Purification and phosphorylation of the effector protein NopL from *Rhizobium* sp. NGR234

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Abstract **Bacterial pathogens use type III secretion systems (TTSSs) to deliver virulence factors into eukaryotic cells. These effectors perturb host-defence responses, especially signal transduction pathways. A functional TTSS was identified in the symbiotic, nitrogen-fixing bacterium *Rhizobium* sp. NGR234. NopL (formerly y4xL) of NGR234 is a putative symbiotic effector that modulates nodulation in legumes. To test whether NopL could interact with plant proteins, in vitro phosphorylation experiments were performed using recombinant *nopL* protein purified from *Escherichia coli* as well as protein extracts from *Lotus japonicus* and tobacco plants. NopL serves as a substrate for plant protein kinases as well as purified protein kinase A. Phosphorylation of NopL was inhibited by the Ser/Thr kinase inhibitor K252a as well as by PD98059, a mitogen-activated protein (MAP) kinase kinase inhibitor. It thus seems likely that, after delivery into the plant cell, NopL modulates MAP kinase pathways.**

Key words: Type III secretion; MAP kinase; Signal transduction; Symbiosis; Legume

1. Introduction

Many pathogenic bacteria possess type III secretion systems (TTSSs) that deliver effector proteins into the cytoplasm of their eukaryotic hosts. Some effectors interfere with intra-cellular signalling cascades of the host cell, while others act on the cytoskeleton [1,2]. TTSSs are also present in bacteria that provoke the formation of nitrogen-fixing nodules on the roots of leguminous plants [3,4]. One such bacterium, *Rhizobium* sp. NGR234, secretes the *n*odulation *o*uter *p*rotein L (NopL) (formerly y4xL), a putative type III effector that promotes nodulation of the legume *Flemingia congesta* [5,6]. A number of sequence repeats and possible phosphorylation sites are present in the 37 kDa NopL protein. Furthermore, the N-terminal amino acid composition of NopL is similar to that of effector proteins of the plant pathogen *Pseudomonas syringae* [7].

Many biological processes are regulated by phosphorylation and/or de-phosphorylation of specific proteins including the mitogen-activated protein (MAP) kinase pathways [8]. Mounting evidence suggests that type III effectors of phytopathogens are translocated into plant cells where they specifically target host proteins [9,10]. We reasoned that proteins like NopL might affect signal transduction pathways during the establishment of symbiosis with legumes. Here we show that purified NopL is an excellent substrate for plant Ser/Thr kinases. Experiments using specific kinase inhibitors indicate that NopL is phosphorylated by MAP kinases.

2. Materials and methods

2.1. Secreted proteins of NGR234

Proteins were isolated from the supernatants of 100 ml cultures of *Rhizobium* sp. NGR234 [11] that had been induced with 10^{-6} M apigenin [5]. After precipitation with 70% w/v $(NH_4)_2SO_4$, the pellets were resuspended in 500 μl of 10 mM Tris–HCl (pH 7.5) containing 50 mM NaCl, and desalted on Microcon-10 concentrators (Millipore, Bedford, MA, USA). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated on 12% sodium dodecyl sulphate (SDS)–polyacrylamide gels and stained with silver nitrate.

2.2. Plant proteins

Extracts possessing protein kinase activity were obtained from young leaves of 20-day old *Lotus japonicus* cv. Gifu and *Nicotiana tabacum* cv. Xanthi plants that were grown under 16 h of light each day at 23°C. Leaves were homogenised in a mortar and pestle with three volumes (w/v) of ice-cold extraction buffer [100 mM Tris–HCl (pH 8.1), 400 mM sucrose, 10% glycerol, 10 mM EDTA, 10 mM KCl] containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitors from Boehringer Mannheim, Mannheim, Germany (2 μg ml^{-1} aprotinin; 0.5 μg ml^{-1} E64; 0.5 μg ml^{-1} leupeptin; and 1 μg ml^{-1} pepstatin). The extracts were centrifuged (13 000 × *g*, 30 min, 4°C), the supernatants collected and aliquots directly used for protein kinase assays.

2.3. Purification and immuno-detection of NopL

nopL (formerly y4xL – accession number AE000106) was amplified by polymerase chain reaction (PCR) using Vent polymerase (Biolabs, Frankfurt am Main, Germany) and the following primers: 5′-GACTGGCGCCATGGATATCAATTCAACCAGC-3′ and 5′-TATCTAGATCAAATGTCAAAATCCACCGA-3′. The PCR product was digested with *Ehe*I and *Xba*I (restriction sites are underlined in the primers) and cloned into the expression vector pPROEX-1 (BRL Life Technologies, Rockville, MD, USA). This vector added an in-frame hexa-histidine tag (6×His) to the N-terminus of NopL. The resulting plasmid was named pPROEX-1*nopL*. Overexpression in *Escherichia*

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Abbreviations: TTSS, type III secretion system; Nop, nodulation outer protein; MAP, mitogen-activated protein; PKA, protein kinase A

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coli DH5α and nickel-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) were carried out according to the manufacturer's instructions. The production and purity of the recombinant NopL was followed using 12% SDS–polyacrylamide gels stained with silver nitrate. In later experiments, the recombinant protein 'NopL–6×His' was only occasionally used. Generally, the hexa-histidine tag was removed from NopL–6×His using rTEV protease (BRL Life Technologies). Control protein fractions were obtained from *E. coli* carrying only the vector pPROEX-1 using the same protocol.

A polyclonal antibody was raised by immunisation of rabbits with recombinant NopL. The antiserum from these rabbits was monospecific for NopL. Secreted proteins from NGR234 or recombinant NopL were separated on SDS–polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore) electrophoretically. After incubation with the antiserum (1:2500 dilution) at room temperature for 2 h and washing with PBS, the immuno-blots were incubated with goat anti-rabbit immuno-globulin G coupled to horseradish peroxidase and developed using the ECL Western blotting analysis system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

2.4. Protein phosphorylation assays

A typical plant protein kinase assay (20 μl final volume) contained 40 mM HEPES (pH 7.4), 4 μg (5.4 μM) of recombinant NopL protein (or 10 μg of secreted NGR234 proteins), 5 μM ATP, 5 μCi/assay [γ-^{33}P]ATP, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 100 μM Na_3VO_4 and the protease inhibitors listed above [10,13]. Reactions were initiated by adding fresh extracts of *L. japonicus* or *N. tabacum* leaves (10 μg of soluble proteins), incubated at 30°C for 30 min, then stopped by adding Laemmli buffer [12] and heated to 100°C for 5 min. The proteins were separated by SDS–PAGE on 12% gels, dried, and exposed to an X-ray-sensitive film. The inhibitors PD98059, bisindolylmaleimide I (GF109203X), and KN62 were obtained from Calbiochem-Novabiochem (San Diego, CA, USA). Okadaic acid, K252a, and sodium vanadate were obtained from Sigma-Aldrich (St. Louis, MO, USA). The incorporation of radioactivity into NopL was quantified using the Gene Genius Bioimaging System (Gene Tools, Syngene, Cambridge, UK).

The protein kinase A (PKA) assay (20 μl final volume) contained 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 0.5 mM EGTA, 100 μM Na_3VO_4, 5 μM ATP, 5 μCi/assay [γ-^{33}P]ATP, protease inhibitors and 1 μg (1.35 μM) of recombinant NopL. The reaction was started by addition of 4 U of PKA from Sigma-Aldrich (approx. 1 μg protein). Samples were incubated at 30°C for 30 min and separated by SDS–PAGE (12%) as described above.

2.5. Determination of Michaelis–Menten constants

Aliquots of NopL (in the concentration range 0–10 μM) were incubated in the corresponding reaction mixtures containing [γ-^{33}P]ATP (5 μM; 5 μCi/assay [γ-^{33}P]ATP). Reactions were initiated by adding 4 U of PKA or plant protein kinase activity (10 μg total protein). Samples were then subjected to SDS–PAGE (12%) as described above. K'_m values were estimated from plots of the rate of [γ-^{33}P] incorporation into NopL against the concentration of NopL in the reaction mixture.

3. Results and discussion

Proteins from supernatants of (apigenin-induced) NGR234 cultures were isolated and analysed by SDS–PAGE. The TTSS mutant NGRΩ*rhcN* [5] was used as a control (Fig. 1A). Concurrently prepared supernatant proteins were used with freshly isolated plant protein extracts and [γ-^{33}P]ATP to study phosphorylation in vitro. Incubation of secreted proteins with extracts from the legume *L. japonicus* resulted in [γ-^{33}P] incorporation into a number of proteins (Fig. 1B). Only background levels of [γ-^{33}P] were incorporated when the proteins were taken from the supernatants of NGRΩ*rhcN* (Fig. 1B, left panel). When the supernatant proteins from NGR234 were co-incubated with the plant proteins, a strong band (marked by an arrow) appeared (Fig. 1B). Experiments

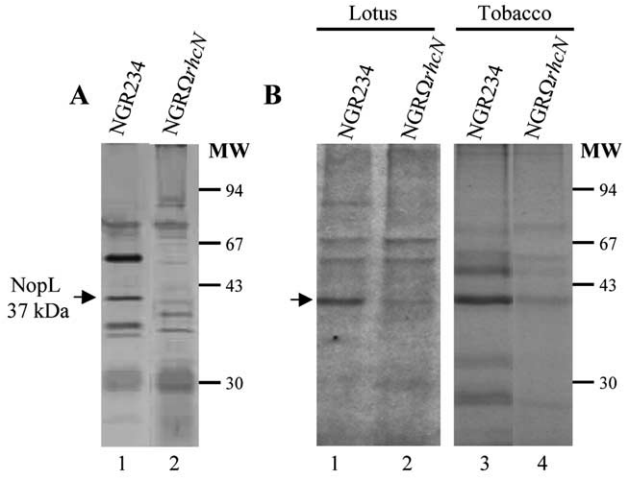

Fig. 1. Phosphorylation of secreted proteins by plant protein extracts. A: SDS–PAGE of secreted proteins from NGR234 (lane 1) and the TTSS null mutant NGRΩ*rhcN* (lane 2) stained with silver nitrate. The arrow shows NopL. B: Autoradiographs showing incorporation of [γ-^{33}P] into proteins. The reaction mixtures were separated by SDS–PAGE and the gels exposed to an X-ray-sensitive film. Lane 1, proteins from *L. japonicus* and NGR234; lane 2, proteins from *L. japonicus* and NGRΩ*rhcN*; lane 3, proteins from *N. tabacum* and NGR234; lane 4, proteins from *N. tabacum* and strain NGRΩ*rhcN*.

with proteins from tobacco rather than *L. japonicus* also resulted in a [γ-^{33}P]-labelled band that was absent when supernatants of NGRΩ*rhcN* were used (Fig. 1B, right panel). It thus seems that plant proteins are required for [γ-^{33}P] incorporation into a TTSS-dependent protein. To facilitate further experimentation, tobacco plants were used as the source of plant protein extracts.

The strongly labelled band (Fig. 1B, lanes 1 and 3) migrated at approximately the same rate as NopL. We hypothesised that the band is NopL, a 37 kDa protein that the NetPhos 2 programme [13] predicted to contain an unusually high number of possible phosphorylation sites. To test whether [γ-^{33}P] can be incorporated into NopL, recombinant NopL was produced. Control protein fractions were prepared from *E. coli* cells harbouring the empty vector (Fig. 2A). Rabbit antibodies raised against recombinant NopL specifically recognised the NopL protein, but control fractions did not give signals on Western blots (Fig. 2B).

Incubation of recombinant NopL with protein extracts from tobacco in the presence of [γ-^{33}P]ATP, followed by SDS–PAGE, resulted in a radioactively labelled band that corresponded to the molecular weight of NopL. Addition of the inhibitor K252a strongly inhibited the phosphorylation reaction, indicating that serine or threonine is the phosphorylated amino acid. When NopL was replaced by the control fraction purified from *E. coli*, or when plant proteins were used alone, only background [γ-^{33}P]-incorporation profiles were obtained. [γ-^{33}P] was not incorporated into NopL in the absence of plant proteins (Fig. 2C). The use of [α-^{32}P]ATP did not lead to any phosphorylated product (data not shown). Further assays were performed with purified PKA, which is a commercially available Ser/Thr kinase. PKA has the capacity to phosphorylate recombinant NopL (Fig. 2D). A slowly migrating band was occasionally seen at the top of the autoradiogram, indicating phosphorylation of an unknown *E. coli*

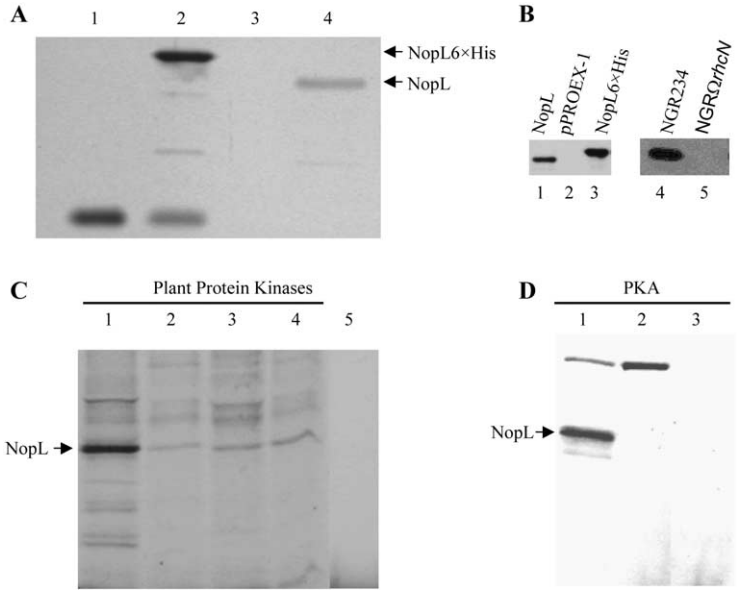

Fig. 2. Purification, immuno-detection and phosphorylation of recombinant NopL. A: Silver nitrate-stained gels (12% SDS–PAGE) of affinity-purified NopL from *E. coli* strain DH5α harbouring pPROEX-1*nopL*. As a negative control, the same procedure was used with DH5α containing the empty vector pPROEX-1. Lane 1, protein fraction from the empty vector pPROEX-1; lane 2, purified 6×HisNopL protein; lane 3, fraction from the strain carrying the empty vector after rTEV protease treatment; lane 4, recombinant NopL after removal of the 6×His tag with rTEV protease. B: Immuno-blot analyses. Recombinant NopL and secreted proteins from bacterial cultures were separated by SDS–PAGE, transferred to PVDF membranes and immuno-detected using the NopL antiserum. Left panel: lane 1, recombinant NopL; lane 2, protein fraction from the strain carrying the empty vector pPROEX-1; lane 3, recombinant 6×His NopL. Right panel: lane 4, secreted proteins from NGR234; lane 5, secreted proteins from NGRΩ*rhcN*. C: Recombinant NopL (1 μg) was phosphorylated by protein kinases of *N. tabacum* (10 μg, 30°C, 30 min) in the presence of [γ-^{33}P]ATP. After separation on SDS–PAGE, the gels were exposed to an X-ray-sensitive film. Lane 1, NopL phosphorylated by proteins of *N. tabacum*; lane 2, same reaction supplemented with 30 μM of the protein kinase inhibitor K252a; lane 3, control reaction with the protein fraction from cells containing the empty vector pPROEX-1; lane 4, *N. tabacum* proteins without NopL; lane 5, incubation of recombinant NopL without *N. tabacum* proteins. D: Phosphorylation reactions with PKA (4 units, 30°C, 30 min). Lane 1, Recombinant NopL and PKA; lane 2, control reaction with the protein from supernatants of the empty vector pPROEX-1; lane 3, PKA without NopL.

protein. These data strongly suggest that NopL is a substrate for plant protein kinases.

Kinetic studies were performed in which the [γ-^{33}P]ATP concentration was kept constant (5 μM) and the NopL concentration varied in the micromolar range (preliminary experiments had shown that the concentration of ATP is saturating under these conditions). The velocity of [γ-^{33}P] incorporation depended upon the concentration of NopL and followed Michaelis–Menten kinetics (Fig. 3). NopL phosphorylation by tobacco protein kinases generated an apparent $K'_m \approx 0.5$ μM. A similar K'_m value was determined for phosphorylation of NopL by PKA ($K'_m \approx 1$ μM).

To further characterise phosphorylation of NopL by tobacco protein extracts, the effects of kinase inhibitors were studied [14,15]. Strongest inhibition was measured for the Ser/Thr kinase inhibitor K252a, whereas KN62, GF-109203X and genistein had no effect (Table 1). Addition of the phosphatase inhibitors to the phosphorylation reaction slightly increased [γ-^{33}P] incorporation into NopL, indicating that protein phosphatases counteracted phosphorylation of NopL by protein kinases. As the Ca^{2+} chelator EGTA did not inhibit [γ-^{33}P] incorporation, Ca^{2+} is not required for the phosphorylation reaction. Interestingly, the MAP kinasae kinase (MEK) inhibitor PD98059 inhibited NopL phosphorylation (Table 1). PD98059 specifically binds to the de-phosphorylated form of MEK and thus blocks subsequent phosphorylation of MAP kinase [16,17]. Hence, plant MAP kinases seem to phosphorylate NopL. After perception of environmental stimuli, MAP kinase pathways are involved in signalling processes, such as those involving wounding and

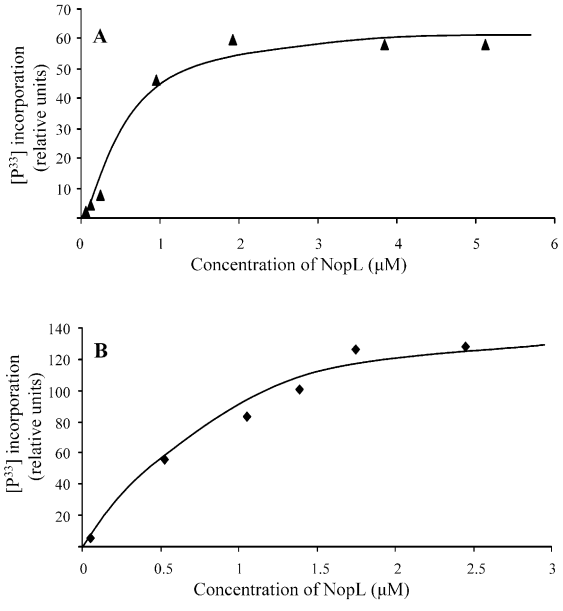

Fig. 3. Apparent Michaelis–Menten constant of PKA and *N. tabacum* protein kinase activity using recombinant NopL as the substrate. NopL (0–10 μM) was incubated with *N. tabacum* extracts containing 10 μg protein (A) or with PKA (4 U) (B) in the presence of [γ-^{33}P]ATP at 30°C for 5 min. The data give an apparent K_m of ≈0.5 μM for *N. tabacum* protein kinase activity and ≈1 μM for PKA.

Table 1
Effects of inhibitors on the phosphorylation of NopL

Inhibitor	Function	Concentration in assay (μM)	[^{33}P] incorporation (% of reaction without inhibitor)
Control	–	–	100
Genistein	Tyrosine kinase inhibitor	30	96
GF109203X	Protein kinase C inhibitor	20	96
KN62	CaM kinase II inhibitor	20	90
PD98059	MAP kinase kinase inhibitor	20	20
K252a	Ser/Thr kinase inhibitor	30	0.8
Okadaic acid	Tyrosine phosphatase inhibitor	15	111
Na_3VO_4	Phosphatase inhibitor	200	124
EGTA	Ca^{2+} chelator	1000	99

Recombinant NopL (1 μg) was incubated with *N. tabacum* proteins (10 μg) and [γ-^{33}P]ATP at 30°C for 30 min. The reaction mixtures were separated by SDS–PAGE and the gels exposed to an X-ray-sensitive film. Phosphorylation of NopL was expressed as the percentage of the amount of [γ-^{33}P] incorporated by the control reaction. Each assay was repeated at least twice.

pathogen-derived elicitors [8,18]. Target proteins recognised by MAP kinases harbour a Ser (or Thr) phosphorylation site followed by a proline residue [19]. The NopL sequence consists of nine predicted Ser phosphorylation sites that fulfill this condition (Ser-7, Ser-17, Ser-52, Ser-73, Ser-89, Ser-139, Ser-148, Ser-187 and Ser-198). Future work is required to test which of these Ser are phosphorylated by MAP kinases.

We suggest that NopL, once translocated into plant cells, might perturb the expression of genes that are activated by MAP kinase pathways, such as plant defence-genes. In this context, it is worth noting that perturbation of the MAP kinase pathway is also a strategy of the (TTSS-possessing) animal pathogen *Yersinia*. The effector protein YopJ is thought to be a protease that compromises signalling pathways, including the MAP kinase cascade [20]. NGR234 has a gene encoding a putative protein (y4lO) that has homology to YopJ [3] and it is possible that y4lO acts synergistically with NopL within host cells.

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