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Translocation of NopP by *Sinorhizobium fredii* USDA257 into *Vigna unguiculata* Root Nodules[▽]

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***Sinorhizobium fredii* is a nitrogen-fixing legume symbiont that stimulates the formation of root nodules. *S. fredii* nodulation of roots is influenced by Nop proteins, which are secreted through a type III secretion system (T3SS). We demonstrate that *S. fredii* injects NopP into *Vigna unguiculata* nodules in a T3SS-dependent manner.**

The mutualistic relationship between rhizobial bacteria and leguminous plants is vital for agriculture. Plants are provided with a usable form of nitrogen, while rhizobia utilize carbohydrates produced by the plant. During the symbiotic interaction, rhizobia invade root hair cells and penetrate into deeper root cortical tissue through thin tubules known as infection threads (12). The bacteria are then engulfed in symbiosomes, or plant membrane-bound compartments, within rhizobia-induced root structures termed nodules. Inside symbiosomes, rhizobia differentiate into nitrogen-fixing cell forms called bacteroids (26).

At the molecular level, root nodule development is a complex process that requires signal exchange between rhizobia and their host plants. Initially, plants release flavonoid compounds that attract rhizobia and activate expression of bacterial genes involved in plant colonization (7). Several of these flavonoid-induced genes encode enzymes that synthesize lipochitooligosaccharide compounds known as nodulation (Nod) factors. Nod factors stimulate several plant responses required for the initiation of rhizobial colonization, including root hair curling and mitosis in root cortical cells (14).

In addition to Nod factors, some (but not all) rhizobial strains secrete proteins that are involved in nodulation (10). Several of these proteins are secreted by a type III secretion system (T3SS) encoded by the *rhc* gene cluster (8). T3SSs are present in many Gram-negative plant and animal pathogens and are known for delivering virulence proteins, collectively called effectors, directly into host cells. Effectors have diverse functions in promoting pathogen virulence, and the repertoire of effector genes varies in each organism with a T3SS.

Effector proteins secreted by rhizobial T3SSs are called Nops (*n*odulation *o*uter *p*roteins). Several studies have shown that Nop proteins can either promote or prevent nodulation, depending on the host plant (8). Two *nop* genes, *nopP* and *nopL*, are found in only a few rhizobial species (*Rhizobium* sp. NGR234, *Sinorhizobium fredii*, and *Bradyrhizobium japonicum*) and are absent from pathogenic bacteria (8). In *Rhizobium* sp. NGR234, *nopP* and *nopL* are required for optimal nodulation of the tropical legumes *Tephrosia vogelii* and *Flemingia congesta* (22, 32). NopP and NopL are also both phosphorylated by plant kinases (3, 32). However, the functions of these and other Nop proteins during nodulation are not well characterized. Some have hypothesized that Nops contribute to suppression of plant innate immune responses or modulate cytoskeletal rearrangements in root cells during nodule development (4, 32, 34). An important first step in determining the roles of Nops during symbiosis is the demonstration that these proteins are delivered into plant roots. However, because roots are not easily infiltrated with large numbers of bacteria and the symbiotic relationship takes time to develop, Nop translocation into roots by rhizobia has been difficult to validate.

T3SS-dependent translocation of effectors from phytopathogens into leaf mesophyll cells has been confirmed using the calmodulin-dependent adenylate cyclase toxin from *Bordetella pertussis* as a reporter (5, 6, 21, 24, 28, 30). When Cya is fused to the C-terminal end of an effector, the resulting hybrid protein is delivered by the T3SS into host cells, where it binds to the eukaryotic protein calmodulin and produces cyclic AMP (cAMP) from ATP (33). In this report, the adenylate cyclase translocation reporter system was optimized for use with *Sinorhizobium fredii* USDA257. This strain nodulates a variety of leguminous plants, including *Glycine max* (soybean) and *Vigna unguiculata* (cowpea, or black-eyed pea), and secretes NopP, NopL, and other Nop proteins when grown in culture (2, 16, 27).

To use Cya as a reporter for Nop translocation into plant roots, the *nopL* and *nopP* genes were amplified by PCR with the relevant primers listed in Table 1 and cloned upstream of *cya* in the plasmid pCPP3214 (30). The resulting plasmids, pJG1 and pEAO1, express *nopL-cya* and *nopP-cya*, respectively. To confirm that these plasmids express Cya fusion proteins, *Escherichia coli* DH5α strains containing pJG1, pEAO1, or pCPP3214 were grown to mid-log phase in LB containing 100 μM isopropyl-β-D-thiogalactoside (IPTG) and 50 μg/ml spectinomycin. Cells were then suspended in amounts of protein sample buffer that were normalized relative to bacterial number as estimated from optical density at 600 nm (OD_{600})

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TABLE 1. DNA oligonucleotide primers used to amplify *nopP* or *nopL*

Gene	Plasmid recipient	Primer sequence	
		5′ (Upstream)	3′ (Downstream)
nopP	pEAO1	CCGTCTAGAAAGGAGAAACAGGATGGA	GTAATACCCGGGAATGTCAAAATCCAGCGATG
nopL	pJG1	CGTCTAGAGGAGTACAAGATCGCCATG	AGAATCCCCGGGCATGAAGTCATCTTCGTAAG
nopP	pLMS149	GCCCATGGTAGGAGTACAAGATCGC	GGTCTAGAATGAAGTCATCTTCGTAAGTC

measurements. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, and Western analysis was carried out using monoclonal anti-Cya antibodies (Santa Cruz Biotechnology), as previously described (30). Proteins of the expected sizes (80.8 kDa for NopL-Cya and 74.8 kDa for NopP-Cya) were identified, although a smaller protein band of ~60 kDa was also visible along with NopP-Cya (Fig. 1). Multiple smaller protein bands have also been observed when other Cya hybrid proteins have been analyzed by Western analysis (30). These bands may result from bacterial processing of the Cya fusion protein or translation from alternative start sites.

Before examining the translocation by *S. fredii* of Nop-Cya proteins into plant roots, we determined whether the fusions were competent for translocation into plants. The phytopathogen *Pseudomonas syringae* pv. tomato DC3000 (hereafter referred to as DC3000) was chosen to test the delivery of NopP-Cya and NopL-Cya into plant tissue because (i) translocation of T3SS effector-Cya hybrid proteins into plant leaves by this organism is well established (19, 25, 30, 31) and (ii) the T3SSs of plant pathogens typically secrete effectors from other bacteria (1, 13, 29). Plasmids were conjugated into

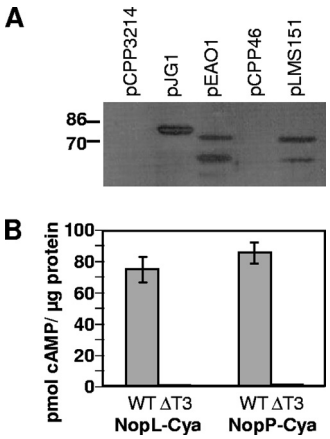

FIG. 1. Expression and T3SS-dependent translocation of Nop-Cya hybrids. (A) Western analysis of extracts from *E. coli* DH5α strains containing plasmids pCPP3214 (vector), pJG1 (expresses NopL-Cya), pEAO1 (expresses NopP-Cya), pCPP46 (vector), or pLMS151 (expresses NopP-Cya). The expected molecular sizes of the proteins are 80.8 kDa for NopL-Cya and 74.8 kDa for NopP-Cya. The sizes of molecular size markers in kilodaltons are shown on the left of the blot. (B) Accumulation of cAMP in *N. benthamiana* leaves infiltrated with wild-type (WT) or Δ*hrpK-hrpR*::ΩCm (ΔT3) DC3000 strains expressing either NopL-Cya or NopP-Cya. The values in the graph represent the average of three quantifications, and error bars indicate the standard deviation. A repeated experiment yielded similar levels of cAMP/μg protein for each strain tested.

wild-type DC3000 and its mutant derivative CUCPB5114 (Δ*hrpK-hrpR*::ΩCm), which lacks the entire T3SS gene cluster and cannot secrete effector proteins (11). The leaves of *Nicotiana benthamiana* were then infiltrated with 10^8 CFU/ml of the DC3000 strains, and cAMP and protein levels were determined in leaf samples collected with a 0.8-cm-diameter cork borer 7 h postinoculation, as previously described (30). Only the wild-type DC3000 strains expressing NopP-Cya or NopL-Cya induced accumulation of cAMP in *N. benthamiana* (Fig. 1B). Thus, both NopL-Cya and NopP-Cya were translocated by DC3000 into plant leaf cells in a T3SS-dependent manner.

Since NopP-Cya was translocated into plant leaves at slightly higher levels than NopL-Cya, we decided to focus on determining the conditions necessary for NopP-Cya delivery into plant roots by *S. fredii*. Our attempts to construct an *S. fredii* USDA257 strain that expresses NopP-Cya from pEAO1 were unsuccessful, indicating that plasmids with an RSF1010 *ori* may not replicate in USDA257. To circumvent this problem, another *cya* fusion plasmid was constructed using the vector pCPP46, which contains the *ori* and *par* loci from the broad-host-range plasmid RK2 and is maintained in the absence of antibiotic selection (9). A pCPP46 derivative expressing *nopP-cya* named pLMS151 was created in several steps by using Gateway cloning technology (Invitrogen). First, the entry vector pLMS149 was constructed by cloning the PCR product of the appropriate primers listed in Table 1 into pENTR11 (Invitrogen). Next, the destination vector pLMS150 was created by subcloning the Gateway B cassette containing the *cya* gene from pCPP3234 into pCPP46 (30). Finally, pLMS151 was created by site-specific recombination (or an LR reaction) between pLMS149 and pLMS150 (Invitrogen). The resulting *nopP-cya* fusion gene in pLMS151 is expressed from the upstream *lac* promoter present in the vector. *E. coli* DH5α strains containing either pCPP46 or pLMS151 were grown in LB supplemented with 100 μM IPTG and 10 μg/ml tetracycline. Samples were then separated by SDS-PAGE, and Western analysis was performed as described above. Anti-Cya antibodies recognized 74.8-kDa and ~60-kDa protein species, as seen for strains containing pEAO1 (Fig. 1A; data not shown). We also observed that *nopP-cya* expression from pLMS151 in *S. fredii* USDA257 did not require IPTG when strains were grown in yeast extract-mannitol (YEM) medium (data not shown). Thus, USDA257 may not bear a functional *lac* repressor, or *S. fredii* LacI may not bind to the *E. coli lac* operator in pLMS151.

To confirm that NopP-Cya is secreted from *S. fredii* USDA257 in a T3SS-dependent manner, culture supernatants from wild-type or *rhcC1* mutant bacteria containing pCPP46 or pLMS151 were analyzed. The *rhcC1* mutant lacks a component of the outer membrane ring of the T3SS and, thus, accumulates Nop proteins in its cytoplasm (23). These *S. fredii* strains were

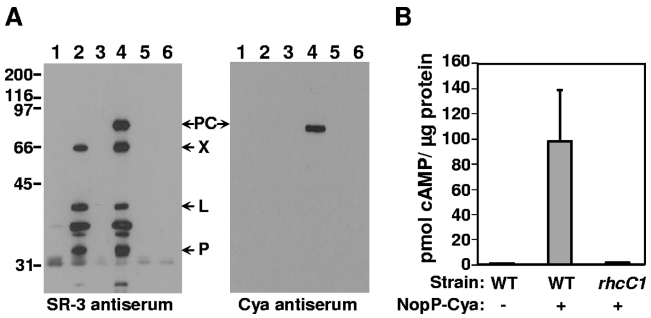

FIG. 2. T3SS-dependent secretion and translocation of NopP-Cya by *Sinorhizobium fredii* USDA257. (A) Western analysis of proteins secreted into culture fluids from *S. fredii* USDA257 strains containing plasmids pCPP46 (vector) or pLMS151 (expresses NopP-Cya). Extracellular protein samples were prepared from strains grown in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 1 μM apigenin. The positions of NopP-Cya (PC), NopX (X), NopL (L), and NopP (P) are indicated by arrows. The sizes of molecular size markers (in kDa) are shown on the left of the figure. Lanes 1 and 2, USDA257 containing pCPP46; lanes 3 and 4, USDA257 containing pLMS151; lanes 5 and 6, USDA257 *rhcC1* containing pLMS151. (B) Accumulation of cAMP in *V. unguiculata* roots infected with wild-type (WT) or *rhcC1* mutant USDA257 containing pCPP46 (vector) or pLMS151 (+NopP-Cya). The values in the graph represent the average of three quantifications, and error bars indicate the standard deviation. A repeated experiment yielded similar results.

grown in YEM medium supplemented with 1 μM apigenin (to induce T3SS genes) for 48 h at 30°C, and culture supernatants were collected as previously described (16, 20, 35). Extracellular protein samples were analyzed by Western analysis using SR-3 polyclonal antisera, which recognize several Nop proteins (2, 15). A protein estimated to be the size of NopP-Cya was detected only in culture supernatants isolated from wild-type USDA257 containing pLMS151 grown in the presence of apigenin (Fig. 2A). Similar results were obtained when the blot was probed with antibodies against Cya (Fig. 2A). The ~60-kDa protein present in whole-cell extracts from bacteria containing pLMS151 (Fig. 1A) was not detected by anti-Cya antibodies in extracellular protein samples (Fig. 2A). Together, these results verify that a functional T3SS is required for *S. fredii* USDA257 to secrete the full-length NopP-Cya hybrid protein.

To examine whether the *S. fredii* T3SS translocates NopP-Cya into plants, *V. unguiculata* roots were dipped into a 10^8 CFU/ml suspension of wild-type or *rhcC1* USDA257 containing either pCPP46 or pLMS151 (17). *V. unguiculata* was selected for this experiment because it can be nodulated by *S. fredii* USDA257 mutants that lack a functional T3SS (16). Thus, T3SS-dependent NopP-Cya translocation could be examined at several stages during symbiosis. In preliminary experiments, roots were collected 7 h, 24 h, and 57 h postinfection, and cAMP and protein levels were determined. At these time points prior to nodule formation, significant differences in cAMP levels were not detected between any of the samples (data not shown). In subsequent experiments, cAMP accumulation was measured in nodules excised 12 days postinfection. The nodules were frozen in liquid nitrogen, ground to a powder, and suspended in 0.1 M hydrochloric acid. cAMP and protein levels were then determined as previously described (30). Root nodules from plants infected with USDA257 expressing NopP-Cya accumulated 99 pmol cAMP/μg protein, whereas the *rhcC1* mutant containing NopP-Cya accumulated only 1 pmol cAMP/μg protein (Fig. 2B). These results suggest that the *S. fredii* T3SS translocates NopP-Cya into root nodules. Because NopP-Cya delivery into nodules required an active T3SS, cAMP accumulation was not merely due to leakage of the hybrid protein from lysed bacteria into root cells.

It is important to note that NopP-Cya secretion into the symbiosome lumen, rather than translocation across the plant cell membrane, could lead to cAMP accumulation in nodules. However, ATP is required for NopP-Cya to produce cAMP, and there is no indication that ATP is present in symbiosomes. Although calmodulin-like proteins are recruited to the symbiosome lumen in *Medicago truncatula*, it is not known whether these proteins are also present in *V. unguiculata* symbiosomes or whether they are capable of activating Cya activity (18).

Since increases in cAMP were not detectable in roots during the early time points following infection with *S. fredii* expressing NopP-Cya, NopP may not facilitate early events in symbiosis, such as root hair curling or formation of infection threads. Alternatively, NopP-Cya may be delivered into root cells early during infection but at levels below the range of detection of the cAMP quantitation assay. It is also possible that cAMP accumulation is inhibited in roots during the early stages of *S. fredii* nodulation. Determination of the timing of NopP delivery during infection will require the use of sensitive fluorescent reporters that can be viewed by microscopy.

During the preparation of the manuscript, a report was published demonstrating delivery by *Bradyrhizobium japonicum* of NopE1-Cya and NopE2-Cya into *Macroptilium atropurpureum* root nodules (36). The levels of cAMP induced by the NopE-Cya fusion proteins in *M. atropurpureum* root nodules were significantly lower than the levels induced by NopP-Cya in *V. unguiculata* nodules. The accumulation of higher levels of cAMP in our experiment may be due to differences in the rhizobial or plant species or higher expression or translocation of NopP-Cya. Nevertheless, our results and those of Wenzel et al. together provide strong evidence that Nops are deployed by the T3SS during rhizobial nodulation of plant roots (36).

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