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Symbiosis-Promoting and Deleterious Effects of NopT, a Novel Type 3 Effector of *Rhizobium* sp. Strain NGR234[▽]

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Establishment of symbiosis between certain host plants and nitrogen-fixing bacteria ("rhizobia") depends on type 3 effector proteins secreted via the bacterial type 3 secretion system (T3SS). Here, we report that the open reading frame y4zC of strain NGR234 encodes a novel rhizobial type 3 effector, termed NopT (for nodulation outer protein T). Analysis of secreted proteins from NGR234 and T3SS mutants revealed that NopT is secreted via the T3SS. NopT possessed autoproteolytic activity when expressed in *Escherichia coli* or human HEK 293T cells. The processed NopT exposed a glycine (G50) to the N terminus, which is predicted to be myristoylated in eukaryotic cells. NopT with a point mutation at position C93, H205, or D220 (catalytic triad) showed strongly reduced autoproteolytic activity, indicating that NopT is a functional protease of the YopT-AvrPphB effector family. When transiently expressed in tobacco plants, proteolytically active NopT elicited a rapid hypersensitive reaction. *Arabidopsis* plants transformed with *nopT* showed chlorotic and necrotic symptoms, indicating a cytotoxic effect. Inoculation experiments with mutant derivatives of NGR234 indicated that NopT affected nodulation either positively (*Phaseolus vulgaris* cv. Yudou No. 1; *Tephrosia vogelii*) or negatively (*Crotalaria juncea*). We suggest that NopT-related polymorphism may be involved in evolutionary adaptation of NGR234 to particular host legumes.

In root nodules of legumes, symbiotic bacteria ("rhizobia" belonging to the *Rhizobiaceae* family) reduce N_2 gas into ammonia by the process termed nitrogen fixation. Nodule formation requires specific bacterial signals and determinants (12). Rhizobial nodulation factors (Nod factors) trigger various host responses, including root hair deformation, expression of symbiosis-related host genes, and cortical cell divisions resulting in nodule formation (31, 32). Host-specific nodulation depends also on surface carbohydrates, such as oligosaccharides released from exopolysaccharides, lipopolysaccharides, cyclic β-glucans, and K antigens (also named capsular polysaccharides). In *Rhizobium* sp. strain NGR234, for example, mutants that do not produce exo-oligosaccharides or flavonoid-inducible lipopolysaccharide are unable to establish symbiosis with various host plants (36, 48). In addition to these symbiotic determinants, host-specific nodulation depends on proteins secreted via a pilus-like secretory apparatus, the type 3 secretion system (T3SS) of NGR234. Recent data from various laboratories provide evidence that T3SSs of certain rhizobial strains modulate establishment of symbiosis and the efficiency of nitrogen fixation (14, 23, 25, 56).

T3SSs from pathogenic bacteria deliver effector proteins (type 3 effectors) into eukaryotic cells to manipulate the host metabolism, e.g., to suppress defense responses. Many reports indicate that type 3 effectors are virulence factors that play a key role in the pathogenesis of humans, animals, and plants (9, 20). On the other hand, eukaryotic host cells developed strategies to perceive type 3 effectors in order to "sense" the invading bacterium. In nonhost plants, specific resistance genes are essential for these recognition events, and a single type 3 effector may act as avirulence factor. As a result, nonhost plants induce a rapid hypersensitive reaction (HR) that prevents pathogen invasion and disease (1).

In response to host-derived flavonoids, rhizobia secret nodulation outer proteins (Nops) in a T3SS-dependent manner (see, for example, references 24, 37, 49, and 56). Mutant analysis indicated that specific Nops modulate the formation of nodules in certain host plants. Nops-dependent nodulation phenotypes have been described for the interaction between *Sinorhizobium fredii* USDA257 and soybeans. The genes determining cultivar-specific nodulation turned out to encode T3SS proteins (see, for example, references 25 and 29). In the closely related strain *Rhizobium* sp. strain NGR234, the abolition of Nops secretion may affect the nodulation of host legumes either positively or negatively (27, 45, 56). Nops of NGR234 are either extracellular components of the T3SS (13, 38, 39) or type 3 effectors secreted via the T3SS. Thus far, only NopL and NopP, have been characterized as type 3 effectors of NGR234 (3, 4, 5, 27, 45).

More than a decade ago, sequencing analysis of pNGR234*a*, the symbiotic plasmid of NGR234, revealed that the open reading frame (ORF) y4zC displays similarities to genes belonging to the YopT-AvrPphB effector family (17). YopT (for *Yersinia* outer protein T), LopT (from *Photorhabdus luminescens*), and AvrPphB (from *Pseudomonas syringae* pv. phaseolicola) are recently investigated representatives from this family. The proteins displayed protease activity, which depended on conserved amino acid residues (C/H/D catalytic triad). YopT from *Yersinia* spp. recognizes prenylated cysteine of Rho family GTPases (RhoA, Rac1, and Cdc42) in human host cells and cleaved the proteins near their C termini. Cleavage resulted in the release of GTPases from membranes and caused cytotoxic effects, such as disruption of the actin cy-

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toskeleton (41, 43). Similarly to YopT, LopT from *P. luminescens*, an insect-pathogenic bacterium living in symbiosis with nematodes, displayed activity on prenylated GTPases (8). AvrPphB from the plant pathogen *P. syringae* pv. phaseolicola is autoproteolytically processed (34, 41). The processed protein exposed an N-terminal glycine predicted for myristoylation, and accumulating data indicate that myristoylated type 3 effectors are targeted to plasma membranes (30, 51). In certain nonhost plants (*Arabidopsis*, tobacco, and beans), AvrPphB functions as avirulence protein and induced an HR (34, 41, 44, 50). In *Arabidopsis*, AvrPphB proteolytically cleaved the protein kinase PBS1. Cleaved PBS1 subsequently activated the disease resistance protein RPS5, which is required for HR induction (2, 42, 57).

We report here that the ORF y4zC of NGR234 encodes a novel rhizobial type 3 effector (termed NopT for nodulation outer protein T). When expressed in *Escherichia coli* or human HEK 293T cells, NopT possessed autoproteolytic activity. Tobacco plants expressing *nopT* elicited an HR, whereas *Arabidopsis* plants transformed with *nopT* showed chlorotic and necrotic symptoms. Nodulation experiments with mutant strains indicated that NopT affected symbiosis either positively or negatively.

MATERIALS AND METHODS

Expression of *nopT* constructs in *E. coli* and purification of recombinant protein. DNA of *nopT* (ORF y4zC of *Rhizobium* sp. strain NGR234; accession no. U00090) was cloned into pET28b (Novagen), generating *E. coli* BL21(DE3)/pET-*nopT*. Point mutation constructs pET-*nopT*-C93S pET-*nopT*-H205A and pET-*nopT*-D220A were generated by site-directed mutagenesis using pET-*nopT* as a template and DpnI for digestion of the amplification products. Constructs were verified by sequencing. *E. coli* strains were grown in Luria-Bertani (LB) medium containing 50 μg of kanamycin ml^{-1} to a density of $A_{600} \approx 0.6$. Protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at either 30 or 37°C. Cells were harvested after 3 h, lysed in the 2× sodium dodecyl sulfate (SDS) loading buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were visualized by Coomassie brilliant blue R-250 staining. Proteins were purified by nickel affinity chromatography according to the manufacturer's protocols under denaturing conditions (Qiagen). Processed NopT (~24 kDa) was used for N-terminal sequencing by the automated Edman degradation method (Jikang Corp., Shanghai, China).

Bioinformatic and statistical analyses. NopT (ORF y4zC) is listed in the MEROPS peptidase database (http://merops.sanger.ac.uk/). The N-terminal sequence of processed NopT was analyzed with the MYR Prediction Server (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm). NopT homologues were aligned with the CLUSTAL W algorithm. The unrooted radial tree was constructed with the MEGA3.1 program using the neighbor-joining method (26). Where indicated, data were statistically analyzed with the nonparametric Kruskal-Wallis rank sum test, which is suitable for unequal replications (PAST software; http://folk.uio.no/ohammer/past/).

Antiserum against NopT and Western blots. NopT protein (nonprocessed protein with a C-terminal His_6 tag) was purified from *E. coli* BL21(DE3)/pET-*nopT* and used to immunize a New Zealand White rabbit. For Western blots, proteins were separated by SDS-PAGE and then blotted onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). To detect NopT protein, the membranes were incubated with the antiserum raised against NopT (1:10,000 dilution) and then with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (Boster, Wuhan, China). Immune blots were developed either with 3,3′-diamino-benzidine (Boster) or with ECL detection reagents (Amersham).

Construction of NGR234 mutant strains. For the construction of NGRΩ*nopT*, an XhoI fragment containing *nopT* was cloned into pBluescript II KS(+). A spectinomycin-resistant (Sp^r) Ω interposon was excised from pHP45Ω (33) with SmaI and ligated into the SphI site of *nopT*. The construct was then cloned into the suicide vector pJQ200SK (35) and *E. coli* crossed with NGR234 in a triparental mating procedure using the pRK2013 "helper" plasmid (16). Replacement of the mutated gene was forced by selecting for the resistance of the interposon marker (Sp^r) to sucrose (5% [wt/vol]). Double-crossover events at homologous sites were confirmed by Southern blot analysis with DNA isolated from mutant strains (10) using a DIG DNA labeling and detection kit from Roche. The NGRΔ*nopT* and NGRΩ*nopL*Δ*nopT* mutants were generated by using a similar approach. After insertion of the Sp^r Ω interposon into the SphI site of *nopT*, a 390-bp fragment (corresponding positions 36 to 167 of NopT) was excised with AccIII. Triparental matings were achieved to transfer suicide vector constructs into NGR234 or NGRΩ*nopL* (27). For strains harboring plasmid p*nopT*, a HindIII and KpnI fragment containing *nopT*, including its upstream promoter region, was cloned into the broad-host-range vector pLAFR6 (D. Dahlbeck and B. Staskawicz, unpublished results). The plasmid (named p*nopT*) was conjugated into NGR234, NGRΔ*nopT*, or NGRΩ*nopT* by using the triparental mating procedure (yielding strains NGRp*nopT*, NGRΔ*nopT*p*nopT*, and NGRΩ*nopT*p*nopT*, respectively).

Isolation of secreted proteins. Secreted proteins from culture supernatants from *Rhizobium* sp. strains NGR234 (wild type), NGRΔ*nopT* (the present study), NGRΩ*nopT* (this study), NGRΔ*nopT*p*nopT* (this study), NGRΩ*nopT*p*nopT* (this study), NGRp*nopT* (this study), NGRΩ*rhcN* (56), NGRΩ*nopL* (27), and NGR*nopB*::*uidA* (38) were isolated according to a previously described procedure (27). Briefly, cultures (RMS medium) were induced with 1 μM apigenin and cultivated on a rotary shaker for approximately 40 h. After centrifugation, proteins in the supernatant were precipitated with ammonium sulfate (60% saturation). Samples were desalted with a Sephadex G-25 column (Amersham Biosciences, Uppsala, Sweden) and then subjected to SDS-PAGE. Secreted proteins were analyzed by silver staining. Western blot analysis with antiserum against NopT was performed with secreted proteins corresponding to ~100 ml of cell culture.

Transcriptional activation of *nopT*. The promoter region of *nopT* (1.2 kb) was cloned upstream of the promoterless *gus* gene of vector pRG960 (54), resulting in pRG-*nopT*p. The plasmid was then mobilized into NGR234 and NGRΩ*ttsI* (56). Mobilization of pRG-*nopT*p into NGRΩ*ttsI* was confirmed by PCR with pRG960 specific primers. Where indicated, cultures (TY medium [6]) were induced with 1 μM apigenin. Equal amounts of cells were harvested at the indicated times and then treated with β-glucuronidase (GUS) extraction buffer (50 mM phosphate buffer [pH 7.0] containing 0.1% Triton X-100, 0.1% sarcosyl, 10 mM EDTA, and 10 mM 2-mercaptoethanol). GUS activity was measured with 2 mM 4-methylumbelliferyl-β-D-glucuronide as substrate. Protein concentrations were determined by the method of Bradford (7).

Expression of *nopT* within eukaryotic cells. A DNA fragment containing *nopT* was cloned into pRT104 (52), which contains the cauliflower mosaic virus (CaMV) 35S promoter and a poly(A) signal. The expression cassette was then excised with HindIII and introduced into the binary vector pCAMBIA2301 (www.cambia.org), yielding pCAMBIA-*nopT*. Derivatives with point mutations and deletions were constructed by PCR-based techniques in a similar way. Amplified point mutant constructs were digested with DpnI. All PCR products were verified by sequencing. Finally, pCAMBIA-*nopT* and derivatives were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. For transient expression in tobacco (*Nicotiana tabacum* cv. Xanthi), agrobacteria were cultivated at 23°C in *Agrobacterium* induction medium [10 mM MES (pH 5.6) containing 10.5 g of K_2HPO_4 $liter^{-1}$, 4.5 g of KH_2PO_4 $liter^{-1}$, 1 g of $(NH_4)_2SO_4$ $liter^{-1}$, 0.5 g of sodium citrate $liter^{-1}$, 1 mM $MgSO_4$, 1 g of glucose $liter^{-1}$, 1 g of fructose $liter^{-1}$, 4 ml of glycerol $liter^{-1}$, and 50 μg of acetosyringone ml^{-1}] on a rotary shaker for 5 to 7 h. The bacteria were then collected by centrifugation and resuspended in infiltration medium (1/2 MS-MES; Duchefa, Harlem, The Netherlands) to reach an optical density at 600 nm of 1.0. Leaves from 3-week-old tobacco plants were infiltrated with a needleless syringe. Leaves were photographed 72 h postinfiltration. Agroinfiltration experiments were repeated at least three times with at least six leaves per experiment.

For transformation of *Arabidopsis thaliana* (ecotype Columbia), plants with floral buds were dipped in a suspension (optical density at 600 nm of ~0.8) containing *A. tumefaciens* strain EHA105 carrying pCAMBIA-*nopT* (or the empty vector), 5% (wt/vol) sucrose, and 500 μg of Silwet L-77 $liter^{-1}$ (11). Plants (T_1 generation) were selected on agar plates containing 50 μg of kanamycin ml^{-1} and then individually cultivated in 350-ml glass jars under similar conditions for 2 weeks. Transgenic plants selected on kanamycin plates showed GUS activity when stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

For the expression of *nopT* in human HEK 293T cells, *nopT* was cloned into the pEGFP-C3 vector (Clontech), resulting in pEGFP-*nopT*. The point mutation construct pEGFP-*nopT*-C93S was obtained in a similar way. HEK 293T cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 80 μg of penicillin ml^{-1}, and 100 μg of streptomycin ml^{-1} at 37°C in 5% CO_2. Transfection of plasmid constructs (4 μg per 6×10^6 cells) was performed

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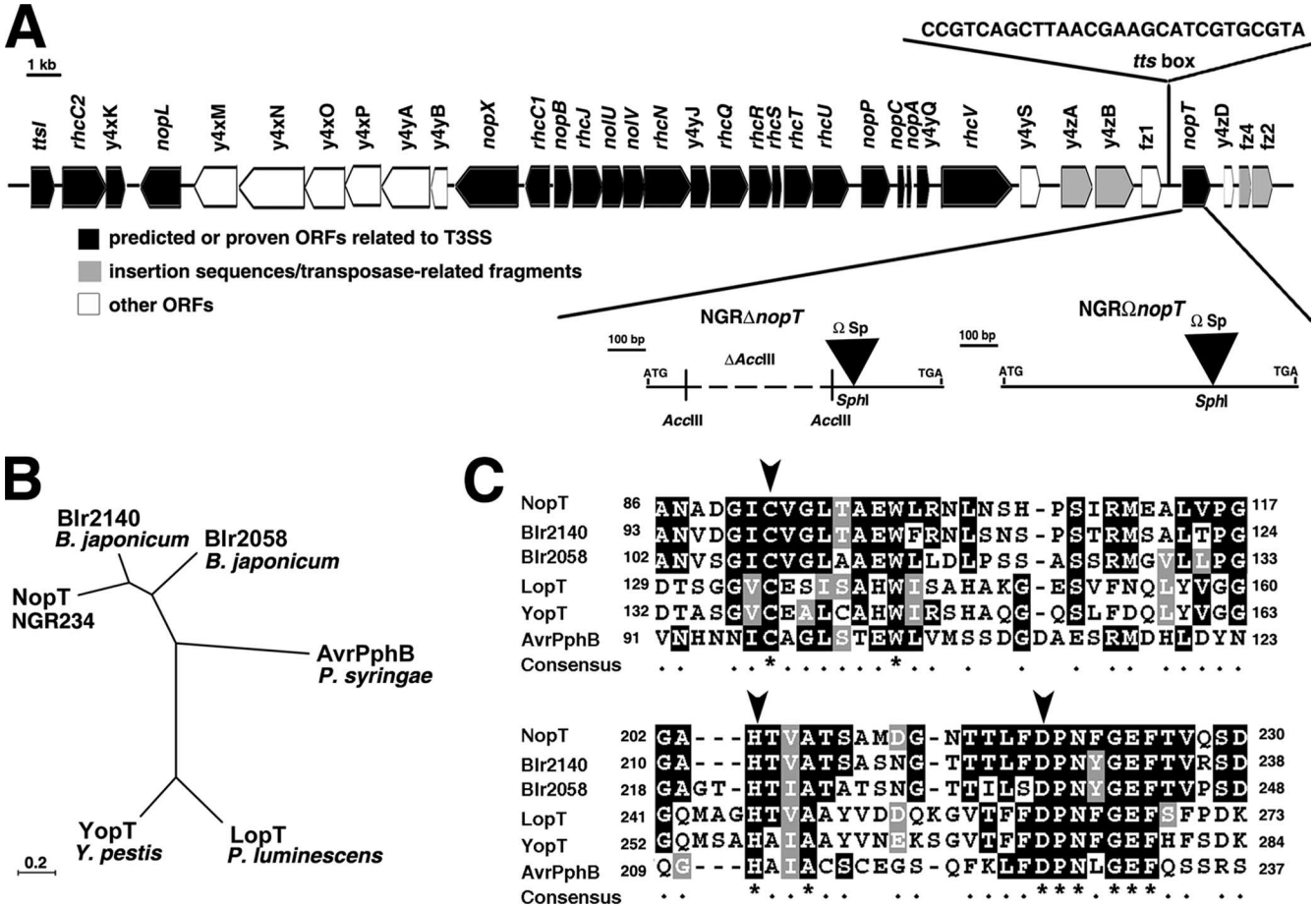

FIG. 1. Characterizarion of *nopT* of *Rhizobium* sp. strain NGR234. (A) Genetic map of the T3SS gene cluster with the neighboring *nopT* (ORF y4zC) of pNGR234*a* (accession no. U00090). The promoter sequence of *nopT* contains a putative *tts* box. The *nopT* gene was mutated by insertion of Ω interposons at the restriction sites indicated (resulting in strains NGRΔ*nopT* and NGRΩ*nopT*). (B) Unrooted phylogenic tree of predicted NopT protein from NGR234 (accession no. AAB91961), predicted proteins from *B. japonicum* USDA110 (Blr2058, accession no. NP_768698; Blr2140, accession no. NP_768780) and recently characterized proteases from *P. syringae* pv. phaseolicola (AvrPphB, accession no. Q52430 [41]), *Yersinia pestis* (YopT, accession no. AL117189 [41]) and *P. luminescens* (LopT, accession no. AAO18078 [8]). The horizontal bar represents a distance of 0.2 substitutions per site. (C) Multiple amino acid sequence alignment with conserved amino acid residues (catalytic triad C/H/D). Only conserved regions flanking the C/H/D residues are shown. Identical residues are in black and similar residues in gray.

with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Nodulation tests. Nodulation tests with beans (*Phaseolus vulgaris* cv. Yudou No. 1), *Tephrosia vogelii*, and *Crotalaria juncea* were performed according to previously described procedures (see, for example, reference 48). Briefly, seeds were surface sterilized and germinated on agar plates, and plantlets were transferred to sterilized 300-ml plastic jar units linked with a cotton wick (a mixture of vermiculite and expanded clay in the upper vessel; nitrogen-free nutrient solution in the lower vessel). Plants (1 plant per jar) were inoculated with $\sim 10^9$ bacteria (grown in TY medium, centrifuged, and resuspended in 10 mM $MgSO_4$). Plants were cultivated at 26 ± 2°C in a plant growth chamber and harvested at different times postinoculation. For determination of N contents, plant material was dried, pulverized, and analyzed with a CHNS analyzer (Elementar Analysensysteme, Hanau, Germany). All data are presented as means ± the SE (standard error).

RESULTS

The ORF y4zC is predicted to encode a protease. The symbiotic plasmid pNGR234*a* of *Rhizobium* sp. strain NGR234 possesses a T3SS gene cluster encoding a functional T3SS (17, 56). The ORF y4zC (encoding a putative protein with 262 amino acids; in the present study termed NopT) is separated from the T3SS cluster by ORFs encoding a putative unknown protein (y4yS), two ORFs (y4zA and y4zB), related to insertion sequences (transposase-related fragments), and an ORF with sequence similarities to serine/histidine decarboxylases (fz1). The short ORF y4zD, downstream of *nopT*, shows no similarities to sequences from other bacteria. The two following ORFs (fz4 and fz2) are putative insertion sequences (Fig. 1A).

The promoter region of *nopT* contains a *tts* box, a putative binding site for the transcriptional activator TtsI (Fig. 1A) (28). Comparisons with sequence databases suggest that *nopT* encodes a protease (family C58 in the CA clan of the MEROPS peptidase database). Proteases from this family have been identified to represent type 3 effectors (19). As shown in Fig. 1B, the NopT sequence of NGR234 is related to two ORFs from *Bradyrhizobium japonicum* USDA110 and known type 3 effectors from pathogenic bacteria, namely, AvrPphB from *P. syringae* pv. phaseolicola, YopT from *Yersinia* spp. and LopT from *P. luminescens*. Other rhizobial strains with T3SS genes,

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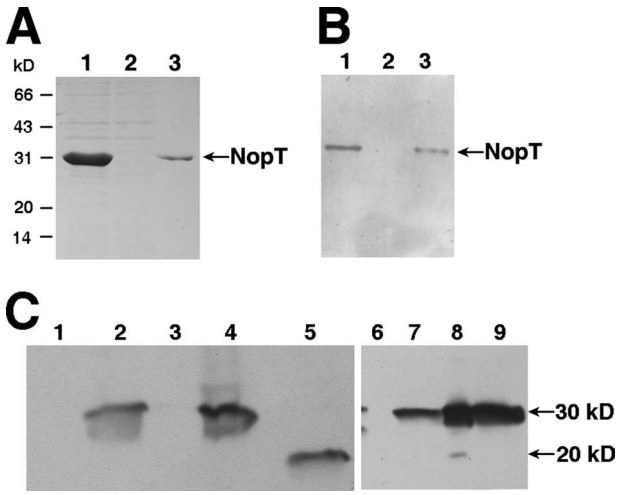

FIG. 2. Purification, immunodetection, and T3SS-dependent secretion of NopT. (A) Expression and purification of recombinant NopT from *E. coli* BL21(DE3)/pET-*nopT*. Proteins from cells grown at 37°C were analyzed by SDS-PAGE. Lane 1, proteins from BL21(DE3)/pET-*nopT*; lane 2, proteins from BL21(DE3) carrying the empty vector pET28b; lane 3, purified NopT with a C-terminal His_6 tag after nickel affinity chromatography. (B) Western blot analysis with the rabbit serum raised against recombinant NopT. Lane 1, proteins from BL21(DE3)/pET-*nopT*; lane 2, proteins from BL21(DE3) carrying pET28b; lane 3, purified NopT with a C-terminal His_6 tag. (C) NopT of *Rhizobium* sp. strain NGR234 is secreted via the T3SS. Secreted proteins from culture supernatants (corresponding to ~100 ml of cell culture) of the indicated strains were subjected to Western blot analysis with the anti-NopT antibodies. Lane 1, NGRΔ*nopT*; lane 2, NGRΔ*nopT*p*nopT* secreting full-length NopT (~30 kDa); lane 3, NGRΩ*rhcN*; lane 4, parent strain NGR234; lane 5, NGRΩ*nopT* secreting a truncated form of NopT (~20 kDa); lane 6, NGR*nopB*::*uidA*; lane 7, NGRΩ*nopL*; lane 8, NGRΩ*nopT*p*nopT* secreting full-length and truncated NopT; lane 9, NGRp*nopT*.

e.g., *Mesorhizobium loti* MAFF303099, lack genes homologous to *nopT*. As shown in Fig. 1C, the amino acid sequence of NopT contains conserved amino acid residues, which are predicted to be required for proteolytic activity (C/H/D residues; invariant catalytic triad in proteins belonging to the YopT-AvrPphB cysteine protease family) (41).

NopT is secreted by the rhizobial T3SS. To identify NopT protein secreted by *Rhizobium* sp. strain NGR234, we used an immunological approach. The sequence encoding NopT (786 bp) was cloned into pET28b, resulting in plasmid pET-*nopT*. When induced by IPTG, *E. coli* cells harboring this plasmid produced recombinant NopT protein with a C-terminal His_6 tag (~30 kDa) (Fig. 2A). The protein was purified by nickel affinity chromatography and then used to raise a rabbit serum against NopT. The antiserum recognized recombinant NopT from *E. coli* cells (Fig. 2B) and secreted NopT in culture supernatants of NGR234 (Fig. 2C). For comparison, we analyzed secreted proteins from two constructed mutant strains with Ω interposons in the *nopT* sequence (strains NGRΩ*nopT* and NGRΔ*nopT*; see Fig. 1A). NopT was not detected in the NGRΔ*nopT* mutant, whereas NGRΩ*nopT* secreted a truncated form of NopT (~20 kDa) (Fig. 2C). A band corresponding to full-length NopT (~30 kDa) appeared when the mutants were complemented with plasmid p*nopT* (strains NGRΩ*nopT*-p*nopT* and NGRΔ*nopT*p*nopT*). NGR234 carrying p*nopT* (strain NGRp*nopT*) secreted increased amounts of NopT (Fig. 2C).

To investigate T3SS-dependent secretion of NopT, we used anti-NopT antibodies to detect NopT in culture supernatants of various mutant strains of NGR234. Mutants lacking a functional T3SS (NGRΩ*rhcN* [56]; NGR*nopB*::*uidA* [38]) did not secrete NopT, indicating that NopT is secreted via the T3SS. In contrast, NopT was secreted by NGRΩ*nopL*, a mutant lacking synthesis of the type 3 effector NopL (27) (Fig. 2C). Similarly, a NopL band was detected, when secreted proteins from NGRΩ*nopT* were probed with anti-NopL antibodies (data not shown). Taken together, these findings indicate that NopT secretion is T3SS-dependent and that mutation of *nopT* does not affect the function of the T3SS.

TtsI-dependent transcriptional activation. To demonstrate flavonoid-dependent transcriptional activation of *nopT*, we cloned a 1.2-kb DNA fragment containing the putative promoter region of *nopT* upstream of a promoterless *gus* gene of vector pRG960 (resulting in pRG-*nopT*p). The construct was then mobilized into wild-type NGR234 or NGRΩ*ttsI* (with mutated *ttsI* gene [56]). Rhizobial cultures were treated with the flavonoid apigenin, which stimulates transcription of *ttsI* in a NodD1-dependent manner (22). Cultures were harvested at the indicated time points and GUS activity was measured to assess the promoter activity of *nopT*. As shown in Fig. 3, GUS activity in NGR234 carrying pRG-*nopT*p was significantly increased after application of apigenin. In the NGRΩ*ttsI* back-

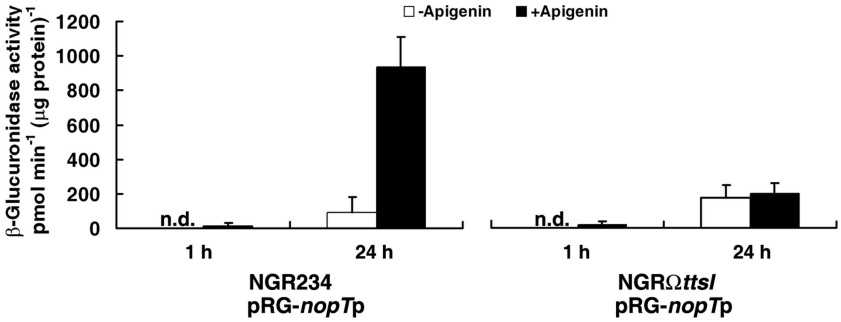

FIG. 3. Transcriptional activation of *nopT* is flavonoid and TtsI dependent. Cultures of NGR234 and NGRΩ*ttsI*, both carrying pRG-*nopT*p (*nopT*-promoter::GUS fusion), were induced with 1 μM apigenin. β-GUS activity was measured 1 and 24 h postinduction. The data indicate means (± the standard error) from three independent experiments (n.d., not detected).

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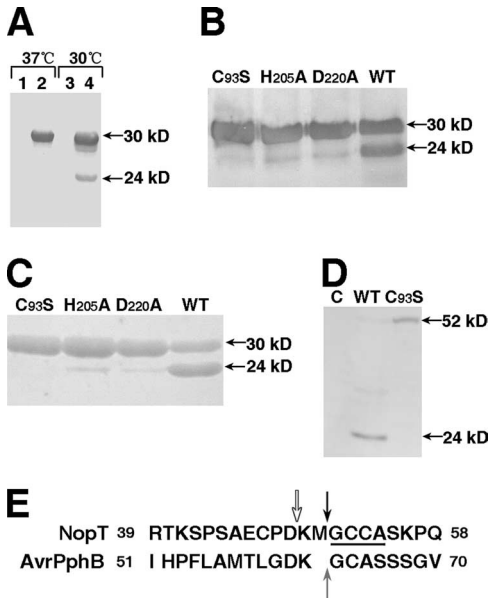

FIG. 4. Autoproteolytic activity of NopT. (A) Immunodetection of NopT protein (with C-terminal His_6 tag) from *E. coli* BL21(DE3) carrying pET-*nopT* cultured at different temperatures (1 mM IPTG for 3 h). Switching the induction temperature from 37 to 30°C resulted in the appearance of an additional band (~24 kDa). Lane 1, BL21(DE3) carrying the empty vector pET28b (37°C); lane 2, BL21(DE3)/pET-*nopT* (37°C); lane 3, BL21(DE3)/pET28b (30°C); lane 4, BL21(DE3)/pET-*nopT* (30°C). Equal amounts of cell lysates were subjected to Western blot analysis with anti-NopT antibodies. (B) NopT proteins mutated in the conserved catalytic triad C/H/D exhibit strongly reduced autoproteolytic activity. Proteins (~1 μg) from *E. coli* cultures (30°C) expressing wild-type NopT (WT) or NopT with the indicated point mutations (C93S, H205A, and D220A) were immunodetected with the anti-NopT antibodies. (C) Wild-type NopT (WT) and mutant protein forms from *E. coli* cultures (30°C) purified by nickel affinity chromatography and analyzed by SDS-PAGE (stained with Coomassie blue R250). (D) Autocleavage of an EGFP-NopT fusion protein in HEK 293T cells. Equal amounts of cell lysates were subjected to Western blot analysis with the anti-NopT antibodies. The 52-kDa EGFP-NopT fusion protein converted to a NopT form (~24 kD), whereas autocleavage was not observed for the EGFP-NopT-C93S fusion protein (52 kDa). Lanes: C, control cells transfected with pEGFP-C3; WT, cells transfected with pEGFP-*nopT*; C93S, cells transfected with pEGFP-*nopT*-C93S. (E) Proposed cleavage site of NopT as determined by Edman sequencing of the 24-kDa form purified from BL21(DE3)/pET-*nopT* (black arrow). Sequenced amino acid residues are underlined. A white arrow marks an alternative cleavage site (with subsequent removal of the M49 by methionine aminopeptidase). For comparison, the cleavage site of AvrPphB is indicated below (gray arrow).

ground, no elevated enzymatic activity was measured in response to apigenin. These experimental data confirm the bioinformatic prediction that *nopT* with a *cis*-acting *tts* box in the promoter region is upregulated in response to flavonoids.

NopT exhibits autoproteolytic activity. When induced at 37°C, *E. coli* BL21(DE3) cells harboring plasmid pET-*nopT* synthesized a protein (~30 kDa), corresponding to NopT with a C-terminal His_6 tag (Fig. 2A and 4A). Reducing the culture temperature to 30°C (or lower) resulted in the appearance of an additional band with an apparent molecular mass of ~24 kDa. Western blot analysis with the anti-NopT antibodies indicated that the 24-kDa protein is a fragment of NopT (Fig. 4A), and anti-His antibodies recognized the C-terminal His_6 tag (not shown). Moreover, a faint band (~6 kDa) was observed, suggesting that NopT was cleaved into two fragments (results not shown). Next, we tested whether the formation of the 24-kDa protein at 30°C depends on the proteolytic activity of NopT. *E. coli* cells carrying pET-*nopT* plasmids with point mutations at position C93, H205, or D220 (see Fig. 1C) produced mainly full-length protein forms, whereas the 24-kDa fragment was barely detectable on Western blots with anti-NopT antibodies (Fig. 4B). Similar results were obtained when the proteins were purified by nickel affinity chromatography and then subjected to SDS-PAGE analysis (Fig. 4C). Hence, the C/H/D residues are essential for autoproteolytic activity of NopT within *E. coli* cells. A similar processing was also observed for HEK 293T cells expressing NopT fused to enhanced green fluorescent protein (EGFP). Western blot analysis with anti-NopT antibodies indicated that a 24-kDa NopT fragment was released from EGFP-NopT, whereas no cleavage was observed for the same construct with a point mutation at position C93 of NopT (Fig. 4D).

To characterize processing of NopT in more detail, the 24-kDa fragment was purified from *E. coli* cells and then subjected to Edman sequencing. The obtained N-terminal sequence GCCA suggests that NopT was cleaved between M49 and G50. Since *E. coli* cells possess methionine aminopeptidase activity (18), it is also possible that NopT was first cleaved between K48 and M49 and then further processed. The two possible autocleavage sites of NopT are highlighted in Fig. 4E, which also shows the cleavage site of AvrPphB (42). In conclusion, proteolytic processing of NopT exposed an N-terminal glycine, which is predicted to be myristoylated in eukaryotic cells (MYR Prediction Server: probability of false-positive prediction for NopT, $1.2e^{-7}$).

NopT induces an HR in tobacco and chlorotic and necrotic symptoms in *Arabidopsis* plants. Expression of type 3 effectors in plant cells is an experimental approach to test their function in planta. An *Agrobacterium*-mediated transformation system was used to transiently express full-length *nopT* under the control of the 35S promoter in tobacco (*N. tabacum* cv. Xanthi). With this agroinfiltration method, the protein of interest is rapidly expressed in the infiltrated leaf area. Agroinfiltration was performed on one side with *A. tumefaciens* EHA105 carrying pCAMBIA-*nopT* and on the other side with EHA105 carrying the empty vector pCAMBIA2301. Western blot analysis with anti-NopT antibodies showed expression of full-length NopT as early as 12 h postinfiltration (not shown). Tobacco cells expressing *nopT* turned grayish and rapidly died at about 2 to 3 days postinfiltration, whereas control regions infiltrated with *A. tumefaciens* carrying pCAMBIA2301 remained green (Fig. 5A). These findings indicate that NopT elicited an HR, a localized programmed cell death response.

We further studied the structural requirements of NopT for HR induction. To test whether proteolytic activity of NopT is associated with HR, DNA encoding an inactive form of NopT (C93 mutated to S) was cloned into pCAMBIA2301. Agroinfiltration with this plasmid did not elicit an HR, indicating that proteolytic activity is required for NopT-induced HR in tobacco leaves (Fig. 5B). Using a similar approach, an HR was induced by NopT lacking 49 N-terminal amino acid residues (Fig. 5C). On the other hand, transformation with DNA en-

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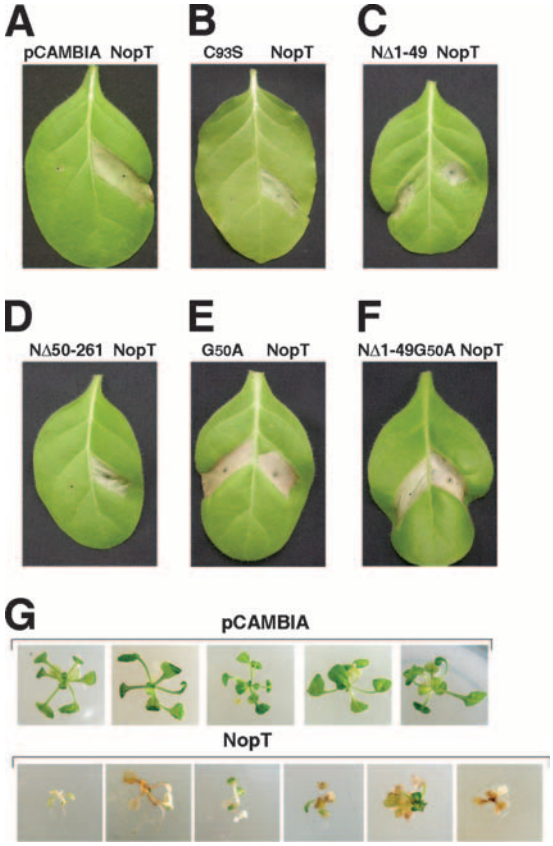

FIG. 5. Expression of *nopT* in plant cells. NopT induces HR in tobacco and chlorotic and necrotic symptoms in *Arabidopsis*. (A to F) Tobacco leaves were infiltrated with *A. tumefaciens* EHA105 carrying pCAMBIA2301 with the indicated constructs. Leaves were photographed 72 h after infiltration. *Arabidopsis* plants were transformed with the floral dip method. (G) Plants (T_1 generation) were selected on agar plates, transferred to glass jars, and photographed 4 weeks after germination. Abbreviations: pCAMBIA, plants transformed with EHA105 carrying the empty pCAMBIA2301 vector (A, left side; G); NopT, plants transformed with the construct pCAMBIA-*nopT* (A to F, right side; G); C93S, mutant construct of NopT with mutation of C93 to S (B, left side); Δ1-49, construct lacking 49 N-terminal amino acid residues of NopT (C, left side); Δ50-261, construct encoding 49 N-terminal amino acid residues of NopT (D, left side); G50A, NopT construct with mutation of G50 to A (E, left side); Δ1-49G50A, NopT construct lacking 49 N-terminal amino acid residues and with mutation of the N-terminal G50 to A (F, left side).

coding the first 49 N-terminal amino acid residues of NopT did not result in an HR (Fig. 5D). Finally, we tested the importance of G50 that is predicted to be myristoylated in eukaryotic cells after processing of NopT. The expression of full-length NopT with G50 mutated to A induced an HR (Fig. 5E). Replacement of G50 with A in NopT lacking the 49 N-terminal amino acid residues also elicited cell death, indicating that G50 is not required for the induction of an HR (Fig. 5F).

Agroinfiltration assays with *A. tumefaciens* carrying pCAMBIA-*nopT* did not induce rapid cell death in leaves of *Arabidopsis* (ecotype Columbia) (data not shown). Chlorotic symptoms were occasionally observed 5 days postinfiltration, however (not shown). We therefore examined *Arabidopsis* plants, which have been stably transformed with pCAMBIA-*nopT*. The expression of NopT in 10 lines was confirmed by Western blot analysis (data not shown). Compared to controls transformed with the empty vector, plants (T_1 generation) transformed with pCAMBIA-*nopT* grew slower and developed gradually chlorotic and necrotic symptoms. Figure 5G shows representative plants (from six lines). Finally, growth was completely arrested, and all plants died about 1 month after germination. Since an HR is generally defined as rapid cell death, it can be concluded that NopT did not elicit an HR but rather induced cytotoxic effects in *Arabidopsis* (Fig. 5G).

NopT is a host-specific determinant of symbiosis. To explore the effects of NopT during symbiosis, nodulation assays were carried out with NGR234 and the constructed mutant strains. *C. juncea* is a host plant of NGR234, but effective nodulation of this legume is blocked by undefined T3SS proteins (27). Under our test conditions, NGR234 induced on *C. juncea* ineffective nodules, which were greenish or brownish. In addition to these ineffective nodules, a few pink nodules were formed that fixed nitrogen and consequently increased the plant's biomass production. When challenged with NGRΔ*nopT*, *C. juncea* formed significantly ($P < 0.05$) more nodules than the parent strain (Table 1). Moreover, the number of pink nodules per plant was strongly increased (NGRΔ*nopT*, 5.5 ± 1.5; NGR234, 1.2 ± 0.5). Nodule biomass per plant was also elevated in plants infected with the mutant strain (Table 1). Similar nodulation data were obtained for *C. juncea* inoculated with the double mutant NGRΩ*nopL*Δ*nopT* (Table 1) and with NGRΩ*nopT* and NGRΩ*nopL*Ω*nopT*, forming a truncated form of NopT (data not shown). Taken together, these findings indicate that NopT had a deleterious effect on the nodule formation of *C. juncea*.

T. vogelii is another host plant of NGR234, and nodulation experiments showed that a double mutant with deleted *nopL* and *nopP* genes induced fewer nodules than the parent strain (45). Compared to NGR234, *T. vogelii* inoculated with NGRΔ*nopT* formed significantly fewer nodules ($P < 0.05$). The nodule biomass per plant was also reduced ($P < 0.05$). The double mutant NGRΩ*nopL*Δ*nopT* induced a similar nodulation phenotype on *T. vogelii* (Table 1). Together, these data indicate that NopT promoted the nodulation of *T. vogelii*.

We next inoculated *P. vulgaris*, since beans have been studied in the interaction with *Pseudomonas* strains secreting AvrPphB, a homolog of NopT (Fig. 1B). In *P. vulgaris* cv.

TABLE 1. Nodulation of *C. juncea* and *T. vogelii* inoculated with strains NGR234, NGRΔ*nopT*, and NGRΩ*nopL*Δ*nopT*

Host plant[a]	Strain	Mean ± SE[b]	
		Nodule no.	Nodule DW (mg)
C. juncea	NGR234	11.2 ± 1.7	6.7 ± 1.4
	NGRΔ*nopT*	28.2 ± 4.1	12.0 ± 2.0
	NGRΩ*nopL*Δ*nopT*	30.4 ± 4.8	12.0 ± 2.5
T. vogelii	NGR234	18.0 ± 2.0	20.8 ± 1.5
	NGRΔ*nopT*	10.0 ± 1.0	17.1 ± 1.0
	NGRΩ*nopL*Δ*nopT*	10.3 ± 0.9	22.3 ± 1.7

[a] Plants (one plant per jar) were inoculated with the indicated strains.
[b] The number of nodules (≥1 mm) and the nodule dry weight (DW) were determined for each plant at the time of harvest (7 weeks postinoculation) ($n \geq 12$).

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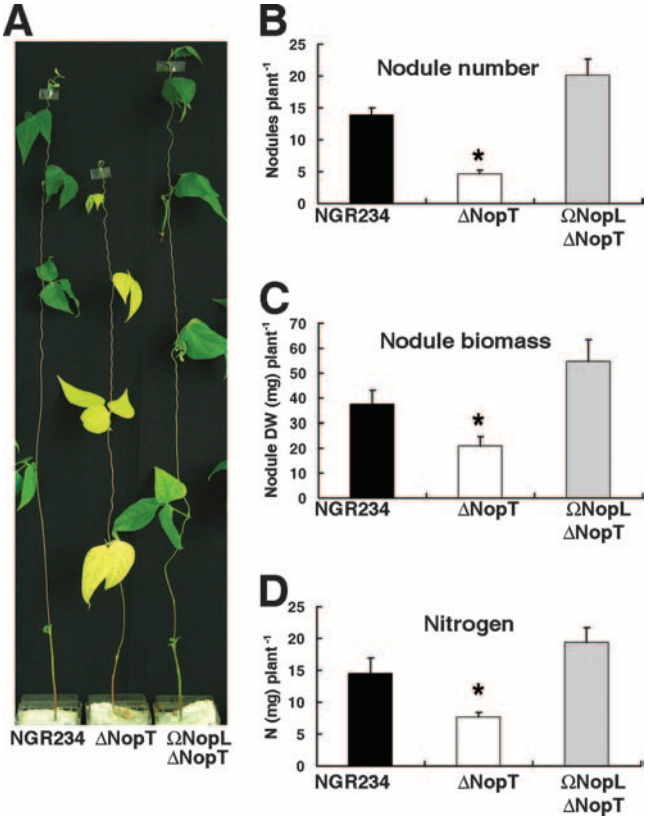

FIG. 6. Nodulation of *P. vulgaris* (cv. Yudou No. 1) inoculated with NGR234, NGRΔ*nopT*, and NGRΩ*nopL*Δ*nopT*. Plants were harvested 4 weeks postinoculation. The data indicate means ± the standard error ($n = 8$). Values topped by asterisks are statistically different compared to those from NGR234-inoculated plants (Kruskal-Wallis test; $P < 0.05$). (A) Aerial part of representative plants photographed at the time of harvest; (B) number of nodules (>1.5 mm) per plant; (C) dry weight (DW) of nodules per plant; (D) amount of nitrogen per plant as measured with the CHNS analyzer.

Tendergreen, AvrPphB elicited an HR, and this in planta response likely depended on the *R3* allele (50). Tendergreen plants inoculated either with NGR234 or with NGRΩ*nopT* formed only greenish nodules that were ineffective (data not shown). We therefore performed nodulation experiments with cv. Yudou No. 1, which established an effective symbiosis with NGR234 (Fig. 6). Plants inoculated with NGR234 formed pink nitrogen-fixing nodules that promoted plant growth. Interestingly, the mutant NGRΔ*nopT* had a lower symbiotic potential than the parent strain. Plants formed fewer nodules (Fig. 6B), and the nodule biomass per plant was also decreased (Fig. 6C), indicating a promoting effect of NopT on nodulation. Although nodules were fully developed and pink, nitrogen fixation was low in nodules induced by NGRΔ*nopT*. Leaves turned yellow (Fig. 6A), and the total amount of nitrogen per plant was significantly reduced compared to plants inoculated with NGR234 (Fig. 6D). Compared to NGR234, plants inoculated with NGRΩ*nopL* grew even better, indicating that NopL had a negative effect on the nodulation of this host plant (L. Zhang and C. Staehelin, unpublished data). When the double mutant NGRΩ*nopL*Δ*nopT* was tested on cv. Yudou No. 1, more nodules were formed compared to inoculation with NGRΔ*nopT*. Plants developed well, and the total amount of nitrogen per plant was high (Fig. 6). These data suggest that NopT secreted by NGR234 attenuated negative effects of NopL in the interaction with cv. Yudou No. 1.

DISCUSSION

This study shows that *Rhizobium* sp. strain NGR234 secretes the protease NopT via its T3SS. Mutation of *nopT* in NGR234 abolished NopT secretion but did not affect the secretion of other Nops, such as NopL. This secretion pattern corresponds to NGR234 derivatives with mutated *nopL* or *nopP* genes, which also retained their ability to secrete Nops via a functional T3SS. Similar to NopT, NopL, and NopP are type 3 effectors that affected nodulation on certain legumes (3, 27, 45).

Our promoter analysis provided evidence that expression of *nopT* is induced in response to flavonoids in a *ttsI*-dependent manner, suggesting that the predicted *tts* box is functional. GUS activity with our pRG-*nopT*p construct was weak (Fig. 3) compared to other flavonoid-inducible promoters of NGR234 (data not shown). Accordingly, NGR234 secreted only low amounts of NopT protein, which were not detectable on SDS-PAGE gels. Nevertheless, using an antiserum raised against recombinant NopT from *E. coli*, we immunologically detected NopT when secreted proteins of NGR234 (corresponding to ~100 ml of culture supernatant) were highly concentrated. This approach seems to be more sensitive than mass spectrometric analysis of proteins from a two-dimensional gel, which has been previously used for the identification of Nops (37, 38, 49).

The present study demonstrates that *nopT* is a functional gene of the YopT-AvrPphB effector protease family. NopT expressed in *E. coli* displayed autoproteolytic activity, which depended on amino acids of the catalytic triad. Autocleavage activity has been reported for the *Pseudomonas* effector AvrPphB (34, 41), whereas no autoproteolytic activities have been described for the *Yersinia* effector YopT and the *Photorhabdus* effector LopT. It is worth noting that only nonprocessed NopT was detected in rhizobial culture supernatants (Fig. 2C), in transgenic tobacco leaves, as well as in *nopT* expressing *Arabidopsis* plants (data not shown). These results raise the question of whether autoproteolytic processing of NopT is an "artifact" of expression systems such as *E. coli* and HEK 293T cells. It is worth noting in this context that agroinfiltration experiments followed by Western blot analysis did not result in detection of AvrPphB in leaves of tomato, bean, tobacco, and *Arabidopsis* plants (30, 50). Similarly, NopT could not be detected with the anti-NopT antibodies when proteins from nodules of *C. juncea* and *T. vogelii* were analyzed (data not shown). These findings suggest that NopT was either produced at low levels or rapidly degraded in legume host cells. Hence, cellular and environmental factors affecting stability and autocleavage of NopT remain to be elucidated.

Similar to AvrPphB, autoproteolytic processing of NopT resulted in an N-terminal glycine (G50), suggesting that NopT could be myristoylated in eukaryotic cells. NopT with an N-terminal alanine (G50 mutated to A) retained the activity to induce HR in tobacco plants, indicating that G50 is not required for induction of this response. These results are similar

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to those from Tampakaki et al. (50), who found that the myristoylation motif in AvrPphB was dispensable for effector action in bean cultivars carrying the *R3* gene allele. Future studies are required to test whether NopT is myristoylated in legume cells and whether myristoylation of type 3 effectors is important for symbiosis with certain host plants.

The rapid HR of tobacco cells induced by expression of *nopT* was used in the present study to assess the activity of different mutant forms of NopT (Fig. 5). We found that the first 49 amino acids at the N terminus of NopT are dispensable for HR-inducing activity. Based on studies with type 3 effectors from pathogenic bacteria (see, for example, reference 46), we suggest that the N-terminal part of NopT is essential for secretion through the pilus of the rhizobial T3SS. Indeed, NGRΩ *nopT* secreted a truncated form of NopT (Fig. 2C), indicating that the C-terminal part is not required for secretion. Similarly, a truncated form of the type 3 effector NopP was secreted by the T3SS of NGR234 (45).

NopT mutated in amino acid residues of the catalytic triad was inactive in inducing an HR in tobacco cells. This is similar to AvrPphB-induced HR in tobacco and certain *Arabidopsis* ecotypes, in which the HR directly depended on proteolytic cleavage of the protein kinase PBS1 by AvrPphB (42). In contrast to AvrPphB (44), NopT expressed in *Arabidopsis* did not elicit an HR but rather induced chlorotic and necrotic symptoms that resulted in growth arrest (Fig. 5G). This observation is in agreement with findings from Zhu et al. (58), who mentioned that PBS1 was cleaved by AvrPphB but not by NopT.

The cytotoxic effects of NopT on *Arabidopsis* are reminiscent of those of YopT on mammalian host cells (21). YopT in human cells cleaved prenylated RhoA and other GTPases near their C termini, resulting in release of the GTPases from membranes and disruption of the cytoskeleton (43). We therefore performed similar experiments with membrane-bound GTPases from legumes and NopT. However, we were unable to demonstrate that prenylated GTPases from plants are substrates for NopT (W. J. Dai and C. Staehelin, unpublished data). We suggest that effector proteins from the YopT-AvrPphB family differ in their substrate specificity toward prenylated GTPases. These differences are supported by the finding that AvrPphB could not cleave prenylated RhoA (41) and that NopT expressed in HEK 293T cells, in contrast to YopT (21, 59), did not induce morphological alterations of the cell shape (images not shown). Hence, it remains an open question of whether NopT targets membrane-bound GTPases from plants.

The nodulation experiments of the present study demonstrate that NopT affected the potential of NGR234 to establish nitrogen-fixing symbiosis with various host plants, indicating that NopT is a type 3 effector involved in host-specific nodulation. Symbiosis-promoting effects of NopT, as measured by the number of nodules per plant, were found for *P. vulgaris* (cv. Yudou No. 1) and *T. vogelii*, suggesting that NopT plays a positive role during the early stages of nodule formation. This is in agreement with the measured *nopT* promoter activity, which was TtsI dependent and induced by flavonoids within 24 h. On the other hand, the NGRΔ*nopT* mutant induced more nodules in the interaction with *C. juncea*. Thus, NopT is a "two-edged sword" that either improves or inhibits nodulation. This is similar to type 3 effectors from pathogenic bacteria that display either virulence or avirulence functions in plant-pathogen interactions (see, for example, reference 1). Based on these parallels, we suggest that certain legumes, such as *C. juncea*, are unable to distinguish between rhizobial type 3 effectors and their related counterparts from pathogenic bacteria. In other words, rhizobial effectors might act as "avirulence proteins" in legumes carrying corresponding resistance genes. We hypothesize that the expression of *nopT* in NGR234 is an evolutionary adaptation to particular host legumes, which established improved symbiosis with rhizobial strains secreting a specific set of type 3 effectors. It is worth noting in this context that sequences homologous to *nopT* of NGR234 have been identified in the genome of *B. japonicum* USDA110 (Fig. 1B) but not in other rhizobial strains. Thus, *nopT* is a strain-specific type 3 effector, and it is tempting to speculate that *nopT* is derived from a pathogenic bacterium. The possibility of gene transfer is supported by the ORFs flanking the *nopT* sequence region, which display similarities to "mobility elements" (Fig. 1C).

Future experiments are required to study the function of mutated NopT proteins within nodules. Our complementation experiment with bacterial cultures demonstrated that the secretion of NopT was restored in NGRΔ*nopT*p*nopT* (Fig. 2B). Complementation with this strain was not obtained in nodulation tests with *T. vogelii* and *C. juncea*, however (data not shown). These findings are in agreement with nodulation data from Deakin et al. (13), who reported that NGR234 lost a pLAFR6 construct in the absence of tetracycline. We conclude that complementation experiments with transconjugants harboring p*nopT* (or corresponding mutant constructs, e.g., p*nopT* with a C93S point mutation) cannot provide conclusive data with respect to the symbiotic role of NopT.

Nodule formation of legumes has been viewed as a "beneficial disease" (53) caused by a "refined pathogen" (15). Legumes may induce transient or localized plant-defense responses, especially during ineffective symbioses (see, for example, references 40, 47, and 55). The findings of the present study indicate that symbiotic rhizobia possess traits that resemble those from pathogenic bacteria. In addition to *nopT*, the symbiotic plasmid pNGR234*a* also harbors the ORFs y4fR (homologous to NopM of *S. fredii* HH103[37]) and y4lO (belonging to the YopJ effector family). It would be interesting to examine the function of these ORFs during symbiosis.

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