REVIEW ARTICLE
Nodulation outer proteins: double-edged swords of symbiotic rhizobia

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Rhizobia are nitrogen-fixing bacteria that establish a nodule symbiosis with legumes. Nodule formation depends on signals and surface determinants produced by both symbiotic partners. Among them, rhizobial Nops (nodulation outer proteins) play a crucial symbiotic role in many strain–host combinations. Nops are defined as proteins secreted via a rhizobial T3SS (type III secretion system). Functional T3SSs have been characterized in many rhizobial strains. Nops have been identified using various genetic, biochemical, proteomic, genomic and experimental approaches. Certain Nops represent extracellular components of the T3SS, which are visible in electron micrographs as bacterial surface appendages called T3 (type III) pili. Other Nops are T3 effector proteins that can be translocated into plant cells. Rhizobial T3 effectors manipulate cellular processes in host cells to suppress plant defence responses against rhizobia and to promote symbiosis-related processes. Accordingly, mutant strains deficient in synthesis or secretion of T3 effectors show reduced symbiotic properties on certain host plants. On the other hand, direct or indirect recognition of T3 effectors by plant cells expressing specific R (resistance) proteins can result in effector triggered defence responses that negatively affect rhizobial infection. Hence Nops are double-edged swords that may promote establishment of symbiosis with one legume (symbiotic factors) and impair symbiotic processes when bacteria are inoculated on another legume species (asymbiotic factors). In the present review, we provide an overview of our current understanding of Nops. We summarize their symbiotic effects, their biochemical properties and their possible modes of action. Finally, we discuss future perspectives in the field of T3 effector research.

Key words: effector, nodulation outer protein, plant defence, root nodule, symbiosis, type III secretion system.

INTRODUCTION

Rhizobia are nitrogen-fixing bacteria that can infect leguminous host plants, usually by entering via root hairs. As a result, an infection thread is formed and the bacteria convert atmospheric nitrogen into ammonia in symbiosomes of formed root nodules. Nodule formation depends on molecular signals and determinants produced by both symbiotic partners. Plant roots secrete flavonoids, which interact with rhizobial NodD proteins to activate symbiotic genes with a conserved *nod* box in the promoter region. Most of these *nod* (*noe*, *nol*) genes encode enzymes required for synthesis of bacterial nodulation signals, called Nod factors (lipochito-oligosaccharidic nodulation factors). Host plants perceive Nod factors by Nod factor receptors (LysM domain receptor kinases) and initiate nodulation signalling to express symbiotic genes required for infection and nodule formation [1]. In addition to Nod factors, nodule formation may be affected by rhizobial surface carbohydrates such as exopolysaccharides, lipopolysaccharides, K-antigens and cyclic β-glucans. These polysaccharides, or oligosaccharides derived from them, are host-specific symbiotic determinants, i.e. they are required for nodulation of certain host plants [2]. Host-specific nodulation may also depend on Nops (nodulation outer proteins) secreted by the rhizobial T3SS (type III secretion system). The T3SS of Gram-negative bacteria is a complex multiprotein secretion apparatus that actively exports proteins through the lumen of needle-like or tubular structures, the T3 (type III) pili. T3SSs of pathogenic or symbiotic bacteria possess the capacity to deliver effector proteins (T3 effectors) directly into eukaryotic host cells and therefore were also named 'injectisomes'. We define Nops as rhizobial proteins that are either extracellular apparatus components of the T3SS or secreted T3 effectors.

The translocated T3 effectors often target components of the host immune system to increase bacterial survival and virulence [3,4]. A function of T3 effectors as bacterial virulence or avirulence factors has been well documented for many pathogenic bacteria, including phytopathogens such as *Pseudomonas syringae* [5] and *Xanthomonas campestris* [6]. In a similar way, rhizobial T3 effectors have been found to play a role in certain symbiotic interactions between rhizobia and legumes. On the basis of mutant analysis and identification of secreted proteins, functional T3SSs have been reported for various nodule-inducing bacteria belonging to the genera *Sinorhizobium* (*Ensifer*) [7–9], *Bradyrhizobium* [10–14], *Mesorhizobium* [15–17] and *Cupriavidus* [18]. Furthermore, recent sequencing data revealed the presence of a T3SS gene cluster in the genome of a nodule-inducing *Burkholderia* strain [19]. Thus the presence of a symbiosis-related T3SS in rhizobia tends to be the rule rather than the exception, particularly for *Bradyrhizobium* strains. As in pathogenic bacteria, rhizobial genes required for synthesis of the T3SS are clustered in the genome. Their genetic organization and the corresponding classification of rhizobial T3SSs into different
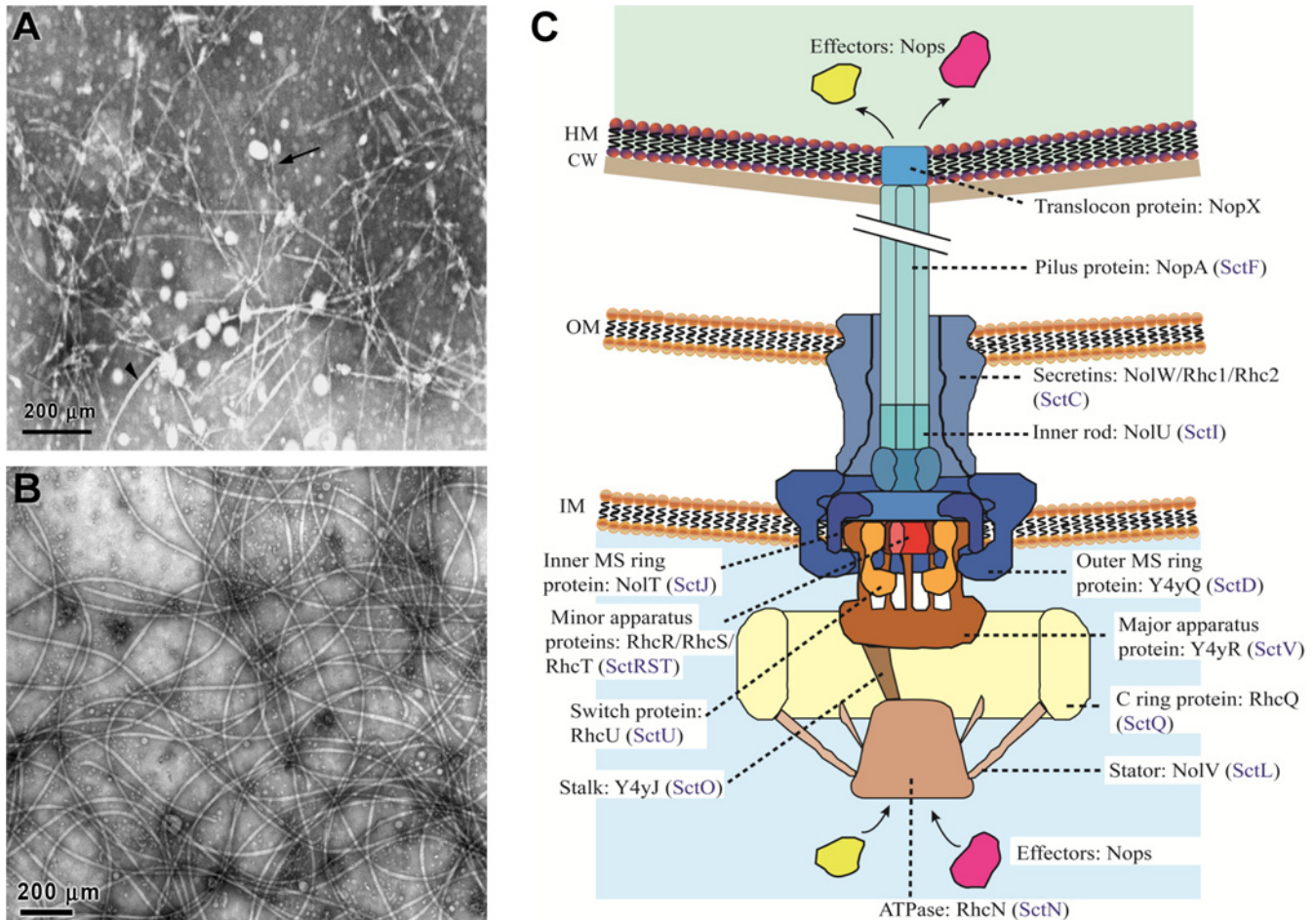

Figure 1   The rhizobial T3SS

Electron micrographs show T3 pili (surface appendages) produced by *S. fredii* USDA257 grown in the presence (**A**) or absence (**B**) of 1 μM apigenin (the arrow points to a T3 pilus and the arrowhead to a flagellum). The proposed model of the rhizobial T3SS (**C**) is based on a recently published T3SS apparatus model [23] and the sequence similarities of indicated rhizobial proteins with T3SS apparatus proteins from other bacteria [20]. Effector proteins (various Nops) are delivered from the bacterial cytoplasma (bottom) through the lumen of the T3 pilus (composed of the pilus subunit NopA and the translocon NopX) into the host cytoplasma (top). Protein names according to the unified nomenclature for T3SS proteins using the Sct (SeCreTion) prefix are indicated in parentheses [20]. Putative pilotins (such as Y4yS [25]) implicated in secretin assembly are not shown. Abbreviations: C ring, cytosolic ring; CW, cell wall; HM, host membrane, IM, inner membrane; MS ring, membrane and supramembrane ring; OM, outer membrane.

categories have been reviewed recently [20,21]. In the present review, we provide an overview on Nops, in particular on rhizobial T3 effectors.

NOPs ARE SECRETED THROUGH THE T3SS APPARATUS IN RESPONSE TO HOST FLAVONOIDS

Bacterial T3SSs show structural similarities to bacterial flagella, suggesting neofunctionalization of flagellum proteins for protein export during evolution [22,23]. Long T3 pili, also known as surface appendages, can be seen clearly on electron microscopy pictures of rhizobia cultured in the presence of host flavonoids [24] (Figures 1A and 1B). The rhizobial T3SS apparatus has been proposed to be similar to that of pathogenic bacteria. Figure 1(C) shows a model of the rhizobial T3SS apparatus on the basis of homology with apparatus proteins of pathogenic bacteria and the conserved structure of bacterial T3SSs. The pilus subunit protein NopA, however, does not show amino acid sequence similarities to pilus proteins from other bacteria. Nevertheless, NopA possesses predicted secondary-structure characteristics of

pilus subunit proteins in other bacteria [26,27]. T3 effectors are actively secreted through the lumen of the pilus with the help of the associated ATPase RhcN. Similarly to T3 effectors of pathogenic bacteria, rhizobial effector proteins can be translocated into host cells. For confirmation of translocation into legume cells, T3 effectors fused to adenylate cyclase were expressed in rhizobia and cAMP formation was measured in infected roots or nodule cells. Using this approach, translocation of the T3 effector NopP from *Sinorhizobium fredii* USDA257 into *Vigna unguiculata* roots was demonstrated [28]. Likewise, the NopE1 and NopE2 effectors of *Bradyrhizobium japonicum* USDA110 were found to be translocated into infected cells of *Macroptilium atropurpureum* nodules [29]. The pilus-associated protein NopX, formerly called NolX, [8,30,31] is a putative translocon protein related to translocon proteins of phyopathogens such as HrpF of *X. campestris* [32] and *PopF1/PopF2* of *Ralstonia solanacearum* [33]. These translocon proteins mediate translocation of T3 effectors into plant cells by forming a pore in the host membrane through which effectors can enter [34]. HrpF possesses lipid-binding activity *in vitro* and can induce pore formation in planar lipid bilayers [32]. The model shown in Figure 1(C) also

implies that rhizobial T3 pili are connected directly to the host cytoplasma. Alternatively, T3 effectors are first secreted into the symbiotic interface and later translocated with the help of NopX. Immunohistochemical localization studies showed that NopX of *S. fredii* USDA257 is mainly accumulated in infection threads [31].

Secretion through the T3SS and subsequent translocation of effectors depends on the N-terminal region of the effector proteins. However, the mechanism of substrate recognition by the T3SS remains largely unknown. Various computational methods have been established to predict T3SS substrates on the basis of N-terminal amino acid sequences [35–39]. However, such algorithms must be treated with caution and require experimental confirmation. Bioinformatic prediction of T3SS substrates is also complicated by the possibility that the information required for secretion may also lie within the mRNA. Frameshift mutations that completely altered the N-terminal amino acid sequence of T3 effectors did not prevent their secretion through the T3SS [40]. Compared with T3 effectors from pathogenic bacteria, Nops seem to possess certain conserved features. Rhizobial proteins related to the T3SS could be clearly discriminated from those of plant pathogenic bacteria when amino acid sequences were analysed using statistical and machine learning methods [41,42]. Nevertheless, Nops known to be secreted by rhizobial T3SS can also be secreted through the T3SS of *P. syringae* [28,43,44], indicating that N-terminal secretion signals are interchangeable among symbiotic and pathogenic bacteria.

Expression of most rhizobial T3SS genes usually depends on TtsI, a transcriptional regulator that controls expression of various flavonoid-inducible genes including those related to the T3SS. TtsI-regulated genes possess a promoter with a *tts* box, a conserved *cis* element required for TtsI-dependent gene expression [10,13,45–47]. For strain *S. fredii* NGR234, direct binding of TtsI to the *tts* box promoter region of two TtsI-regulated genes has been demonstrated by electrophoretic mobility-shift and DNase I cleavage protection assays [47]. In *Bradyrhizobium elkanii* SEMIA587, but not other strains, TtsI can also up-regulate its own expression since the *ttsI* promoter region contains a functional *tts* box [13]. Like *nod* genes required for Nod factor synthesis, *ttsI* genes possess a *nod* box in their promoter. Accordingly, expression of *ttsI* (as determined by promoter–*lacZ* reporter gene fusion constructs) is strongly induced by flavonoids in a NodD-dependent manner [10,48,49]. Negative regulation of *ttsI* gene expression by the regulatory protein NolR has been also reported. NolR appears to bind to a specific operator sequence in the *ttsI* promoter and thereby interferes with the action of NodD transcriptional activators [49,50]. As expression of *ttsI* usually depends on NodD proteins and specific host flavonoids, T3 pilus formation (Figures 1A and 1B) and subsequent secretion of T3 effectors through the T3SS are strongly induced during the rhizobial infection process.

IDENTIFICATION OF NOPs

Plant flavonoids of host plants are important signals for NodD-dependent activation of rhizobial *nod* genes and subsequent Nod factor production [2]. In the course of these *nod* gene studies, flavonoid-responsive promoters were identified by random insertion of a promoterless galactosidase reporter gene into the rhizobial genome. Using this method, two flavonoid-induced genes (host-inducible genes A and B) were identified in *S. fredii* USDA201. The mutants obtained showed reduced nodulation competitiveness on soya bean (or soybean, *Glycine max*) plants, i.e. were outcompeted by the wild-type strain [51]. Activation

of host-inducible gene B (later named *nolJ*) by flavonoids was found to be dependent on a functional *nodD1* gene. Nodulation experiments with the *nolJ* mutant and soya bean plants resulted in delayed nodule formation indicating a positive effect of this gene on symbiosis [52]. Furthermore, a gene of *Rhizobium etli* strain CNPAF512 showed sequence similarities to the host-inducible gene A of USDA201. A corresponding mutant induced delayed nodule formation on common beans (*Phaseolus vulgaris*) and reduced nodulation competitiveness compared with the wild-type strain [53]. At that time, the scientists reporting these findings could not know that they had characterized *nop* genes (encoding NopP and NopC respectively).

Other genes related to the T3SS were identified in the context of the phenomenon that certain *S. fredii* strains nodulate soya bean in a cultivar-specific manner. It was observed that soya bean cultivar McCall did not induce nodules when inoculated with *S. fredii* USDA257 [54] (Figure 2A), a strain that efficiently nodulates other soya bean cultivars such as Hardee [59]. Subsequent Tn5 mutagenesis of USDA257 resulted in mutant derivatives that gained the ability to induce nodules on McCall (Figure 2B). These findings showed that specific genes in USDA257 blocked nodulation of McCall [55]. The blockage of nodulation was found to occur at the stage of infection, as USDA257 induced on McCall only few aberrant infection threads [60]. Sequencing of the Tn5-tagged locus in USDA257 and further mutant analysis resulted in identification of a cluster of rhizobial genes involved in host-specific nodulation (*nolXWBTUV*; for synonymous gene names, see Supplementary Table S1) [61]. A nearly identical locus was later identified in the broad-host-range strain *S. fredii* NGR234 [62]. Similarly to *nolJ* of USDA201 [52], expression of these genes was found to be dependent on NodD proteins and flavonoids. When bacteria were grown in the presence of flavonoids, the NolX (later renamed NopX) and NolT proteins could be immunologically detected with raised antibodies [7,30,63]. As specific proteins in USDA257 and NGR234 were found to accumulate in bacterial culture supernatants in response to flavonoids [64], it was tested whether secretion of flavonoid-inducible proteins depends on the *nolXWBTUV* locus. This was indeed the case for five extracellular proteins, called SR (signal-responsive) proteins. [7]. The SR proteins were later renamed Nops (Table 1). Identification of T3SSs in pathogenic bacteria and sequencing of corresponding rhizobial genes (*rhc* genes) downstream of the *nolXWBTUV* locus (accession number L12251.1 for USDA257; accession number U00090.1 for NGR234) indicated that a rhizobial T3SS is required for secretion of Nops [30,65]. Similarly to the T3SS-deficient mutants of USDA257, a T3SS-knockout derivative of NGR234 (mutation in the *rhcN* ATPase gene) failed to secrete Nops. N-terminal amino acid sequencing resulted in identification of two Nops of NGR234, namely the previously identified NolX (NopX) and NopL (y4xL locus) [8]. In addition to USDA257 and NGR234, functional T3SSs and corresponding Nops were subsequently characterized in various other rhizobial strains (Table 1). Most Nops were identified by comparing extracellular protein profiles of wild-type bacteria with those of mutant strains with non-functional T3SSs. Alternatively, T3 pili (surface appendages) were purified from bacterial culture supernatants. Proteins were subsequently used for microsequencing or mass spectrometry analysis. Comparisons of extracellular protein profiles of flavonoid-induced with those of non-induced cultures also helped to identify Nops as it turned out that expression of *nop* genes is tightly regulated by TtsI and NodD proteins. An exception is *B. elkanii* USDA61, which secretes Nops independently of flavonoid inducers [12]. In many cases, T3SS-dependent secretion of Nops (wild-type compared with T3SS-knockout mutant) was
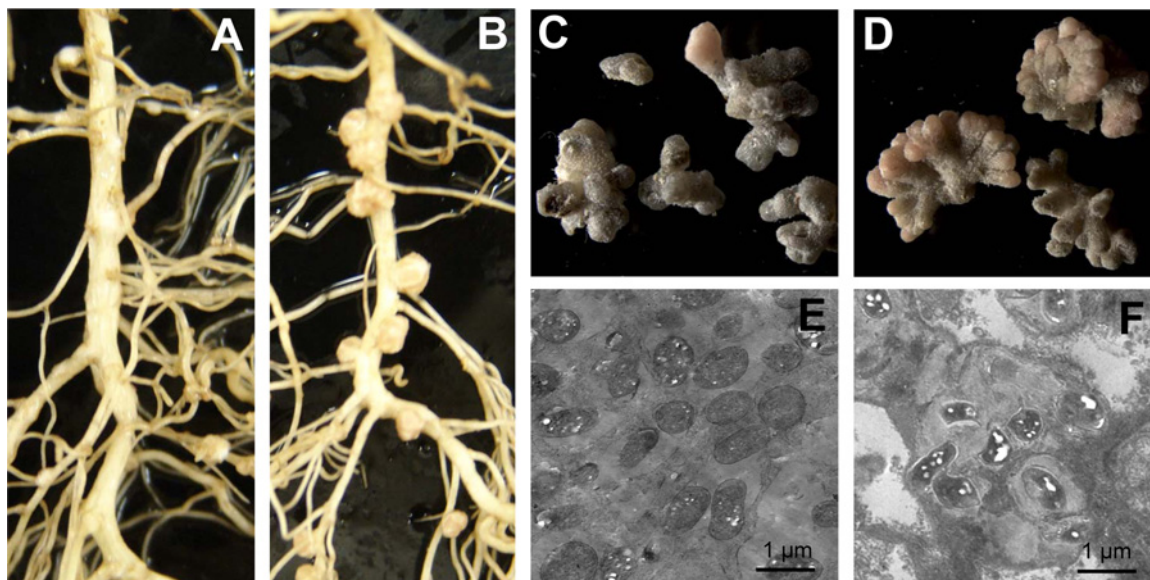
Figure 2   Symbiotic phenotypes of *nop* mutants

The examples shown indicate negative (**A–D**) and positive (**E** and **F**) effects of Nops on the nodule symbiosis. (**A**) *S. fredii* USDA257 (wild-type) secreting Nops on soya bean cv. McCall showing nodulation blockage. (**B**) USDA257 T3SS-knockout mutant (DH4; *rhcU* mutant) on soya bean cv. McCall forming normal effective (nitrogen-fixing) nodules [55]. (**C**) *S. fredii* NGR234 (wild-type) on *C. juncea* inducing mainly ineffective nodules (NopT functions as asymbiotic factor). (**D**) NGR234 *nopT* mutant on *C. juncea* inducing mainly effective nodules [56,57]. (**E**) Bacteroids of NGR234 (wild-type) in infected cells of mature *P. vulgaris* cv. Tendergreen nodules (NopL functions as symbiotic factor). (**F**) Bacteroids of the NGR234 *nopL* mutant in infected cells of Tendergreen nodules showing premature senescence (necrotic nodules) [58].

confirmed by immunoblot analysis using a specific antiserum, either raised against a given protein expressed in *Escherichia coli* or against a corresponding synthetic peptide. Using already available antisera against other Nops, it could be examined whether Nop secretion is maintained or abolished in a constructed *nop* gene mutant. In this way, it was found that the NopB proteins of USDA257 and NGR2334 are required for a functional T3SS [68,69], whereas other Nops with putative effector functions are not essential for T3SS-dependent protein secretion. Examples of Nops characterized in this way are NopL [56,69,74], NopP [77], NopT [56,57] and NopM [57] of NGR234. Antisera against Nops were also extremely useful for biochemical characterization of purified T3 pili. NopA, NopB and NopX proteins were co-purified with pili of *S. fredii* strains [24,26,68,69] and it was later found that these three proteins interact directly with one another [83].

Certain Nops such as NopT and NopM are similar to T3 effectors of pathogenic bacteria (Table 1). In fact, sequence comparisons with existing effector gene databases are helpful when searching for novel *nop* genes in rhizobial genomes. Candidate *nop* genes can also be identified on the basis of information from whole-genome gene expression data (genes induced by flavonoids) and location in the T3SS gene clusters [21]. Moreover, *nop* gene candidates can be predicted on a whole-genome scale by identification of *tts* boxes in promoter regions [10,16,43–47] and computational prediction of N-terminal T3SS-specific secretion signal sequences [35–39]. Nop candidate proteins (or the N-terminal domain containing the putative secretion signal sequence) fused to C-terminal tags (e.g. GFP or FLAG tag) can be expressed in wild-type rhizobia and a T3SS-deficient mutant for further analysis. T3SS-dependent secretion of such test proteins can then be analysed by fluorescence microscopy or immunological methods [16,68,72]. By definition, Nops should be secreted by wild-type bacteria, but not by a T3SS-knockout mutant. Using this approach, four FLAG-tagged candidate effectors of *Mesorhizobium loti* MAFF303099

were found to be secreted via the T3SS [16,72]. Another way to characterize candidates is to test them in a heterologous *Pseudomonas–Arabidopsis* translocation system. A given Nop candidate fused to Δ79AvrRpt2 (*P. syringae* effector AvrRpt2 lacking the N-terminal secretion signal) is expressed in *P. syringae* strain *Pto*DC3000 (or its T3SS-deficient mutant Δ*hrcC* as control). In the case of translocation of the hybrid protein, infiltrated leaves will induce rapid cell death caused by AvrRpt2 action. Tests performed in this way indicated translocation for most previously identified Nops (Table 1). In addition, many novel proteins from various rhizobial strains were found and designated as Nops [43,44]. In our opinion, such classification is somehow too early as secretion of these proteins through a rhizobial T3SS was not tested. In the present review, we excluded all Nops from Table 1 that were only tested in the heterologous *Pseudomonas–Arabidopsis* translocation system and instead list them separately in Supplementary Table S1. Future work is required to confirm whether these proteins are *bona fide* Nops secreted via a rhizobial T3SS. Remarkably, NopB, a pilus-associated Nop required for translocation of other rhizobial Nops [68,69], was found to be a translocated effector according to the *Pseudomonas–Arabidopsis* translocation system. Surprisingly, the predicted apparatus proteins NolU, NolV, y4yQ, y4yJ and NolT (Figure 1C) were found to be translocated in a similar way and designated as Nops [43]. Nop translocation results obtained from heterologous translocation systems should therefore be interpreted with caution, and future experiments are required to clarify these discrepancies.

SYMBIOTIC PHENOTYPES OF *nop* GENE MUTANTS

Construction of a T3SS-knockout mutant (genes encoding T3SS apparatus proteins, *nopA*, *nopB* or the regulatory gene *ttsI*) followed by nodulation tests on leguminous host and non-host plants was frequently conducted to elucidate the symbiotic

Table 1 Identification of Nops by various methods: overview of *bona fide* Nops

Protein names (or identification numbers) of synonymous or related proteins are indicated in parentheses. Strain names: *Sinorhizobium* (*Rhizobium*, *Ensifer*) *fredii* NGR234, USDA207, USDA257, USDA191 and HH103; *Bradyrhizobium japonicum* (*Bradyrhizobium diazoefficiens*) USDA6, USDA110, USDA122, USDA123 and USDA124; *Bradyrhizobium elkanii* USDA61 and SEMIA587; *Mesorhizobium loti* MAFF303099. The following protein detection methods were used. Im, immunodetection: protein (or the N-terminal part of the protein with a C-terminal tag) immunodetected in the supernatant of wild-type bacteria, but not in the supernatant of a mutant strain deficient in Nop secretion. Alternatively, proteins from wild-type bacteria grown with and without flavonoids were compared. Ma, mass spectrometry: protein identified by comparative 2D gel electrophoresis followed by mass spectrometry analysis using culture supernatants from wild-type bacteria and from a mutant strain deficient in Nop secretion. Alternatively, purification of flavonoid-induced pili followed by mass spectrometry was performed (USDA257, USDA191) [67]. Cy, adenylate cyclase translocation reporter systems with Nop–Cya fusion proteins: translocation by rhizobial T3SS into legume cells confirmed for NopP (USDA257), NopE1 and NopE2 (USDA110); NopL (USDA257) and NopP (USDA257) were translocated into *N. benthamiana* leaf cells via the T3SS of *P. syringae* pv. tomato DC3000 [28,29]. Tr, translocation analysis performed using *P. syringae* pv. tomato DC3000 expressing Nop candidates fused to the Δ79AvrRpt2 reporter and *Arabidopsis* leaves. Nop fusion proteins causing cell death were considered to be translocated [43,44]. At least one candidate of the rhizobial strains listed was tested. Mi, microsequencing: N-terminal amino acid sequencing of differently expressed proteins of culture supernatants (wild-type compared with NGRΩ*rhcN* mutant) [8]. Ra, radioactively labelled protein detected in culture supernatant. Ph, protein identified by phage display [77].

Protein names	Strains	Proposed function	Methods	Related proteins	References
NopA (SR5, Nop7)	NGR234 USDA257 USDA191 HH103 USDA61 SEMIA587 MAFF303099	Pilus subunit	Im, Ma, Ra	Rhizobial homologous	[7,9,12,13,16,24,26,49,66,67]
NopB (NolB, SR4; blr1812; mlr8763)	NGR234 USDA207 USDA257 USDA191 USDA110 USDA61 MAFF303099	Pilus component and/or effector	Im, Ma, Ra, Tr	Rhizobial homologous; C-terminal domain related to the flagellar protein FlgK; weak similarities to DNA polymerase III subunits γ and τ (PRK07994)	[7,12,17,43,44,66–70]
NopC (NolJ; host-inducible protein B)	NGR234 HH103 USDA6 USDA207 USDA257 USDA191	Pilus component and/or effector	Im, Ma, Tr	Rhizobial homologous	[26,43,67,71]
NopD (SFHH103_04358; NopBG, mlr6316)	HH103 MAFF303099	Effector	Ma, Im, Tr	Repeat-rich effector proteins with a C-terminal C48 (SUMO) peptidase domain (XopD T3 effector family)	[44,71,72]
NopE (NopE1, blr1806; NopE2, blr1649)	USDA6 USDA110 USDA122 USDA123 USDA124	Effector	Ma, Cy, Tr	Proteins with DUF1521 domains (including a putative T3 effector in *Vibrio coralliilyticus*) [73]	[11,14,29,43,70]
NopF (NopAG, bll1862 or bll8201)	USDA6 USDA110 USDA122 USDA123 USDA124	Effector	Ma, Tr	Proteins with putative acetyltransferase domain (PRK10562); HopBG T3 effector family (e.g. HopBG1, ADQ74901 of *P. syringae* pv. *maculicola*)	[11,14,43,70]
NopJ (y4lO)	NGR234	Effector	Tr	YopJ family T3 effectors with serine/threonine acetyltransferase or protease domain (pfam03421)	[43,57]
NopL (y4xL; SR2, Nop38)	NGR234 USDA207 USDA257 USDA191 USDA61 HH103 SEMIA587	Effector	Im, Ma, Mi, Ra, Cy, Tr	Rhizobial homologous; weakly similar to DNA polymerase III subunits γ and τ domain (PRK07764)	[7,8,12,13,24,28,43,66,67,71,74]
NopM (y4fR, blr1676)	NGR234 HH103 USDA110 USDA122 USDA124	Effector	Im, Ma, Tr	IpaH (SlrP) family T3 effectors with NEL (novel E3 ligase) domain and N-terminal leucine-rich repeat (LRR 8) domain (pfam13855)	[14,43,57,71,75]
NopP (y4yP, SR3, Nop34, blr1752; host-inducible protein A)	NGR234 USDA207 USDA257 HH103 USDA110 USDA61	Effector	Im, Ma, Ra, Ph, Cy, Tr	Rhizobial homologous (including the NopAH candidate); similar to a protein (ACQ68626) of *Hamiltonella defensa*, a bacterial endosymbiont of aphids [76]	[7,11,12,28,43,67,70,71,77–79]
NopT (y4zC, NopT1, Blr2140)	NGR234 USDA110	Effector	Im, Ma	YopT T3 effector family; proteins with cysteine protease domain (TIGR01586; peptidase C58 domain, pfam03543)	[56,57,70]

Table 1   Continued

Protein names	Strains	Proposed function	Methods	Related proteins	References
NopX (SR1, NolX, SR1; mll6337)	NGR234 USDA207 USDA257 USDA191 USDA61 HH103 MAFF303099	Translocon protein or effector	Im, Ma, Mi, Ra, Tr	Proteins with NolX domain (pfam05819); related to translocon proteins such as HrpF of *X. campestris* [32]	[7,8,12,16,17,24,30,31,43,44,62, 63,67,71,74]
NopAA (GunA2, blr1656)	USDA207 USDA257 USDA6 USDA110 USDA122 USDA123 USDA124	Effector	Ma, Tr	Glycoside hydrolase family 12 proteins (pfam01670)	[11,14,43,70,80]
NopAC (Pgl, blr1993)	USDA6 USDA110 USDA122 USDA124	Effector	Ma, Tr	Polygalacturonases (glycoside hydrolase family 28, pfam00295)	[43,70,80]
NopAR (bll1840, ORF17)	USDA110 USDA122	Effector	Ma, Tr	Rhizobial homologous	[14,43]
NopBX (mlr6361) and NopBV (mlr6331)	MAFF303099	Effector	Im, Tr	Proteins with shikimate kinase domain (pfam01202); proteins with conserved multidomain region PRK09196 such as XopAD of *X. campestris* and Skwp2 of *R. solanacearum* (Hpx30 effector family with SKWP repeat) [81]	[16,17,44,72]
NopBW (mlr6358)	MAFF303099	Effector	Im, Tr	C-terminal domain of NopBW is related to T3 effectors such as XopP of *X. oryzae* [82]; N-terminal domain of NopBW is similar to NopBV (mlr6361)	[16,44]

role of the whole T3 effector arsenal of a given strain. In most cases, specific legume species were identified that nodulated either better or worse with such T3SS knockouts. It was also observed that various mutant strains showed altered competiveness in co-inoculation experiments with the parent strain. Symbiotic phenotypes of T3SS-knockout mutants have been described in detail for various strains, namely *S. fredii* (NGR234 [8,26,45,69,74], HH103 [9,49] and USDA257 [24,27,61,68]), *B. japonicum* (USDA110 [10] and USDA122 [14]), *B. elkanii* (USDA61 [12,84] and SEMIA58 [13]), *M. loti* (MAFF303099 [15,17,72]) and *Cupriavidus taiwanensis* (LMG19424 [18]). Analysis of *S. fredii* strains mutated in the translocon gene *nopX* resulted in symbiotic phenotypes that were similar to those of the T3SS-knockout mutants, providing clues to the role of NopX in effector translocation [30,74].

When single T3 effector genes were mutated in rhizobial strains, positive, negative or no effects on symbiosis were observed (Figures 2C–2F). Altered symbiotic phenotypes that can be attributed to the lack of a specific T3 effector have been reported for *nopL* [58,74], *nopM* [57,75] and *nopT* [56,57] of *S. fredii* NGR234, as well as for *nopBG* (mlr6316) and *nopBX* (mlr6361) of *M. loti* MAFF303099 [15–17,72]. Symbiotic phenotypes were also observed for *nopP* mutants of various *S. fredii* strains [51,77–79] as well as for *Rhizobium etli* CNPAP512 [53]. In other cases, however, no or only minor effects on symbiosis were observed when strains lacking a single effector gene were tested in nodulation experiments. Examples for such effector genes are *nopJ* of NGR234 [57], *nopBV* (mlr6331) and *nopBW* (mlr6358) of *M. loti* MAFF303099 [17,72], as well as *nopAA* (gunA2), *nopAC* (pgl), *nopAD* (pme), *nopE1* and *nopE2* of *B. japonicum* USDA110 [29,80]. This prompted researchers to mutate further already constructed T3 effector

gene mutants. Accordingly, cumulative or synergistic effects were observed in certain double or triple mutants [29,56,57,72,78]. The double mutant *nopE1/nopE2* of USDA110, for example, showed increased nodulation with *Vigna radiata* cv. KPS2 and reduced nodule number with soya bean (cv. Amphor) and *Macroptilium atropurpureum* [29]. Likewise, evidence for symbiotic or asymbiotic effects of *nopBG*, *nopBV* and *nopBW* in MAFF303099 was obtained by comparing the nodulation competitiveness of various mutants on *Lotus japonicus* and *Lotus tenuis*. Test plants were co-inoculated with two strains in equal amounts and nodule occupancy (percentage of nodules induced by each strain) was determined at the time of harvest [72].

FUNCTION OF T3 EFFECTORS

Rhizobia appear to express most Nops at early symbiotic stages (infection thread formation), and also in fully developed nodules [27,31,46,58,62,85]. As in plant–pathogen interactions, symbiotic effects of rhizobial Nops are considered to be due to T3 effectors translocated into host cells. However, before translocation, certain Nops are likely to be first secreted into the symbiotic interface and thus may come into direct contact with cell wall materials in infection threads and the surface of the host plasma membrane. In fact, some Nops (or Nop candidates) are putative cell-wall-degrading proteins that could facilitate effector translocation into host cells [34]. In *B. japonicum* USDA110, for example, *nopAA* (gunA2) encodes a glycoside hydrolase family 12 enzyme (protein with putative endoglucanase, xyloglucan hydrolase, β-1,3-1,4-glucanase or xyloglucan endotransglycosylase activity). When expressed in *E. coli*, recombinant NopAA protein efficiently cleaved carboxymethylcellulose [80]. The *nopAC* (pgl) and *nopAD* (pme) genes of USDA110 encode proteins with

predicted polygalacturonase (glycoside hydrolase family 28) and pectin esterase activity respectively. However, the triple mutant *nopAA*/*nopAC*/*nopAD* showed no obvious nodulation phenotype on test plants [80]. This might be due to the fact that plant-cell-wall-cleaving enzymes related to symbiosis may be secreted by other bacterial protein-secretion systems [86]. Moreover, proteins involved in infection-related cell wall modifications are also produced by host plants. In *L. japonicus*, for example, a pectate lyase is essential for infection by *M. loti* [87].

Compared with T3 effectors of pathogenic bacteria, little is known about the function of rhizobial T3 effectors delivered into plant cells. Besides identification (Table 1), mutant characterization (see above) and translocation into plant cells [28,29,43,44], only five rhizobial T3 effector proteins have been biochemically characterized in detail, namely NopE1/NopE2 (*B. japonicum* USDA110), NopL (*S. fredii* NGR234), NopM (*S. fredii* NGR234), NopP (*S. fredii* NGR234) and NopT (*S. fredii* NGR234; *B. japonicum* USDA110).

NopE

The NopE proteins are typical bradyrhizobial effectors. The proteins NopE1 and NopE2 of *B. japonicum* USDA110 have been characterized in two publications [29,88]. Both proteins possess two C-terminal DUF1521 domains. DUF1521 contains an EF-hand-like motif required for calcium binding. Remarkably, non-enzymatic autoproteolysis of DUF1521 was observed in the presence of Ca^{2+} ions, whereas other divalent cations showed no effects. Except for EDTA, none of the protease inhibitors tested blocked proteolysis. Each DUF1521 domain contains the cleavage site GD-PHVDA. The substitution of the aspartate and proline residues next to the cleavage site by alanine resulted in a non-cleavable DUF1521 domain. Truncated DUF1521 domain constructs were not cleaved, indicating that a domain length of ∼140 amino acids in length is required for self-cleavage. Nodulation tests with a *nopE1*/*nopE2* double mutant expressing a non-cleavable NopE1 form and the host plant *Vigna radiata* (KPS2) revealed that autocleavage of the protein is required for effector activity in this plant [29,88].

NopL

Construction of transgenic plants expressing a given *nop* gene is an approach to test effector function within plant cells. When tobacco (*Nicotiana tabacum*) and *L. japonicus* plants expressing the *nopL* gene of *S. fredii* NGR234 were tested in this way, expression of pathogen-related proteins (class I chitinase and class I glucanase) was reduced compared with control plants. These findings indicate that NopL can suppress the plant's innate immunity [89] and are thus consistent with the observation that a *nopL*-knockout mutant of NGR234 (strain NGRΩ*nop*L) induces fewer nodules on the legume *Flemingia congesta* [74]. Mutant analysis also revealed a positive effect of NopL in nodules of certain bean cultivars (e.g. *P. vulgaris* cv. Tendergreen). Nodules induced by the *nopL* mutant turn necrotic, and bacteroids of the *nopL* mutant show typical senescence symptoms, indicating that NopL suppresses premature nodule senescence in these bean plants (Figures 2E and 2F) [58]. Work with recombinant NopL revealed that NopL can be phosphorylated *in vitro* by crude plant protein extracts. The addition of the MAPKK (mitogen-activated protein kinase kinase) inhibitor PD98059 to the reaction mixture resulted in reduced phosphorylation of NopL, providing the first clues that NopL phosphorylation is associated with MAPK (mitogen-activated protein kinase) signalling in eukaryotic cells

[90]. Further analysis showed that NopL expressed in tobacco or yeast (*Saccharomyces cerevisiae*) is indeed phosphorylated *in vivo* and four phosphoserine residues in NopL were identified by mass spectrometry [58]. The phosphorylation sites in NopL possess a conserved serine-proline motif, which is typical for phosphorylation sites in MAPK substrates. Remarkably, NopL expressed in eukaryotic cells impaired the induction of MAP-mediated responses such as mating pheromone (α-factor) signalling in yeast and MAPK (SIPK[DD])-mediated cell death in tobacco. Hence it was concluded that NopL suppresses plant defence reactions by interfering with MAPK signalling [58]. Recent work confirmed that NopL is indeed a MAPK substrate (Ge, Y.-Y., Xiang, Q.-W., Wagner, C., Xie, Z.-P. and Staehelin, C., unpublished data).

NopM

The NopM effectors belong to the NEL (novel E3 ubiquitin ligase) domain effector family and possess an N-terminal LRR (leucine-rich repeat) and a C-terminal E3 ubiquitin ligase domain. The first NEL domain effector described is the IpaH9.8 protein of the human pathogen *Shigella flexneri* [91]. E3 ubiquitin ligases in eukaryotic cells facilitate the covalent conjugation of ubiquitin from an ubiquitin-loaded E2 to one or more lysine residues in a given protein substrate. Bacterial E3 ubiquitin ligases delivered into host cells mimic the activities of host E3 ubiquitin ligases and ubiquitinate specific target proteins. NopM of *S. fredii* NGR234 was characterized in detail [75]. Using recombinant protein and proteins required for an *in vitro* ubiquitination reaction, E3 ubiquitin ligase activity of NopM was demonstrated. The reaction resulted in formation of polyubiquitination chains in the range 24–200 kDa. When the catalytic residue Cys^{338} in the NEL domain of NopM was replaced by alanine, a dysfunctional protein lacking activity was obtained. In contrast with the NopM-expressing parent strain NGR234, a mutant producing NopM with the C338A substitution induced fewer nodules on the host plant *Lablab purpureus*, indicating that ubiquitination of an unknown target protein is required for optimal nodulation. NopM directly expressed in the non-host *Nicotiana benthamiana* dampened generation of ROS (reactive oxygen species), which are formed in response to the elicitor peptide flagellin (a component of the bacterial flagellum) [75]. These findings point to the possibility that NopM promotes nodule initiation of host plants [57,75] by reducing the levels of harmful ROS during the rhizobial infection process.

NopP

Protein kinase assays performed with the microsomal fraction of various host legumes demonstrated that NopP of *S. fredii* NGR234 can be phosphorylated *in vitro* by plant protein kinases. A truncated NopP form consisting of only 62 N-terminal amino acid residues was also phosphorylated, indicating that the secretion signal sequence of NopP contains phosphorylation sites. The phosphorylation reaction was inhibited by the protein kinase inhibitors genistein (a tyrosine kinase inhibitor) and K252a (a serine/threonine kinase inhibitor) [78]. Whether NopP is also phosphorylated *in planta* and whether NopP phosphorylation is required for effector activity remains unknown. During the infection process, NopP probably interferes with activation of plant defence reactions as nodulation tests with NGR234 mutants and certain host plants revealed synergistic effects for NopP and NopL [78]. However, when a *nopP* mutant of *S. fredii* HH103 was inoculated on soya bean (cv.

Williams), roots showed slightly enhanced nodule formation and lower transcript levels of the defence gene *PR1* (assumed to be a marker for systemic acquired resistance) than with the parent strain, indicating that NopP has a negative effect on symbiosis with this host plant [79].

NopT

The NopT effectors belong to the YopT effector proteinase family that includes AvrPphB of the pathogen *P. syringae* (pv. *phaseolicola*). These proteins are cysteine proteinases (family C58 in the CA clan of the MEROPS peptidase database) that contain three well-conserved catalytic amino acid residues (catalytic triad). Recombinant NopT proteins of *S. fredii* NGR234 or *B. japonicum* USDA110 (NopT1 and NopT2) expressed in *E. coli* possess autoproteolytic activity and recognize a DKM motif in the N-terminal region of the protein [56,57,92]. For the two NopT proteins of USDA110, proteolytic activity was also demonstrated with resorufin-labelled casein as substrate [92]. Substitution of amino acids of the catalytic triad in NopT proteins dramatically reduced the proteolytic activity [56,57,92]. Accordingly, symbiotic activity of NopT (e.g. negative effects on *Crotalaria juncea*) was abolished in a mutant of NGR234 producing NopT with a D220A substitution [57]. The autoproteolytically processed NopT forms lack the N-terminal secretion signal sequence and possess an N-terminus with glycine and cysteine residues, which represent potential myristoylation and palmitoylation sites respectively [56,92,93]. For NopT of NGR234, effector lipidation was demonstrated for *in vitro*-transcribed protein and for the protein expressed in yeast [93]. Effector lipidation in plant cells appears to influence the subcellular localization of NopT. When NopT of NGR234 with a C-terminal fluorescence tag was expressed in Chinese cabbage (*Brassica campestris* subsp. *napus*), the protein was found to be localized to the plasma membrane. In contrast, a NopT protein form lacking the myristoylation and palmitoylation sites (G50A/C51A/C52A substitutions) expressed in the same plant accumulated in the cytosol [93]. NopT of NGR234 [56] and NopT1 of USDA110 [92] expressed in tobacco both induced an apoptosis-like cell death (hypersensitive reaction) that can be considered as rapid plant defence response. Analysis of NopT forms with substituted amino acids indicated that induction of cell death depends on autocleavage and subsequent palmitoylation of NopT, whereas an intact myristoylation motif is apparently not essential [56,92].

EFFECTOR-TRIGGERED PLANT RESPONSES: NOPS ARE DOUBLE-EDGED SWORDS

Rhizobial Nops possess traits that resemble those from pathogenic bacteria. Once delivered to host cells, most rhizobial Nops probably manipulate cellular processes to suppress defence responses against invading bacteria. As mentioned above, NopL of *S. fredii* NGR234 is the prototype of such a toxin-like T3 effector [58,89]. Increasing evidence is provided that suppression of plant defence responses has a positive effect on establishment and maintenance of symbiosis [94]. Accordingly, compared with the parent strain *S. fredii* HH103, reduced nodule formation of soya bean (cv. Williams) and a higher induction of the defence gene *PR1* were observed in response to inoculation with a T3SS-deficient mutant lacking secretion of Nops [9,79].

Other Nops perhaps promote symbiosis-related processes directly by interfering with nodulation signalling in host cells, which is initiated by perception of rhizobial Nod factors.

Support for such a mechanism was obtained from the interaction between *B. elkanii* strain USDA61 and soya bean genotypes (Clark-*rj1*; En1282) that are deficient in a Nod factor receptor gene (*GmNFR1α*) [95]. In this system, occasional rhizobial infections do not depend on activation of nodulation signalling by Nod factors [84]. However, nodule formation with a T3SS-knockout mutant of USDA61 is nearly abolished, indicating that Nod-factor-independent nodule formation only occurs as long the bacteria deliver T3 effectors into the host plant [12,84]. Comparative microarray analysis showed that the parent strain USDA61, but not the T3SS-knockout mutant, stimulated nodulation signalling as analysed by transcript levels of marker genes such as *NIN* (nodule inception protein) [84]. These findings suggest that at least one T3 effector of USDA61 manipulates the host cell to activate nodulation signalling in the absence of Nod factors. One possibility would be that a T3 effector causes cellular changes that increase levels of the plant hormone cytokinin. In fact, cytokinin applied to *L. japonicus* roots can trigger expression of *NIN* in the absence of Nod factors [96]. Such a mechanism would be reminiscent of the T3 effector HopQ1 of *P. syringae* pv. tomato, which is able to induce cytokinin signalling in *Arabidopsis thaliana* [97].

Nops are double-edged swords that may promote establishment of symbiosis with one legume (symbiotic factors) and impair symbiotic processes with another legume species (asymbiotic factors). Direct or indirect recognition of Nops by plant cells can result in plant defence reactions that negatively affect rhizobial infection and nodule formation. Such plant defence responses are likely to be similar to the phenomenon of ETI (effector-triggered immunity) in plant–pathogen interactions [98]. The cell-death-inducing effects of NopT in tobacco plants [56,92], for example, appear to be identical with those caused by a NopT homologue, the avirulence protein AvrPphB of *P. syringae* pv. *phaseolicola* [99]. Likewise, negative effects of NopT of NGR234 in nodules of the host plant *C. juncea* [56,57] are likely to be due to an ETI-like defence response culminating in necrotic nodule cells (Figures 2C and 2D).

Negative effects of Nops can result in complete blockage of nodulation in certain strain–plant combinations. In soya bean, certain dominant *Rj* genes (*Rj2/Rfg1* and *Rj4*) can play a crucial role in Nop-related nodulation blockage. The allelic genes *Rj2* and *Rfg1* have been identified by positional cloning [100], taking advantage of the sequenced soya bean genome and various previous studies [101]. Soya bean cultivars with the *Rj2* genotype (e.g. Hardee) show nodulation blockage with *B. japonicum* strains such as USDA122 [102], whereas those with the *Rfg1* genotype [103], such as McCall, are incompatible with certain *S. fredii* strains, namely USDA257 (Figure 2A). The gene products of *Rj2* and *Rfg1* are R (resistance) proteins belonging to the TIR (Toll/interleukin-1 receptor)-NBS (nucleotide-binding site)-LRR class. Three allele types, namely Rj2 (rfg1), rj2 (rfg1) and rj2 (Rfg1) with corresponding amino acid polymorphisms have been identified [100]. Nodulation tests with T3SS-knockout mutants of USDA257 [100] and USDA122 [14] confirmed the involvement of Nops as asymbiotic factors. The loss of the T3SS resulted in compatible interactions, i.e. nitrogen-fixing nodules were formed. These data indicate that incompatible rhizobial strains fail to infect soya beans depending on distinct amino acids in the Rj2/Rfg1 protein [100]. It likely that an as yet unknown T3 effector is directly or indirectly perceived by a specific Rj2/Rfg1 protein variant. Such a recognition process would then culminate in an R-protein-mediated defence reaction (ETI), which blocks bacterial infection and nodule formation.

Another dominant soya bean gene involved in genotypic nodulation is *Rj4* [101]. Soya beans with the *Rj4* genotype

(e.g. cv. Hill) show T3SS-dependent nodulation blockage with *B. elkanii* USDA61 [12]. A T3SS-knockout mutant of USDA61 could overcome the nodulation blockage in cv. Hill, suggesting the presence of an asymbiotic T3 effector in the wild-type strain. Identification of a candidate gene encoding such a T3 effector was recently announced for strain *B. japonicum* Is-34, which is also incompatible with *Rj4* soya beans [104]. A detailed characterization of this NopD/NopBG-related gene has not yet been published. Positional cloning efforts resulted recently in the identification of the *Rj4* gene. Surprisingly, *Rj4* does not encode an R protein but a thaumatin-like protein [105,106]. Thaumatin-like proteins can be considered as PR (pathogen-related) proteins (PR protein family 5). Purified proteins often possess antimicrobial activity, particularly when tested on fungi. Remarkably, some of them bind to other proteins (enzyme inhibitors) or may possess enzymatic activity (β-1,3 glucanase) [107]. Identification of the *Rj4* gene now forms the basis to elucidate the molecular mechanisms of the Rj4 protein in nodulation blockage.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Results from various laboratories working on different rhizobial strains highlight the symbiotic role of Nops in various strain–host plant interactions. Beyond Nops, proteins secreted by other bacterial protein secretions may also represent symbiotic determinants in certain interactions and should be explored in future. For example, rhizobial proteins secreted by the bacterial type VI protein secretion system of *Rhizobium leguminosarum* strain RBL5523 may function as asymbiotic factors in the interaction with *Pisum sativum* [108]. Likewise, mutant analysis indicated that type IV effectors secreted by the type IV protein secretion system of *M. loti* strain R7A promote nodule formation on the host *Lotus corniculatus* but negatively affect establishment of symbiosis with *Leucaena leucocephala* [15].

Conclusions on Nops and their dilemma as to function either as symbiotic or asymbiotic factors were mainly drawn from inoculation tests with mutant strains. On the protein level, however, characterization of Nops remains in its infancy. Further research on Nops and Nop candidates is needed to investigate their structural and biochemical properties. Future efforts are required to elucidate the molecular function of rhizobial effectors within host cells. Identification of plant proteins that are either targets of symbiosis-promoting effectors or components of the defence system against asymbiotic effectors will be of particular interest. Special attention should be paid to the possibility that effector activities in plant cells may depend on subcellular localization and post-translational events such as cleavage (NopT and NopE), phosphorylation (NopL and NopP) and lipidation (NopT). Finally, further characterization of effectors in plant cells could inspire medical researchers to test the possible pharmaceutical potential of Nops in human cells.

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