Functional Analysis of the Type 3 Effector Nodulation Outer Protein L (NopL) from Rhizobium sp. NGR234

SYMBIOTIC EFFECTS, PHOSPHORYLATION, AND INTERFERENCE WITH MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING*†

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Pathogenic bacteria use type 3 secretion systems to deliver virulence factors (type 3 effector proteins) directly into eukaryotic host cells. Similarly, type 3 effectors of certain nitrogen-fixing rhizobial strains affect nodule formation in the symbiosis with host legumes. Nodulation outer protein L (NopL) of Rhizobium sp. strain NGR234 is a Rhizobium-specific type 3 effector. Nodulation tests and microscopic analysis showed that distinct necrotic areas were rapidly formed in ineffective nodules of Phaseolus vulgaris (cv. Tendergreen) induced by strain NGR234 mutated in nopL, indicating that NopL antagonized nodule senescence. Further experiments revealed that NopL interfered with mitogen-activated protein kinase (MAPK) signaling in yeast and plant cells (Nicotiana tabacum). Expression of nopL in yeast disrupted the mating pheromone (α-factor) response pathway, whereas nopL expression in N. tabacum suppressed cell death induced either by overexpression of the MAPK gene SIPK (salicylic acid-induced protein kinase) or by SIPKDD (mutation in the TXY motif resulting in constitutive MAPK activity). These data indicate that NopL impaired function of MAPK proteins or MAPK substrates. Furthermore, we demonstrate that NopL was multiply phosphorylated either in yeast or N. tabacum cells that expressed nopL. Four phosphorylated serines were confirmed by mass spectrometry. All four phosphorylation sites exhibit a Ser-Pro pattern, a typical motif in MAPK substrates. Taken together, data suggest that NopL mimics a MAPK substrate and that NopL suppresses premature nodule senescence by impairing MAPK signaling in host cells.

Rhizobia are nitrogen-fixing bacteria that induce nodules on roots of legume host plants. Nodule initiation is triggered by rhizobial Nod factors, i.e. lipo-chitooligosaccharidic nodulation signals produced in response to host flavonoids in the rhizosphere (1). Additional symbiotic determinants produced by specific rhizobial strains often influence establishment of symbiosis in a host-specific manner. For example, rhizobial effector proteins secreted by bacterial secretion systems can significantly affect nodule initiation and nitrogen fixation on certain host plants (2). Various rhizobial strains possess a type 3 secretion system and deliver type 3 effector proteins into eukaryotic host cells (3, 4), a strategy common to many pathogenic Gram-negative bacteria (e.g. Yersinia spp., Shigella spp., Salmonella spp., and Pseudomonas spp.; Ref. 5).

Once delivered into host cells, many type 3 effectors of pathogenic bacteria function as virulence factors, which manipulate the host cell by circumventing or suppressing innate immunity. Type 3 effectors may target the ubiquitination-26 S proteasome pathway, alter RNA metabolism, or interfere with protein kinases in host cells (6–8). Various type 3 effectors target components of mitogen-activated protein kinase (MAPK) pathways. For example, YopJ of Yersinia spp. inactivates human MAPK kinase by acetylation of amino acid residues in the activation loop (9) and the IpaH30 effector of Shigella spp. is an E3 ubiquitin ligase that targets the MAPK kinase Ste7 in yeast and interferes with nuclear factor κB signaling in human cells (10, 11). Furthermore, a number of type 3 effectors (HopA1/OspF/SpvC family) from plant and animal pathogens inactivate MAPKs by cleaving the C–OP bond of phosphothreonine at their TXY motif (Thr–Xaa–Tyr, where Xaa is any amino acid) in the activation loop (12). MAPK signal pathways are major elements of the innate immunity of the plant and play crucial roles in recognition of pathogens as well as in activation of plant defense responses (13, 14). Overexpression of genes involved in MAPK signaling can result in induction of a hypersensitive response (HR), i.e. rapid localized cell death. Overexpression of SIPK (salicylic acid-induced protein kinase) and NtMEK2 (the MAPK kinase upstream of SIPK) induced necrosis of tobacco leaves within a few hours (15–17). MAPKs are the terminal protein kinases of a given MAPK signal cascade. Upstream MAPK kinases activate MAPK by dual phosphorylation of their TXY motif in the activation loop. Aspartic acid or glutamic acid can mimic the phosphorylation status of certain protein kinases. Mutant proteins, such as NtMEKDD of tobacco, are constitutively activated (15, 16). Activated MAPKs phosphorylate target proteins, such as transcription factors.

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3 The abbreviations used are: HR, hypersensitive response; MAPK, mitogen-activated protein kinase; NGR234, Rhizobium sp. strain NGR234; NopL, nodulation outer protein L; NI-NTA, nickel-nitrilotriacetic acid; SC, synthetic complete (medium); SD, selection dropout (medium); SIPK, salicylic acid-induced protein kinase; APase, alkaline phosphatase.
MAPK signaling cascades are conserved in animals, plants, and fungi. Although often associated with signaling in response to external stress factors, MAPKs may be essential for developmental processes. The mating pheromone response pathway for example, a well characterized MAPK signaling pathway in yeast, is specifically activated by the α-factor pheromone (18). In the Rhizobium-legume symbiosis, transient activation of MAPKs pointed to a possible role of MAPK signaling during the bacterial infection process (19).

The broad host range Rhizobium (Sinorhizobium) sp. strain NGR234 (20) possesses a functional type 3 secretion system (21), which can affect nodulation of host plants either positively or negatively. Up to now, four nodulation outer proteins (Nops) of NGR234 have been identified as type 3 effectors, namely NopL (21–24), NopP (25, 26), NopT (27, 28), and NopM (28). Their functions remain unclear, however. Some of these effectors share sequence similarity with effectors of pathogenic bacteria. NopT, a cysteine protease, is homologous to YopT of Yersinia spp. and contains ubiquitin ligase motifs that share sequence similarity with effectors of pathogenic bacteria. NopT, a cysteine protease, is homologous to YopT of Yersinia spp. and contains ubiquitin ligase motifs that are present in the E3 ubiquitin ligase IpA_H8 of Shigella spp. (28). On the other hand, proteins homologous to NopL have not been identified in pathogenic bacteria, indicating that NopL is a Rhizobium-specific effector.

Previous studies showed that transgenic plant cells expressing the type 3 effector gene nopL of NGR234 showed effects on expression of defense genes, suggesting that NopL can suppress the innate immunity of the plant (24). These findings were consistent with the observation that a nopL mutant of NGR234 (strain NGR1nopL) induced fewer nodules on the legume Flemingia congesta (22). Experiments with recombinant NopL protein expressed in Escherichia coli revealed that NopL could be phosphorylated at unknown sites by nondefined protein kinases from crude plant protein extracts (23, 24). Phosphorylation of NopL in vitro was reduced by addition of PD98059, a MAPK kinase inhibitor (23).

In this work, we characterized NopL of strain NGR234 in detail. Inoculation experiments with NGR234 and the mutant strain NGR1nopL indicated that NopL suppressed premature senescence of infected cells in nodules of the host plant Phaseolus vulgaris (cv. Tendergreen). To study the effect of NopL within eukaryotic cells, nopL was expressed in yeast and tobacco cells. In both cases, NopL blocked responses induced by MAPK signaling, indicating that NopL is a type 3 effector that targets MAPK pathways. Finally, we found that NopL was phosphorylated in planta and identified four phosphorylation sites in recombinant NopL purified from nopL expressing yeast cells.

**EXPERIMENTAL PROCEDURES**

**Nodulation Tests**—Nodulation tests with P. vulgaris (cv. Tendergreen) were performed according to previously described procedures (29). Surface-sterilized seeds were left to germinate on agar plates and then transferred to sterilized 300-ml plastic jars (1 plant per jar) with units linked with a cotton wick (3:1 v/v mixture of vermiculite and expanded clay in the upper vessel; nitrogen-free nutrient solution in the lower vessel). Plants were either inoculated with ~10⁹ bacteria of Rhizobium (Sinorhizobium) sp. strain NGR234 or its nopL mutant NGR1nopL (22). For complementation analysis, plasmid pLAFR-pnopL (supplemental Table S1) was constructed and mobilized from E. coli DH5α into NGR1nopL by a triparental mating procedure using the pRK2013 “helper” plasmid. Plants were grown in an air-conditioned growth room at 24 ± 2 °C and harvested at the indicated time points postinoculation. Where indicated, nodules were cut into two parts and photographed with a ruler. Areas of central infected zones and dark necrotic areas within these zones were quantified for each nodule using the ImageJ software (rsb.info.nih.gov/ij). For determination of nitrogen contents, plant material was dried, pulverized, and analyzed with a CHNS analyzer (Elementar Analysensysteme, Hanau, Germany).

**Microscopy**—Nodules were excised from roots, sliced manually, and immediately fixed in 67 mM sodium phosphate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde (4 °C, overnight). Samples were then post-fixed and embedded. An ultramicrotome equipped with a diamond knife was used to obtain ultrathin sections (~100 nm thick). Sections were stained with uranyl acetate and lead citrate and finally observed in a transmission electron microscope (JEM-2100HR). For light microscopy observations, semi-thin sections (1 μm thick) from the same samples were stained with 0.6% (w/v) toluidine blue.

**Yeast Strains, Plasmids, and Media**—Saccharomyces cerevisiae haploid strains W303-1A (MATa) and W303-1B (MATα) (30) were used in this study. The pYES2 vector (Invitrogen), a galactose-inducible plasmid, was used for NopL protein expression in S. cerevisiae. The pYES2 derivatives with mutated nopL sequences are listed under supplemental Table S1. For purification of recombinant His-tagged NopL (NopLHis), a DNA fragment containing the coding sequence of nopL fused to a C-terminal hexahistidine (His₆) tag was amplified by PCR and then cloned into the pYES2 vector yielding plasmid pYES-nopLHis (supplemental Table S1). Yeast transformation was performed with the lithium acetate/polyethylene glycol method according to the Yeast Protocols Handbook (Clontech). Yeast cells were cultured at 30 °C in YPD, SD, or SC medium (for media, see Yeast Protocols Handbook, Clontech and pYES2 Handbook, Invitrogen) containing 2% (w/v) glucose, raffinose, or galactose in the presence or absence of 1 M sorbitol. Uracl was excluded from the media (−Ura; without uracil).

**Halo Assays with Mating Pheromone**—Yeast cultures expressing nopL were grown to stationary phase and diluted to 2.5 × 10⁶ cells ml⁻¹ with H₂O. SD/-Ura medium plates (9 cm in diameter) containing 2% (w/v) galactose were overlaid with 1 ml of the yeast cell suspension. Filter disks were impregnated with 8 µg of the mating pheromone α-factor (Sigma, dissolved in 8 µl of H₂O) and placed onto the overlay. The plates were sealed and incubated at 27 °C for 1 week. Strain W303-1A (MATa) carrying either plasmid pYES-nopL or empty vector pYES2 was used for this assay.

**Agrobacterium-mediated Transient Transformation of Tobacco Leaves**—A cDNA clone of SLPK (accession number U94192) was kindly provided by Shuqun Zhang (University of Missouri, Columbia, MO). Thr³¹⁸ and Tyr²²⁹ residues in the
conserved TXY motif of SIPK were mutated into Asp by PCR-based site-directed mutagenesis. SIPK and the mutated SIPK sequence were predicted into the dexamethasone-inducible binary vector pTA7002 (31), yielding plasmids pTA-SIPK and pTA-SIPK<sup>Δ20</sup>, respectively (supplemental Table S1). DNA encoding NopL of *Rhizobium* sp. NGR234 was also cloned into pTA7002 and the plasmid was named pTA-nopL (supplemental Table S1). Transient expression studies with tobacco (*Nicotiana tabacum* cv. Xanthi) were performed according to previously described procedures (16) with the following modifications. Cultures of *Agrobacterium tumefaciens* strain AGL1 carrying different constructs were grown without acetylserine, resuspended to *A*<sub>600</sub> = 0.6 in 10 mM MgSO<sub>4</sub> supplemented with 5 μM acetylserine, and then used for leaf infiltration. Necrotic areas and infiltrated areas of tobacco leaves were quantified with the Adobe Acrobat 8 program. Where indicated, transformation of tobacco leaves was performed with strain AGL1 harboring plasmid pPZP-nopL (24), which resulted in *nopL* expression driven by the CaMV<sup>35S</sup>-promotor.

**Bioinformatic Analysis**—Putative phosphorylation sites of *nopL* were predicted with the NetPhosK 1.0 Server and kinase-specific phosphorylation sites of *nopL* with the NetPhosK 1.0 Server from the Technical University of Denmark. Potential phosphorylation sites in NopL predicted to be phosphorylated by proline-dependent serine/threonine kinases were searched phosphorylation sites in NopL predicted to be phosphorylated by proline-dependent serine/threonine kinases were searched using MotifScan of the Scansite 2.0 Server from the Massachussetts Institute of Technology. Clustal W algorithm was performed with the sequence alignment editor BioEdit.

**Purification of Recombinant His-tagged NopL Protein from Yeast Cells**—*S. cerevisiae* strain W303-1B harboring plasmid pYES-nopLHis (supplemental Table S1) was grown in SC medium (−Ura) and expression of His-tagged NopL (NopLHis) was induced with 2% (w/v) galactose. Extraction of soluble proteins from yeast cells was performed according to the pYES2 Handbook (Invitrogen). Nickel-nitritriacetic acid (Ni-NTA) affinity chromatography (Ni-NTA His-Bind<sup>®</sup> Resin, Novagen) was carried out according to the manufacturer’s instructions under denaturing conditions. Proteins binding to the Ni-NTA column were eluted with 150 mM imidazole. Purified NopLHis was desalted and concentrated by a Microcon-30 microconcentrator (Millipore).

**Treatment of NopL with Alkaline Phosphatases (APases)**—Tobacco leaves were infiltrated with *A. tumefaciens* AGL1 carrying pPZP-nopL (24). Three days later, proteins were extracted from tobacco leaf discs as previously described (23) without EDTA, using 10 mM MgCl<sub>2</sub> instead of KCl. Extracted soluble proteins (22 μl, corresponding to 7.3 mg of leaf FW) were directly treated with 2 units of 1 μl<sup>−1</sup> of APase (calf intestinal alkaline phosphatase, Fermentas) at 37°C for 1 h. NopLHis protein (∼5 μg) purified from yeast cells was incubated with 10 units of 1 μl<sup>−1</sup> of APase (FastAP<sup>TM</sup>, Fermentas) in a volume of 50 μl containing 1× APase buffer (Fermentas) at 37°C for 1 h. Where indicated, the phosphatase inhibitor Na<sub>3</sub>VO<sub>4</sub> (10 mM final concentration) was added.

**Two-dimensional Electrophoresis**—Procedures were similar to those described previously (32). Isoelectric focusing (IEF) was performed in a Bio-Rad Protein IEF Cell using 7-cm Ready-Strip IPG strips (Bio-Rad) with a linear pH gradient from 4 to 7 according to the manufacturer’s protocols. Proteins were separated by 12% SDS-PAGE gels and visualized by Coomassie Brilliant Blue R-250 staining or Western blot analysis.

**Western Blot Analysis**—Plasmid pPROEX-1nopL (23) was introduced into *E. coli* strain BL21(DE3) and His-tagged recombinant NopL was purified by Ni-NTA affinity chromatography. The purified protein was then used to raise a rabbit antiserum against NopL (at 1:4,000 or 1:8,000 dilutions). Anti-His monoclonal antibody was purchased from Invitrogen (1:1,000 dilution). Blots were treated with corresponding horseradish peroxidase-conjugated second antibodies and developed with 3,3′-diaminobenzidine according to the supplier’s instructions (Boster, Wuhan, China).

**Identification of Phosphorylation Sites by Mass Spectrometry**—Phosphorylated NopLHis protein from yeast cells was purified by Ni-NTA affinity chromatography. Spots obtained after two-dimensional electrophoresis were excised, mixed, and in-gel digested with trypsin (Promega). Phosphopeptides were enriched with a Titanium Dioxide MicroTrap (Michrom Bioresources), and analyzed by mass spectrometry (MALDI-TOF/TOF 4800 PLUS; Applied Biosystems, ABI) at the Mass Spectrometry Analysis Center of Shenzhen Graduate School, Peking University, Shenzhen, China. The mass spectral data were analyzed using the Mascot searching algorithm (Matrix Science), an in-house version of Mascot version 2.1. Phosphorylated residues were then determined by manual inspection of the MS/MS data using Data Explorer version 4.5 (ABI).

**RESULTS**

**NopL Antagonizes Senescence of P. vulgaris (cv. Tendergreen)**

**Nodules**—*P. vulgaris* (cv. Tendergreen) plants were inoculated either with *Rhizobium* sp. NGR234 or with a *nopL* mutant derivative, strain NGR<sub>ΔnopL</sub>. Inoculation with NGR<sub>ΔnopL</sub> led to the formation of an increased biomass of nodules on cv. Tendergreen as compared with the parent strain NGR234 (supplemental Table S2). Young plants (22 days postinoculation) inoculated either with NGR234 or NGR<sub>ΔnopL</sub> formed pink nodules, suggesting expression of leghemoglobin (Fig. 1A). However, initially green leaves turned rapidly yellow (Fig. 1C). Nitrogen contents of harvested total plants inoculated with both strains were similar and showed low values (11.6 ± 0.5 mg of nitrogen per g (DW) with NGR234; 11.2 ± 0.6 mg of nitrogen per g (DW) with NGR<sub>ΔnopL</sub>). Hence, NGR234 and NGR<sub>ΔnopL</sub> established an ineffective symbiosis with cv. Tendergreen.

Interestingly, nodules induced by NGR<sub>ΔnopL</sub> harvested at later time points displayed an increasing number of dark necrotic lesions, which were less frequent in nodules induced by the parent strain NGR234 (Fig. 1A). To substantiate this observation, nodules from a time course experiment were cut into two parts and their cutting areas were quantitatively analyzed with the image processing software ImageJ. For each nodule, the dark necrotic area of infected zones was expressed as percentage of the total area of infected zones. Compared with the parent strain NGR234, nodules containing NGR<sub>ΔnopL</sub> showed higher values, indicating an accelerated formation of necrotic lesions over time (Fig. 1B).
In a complementation test, NGR\textsuperscript{nopL} carrying plasmid pLAFR-\textit{pnopL} showed significantly reduced nodule necrosis in comparison with NGR\textsuperscript{nopL}, albeit values were not completely reaching those of the parent strain NGR234 (supplementary Table S3).

To obtain more information on these necrotic lesions, microscopic analysis was performed with selected nodules, which were either completely pink or necrotic in their central nodule tissue (Fig. 2 and supplementary Fig. S1). Central zones in pink nodules induced by NGR234 or NGR\textsuperscript{nopL} were rich in infected cells filled by intact symbiosomes, \textit{i.e.} bacteroids were surrounded by symbiosome membranes of the host plant. All microscopic pictures obtained from nodules with necrotic tissue showed typical features of senescence. In infected cells of these nodules, most bacteroids of NGR234 or NGR\textsuperscript{nopL} lacked surrounding symbiosome membranes, which were apparently degraded or fused, a typical symptom of nodule senescence. Furthermore, bacteroids appeared to be rapidly degraded in necrotic nodules. Taken together, the observed dark areas in nodules represented necrotic lesions and NopL antagonized nodule senescence in the examined interaction with \textit{P. vulgaris} (cv. Tendgreen).
Characterization of NopL

**Cytostatic Effects of NopL and Mutant Proteins in Yeast Cells**—The yeast *S. cerevisiae* is a eukaryotic model system for functional analysis of bacterial virulence proteins, including type 3 effectors (33). To study effects of NopL within yeast cells, the coding sequence of *nopL* was cloned into the vector pYES2 and the resulting plasmid pYES2-nopL was introduced into *S. cerevisiae*. The haploid strains W303-1A (MATα) and W303-1B (MATα) used in this study have the ade2-1 phenotype, in which red pigments are accumulated when yeast cells are grown under adenine-limiting conditions (34).

Compared with control cells carrying the empty vector pYES2, expression of *nopL* after galactose induction in W303-1B carrying pYES-nopL resulted in delayed formation of colonies, which were smaller and faint pink when grown on agar plates with the adenine-limiting medium SD/-Ura. Smaller colonies were also observed when NopL was expressed in W303-1B grown on adenine-sufficient medium SC/-Ura, where colonies did not turn pink (Fig. 3A). Western blot analysis with anti-NopL serum confirmed that NopL under control of the GAL1 promoter was strongly expressed in yeast strain W303-1B harboring pYES-nopL (Fig. 3B).

As NopL displayed a cytostatic activity, expression of *nopL* in yeast cells (especially in W303-1B under adenine-limiting conditions) could be further used as a bioassay to characterize NopL mutant proteins that lack this cytostatic activity. NopL forms deleted either at the N or C terminus were expressed in yeast and the amounts of produced mutant protein were determined by Western blot analysis with anti-NopL serum (Fig. 3C). NopL bands at expected molecular weights were clearly visible, but reduced protein levels were observed for NopL(D271A), NopL(S240A/S245A), and NopL(D271A). Yeast cells expressing these three mutant proteins exhibited delayed growth (Fig. 3C), suggesting that NopL is either not a protease or inhibited yeast growth independently of this activity.

**NopL Interferes with the Yeast Mating Pheromone Response Pathway**—The mating pheromone response pathway in yeast is one of the well-characterized MAPK signaling pathways. Treatment of strain W303-1A (MATα) with the α-factor pheromone results in specific activation of this pathway, which triggers a subsequent cell cycle arrest (18). To study the effect of *nopL* on this pathway, a halo assay with α-factor was performed with strain W303-1A (MATα) carrying pYES-nopL. Colonies of strain W303-1A harboring pYES-nopL expressed NopL under the tested conditions and grew slower than those containing the empty vector pYES2. Due to the cytostatic activity of NopL, the color of formed colonies was different for the two strains (dark colonies in Fig. 4A indicate fast growth, whereas white colonies reflect slower growth). However, strain...
**Characterization of NopL**

W303-1A expressing *nopL* could overcome the cell cycle arrest induced by the α-factor pheromone, indicating that NopL inhibited the mating pheromone response pathway (Fig. 4A). NopL Impairs Cell Death in Tobacco Leaves Induced by Overexpression of SIPK or SIPK<sup>DD</sup>—Agrobacterium-mediated overexpression of the MAPK SIPK gene in tobacco leaves triggers HR cell death, resulting in rapid formation of necrotic leaf zones (16). Co-expression of *nopL* and SIPK in tobacco leaves partially resulted in reduced SIPK-induced HR and the necrotic area of zones infiltrated with *A. tumefaciens* was significantly smaller (Fig. 4B). Hence, NopL interfered also with MAPK signaling in plants.

We further examined the effects of overexpression of SIPK<sup>DD</sup>, a mutant form of SIPK, in which Thr<sup>218</sup> and Tyr<sup>229</sup> in the activation loop were replaced by aspartic acid (Fig. 4C). As shown in Fig. 4B, *Agrobacterium*-mediated expression of SIPK<sup>DD</sup> induced an HR in tobacco leaves. Compared with SIPK (wild-type gene) expression, the HR was weaker (Fig. 4B). Nevertheless, these findings indicate that SIPK<sup>DD</sup> is a functional MAPK, which does not require activation of the upstream MAPK kinase NtMEK2 (Fig. 4C). Interestingly, co-expression of *nopL* with SIPK<sup>DD</sup> totally suppressed the HR induced by SIPK<sup>DD</sup> (Fig. 4B). These data indicate that NopL impaired MAPK signaling by inhibiting SIPK or downstream transcription factors.

**NopL Is Multiply Phosphorylated**—In vitro experiments showed that radioactively labeled ATP could be incorporated into NopL (23, 24). We therefore asked whether protein kinases phosphorylate *NopL* in vivo. To answer this question, we examined phosphorylation of NopL in transgenic tobacco leaves transformed with *A. tumefaciens* AGL1 pPZP-nopL. Antibodies directed against NopL recognized two protein bands on Western blots (Fig. 5A). Commercial purified APase was then used to dephosphorylate NopL in tobacco protein extracts. Western blot analysis revealed that incubation with APase converted the two NopL bands to a single band with a lower molecular weight. No shifts were observed when the APase inhibitor Na<sub>3</sub>VO<sub>4</sub> was added to the reaction mixture (Fig. 5B). These data provide evidence that NopL was phosphorylated in plant cells.

Next, we tested whether NopL is also phosphorylated in yeast cells. NopLHis (NopL with a C-terminal His<sub>6</sub> tag) from strain W303-1B carrying pYES-nopLHis was purified on a Ni-NTA column. Staining of SDS-PAGE gels showed that purified NopLHis corresponded to various smeared protein bands. After incubation with APase, NopLHis appeared as a sharp band on the gel, whereas addition of Na<sub>3</sub>VO<sub>4</sub> blocked formation of the sharp NopLHis band (Fig. 5C). Thus, NopLHis was also phosphorylated in yeast cells.

NopLHis from yeast cells purified by Ni-NTA affinity chromatography was further analyzed by two-dimensional electrophoresis. At least nine protein spots of purified NopLHis were observed on two-dimensional gels, which were absent in control protein samples purified from yeast cells carrying the empty vector pYES2 (Fig. 5, D and E). NopLHis spots with a lower isoelectric point (pl) had a higher apparent molecular weight (Fig. 5D), suggesting that these spots represent different phosphorylated protein forms of NopLHis (phosphoryl groups are negatively charged). Western blot analysis with an anti-His monoclonal antibody confirmed that phosphorylated NopLHis was separated into at least 9 different protein forms. Furthermore, a similar pattern was observed for an additional NopLHis form with a slightly lower molecular mass (ΔΔ kDa), which was presumably formed by proteolytic degradation in the N-terminal region of NopLHis (Fig. 5F).

**Identification of Phosphorylation Sites in NopL**—Phosphorylated NopLHis from yeast cells (strain W303-1B pYES-nopLHis) was purified by Ni-NTA affinity chromatography and two-dimensional electrophoresis. NopLHis protein spots were cut off from the two-dimensional gels and digested with trypsin. Phosphopeptides were then enriched and subjected to MALDI-TOF/TOF analysis. Four phosphorylation sites of NopL were confirmed by observation of a sufficient number of *b*<sub>n</sub> ions (N terminus-derived fragment ions) and *y*<sub>n</sub> ions (C terminus-derived fragment ions). The obtained data indicate that Ser<sup>89</sup>, Ser<sup>139</sup>, Ser<sup>148</sup>, and Ser<sup>198</sup> of NopLHis were phosphorylated (Fig. 6). Furthermore, precursor ions corresponding to two additional NopLHis peptides obtained by nonspecific MALDI-TOF/TOF analysis did not result in detectable ions, suggesting that additional phosphorylation sites are present in NopLHis.

**FIGURE 5.** NopL is multiply phosphorylated in tobacco (A and B) and yeast cells (C to F). A, Western blot analysis of NopL expressed in tobacco cells. Total soluble proteins were extracted from leaves infiltrated with *A. tumefaciens* AGL1 carrying either pPZP-nopL or the empty vector pPZP112. Equal amounts of isolated proteins (corresponding to 5 mg of leaf FW) were treated with APase. Control reactions were performed without APase or with APase in the presence of 10 mM Na<sub>3</sub>VO<sub>4</sub>. Proteins were then separated by SDS-PAGE and NopL was visualized on a Western blot with an APase inhibitor Na<sub>3</sub>VO<sub>4</sub> was added. No shifts were observed when the APase inhibitor Na<sub>3</sub>VO<sub>4</sub> was added to the reaction mixture (Fig. 5B). These data provide evidence that NopL was phosphorylated in plant cells. B, Western blot analysis of NopLHis. Ni-NTA chromatography was further analyzed by two-dimensional electrophoresis and immune detected using an anti-His monoclonal antibody. Purified NopLHis was separated into at least 9 protein spots.
mature nodules. suggesting that NopL could suppress HR-like cell death in nodules induced by NGR.

As cell death in plants can be induced by prolonged activation of MAPK pathways (15–17), we wondered whether NopL interferes with MAPK signaling in eukaryotic cells. When expressed in yeast, NopL counteracted the cell cycle arrest induced by the α-factor pheromone response pathway, a specific MAPK signaling pathway (18). Moreover, nopL expression in tobacco cells had negative effects on the HR cell death mediated by SIPK overexpression (Fig. 4B), providing evidence that NopL interfered also with MAPK signaling in plant cells. Interestingly, coexpression of nopL and SIPKDD in tobacco resulted in total suppression of the SIPKDD-induced HR (Fig. 4B). As the upstream MAPK kinase NtMEK2 is not required for SIPKDD activity (Fig. 4C), NopL seems not to target NtMEK2 or upstream elements of the MAPK signaling pathway. We suggest that NopL either directly inhibits action of SIPK or downstream MAPK substrates.

DISCUSSION

Type 3 effectors of phytopathogenic bacteria function as tox-in-like virulence factors that weaken and manipulate host defense responses. Certain host plants perceive type 3 effectors by plant resistance (R) protein-mediated recognition mechanisms and respond with an HR cell death, which prevents bacterial invasion (36). Thus, specific type 3 effectors can function as avirulence factors on certain host plants. Similarly, rhizobial type 3 effectors, such as NopT of NGR234, possess either a positive or negative role in the symbiosis with legume host plants (27, 28), and R-proteins control host-specific nodulation in the interaction between soybean and specific rhizobial strains (37). In this study, the nopL mutant NGRΔnopL was compared with the parent strain NGR234 in nodulation tests with the host plant P. vulgaris (cv. Tendergreen). An interesting effect of NopL on symbiosis was observed once infected cells with symbiosomes were formed. Pink zones of mature nodules induced by strain NGRΔnopL rapidly turned necrotic within 22 to 42 days postinoculation, whereas mature nodules induced by NGR234 remained pink over a prolonged period (Fig. 1B). Electron microscopy analysis confirmed that infected cells in necrotic nodules exhibited typical features of senescence (38), indicating that NopL antagonized premature nodule senescence in this host plant. The rapid formation of necrotic lesions in nodules induced by NGRΔnopL is reminiscent of an HR, suggesting that NopL could suppress HR-like cell death in mature nodules.

As cell death in plants can be induced by prolonged activation of MAPK pathways (15–17), we wondered whether NopL interferes with MAPK signaling in eukaryotic cells. When expressed in yeast, NopL counteracted the cell cycle arrest induced by the α-factor pheromone response pathway, a specific MAPK signaling pathway (18). Moreover, nopL expression in tobacco cells had negative effects on the HR cell death mediated by SIPK overexpression (Fig. 4B), providing evidence that NopL interfered also with MAPK signaling in plant cells. Interestingly, coexpression of nopL and SIPKDD in tobacco resulted in total suppression of the SIPKDD-induced HR (Fig. 4B). As the upstream MAPK kinase NtMEK2 is not required for SIPKDD activity (Fig. 4C), NopL seems not to target NtMEK2 or upstream elements of the MAPK signaling pathway. We suggest that NopL either directly inhibits action of SIPK or downstream MAPK substrates.

DISCUSSION

Type 3 effectors of phytopathogenic bacteria function as tox-in-like virulence factors that weaken and manipulate host defense responses. Certain host plants perceive type 3 effectors by plant resistance (R) protein-mediated recognition mechanisms and respond with an HR cell death, which prevents bacterial invasion (36). Thus, specific type 3 effectors can function as avirulence factors on certain host plants. Similarly, rhizobial type 3 effectors, such as NopT of NGR234, possess either a positive or negative role in the symbiosis with legume host plants (27, 28), and R-proteins control host-specific nodulation in the interaction between soybean and specific rhizobial strains (37). In this study, the nopL mutant NGRΔnopL was compared with the parent strain NGR234 in nodulation tests with the host plant P. vulgaris (cv. Tendergreen). An interesting effect of NopL on symbiosis was observed once infected cells with symbiosomes were formed. Pink zones of mature nodules induced by strain NGRΔnopL rapidly turned necrotic within 22 to 42 days postinoculation, whereas mature nodules induced by NGR234 remained pink over a prolonged period (Fig. 1B). Electron microscopy analysis confirmed that infected cells in necrotic nodules exhibited typical features of senescence (38), indicating that NopL antagonized premature nodule senescence in this host plant. The rapid formation of necrotic lesions in nodules induced by NGRΔnopL is reminiscent of an HR, suggesting that NopL could suppress HR-like cell death in mature nodules.

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Studies with alkaline phosphatase showed that NopL is phosphorylated in yeast or plant cells that expressed nopL. When NopLHis was expressed in yeast, at least nine protein spots were separated by two-dimensional electrophoresis, suggesting that the protein was incompletely phosphorylated at different phosphorylation sites. These findings indicate that there are at least eight phosphorylated residues in NopL. The four phosphorylation sites of NopL confirmed by mass spectrometry are located in the N-terminal part of NopL (residues 1–198). Interestingly, all phosphorylated serine residues in NopL are followed by a proline residue (Fig. 7).

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FIGURE 6. MS/MS analysis of phosphorylation sites in NopLHis purified from yeast (strain W303-1B carrying pYES-nopLHis). Data indicate enlarged tandem mass spectra of phosphopeptides with the indicated sequences and mass-to-charge (m/z) values. A sufficient number of b ions, N terminus-derived fragment ions) and y ions (C terminus-derived fragment ions) provide evidence that Ser139 (A), Ser139 (C and E), Ser148 (D and E), and Ser198 (B) of NopL are phosphorylated. The asterisks indicate partial peaks. Panels C and D show the same tandem mass spectrum, which corresponds to a mixture of two phosphopeptides with identical m/z value (2934.46).

FIGURE 7. Alignment of the two tandem repeat sequences in NopL with the Clustal W algorithm. The four identified phosphorylation sites of NopL confirmed by MS/MS analysis are indicated. NopL-N1, first repeat of NopL (residues 1–142); NopL-N2, second repeat of NopL (residues 143–258).

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was suppressed in transgenic tobacco plants expressing nopL (24). Similarly, the nodulation tests in this study indicate that NopL suppressed HR-like senescence in nodules of P. vulgaris cv. Tendergreen, thereby prolonging the lifespan of infected host cells. Oxidative stress might play a key role in triggering early nodule senescence (38). We suggest that formation of reactive oxygen species in nodules is linked with activation of MAPK pathways, particularly in ineffective nodules. In fact, premature HR-like nodule senescence was not observed for the effective symbiosis between NGR1nopL and P. vulgaris cv. Yudou No. 1 (data not shown).

As there are at least eight phosphorylation sites in NopL, it is likely that a single mutation of a phosphorylation site is not sufficient to affect NopL function. Indeed, single mutations in two confirmed phosphorylation sites (Ser$^{89}$ and Ser$^{139}$ mutated to Ala) did not influence the activity of NopL to inhibit yeast growth. Single mutations in three putative phosphorylated serine residues followed by proline (Ser$^{7}$, Ser$^{25}$, and Ser$^{73}$) also did not reduce the cytostatic activity (data not shown). Future experiments are required to test whether phosphorylation of NopL is required for symbiotic effects in the interaction with host legumes. Phosphorylation within host cells can be essential for the function of certain type 3 effectors. The plant pathogen P. syringae DC3000 delivers the type 3 effector AvrPtoB into plant host cells, where functions of protein kinases are inhibited (8, 41). Phosphorylation of a specific serine residue (Ser$^{250}$) in AvrPtoB considerably promoted the virulence activity of P. syringae DC3000 (42).

NopLHis was strongly phosphorylated in yeast (Fig. 5), but displayed only weak cytostatic activity (data not shown). Similarly, NopL without its C-terminal part (NopL$^{Delta 265-338}$) lacked cytostatic activity (Fig. 3C). These data indicate that phosphorylation of NopL per se is not causing yeast growth inhibition and that a C-terminal domain of NopL is crucial for the cytostatic activity of NopL. It is worth noting in this context that levels of NopL without the 6 C-terminal amino acids (NopL$^{Delta 333-338}$) were reduced in yeast, suggesting that these residues are important for stability or correct protein folding. Future experiments are required to test whether phosphorylation and cytostatic activity of NopL correlate with the symbiotic activity of NopL to suppress HR-like cell death in legume host plants.

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