substrates for plant kinases in vitro (2, 33, 107, 141), with NopM and NopL also shown to be phosphorylated *in planta* by the MAPK salicylic acid–induced protein kinase (SIPK) of *Nicotiana tabacum* (NtSIPK) (33, 140, 141, 147). Physical interactions of NopL and NtSIPK in the plant cell nucleus were also reported (33). Disruption of signaling of MAPKs in legume cells is probably the main function of NopL, since inactivation of all its multiple phosphorylation sites resulted in a symbiotic phenotype similar to that of a *nopL* knockout mutant, with premature nodule senescence in *Phaseolus vulgaris* (33). In contrast to NopL, which can be hyperphosphorylated (33), NopM carries a single serine residue found to be phosphorylated in vitro as well as in vivo. As NopM is also an E3 ubiquitin ligase, it may interfere with both MAPK signaling and protein degradation processes.

Proteolytic Activities of Rhizobial T3Es

Several effectors of plant pathogens have been shown to cleave proteins once inside host cells (15, 23). Among them, the C58 cysteine protease AvrPphB of *P. syringae* was shown to undergo self-cleavage and also to cleave several plant receptor-like cytoplasmic kinases, such as BIK1 and PBS1 of *A. thaliana*, which are involved in transducing PTI and ETI responses, respectively (25, 146). By homology, NopT effectors of rhizobia belong to the large AvrPphB/YopT family of T3Es. Several studies have indicated that, once translocated into a host cell, NopT undergoes proteolytic self-cleavage that exposes residues required for lipid acylation modification (N-myristoylation and S-palmitoylation), which are needed to subsequently target the plant plasma membrane (25, 29, 67). The typical catalytic triad residues C/H/D of cysteine proteases that were identified in NopT effectors were shown to be required for optimal self-cleavage as well as for induction of a hypersensitive response in transformed tobacco cells (19, 25, 29, 52, 67). Interestingly, the sites for autoprocessing in AvrPphB and for the AvrPphB-dependent cleavage of PBS1 share identical amino acids (25). By analogy, the DKM amino acid motif that is needed for self-cleavage of NopT of strain NGR234 is possibly also conserved in the plant proteins targeted by NopT.

When incubated in vitro in the presence of Ca^{2+}, the purified NopE1 proteins of *B. diazoefficiens* USDA110 also displayed autoproteolytic activity, but via two metal ion-inducible autocleavage (MIIA) domains (102, 136). As a noncleavable form of NopE1 did not complement a *nopE1 nopE2* double mutant of USDA110 to restore the wild-type detrimental symbiotic phenotype on *Vigna radiata* plants (136), the self-cleavage property of NopE1 appears to be required for its activity *in planta*. Whether NopE1 and NopE2 of USDA110 act as bona fide effectors by cleaving specific target proteins or play a role in the translocation of rhizobial T3Es across the host plasma membrane as proposed by Teulet et al. (122) remains to be determined.

TARGETS OF RHIZOBIAL T3Es

Besides MAPKs such as SIPK that are targeted by NopL and NopM, few other legume proteins have been identified as specific substrates for rhizobial T3E activity. Recently, *Robinia pseudoacacia*

proteins susceptible to being targeted by NopT and NopP of *Mesorhizobium amorphae* CCNWGS0123 were identified (62, 67). In yeast two-hybrid combinations of bait (NopT or NopP) and prey (cDNA of *R. pseudoacacia*), the membrane-associated ATP-CSACP2 (ATP-citrate synthase alpha chain protein 2) and HIRP (hypersensitive-induced response protein) interacted with NopT, while a TRAPPC13 (trafficking protein particle complex subunit 13)-like protein interacted with NopP. Although interaction of NopT and NopP with these plant proteins has been validated by bimolecular fluorescence complementation assays in *N. benthamiana*, further studies are needed to determine how these T3E/target(s) interactions modulate the host cellular processes and symbiotic properties of secreting strains.

Quantitative trait locus (QTL) analyses of modifications in the number and dry weight of nodules on soybean plants also led to the identification of possible targets for NopD, NopL, and NopP of *E. fredii* HH103 (131, 132, 148). QTL mapping in soybean showed that NopD was associated with a locus coding for one F-Box/LRR-repeat protein (132), NopL was associated with two soybean genes coding for a protein phosphatase 2C (PP2C)-related protein and a receptor protein-tyrosine kinase (RPK) (148), and NopP was associated with a thaumatin-like protein (TLP) and MAPK3 (131). While the putative functions assigned to these candidate loci are consistent with T3Es interfering with plant immunity, further studies are needed to confirm whether the interactions between NopD, NopL, and NopP and the candidate plant proteins are direct or indirect.

T3Es AND EFFECTOR-TRIGGERED IMMUNITY IN LEGUMES

An increasing number of reports confirm that rhizobial T3Es can trigger ETI responses in legumes, thus restricting the host range of strains and/or limiting symbiotic proficiency to specific cultivars (74, 114). Products of dominant alleles have been known for decades to restrict nodulation of specific strains of *Glycine max* (16). Yet, genetic analyses only recently revealed the roles in host specificity of cytosolic plant receptors and of the corresponding T3Es. For example, the *Rj2* and *Rfg1* alleles of the same locus on soybean chromosome 16 code for nearly identical 1,052-residue-long Toll-interleukin receptor (TIR)-nucleotide-binding site (NBS)-LRR proteins involved in the contrasted specificities of *Bradyrhizobium japonicum* USDA122 and *E. fredii* USDA257 (142). Inoculation of the *Rj2*-genotype *G. max* cultivar Hardee with natural variants of strain USDA122 showed that NopP determines the symbiotic incompatibility, with 3 of its 277 amino acids being critical for the *Rj2*-mediated transient activation of defense markers such as the *PR-2* gene at two days after inoculation (116). Remarkably, in host plants, a single–amino acid difference between *Rj2* alleles may result in symbiotic incompatibility (117). While NopP of *B. diazoefficiens* USDA110 did not elicit ETI on *G. max* cultivar Hardee (116), it blocked nodulation via root hairs on soybean accessions with a functional *Gm*NNL1 locus, which encodes another TIR-NBS-LRR protein also capable of detecting NopP variants (145). Interestingly, disruption of *Gm*NNL1 by a 179-bp short interspersed nuclear element (SINE)-like transposon improved nodulation via root-hair infection and consequently nitrogen fixation by previously rejected strains. This could explain why today most cultivated soybeans carry an inactivated *Gm*NNL1 locus, which was inadvertently selected by breeders when they screened for improved nitrogen fixation and biomass accumulation (145).

In *G. max* cultivar BARC-2 of the *Rj4*/*Rj4* genotype, *B. elkanii* strain USDA61 failed to form root-hair curling and infection threads, while roots accumulated salicylic acid, H_2O_2, and products of defense-related genes, some of which were shown to act during ETI in *Arabidopsis* (143). However, the *Rj4* dominant allele of soybean chromosome 1 that restricts nodulation by USDA61 does not code for an NBS-LRR but rather for a thaumatin-like protein (120) that mediates symbiotic incompatibility when challenged by the SUMO-protease effector Bel2-5 (27).

T3E-mediated symbiotic incompatibility is not restricted to *Glycine soja* and *G. max* cultivars, as *V. radiata* accessions KPS1 and SUT1 were shown to be incompatible with the *B. elkanii* USDA61 and *B. vignae* ORS3257 strains, respectively. Interestingly, two distinct effectors, NopP of strain ORS3257 and InnB of strain USDA61, were shown to mediate this incompatibility in *V. radiata* (80, 108). The identification of Rj2 orthologs in *V. radiata* suggests that the mechanism responsible for NopP recognition by the soybean Rj2 system may also mediate strain compatibility in *Vigna* spp. As additional T3Es such as InnB and Bel2-5 can also provoke symbiotic incompatibility via recognition by distinct R-proteins, a diverse range of controls appear to be used by legumes to select specific rhizobia.

That legumes may block nodulation of proficient rhizobia raises the question of what can lead to such a deadlock in an interaction that would otherwise be beneficial to the host. One possible explanation is collateral damage caused by the immune system programed to repel microbial infections. Some studies aiming at large-scale identification of effector targets have revealed the existence of a core hub of plant proteins likely to be targeted by a wide range of phytopathogens (76, 137). It is tempting to speculate that some T3Es secreted by rhizobia may also target plant proteins belonging to this so-called immune hub to promote symbiotic infections. If so, some of the NBS-LRR proteins that guard the immune hub against pathogen effectors could recognize rhizobial T3Es and trigger a response, thereby limiting infection of proficient rhizobia.

T3SS ALTERNATIVES TO NOD FACTOR–DEPENDENT NODULATION

The long-established paradigm of nodulation depending exclusively on NFs was challenged when it was shown that photosynthetic *Bradyrhizobium* strains forming nodules on both roots and stems of tropical *Aeschynomene* species did not possess the canonical *nod* genes required for NF synthesis (35). Initially considered an exception to the rule, nodulation in the absence of NFs was recognized as being less exceptional when legume species other than *Aeschynomene* were found to form nodules when inoculated with rhizobia mutants unable to synthesize NFs (68, 84). Remarkably, nodulation of *G. max* cultivar Enrei by a *nodC* mutant of the nonphotosynthetic *B. elkanii* USDA61 strain was shown to depend upon a functional T3SS (84). As several symbionts of *Aeschynomene* species are photosynthetic bradyrhizobia that do not produce NFs and do not possess T3SSs (e.g., strains ORS278 and BTAi1), it now appears that at least two alternative NF-independent pathways may support nodule formation, one of which involves T3Es (85). A feature shared by these NF-independent nodulation processes is that infection of legume tissues by rhizobia is not mediated via infection threads, which is the case in most legume crops. Instead, rhizobia use an intercellular invasion mechanism that still occurs in approximately 25% of known legume species and that is suggested to predate infection threads (12, 84, 113).

Apparently, the use of T3Es to nodulate legumes is widespread in bradyrhizobia, as a wide range of nonphotosynthetic *Bradyrhizobium* strains can elicit nodules on *A. indica* in a T3SS-dependent manner (85). Interestingly, depending on the inoculated strain, infection of nodule cells is not necessarily achieved. For *B. elkanii* strain USDA61, proliferation is restricted to the intercellular space between nodule cells, whereas *B. vignae* strain ORS3257 infects nodule cells intracellularly (**Figure 3**). Although differences in the composition of cocktails of secreted T3Es may contribute to these distinct phenotypes, intracellular ORS3257 bacteria did not fix enough nitrogen to foster plant growth, possibly because bacteroid differentiation was blocked and/or premature degradation of intracellular bacteria occurred (85). In the case of strain ORS3257, at least five T3Es were found to play synergistic and complementary roles in nodulation of *A. indica*: (*a*) A rhizobial-specific T3E named ErnA (effector required for nodulation A) controlled nodule organogenesis, (*b*) NopT and NopAB were both needed for infection of nodules, and (*c*) NopM1 and NopP1 were required to maintain chronic infection of nodule cells (121). However, whether these successive

T3SS-dependent steps in nodulation and infection suffice to secure proficient symbioses in natural environments remains to be demonstrated. In this respect, so far only a few strains such as *Bradyrhizobium mercantei* SEMIA6399, *Bradyrhizobium liaoningense* CCBAU83689, and *Bradyrhizobium* sp. Y36, all of which were isolated from field nodules, have been confirmed to lack *nod* genes and to carry T3SS loci. It is therefore possible that in those cases, T3Es act as the main nodulation determinants (122).

RHIZOBIAL T3Es CAPABLE OF TRIGGERING NODULATION

The importance of ErnA in nodulation of legumes was confirmed using two approaches (121). First, the transfer of *ernA* was shown to extend the host range of a recipient *Bradyrhizobium* strain to *A. indica*. Second, transgenic roots of *A. indica* that expressed *ernA* ectopically formed nodule-like structures (**Figure 3**). The 370-amino-acid-long ErnA displays no homology to known functional domains, except for a nuclear localization signal (NLS) needed for targeting the plant nucleus. As Förster resonance energy transfer (FRET)-fluorescence lifetime imaging (FLIM) data confirmed the association between ErnA and nuclear nucleic acids *in planta*, it was proposed that ErnA modulates plant gene expression and in this way activates the nodule developmental program. In support of this hypothesis, several T3Es from plant pathogens were shown to modulate plant gene expression (15), including the transcription activator–like (TAL) effectors from *Xanthomonas* spp. (e.g., members of the AvrB family) and the two paralogous T3Es from *Pantoea agglomerans* called HsvG and HsvB (7, 8, 15, 81, 82). Interestingly, HsvG and HsvB were found to induce the formation of galls that, like in root nodules, involve the reactivation of the cell cycle in the targeted plant cells.

However, ErnA of strain ORS3257 is not the only rhizobial T3E capable of triggering nodule formation. Notably, while the 1,328-amino-acid-long Bel2-5 of *B. elkanii* USDA61 blocks symbiosis with *G. max* cultivar BARC-2, it was shown to be required for nodulation of the *G. max* cultivar Enrei *nfr1* mutant that is unable to perceive NFs (93). Since Bel2-5 targets plant cell nuclei and carries a SUMO protease domain needed for nodulation (93), Bel2-5 might alter gene expression in legume cells by deSUMOylation of some plant proteins, in much the same way as XopD of *X. campestris* does in *Arabidopsis* and tomato (54, 119). If Bel2-5 and ErnA most probably trigger nodulation using different mechanisms, they both may target conserved determinants in the NF-signaling pathway such as the nuclear regulators CCaMK, CYCLOPS, NIN, LBD16, NF-Y, and CRE1, which, when overexpressed in various legumes, induce nodule-like structures similar to those triggered by ErnA on *A. indica* (26, 36, 101, 106, 111, 112, 123, 124). Alternatively, ErnA and/or Bel2-5 could alter the levels of cytokinin and auxin phytohormones, both of which contribute to nodule formation (9). The fact that T3E HopQ1 of *P. aeruginosa* was shown to activate cytokinin signaling supports this hypothesis (41).

Unlike Bel2-5, which has been found in only a few strains of *B. diazoefficiens*, *B. elkanii*, and *B. japonicum* (93), *ernA* homologs were identified in the genomes of more than two-thirds of the *Bradyrhizobium* strains that carry a T3SS (121, 122). Surprisingly, all of the strains that carried *ernA* and/or *bel2-5* also carried genes for NF synthesis and were isolated from legumes known to depend upon compatible NFs for nodulation. This raises the question of the roles of ErnA and Bel2-5 in conventional NF-dependent symbioses and whether these proteins act synergistically with NFs to reinforce the nodulation properties of a strain. Alternatively, ErnA and Bel2-5 may have been recruited by rhizobia to gain access to legumes that do not respond to the NFs they secrete.

CONCLUSIONS

Initially thought to be characteristic features of pathogens needed for full virulence, T3SSs are now also recognized as hallmarks of beneficial microbes such as rhizobia. In addition to the fact

that the T3SS machineries of plant pathogens and rhizobia share structural and functional homologies, symbiotic and pathogenic T3Es act in surprisingly similar ways on the plant cells they target. The fact that T3SS clusters were found to have coevolved with common nodulation genes in most bradyrhizobia (122) suggests that the switch from a free-living to a symbiotic state originally depended on the co-acquisition of T3SSs and NF-biosynthetic functions. Alternatively, in the context of primal rhizobium-legume interactions, the activity of T3SSs was under strong positive selection, at least in members of the *Bradyrhizobium* genus. Either way, and perhaps except for ErnA and Bel2-5, which are capable of triggering nodulation even in the absence of NFs, symbiotic T3Es are mostly involved in specifying the host range of rhizobia, much in the same way as effectors do for plant and animal pathogens. When translocated into plant cells not equipped for their detection, T3Es of rhizobia appear to promote colonization of plant tissues mostly by interfering with the plant systems needed to locally fight and repel microbial infections (e.g., MAPKs in PTI signaling). By contrast, in legume hosts capable of detecting one or several of the secreted T3Es, plant immune responses may halt nodulation. That cocktails of T3Es eventually determine the symbiotic outcome is thus fully consistent with a multifactorial checks-and-balance system that allows plants to carefully choose the bacteria that will be allowed access to the secluded ecological niche formed by nodules.

The lowering of plant defenses below a threshold that would block infection by rhizobia appears to be a common phenomenon in many—if not most—legume-rhizobia associations. Since the infectious perimeter to which symbiotic rhizobia are confined is strikingly circumscribed in both time and space (e.g., infection threads, infection pockets, and some nodule cells), the control of legumes over rhizobium penetration and dissemination is extremely tight. In plant-pathogen research, the continuous evolution of T3E inventories needed for successful infections versus the associated plant defense mechanisms needed to repel pathogens has often been compared to an arms race. Despite the obvious growth benefits legumes obtain by associating with nitrogen-fixing bacteria, host plants and rhizobia also appear to be engaged in a struggle in which bacteria search to secure all-inclusive accommodations while legumes try to protect resources and to maximize nodule outputs. Both ErnA and Bel2-5 may thus be regarded as rhizobial attempts to force the issue toward nodulation, possibly together with other T3Es whose role in triggering nodule organogenesis may have thus far been overshadowed by the more effective NFs.

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