The Type III–Secreted Protein NopE1 Affects Symbiosis and Exhibits a Calcium-Dependent Autocleavage Activity

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Submitted 25 March 2009. Accepted 3 September 2009.

The type III–secreted proteins NopE1 and NopE2 of *Bradyrhizobium japonicum* contain a repeated domain of unknown function (DUF1521), which is present in a few uncharacterized proteins. A *nopE1/nopE2* double mutant strain exhibited higher nodulation efficiency on *Vigna radiata* KPS2 than the wild type or single *nopE1* or *nopE2* mutants. This indicates that both proteins are effectors that functionally overlap. To test translocation into the plant cell compartment during symbiosis, NopE1 and NopE2 were fused with adenylate cyclase (*cya*) as reporter. A fusion with the full-length proteins or N-terminal peptides resulted in increased cAMP levels in nodules, indicating translocation. Purified NopE1 exhibited self-cleavage in the presence of Ca^{2+} . Two identical cleavage sites (GD'PHVD) were identified inside the DUF1521 domains. The C-terminal cleavage site was analyzed by alanine scanning. Protein variants in which aspartate or proline next to the cleavage sites was substituted displayed no cleavage. A noncleavable protein was obtained by exchange of the aspartate residues preceding both cleavage sites. Complementation analysis with the noncleavable NopE1 variant did not restore wild-type phenotype on *Vigna radiata* KPS2, indicating a physiological role of NopE1 cleavage in effector function.

The type III secretion system (T3SS) is a complex protein transport system of gram-negative bacteria. Many rhizobia, e.g., *Bradyrhizobium japonicum* USDA110, *Sinorhizobium fredii* USDA257, *Sinorhizobium fredii* HH103, *Rhizobium* sp. strain NGR234, and *Mesorhizobium loti* MAFF303099 possess a functional T3SS (de Lyra et al. 2006; Göttfert et al. 2001; Kaneko et al. 2000; Krishnan et al. 2003; Viprey et al. 1998). Whether the secreted proteins, which are called nodulation outer proteins (Nop), influence symbiosis positively or negatively depends on the host (Krause et al. 2002a; Marie et al. 2003; Meinhardt et al. 1993).

Little is known about the activity or function of secreted proteins. NopA, NopB, and NopC are components of extracellular structures of the type III apparatus (Deakin et al. 2005; Lorio et al. 2004; Saad et al. 2005). NopX is postulated to form the translocon pore in the host cell membrane (Marie et al. 2003). NopD, NopL, NopM, NopP, and NopT are supposed

to be effector proteins, which are translocated into eukaryotic host cells. NopL and NopP can be phosphorylated by plant protein kinases (Bartsev et al. 2003; Skorpil et al. 2005). They are thought to interfere with host signaling cascades. NopD shows similarity to C48 cysteine peptidases (Rodrigues et al. 2007), and NopM is similar to YopM, an effector protein of *Yersinia* spp., which moves to the nucleus of host cells (Heusipp et al. 2006; Rodrigues et al. 2007). NopT might be a cysteine protease (Dai et al. 2008; Kambara et al. 2009).

After induction by genistein, *Bradyrhizobium japonicum* secretes at least 10 Nops (Hempel et al. 2009; Krause et al. 2002b; Süß et al. 2006), including NopE1 (formerly Blr1806) and NopE2 (formerly Blr1649). A translational *nopE1-lacZ* reporter gene fusion is expressed in mature nodules of *Macroptilium atropurpureum* (Zehner et al. 2008), suggesting a function of NopE1 during symbiosis. NopE1 has no obvious similarity to characterized proteins. The closest homolog is NopE2 of *B. japonicum* (77% identity). Both proteins contain two conserved domains of unknown function (DUF1521). This domain is also present in hypothetical proteins of the plant-associated bacterium *Burkholderia phytofirmans* PsJN (accession YP_001888935) and *Sphingomonas* sp. strain SKA58 (accession Q1NCW0).

Here, the effector proteins NopE1 and NopE2 are studied in detail. We show their impact on symbiosis with different host plants. The translocation of these effectors into nodule cells is shown using the adenylate cyclase (*cya*) reporter assay. We demonstrate that purified NopE1 exhibits calcium-dependent self-cleavage, which seems to be important for effector function in vivo.

RESULTS

NopE1 and NopE2 are responsible for a host-specific nodulation phenotype.

To test whether NopE1 and NopE2 influence nodulation, single and double mutant strains were created. Nodule formation was examined on soybean, mungbean, and *Macroptilium atropurpureum*. No significant difference in nodule number per plant was found between wild type and the *nopE1* (BJDΔ718) and *nopE2* (BJD215) single mutant strains (Fig. 1). Nodule numbers were significantly reduced ($P < 0.01$) when soybean and *M. atropurpureum* were inoculated with a *nopE1/nopE2* double mutant (BJDΔ718-215), suggesting that NopE1 and NopE2 have a positive effect on nodulation of these host plants.

B. japonicum shows weak nodulation capacity on the mungbean cultivar KPS2. Only six of 39 plants (15%) were nodulated by *B. japonicum* wild type (Table 1). In contrast, 86% of the tested plants formed nodules when KPS2 was inoculated with the *nopE1/nopE2* double mutant (BJDΔ718-215), indicating that

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables are published online.

NopE1 and NopE2 are negative effectors on this mungbean variety. In order to test whether translocation of the effectors is needed for symbiotic activity, the type III secretion mutant $\Delta 132$ was used for inoculation of mungbean. In strain $\Delta 132$, genes encoding conserved components of the T3SS are deleted and the secretion of effector proteins is precluded (Krause et al. 2002a; Süß et al. 2006). With mutant $\Delta 132$, 96% of mungbean plants are nodulated, suggesting that a functional T3SS is essential for NopE1 and NopE2 to act as effectors in symbiosis.

NopE1 and NopE2 are translocated into plant cells.

Although several type III–secreted rhizobial proteins have been described, formal proof that these proteins are translocated into plant cells is still lacking. Because bacteria do not

activate the calmodulin-dependent cya of *Bordetella pertussis*, it can be used as reporter (Sory and Cornelis 1994). The cya reporter was fused to amino acids 1 (strain BJD727), 111 (strain BJD728), and 484 (strain BJD729) of NopE1 (Fig. 2A). The reporter gene constructs were integrated into the chromosome at the original *nopE1* locus by homologous recombination, which leads to the expression of the fusion proteins from the native *nopE1* promoter. This promoter was previously shown to be active in nodules of *M. atropurpureum* (Zehner et al. 2008). The activities of the cya reporter strains were measured from nodules of *M. atropurpureum* 14 days after inoculation (Fig. 2B). Nodules formed by strain BJD727 contained very low levels of cAMP, similar to wild-type infected nodules, indicating that the reporter protein resides in the bacterial cell. Significantly more reporter activities were observed in nodules formed by the strains BJD728 and BJD729 ($P < 0.05$),

Fig. 1. Nodulation properties of *Bradyrhizobium japonicum* wild type and mutant strains. All tested plants formed nodules. Nodule numbers per plant (average ± standard deviation) were determined from at least 17 plants per strain. Results are from two independent experiments. Nodule numbers with the double-mutant strain are significantly lower than with the wild type or single mutants ($P < 0.01$).

Fig. 2. cAMP levels in nodules of *Macroptilium atropurpureum* infected with wild type or strains encoding different NopE1- and NopE2-cya fusions. **A**, Schematic representation of the fusion sites. The numbers indicate the amino acid residues that are deleted within the respective fusion. **B**, cAMP levels measured with nodules 14 days after inoculation with the indicated strain. Values obtained for the wild type infected nodules are in the same range as reported for other legumes (Terakado et al. 1997). The experiments were performed in triplicate. Wt = wild type containing no cya reporter.

Table 1. Nodulation properties of *Bradyrhizobium japonicum* wild type and mutant derivatives on *Vigna radiata* cv. KPS2^a

Strain used for inoculation	Nodulated plants	Nonnodulated plants	Plants tested	Nodulation efficiency (%)
Wild type	6	33	39	15
BJDΔ718	3	39	42	7
BJD215	1	43	44	2
ΔBJDΔ718-BJD215	32	5	37	86
BJDΔ718-Δ644	21	2	23	91
Δ132	22	1	23	96
BJD735	5	19	24	21
BJD740	21	3	24	88

^a Nodulation efficiency (%) = (number of nodulated plants/number of tested plants) × 100. Results are from at least two independent experiments. Nodules were counted 25 days after inoculation. All observed nodules were pink and of similar size.

suggesting that fusion proteins containing the first 111 amino acids of NopE1 or the full-length protein are translocated into the plant cell compartment. These results also indicate that the secretion signal of NopE1 locates in the N-terminal part of the protein. Similar experiments were performed with NopE2. The cya reporter was fused to amino acids 1 (strain BJD730), 88 (strain BJD731), and 512 (strain BJD732) of NopE2 and integrated at the original *nopE2* locus (Fig. 2A). The reporter activity in nodules formed by strain BJD730 was significantly less ($P < 0.01$) than in nodules formed by BJD731 and BJD732 (Fig. 2B), indicating that NopE2 is translocated into the host cell compartment in the presence of an N-terminal signal that is likely to be located in the first 88 amino acids.

As control, the full-length NopE1- and NopE2-cya fusions were integrated into type III secretion mutant $\Delta 132$ (Fig. 2B, strains $\Delta 132$ -729 and $\Delta 132$ -732). As expected, the cAMP values in nodules were significantly lower than for the same fusions in the wild-type background (BJD729 and BJD732; $P < 0.01$ and 0.02 , respectively), indicating that NopE1 and NopE2 require the T3SS for transport into the host cell compartment.

NopE1 is a secreted protein with two specific autoproteolytic cleavage sites.

Fragments of NopE1 can be found in the supernatant of *B. japonicum* cultures (Süß et al. 2006). For further biochemical characterization of the full-length protein, NopE1 was expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion protein, and the GST tag was removed after purification. Because bioinformatic analysis with the SMART program (Letunic et al. 2006; Schultz et al. 1998) indicated that NopE1 contains two EF hand-like calcium-binding motifs within the DUF1521 domains (at amino acid positions 149 to 167 and 361 to 391), NopE1 was incubated with Ca^{2+} , revealing that NopE1, which has a molecular mass of 51 kDa, is cleaved into three fragments in the presence of free Ca^{2+} , one of about 23 kDa, and two of about 14 kDa (Fig. 3). The cleavage can be inhibited by addition of EDTA or EGTA but not by the Complete-Mini protease inhibitor mix or the protease inhibitor 1,10-phenanthroline (data not shown).

Because the two smaller fragments are not separated well by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the following experiments were done with the full-length GST-NopE1 fusion protein, which gave three well-distinguished fragments (Fig. 4). Internal cleavage sites were analyzed by Edman degradation of fragments c and d (Fig. 4A), which yielded the sequence PHVDA in both cases. Both sequences are located inside the conserved DUF1521 domains (Fig. 4B). The cleavage sites of NopE1 were further analyzed by site-specific mutagenesis. Eight amino acids surrounding

Fig. 3. Fragmentation of NopE1 in the presence of Ca^{2+} . Purified NopE1 was incubated in TKE₁₀ buffer (lane 1) containing 20 mM CaCl_2 (lane 2).

the C-terminal cleavage site (IKGD₃₅₉'PHVD) were replaced individually by alanine (Fig. 4B and C). The substitution of aspartate (D₃₅₉) or proline (P₃₆₀) prevented autoproteolysis at this position, resulting in only two fragments. The replacement of the residues I₃₅₆, K₃₅₇, G₃₅₈, H₃₆₁, V₃₆₂, D₃₆₃, and H₁₄₅ did not inhibit fragmentation. Simultaneous exchange of residues D₁₄₇ and D₃₅₉ to alanine resulted in a noncleavable NopE1 derivative (Fig. 4C).

Relevance of NopE1 cleavage in symbiosis.

To test if cleavage is essential for the physiological function of NopE1, plasmids encoding the wild-type NopE1 (pBJD735) or a noncleavable NopE1 derivative (pBJD740) were integrated into the chromosome of the *nopE1/nopE2* deletion mutant BJD $\Delta 718$ - $\Delta 644$. The resulting strains were used to inoculate *Vigna radiata* KPS2, and nodulation efficiency was analyzed 25 days after inoculation (Table 1). Plants inoculated with strain BJD735 (complementation with wild-type *nopE1*) showed weak nodulation efficiency (21%), which is similar to *B. japonicum* wild type (15%). In contrast, strain BJD740 induced formation of nodules on 88% of the tested plants. A similar nodulation efficiency was observed when plants were inoculated with the *nopE1/nopE2* deletion mutant (91%). These results suggest that the noncleavable NopE1 variant is not active.

Fig. 4. Characterization of the NopE1 cleavage sites. **A**, Scheme of GST-NopE1 fragmentation. Sizes of fragments a-d are depicted. Filled arrowheads indicate the position of the identified cleavage sites. **B**, Amino acid sequence at the cleavage sites of both DUF1521 domains. Sites were determined by Edman degradation. **C**, Amino acid exchanges at the C-terminal cleavage site of NopE1 revealed two essential residues D₃₅₉ and P₃₆₀. Other amino acid substitutions did not prevent cleavage. Concurrent exchange of D₁₄₇ and D₃₅₉ lead to a non-cleavable variant. Full length protein GST-NopE1 is labeled with f, released fragments are labeled with a, b, c, and d as depicted in A. Fragments a and c consistently showed a slightly aberrant mobility. WT, wild-type protein.

DISCUSSION

The presence of a functional T3SS was found to affect nodulation with soybean and *M. atropurpureum* positively (Krause et al. 2002a). Nodulation phenotype of a *nopE1/nopE2* double mutant suggests that the positive effect is at least partially due to the activity of the NopE proteins. Plant assays with *V. radiata* KPS2 indicate that NopE proteins are major negative effectors for nodulation with this plant variety. In this case, they might be recognized as “pathogen-associated molecular patterns” that induce a defense reaction. That effector proteins can act positively on one host and negatively on another host has also been shown for *Rhizobium* sp. strain NGR234 (Kambara et al. 2009). Because single mutants in *nopE1* or *nopE2* displayed a phenotype similar to the wild type, NopE1 and NopE2, which are 77% identical on the amino acid level, are likely to overlap functionally.

In previous work, NopE1 and NopE2 were found solely in fragmented form in the supernatant of *B. japonicum* cultures, which suggests that post-secretion processing or degradation, or both, takes place (Süß et al. 2006). Interestingly, NopE1 could be isolated in full-length form from *E. coli*, and cleavage could be induced after purification. Because of the elaborate purification procedure and the insensitivity to protease inhibitors, it seems unlikely that cleavage is caused by a specific but unknown protease that is copurified from *E. coli* and also present and secreted in *B. japonicum*. The dependency of the cleavage on calcium ions and the prediction of calcium-binding motifs in NopE1 suggest that NopE1 has autoproteolytic activity. Moreover, complementation analysis indicates a physiological requirement for the cleavage of NopE1. Examples of type III-secreted proteins that are activated by an autoproteolytic cleavage event are HopAR1 (formerly AvrPphB) of *Pseudomonas syringae* pv. *phaseolicola* (Puri et al. 1997; Shao et al. 2002, 2003) and AvrRpt2 of *Pseudomonas syringae* DC3000 pv. *tomato* (Coaker et al. 2005, 2006; Mudgett and Staskawicz 1999). Both proteins are members of cysteine protease families and involved in pathogenic interaction with host plants. In rhizobia, only NopT, which is a YopT-like protein, has been shown to exhibit some autoproteolytic activity (Dai et al. 2008; Kambara et al. 2009).

NopE1 is cleaved at two sites between aspartate and proline, which are within a conserved sequence context close to the predicted EF hand-like motifs (Fig. 5). The sequence of the cleavage sites is conserved in NopE2, suggesting an identical processing. Alanine scanning of the C-terminal cleavage site of NopE1 showed that only the aspartate and proline next to the cleavage site are essential for cleavage. However, the sequence motif “DP” is not sufficient, as it is not cleaved at another position in NopE1.

NopE1 has no obvious similarity to known protease families and cleavage may be the result of a nonprotease reaction. Such mechanisms have been proposed for several nonhomologous proteins of different functions. A calcium-dependent “clip and link” mechanism was described for FrpC of *Neisseria meningitidis* (Osička et al. 2004), which is also cleaved between aspartate and proline. In this case, the processing is concerted with a linkage of the aspartate to an ϵ -amino group of lysine, resulting in an intermolecular protein cross-linking. This is in contrast to NopE1, for which higher molecular-weight fragments were never observed. YscU-like proteins, representing a conserved component of the type III and flagellar secretion apparatus were also shown to be processed by a nonprotease mechanism (Ferris et al. 2005; Wiesand et al. 2008).

Calcium is important for signal transduction in eukaryotic cells (Trewavas and Malhó 1998). Legume root hairs, for example, respond to Nod factors with calcium spiking (Ehrhardt et

al. 1996; Oldroyd and Downie 2004). NopE1 might be transported as a preprotein into the plant cytosol and activated by calcium-dependent processing. In *E. coli* cells, NopE1 is produced as full-length protein in soluble form. This suggests that either the estimated intracellular calcium concentration of about 90 nM (Gangola and Rosen 1987) is too low to induce cleavage or that the intracellular NopE1 does not have a conformation that is required for cleavage. In plant cells, calcium concentrations can be considerably higher and reach the micromolar range if signaling is induced by an extracellular stimulus (Ehrhardt et al. 1996; Pauly et al. 2000).

The transport of NopE1 and NopE2 into the nodule cell compartment was proven by *cya* reporter assay. The activity of the reporter protein is dependent on ATP and calmodulin-like proteins. Both components are present in the plant cell cytosol. Calmodulin-like proteins were also reported to be present in the symbiosome space that immediately surrounds the bacterium (Liu et al. 2006; Webb et al. 2000). There is no evidence that ATP is available in the symbiosome space. Thus, the observed increased cAMP levels with constructs in which the reporter was fused to N-terminal peptides of the NopE proteins or the full-length proteins are most likely derived from the plant cytosol.

NopE1 and NopE2, so far known as secreted proteins of *B. japonicum* (Süß et al. 2006), were identified as positive and negative effectors in the interaction with different legumes. Using the *cya* reporter, the transport of effector proteins into the plant cell compartment of nodules was demonstrated for the first time. An unusual autocleavage activity of the NopE1 protein is required for effector function in the plant. Further studies are needed to identify interacting molecules within the cell.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

Strains and plasmids used in this study are listed in Supplementary Table S1. For expression of proteins, *Escherichia coli* cells were grown in Luria-Bertani (LB) broth at 30°C with appropriate antibiotic until the optical density at 600 nm reached 0.7. Cells were induced by adding 200 μ M isopropyl- β -D-thiogalactoside and were further incubated for 3 to 4 h. *B. japonicum* was grown in peptone salts yeast extract medium (Regensburger and Hennecke 1983) for conjugation and plant assays or in arabinose-gluconate medium (Sadowsky et al. 1987) for routine growth.

Generation of *nopE1* and *nopE2* mutants and of constructs for complementation analyses.

For deletion of *nopE1*, pBJD113 was cut with *Bgl*II and *Spl*II, thereby removing the first 1,317 bp of *nopE1*. The protruding ends were filled in with Klenow enzyme, and a *Sma*I fragment containing the *aadA* gene of pHP45 Ω (Prentki and Krisch 1984) was inserted. The modified insert of pBJD113 was cloned into pSUPPOL2SCA (Krause et al. 2002a), generating pBJD718. The plasmid was transferred into *B. japonicum* by conjugation as described previously (Krause et al. 2002a). Double crossover resulted in the streptomycin-resistant mutant

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NopE1 144..IHGDPHVDADGDGKDDFDFKKDMT..167
NopE1 356..IKGDPHVDADGDGKVDFDFKKNMT..359
NopE2 153..IHGDPHVDADGDGKDDFDFKKDMT..176
NopE2 365..IHGDPHVDADGDGKDDFDFKKGMT..388
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Fig. 5. Comparison of NopE1 cleavage sites with NopE2. Cleavage takes place between the conserved amino acids D and P (underlined). Conserved amino acids are in bold. Numbers depict amino acid positions in the proteins.

BJD Δ 718. For disruption of *nopE2* (formerly *blr1649*), an internal 466-bp fragment was amplified by polymerase chain reaction (PCR) and was cloned into pSUPPOL2SCA (Krause et al. 2002a), resulting in plasmid pBJD215. The plasmid was transferred into *B. japonicum* by conjugation. Homologous recombination resulted in plasmid cointegration and disruption of *nopE2* Δ (strain BJD215). The double mutant strain BJD Δ 718-215 was generated by cointegration of plasmid pBJD215 in strain BJD Δ 718.

For deletion of *nopE2*, two fragments adjacent to the *nopE2* locus were amplified and cloned into pSUPPOL2SCA. A kanamycin-resistance cassette was cloned between these fragments, resulting in plasmid pBJD644. The plasmid pBJD644 was mobilized into the *nopE1* deletion strain *B. japonicum* BJD Δ 718 as described previously (Krause et al. 2002a). Homologous recombination resulted in strain *B. japonicum* BJD Δ 718- Δ 644, in which *nopE1* and *nopE2* are deleted.

For complementation, a 3-kb *Cla*I fragment of pBJD113 containing *bll1804*, *bll1805*, and *nopE1* was cloned into pSUPPOL2SCA. The resulting plasmid pBJD735 was integrated into the genome of BJD Δ 718- Δ 644, thereby generating strain BJD735. Correct integration was confirmed by PCR. A *Fse*I-*No*I fragment, encoding a noncleavable NopE1 variant, was excised from the plasmid pBJD216_{D147A, D359A} and was used to replace the *nopE1* gene of pBJD735, resulting in plasmid pBJD740. Correct cloning was confirmed by sequencing. The plasmid was integrated into the genome of BJD Δ 718- Δ 644 resulting in strain BJD740.

Nodulation assays.

Seeds of soybean (*Glycine max* cv. Amphor) and *Macroptilium atropurpureum* cv. Aztec Atro were obtained from Rustica Semences (Blagnac, France) and Southedge Seeds (Mareeba, Australia), respectively. Seeds of mungbean (*V. radiata* cv. KPS2) were a gift from N. Teaumroong (Suranaree University of Technology, Thailand). Seeds were surface-sterilized, germinated, and inoculated with *B. japonicum* as described previously (Krause et al. 2002a). The statistical significance of the results was calculated using analysis of variance between groups ($\alpha = 0.01$).

Construction and chromosomal integration of translational *nopE1*- and *nopE2*-*cya* fusions.

The *cya* reporter gene from pMS107 (Sory and Cornelis 1994) was amplified by PCR and was cloned into the *Xba*I and *No*I sites of the vector pSUPPOL2SCA, thereby generating plasmid pSLC5. Fragments of *nopE1* and *nopE2* genomic regions were amplified by PCR and were cloned into the *Eco*RI and *Xba*I sites of pSLC5, resulting in plasmids pBJD727, pBJD728, and pBJD729 for *nopE1* fusions and pBJD730, pBJD731, and pBJD732 for *nopE2* fusions. The inserts were confirmed by DNA sequencing. Recombinant plasmids were transferred into *B. japonicum* wild type and Δ 132 by biparental conjugation as described previously (Krause et al. 2002a). The correct integration of the plasmids into the chromosome of *B. japonicum* was confirmed by PCR.

Cya assays.

To determine cAMP levels, root nodules were collected 14 days after inoculation, were frozen in liquid nitrogen, were ground to a powder, and were suspended in 10 $\times$ volume of 0.1 M HCl (per nodule weight). The suspension was centrifuged and the supernatant was used for cAMP measurement with the cyclic AMP (direct) EIA kit (Assay Designs Inc., Ann Arbor, MI, U.S.A.) according to the manufacturer's instructions. Each sample was diluted for quantification to measure cAMP concentration in the detection range of the assay.

Expression of NopE1

as glutathione S-transferase fusion protein.

nopE1 was amplified by PCR and was cloned into the vector pGEX-4T3 resulting in plasmid pBJD216 (*gst-nopE1*), which was transformed into BL21(DE3). For protein purification, cells were grown as described above, were harvested, and were washed with phosphate buffered saline. The cells were resuspended in TKE₁₀-buffer (100 mM Tris-Cl, 200 mM KCl, 10 mM, EDTA pH 7.5), were incubated with 1% lysozyme for 30 min on ice, and were disrupted by sonication. Cell debris was removed by centrifugation (10,000 $\times$ g, 10 min, 4°C). The cleared lysate was filtered through a 0.25- μ m filter and was loaded on a "GSTrap FF" affinity column (GE Healthcare, Chalfont St. Giles, U.K.). The column was washed to remove unbound protein. By adding 80 U thrombin (GE Healthcare) to the column and with overnight incubation, NopE1 was cleaved from GST and eluted from the column. For purification of GST-NopE1 fusion protein, thrombin cleavage was omitted and elution was performed with 10 mM reduced glutathione in TKE₁₀ buffer. Eluates were collected and stored at 4°C until use. Alternatively, GST-NopE1 variants were purified using Glutathione Sepharose 4 Fast Flow (GE Healthcare) slurry. The supernatant, wash buffer, and eluates were separated from the slurry by centrifugation (400 $\times$ g, 4°C).

Determination of N-terminal sequences by Edman degradation.

Purified protein was incubated with 50 mM CaCl₂ for 10 min at 20°C and was subsequently loaded on a 12% SDS polyacrylamide gel. The gel-separated bands were blotted onto a polyvinylidene difluoride membrane (Hybond P, GE Healthcare) in a semidry blotting chamber (1.5 mA/cm², 1 h) in a discontinuous buffer system containing Tris-CAPS (cleaved amplified polymorphic sequence) buffer (60 mM Tris, 40 mM CAPS). The membrane was stained with Coomassie brilliant blue R250 and dried. The bands were cut out and analyzed by ChromaTec GmbH (Greifswald, Germany).

Site-directed mutagenesis of NopE1.

For site-directed mutagenesis, the QuikChange strategy (Stratagene, Cedar Creek, TX, U.S.A.) was used with pBJD216 as target and high-performance liquid chromatography-purified oligonucleotides containing the desired nucleotide changes (Supplementary Table S2). The modified sequence of the resulting derivatives was confirmed by sequencing.

Biochemical characterization of NopE1.

To test the effect of protease inhibitors, NopE1 was incubated 30 min at room temperature with 10 mM 1,10-phenanthroline or Complete-Mini protease inhibitor mix (Roche, Penzberg, Germany), followed by the addition of 1/10 volume of 0.25 M CaCl₂ solution. Protein fragmentation was subsequently analyzed by SDS-PAGE.

ACKNOWLEDGMENTS

We thank G. Cornelis (University of Basel, Switzerland) for providing plasmid pMS107 and N. Teaumroong (Suranaree University of Technology, Thailand) for kindly providing mungbean seeds. We are grateful to S. Okazaki (Nara Women's University, Nara, Japan) for helpful discussions. This work was supported by the Department of Biology, Dresden University of Technology.

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