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A *nopA* Deletion Mutant of *Sinorhizobium fredii* USDA257, a Soybean Symbiont, is Impaired in Nodulation

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Abstract *Sinorhizobium fredii* USDA257 employs type III secretion system (T3SS) to deliver effector proteins into the host cells through pili. The *nopA* protein is the major component of USDA257 pili. The promoter region of USDA257 *nopA* possesses a well conserved *tts* box. Serial deletion analysis revealed that the *tts* box is absolutely essential for flavonoid induction of *nopA*. Deletion of *nopA* drastically lowered the number of nodules formed by USDA257 on cowpea and soybean cultivar Peking. In contrast to the parental strain, the USDA257 *nopA* mutant was able to form few nodules on soybean cultivars McCall and Williams 82. Light and transmission electron microscopy examination of these nodules revealed numerous starch grains both in the infected and uninfected cells.

Introduction

Sinorhizobium fredii, primarily known as a soybean symbiont, nodulates a diverse group of legumes [24]. Of particular interest is the strain USDA257 that exhibits remarkable cultivar specificity [3]. USDA257 elicits nodules on primitive soybean cultivars such as "Peking" but is unable to induce nodules on agronomically improved "McCall" cultivar [12]. Molecular studies have enabled the identification, isolation, and characterization of soybean cultivar-specificity locus located on the symbiosis plasmid of USDA257 [12, 13, 21]. Additional studies [15, 16, 20] have confirmed that type III protein secretion system (T3SS) is a part of the soybean cultivar-specificity locus.

T3SS is a specialized protein secretion system present among many Gram-negative bacteria [9, 11]. The T3SS is used by pathogenic bacteria to deliver effector proteins into eukaryotic host cells [11]. Effector proteins, which act as the virulence factors, are responsible for suppressing activation of genes responsible for the host defense. The T3SS apparatus is highly conserved among bacteria and is composed of about 20 proteins that span the inner and outer bacterial membranes [11]. The effector proteins are delivered to the host cells by means of T3SS-dependent extracellular appendages (pili/needle complexes) that serve as conduits [9, 11]. Unlike the components of the T3SS apparatus, which are highly conserved among diverse bacteria, the effectors are diverse.

Even though the T3SS was initially reported to be exclusive to pathogenic bacteria, it is now well documented that some symbiotic rhizobia also employ T3SS to influence nodulation of legume plants in a host-dependent manner [27]. The presence of functional T3SS have been reported in *S. fredii* USDA257 [16], *S. fredii* HH103 [7], *Rhizobium* strain NGR234 [27], *Bradyrhizobium japonicum* USDA110 [14], *Bradyrhizobium elkanii* [22], and *Mesorhizobium loti* MAFF303999 [23]. In addition, the presence of a T3SS cluster of unknown function was discovered in the genome sequence of a β-rhizobium *Cupriavidus taiwanessis* LMG 19424, a symbiont of *Mimosa* spp. [2]. In α-rhizobia, genes encoding the type III secretion apparatus are clustered within a 30–40 kb region and are under the control of the transcriptional regulator TtsI [14]. Proteins secreted by rhizobia via T3SS are referred to as nodulation outer proteins (Nops). USDA257 when grown in presence of *nod* gene-inducing flavonoids secretes several proteins into the extracellular medium

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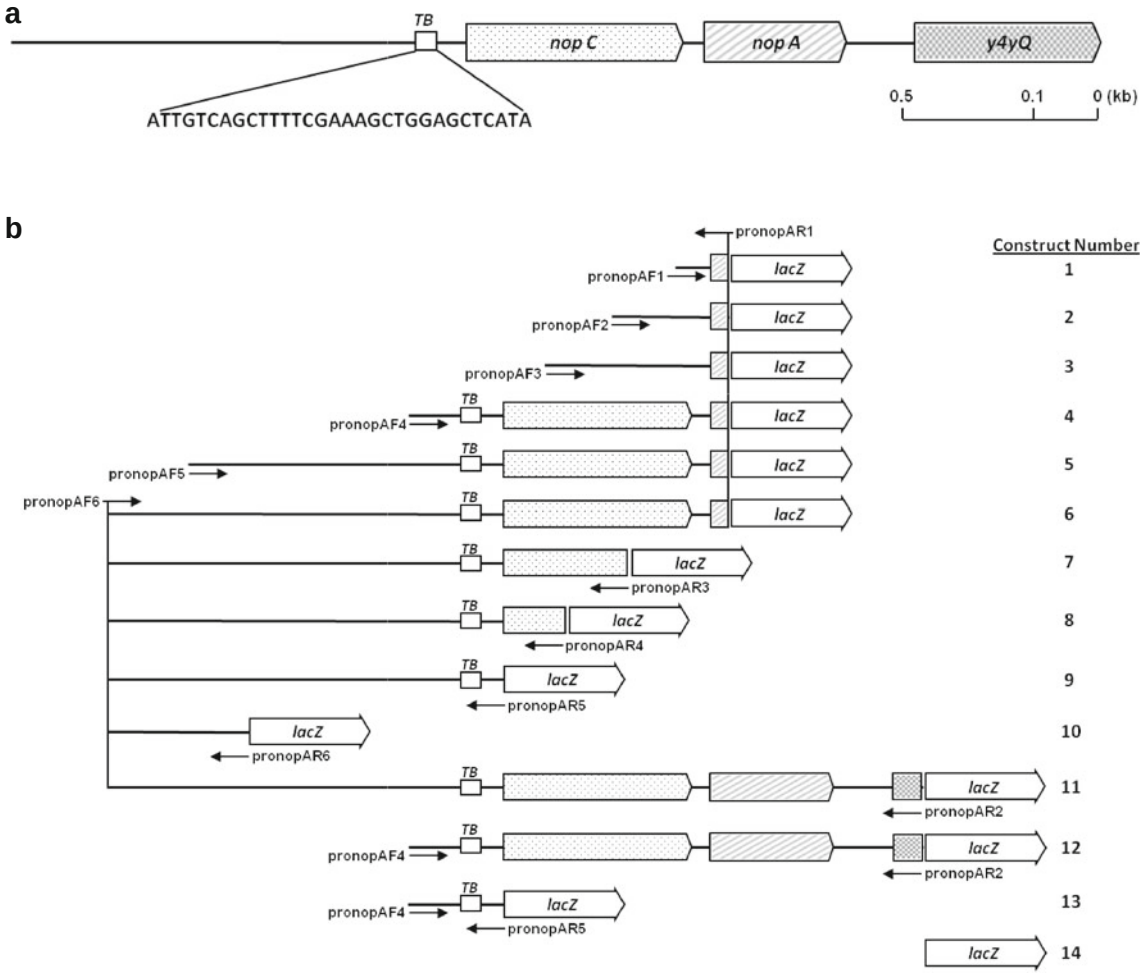
a
TB
nop C
nop A
y4yQ
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0.5
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0 (kb)
b
Construct Number
pronopAR1
pronopAF1
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lacZ
TB
pronopAR3
pronopAR4
pronopAR5
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12
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c
β - galactosidase activity
AP-
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2500
2000
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0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Construct Number

◀ **Fig. 1** **a** Genetic map of *nopA* and flanking DNA region encoding part of type 3 secretion system locus of *Sinorhizobium fredii* USDA257. *Arrow-shaped boxes* indicate the location and orientation of *nopC*, *nopA*, and y4yQ. The small rectangular box in front of *nopC* represents the *tts* box (TB). The nucleotide sequence of the *tts* box is also shown. **b** Schematic representation of plasmid constructs of transcriptional fusions used for monitoring promoter activity. Serially deleted DNA fragments were amplified by PCR using specific primers designed with restriction sites for facilitating directional cloning. PCR amplified DNA fragments were individually cloned into a promoter probe vector (pMP220) and mobilized into *S. fredii* USDA257 by triparental mating. **c** Transcriptional activity measurement. A total of 14 constructs were mobilized into USDA257 and were grown either in the presence or absence of 1 μM apigenin. The promoter activity was monitored by measuring β-galactosidase activity and expressed in Miller units. Data represents the means (±standard errors) of three independent experiments

[15]. Several of the USDA257 Nops that are secreted by T3SS (*nopA*, *nopB*, *nopC*, *nopL*, *nopP*, and *nopX*) have also been identified in other rhizobia possessing T3SS [5]. Putative regulatory motifs termed *tts*-boxes are located upstream of *nopB*, *nopC*, *nopL*, *nopP*, and *nopX* [11]. Nops can either have a positive or negative effect on the ability of rhizobia to nodulate legumes in host-specific manner [5].

Transmission electron microscopy observation has shown that when USDA257 was grown in the presence of *nod* gene-inducing flavonoids it produces thin filaments termed pili [17]. Peptide mass fingerprinting of partially purified USDA257 pili revealed these surface appendages were made up of several Nops including *NopA*, *NopB*, and *NopX* [18]. *nopA*, a 7 kDa protein, was shown to be the major component of the pili [6, 17]. In this study, we have investigated the role of *nopA* in regulating soybean cultivar-specific nodulation. Our results reveal deletion of *nopA* alters its ability to nodulate soybean in a cultivar-specific manner.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Escherichia coli was cultured in Luria–Bertani broth at 37° C, while rhizobia were grown in yeast extract mannitol (YEM) medium at 30° C on a reciprocal shaker. When appropriate, antibiotics were added at the following concentrations in μg/mL: kanamycin, 50; tetracycline, 10; spectinomycin, 50; ampicillin, 100; gentamycin, 10; and trimethoprim, 10.

Construction of *nopA* Mutant

A 1.69 and 1.18 kb DNA region immediately upstream and downstream of *nopA* start and stop codon, respectively, were amplified from the genomic DNA of USDA257 using appropriate primers by polymerase chain reaction. The amplified DNA fragments were sequenced to verify PCR fidelity. These two fragments were combined together utilizing an introduced *Eco*RI site. The resulting 2.87 kb DNA fragment without the *nopA* coding region was cloned into the *Cla*I and *Bam*HI site of pBluescript KS to give rise to pBSΔ*nopA*. The neomycin phosphotransferase II (*nptII)* without its *rho*-independent transcriptional terminator [1], was then inserted into the *Eco*RI site producing pBSΔ*nopA*:*nptII*. Subsequently, the Δ*nopA*:*nptII* replacement fragment was subcloned into pJQ200 [25] and mobilized into USDA257 by triparental mating with helper plasmid pRK2013. Double recombination events were selected by plating the matting mixture on YEM medium containing 5 % sucrose supplemented with appropriate antibiotics. *nopA* deletion was confirmed by Southern blot hybridization.

Nodulation Assays

Seeds of *Glycine max* (L.) Merr. cultivars Williams 82, McCall and Peking and seeds of *Vigna unguiculata* (L.) Walp. were surface sterilized and germinated on water agar plates. Nodulation assay and microscopy was performed as described earlier [12]. Nodulation assay was repeated three times with four plants per treatment in each experiment.

Light and Transmission Electron Microscopy

Soybean nodules harvested at 20 days after inoculation were embedded in paraffin. The embedded nodules were sectioned (10 μm thick) with a microtome and stained either with hematoxylin and eosin or stained for starch using potassium iodide. For ultrastructural analysis, soybean nodules were first cut into 2–4 mm pieces with a razor blade and transferred into glass vials containing 2.5 % glutaraldehyde in 50 mM sodium phosphate, pH 7.2. The

Table 1 Nodulation properties of *S. fredii USDA257* and the *nopA* mutant

Rhizobium	Host			
	Williams 82 Number of nodules	Peking Number of nodules	McCall Number of nodules	Cowpea Number of nodules
USDA257	0	21.33 ± 6.68	0	39.4 ± 17.04
USDA257 *nopA* mutant	1.66 ± 0.94	9.50 ± 6.24	2.50 ± 0.71	7.67 ± 3.21

tissue was fixed for 4 h at room temperature. Following this, the samples were repeatedly washed with 50 mM sodium phosphate buffer pH 7.2, post-fixed with 2 % aqueous osmium tetroxide and infiltrated with Spurr's resin. Thin sections were stained with 0.5 % aqueous uranyl acetate and 0.4 % aqueous lead citrate and viewed with a JEM 100B (JEOL Ltd., Tokyo, Japan) electron microscope with an accelerating voltage of 100 kV.

Results

tts Box is Essential for Flavonoid Induction of *nopA*

The complete genome sequence of USDA257 was recently reported [26]. An analysis of the DNA sequence encoding the T3SS of USDA257 reveals that *nopA* is a part of an operon that includes *nopC*-*nopA*-y4yQ-*rhcV*-y4yS (Fig. 1). The coding region of *nopC* is composed of 297 bp and encodes a 99 amino acid protein with a theoretical molecular weight of 9.8 kDa. The stop codon of *nopC* and the start codon of *nopA* are separated by 70 bp. The coding region of *nopA* is composed of 216 bp encoding a 6.9 kDa protein. BLAST search analysis showed that both *nopC* and *nopA* are identical to similar proteins from NGR234 and HH103. The organization of this operon is very similar in all three *Sinorhizobium* strains. As in NGR234 and HH103, the promoter region in front of *nopC* contains a well conserved *tts* box (Fig. 1a). The *tts* box is located 43 bp upstream of the start codon of *nopC*. To examine the role of the *tts* box and other DNA sequences in the flavonoid-dependent induction of *nopA*, we created a transcriptional fusion with *lac*Z by cloning a 1,455 bp DNA region in pMP220 (construct 11), a promoter probe vector (Fig. 1b). β-Galactosidase activity measured from USDA257 carrying this construct increased 7.5-fold in the presence of apigenin (Fig. 1c). We sequentially deleted sequences either from the 3′ or the 5′ region of the cloned DNA fragment and mobilized these constructs into USDA257 (constructs 1–13). β-Galactosidase activity measurement from these transconjugants clearly established that the *tts* box is absolutely necessary for the flavonoid induction of *nopA*. Our results are consistent with the earlier observations reported for *S. fredii* NGR234 and HH103 [5–7]. Interestingly, deletion of DNA sequences encoding *nopC* region resulted in greater basal level of β-galactosidase activity (constructs 7 and 8) even in the absence of flavonoid induction (Fig. 1c).

Symbiotic Phenotype of USDA257 *nopA* Mutant

Previous studies have shown that Nops play an important role in regulating nodulation in a host-specific manner [7, 14, 16, 22, 23, 27]. To ascertain the symbiotic ability of USDA257 and the *nopA* mutant we inoculated them on cowpea and soybean cultivars Peking, Williams 82, and McCall. As expected, USDA257 did not nodulate Williams 82 and McCall while the USDA257 *nopA* mutant was able to initiate a limited number of nodules on these soybean cultivars (Table 1). Even though the USDA257 *nopA* mutant was able to form nodules on McCall and Williams 82, the number of nodules produced were significantly lower than that of USDA191 (29.7 ± 10.3 nodules/plant), a *S. fredii* strain which does not discriminate between primitive and agronomically improved soybean cultivars. Earlier studies have established *S. fredii* USDA191 forms nitrogen-fixing nodules on roots of these legumes [3, 17, 21] and was used in this study for the purpose of relative comparison of nodulation. Interestingly, USDA257 *nopA* mutant produced a drastically lower number of nodules on Peking and Cowpea when compared to the parental strain, indicating that the absence of Nops have a negative effect on these legume hosts.

Nodules Initiated by USDA257 *nopA* Mutant Accumulate Starch Grains

To investigate if there were any anatomical changes in the nodules initiated by USDA257*nopA* mutant we examined thick sections of paraffin-embedded nodules under a light microscope. Since the USDA257 does not form nodules on agronomically improved North American soybean cultivars we compared the anatomy of nodules induced by USDA257 *nopA* mutant to that of nodules produced by USDA191, which forms nitrogen-fixing nodules. Nodules induced by USDA191 and USDA257 *nopA* mutant revealed a central infected zone which was surrounded by an outer cortex (Fig. 2). The cortex region contained several vascular bundles. The outer cortex was enclosed by a sclerenchymatous layer. Unlike the USDA191 nodules, the central zone in nodules initiated by USDA257 *nopA* mutant contained several uninfected cells and the presence of numerous starch grains (Fig. 2). The accumulation of starch grains was visualized by iodine stain which clearly showed the preponderance of starch accumulation in the cell layers immediately surrounding the infected central zone (Fig. 2). In contrast to nodules initiated by USDA191, USDA257 *nopA* mutant initiated nodules contained numerous starch grains distributed throughout the infected central zone (Fig. 2). This is clearly seen by the difference in the staining intensity of cells in the infected zones of USDA191 and USDA257 *nopA* mutant initiated nodules. Light microscopy observation of semi-thin sections of nodules initiated by USDA257 *nopA* mutant on the roots of soybean cultivar Williams 82 revealed structural features typical of a

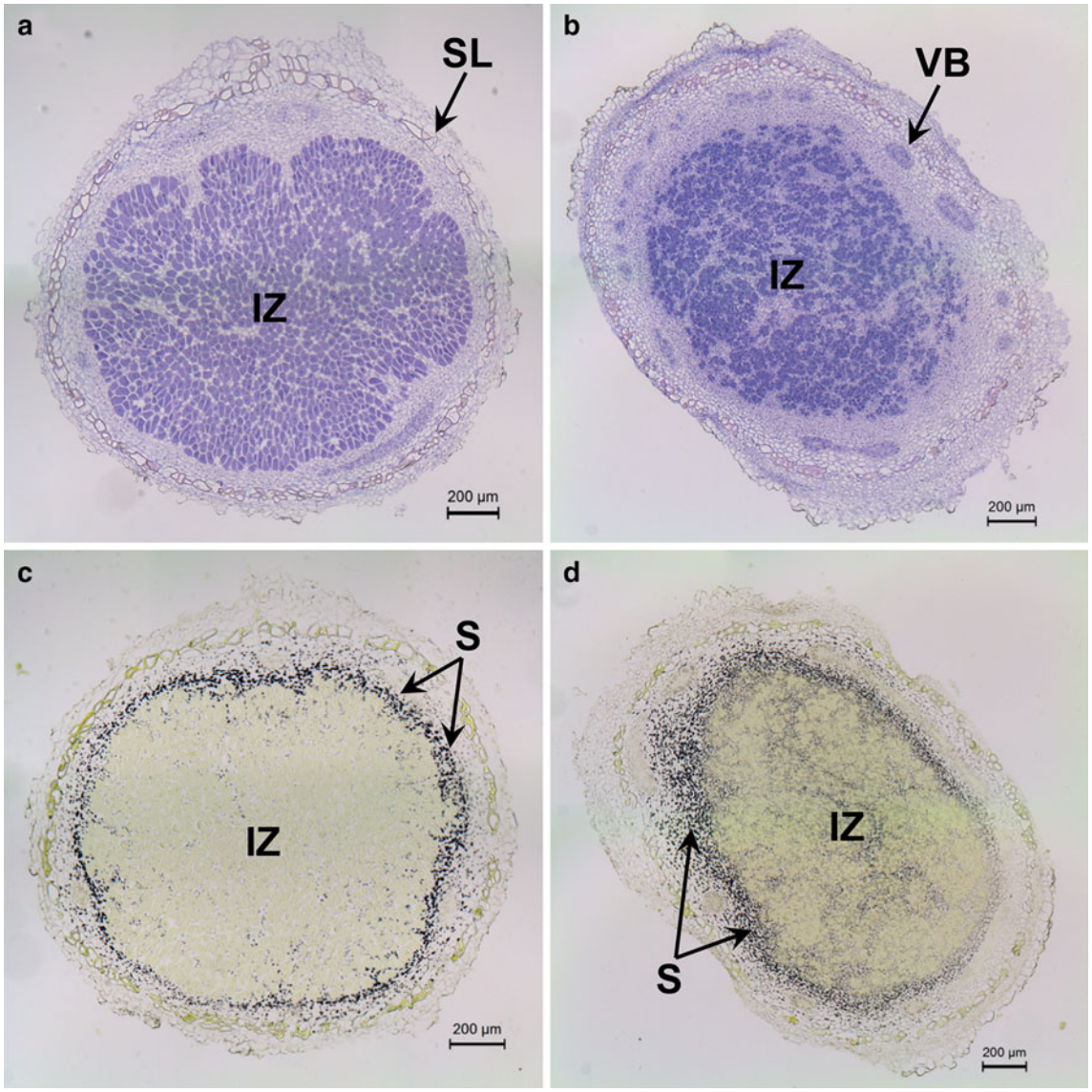

Fig. 2 Detection of starch in *Glycine max* cv. Williams 82 nodules. Photomicrographs of cross section of paraffin-embedded nodules at 20 days after inoculation stained with hematoxylin (**a**, **b**) or iodine (**c**, **d**). **a**, **c** nodules formed by USDA191; **b**, **d** nodules formed by 257Δ*nopA*. *IZ* infected zone; *S* starch grain; *SL* sclerenchyma layer; *VB* vascular bundle

determinate nodules. The infected zone of USDA191 initiated nodules were completely filled with bacteria (Fig. 3a). In contrast the infected zone in nodules formed by USDA257 *nopA* mutant contained cells that were not completely filled with bacteria (Fig. 3b). Ultrastructural analysis of the USDA191 infected cells revealed the presence of numerous bacteroids that were enclosed in peribacteroid membranes (Fig. 3c). However, USDA257 *nopA* mutant infected cells contained prominent vacuoles along with bacteroids. Interestingly, these cells also contained prominent starch inclusions near the cell walls (Fig. 3d). In contrast, no starch accumulation was found in infected cells of nodules initiated by USDA191 (Fig. 3c).

Discussion

The results of our study demonstrate an important role for USDA257 *nopA* in regulating soybean cultivar specificity. An interesting feature of soybean nodules initiated by USDA *nopA* mutant on agronomically improved soybean cultivars is the accumulation of starch in both infected and uninfected nodule cells. Previous studies have shown a correlation between high starch accumulation and reduced nitrogen fixation. Rhizobia that are defective in the transport of dicarboxylic acids [8], or amino acid import/export [18], or glutathione synthesis [10], all form nodules with high starch accumulation. A role for Nops in plant defense

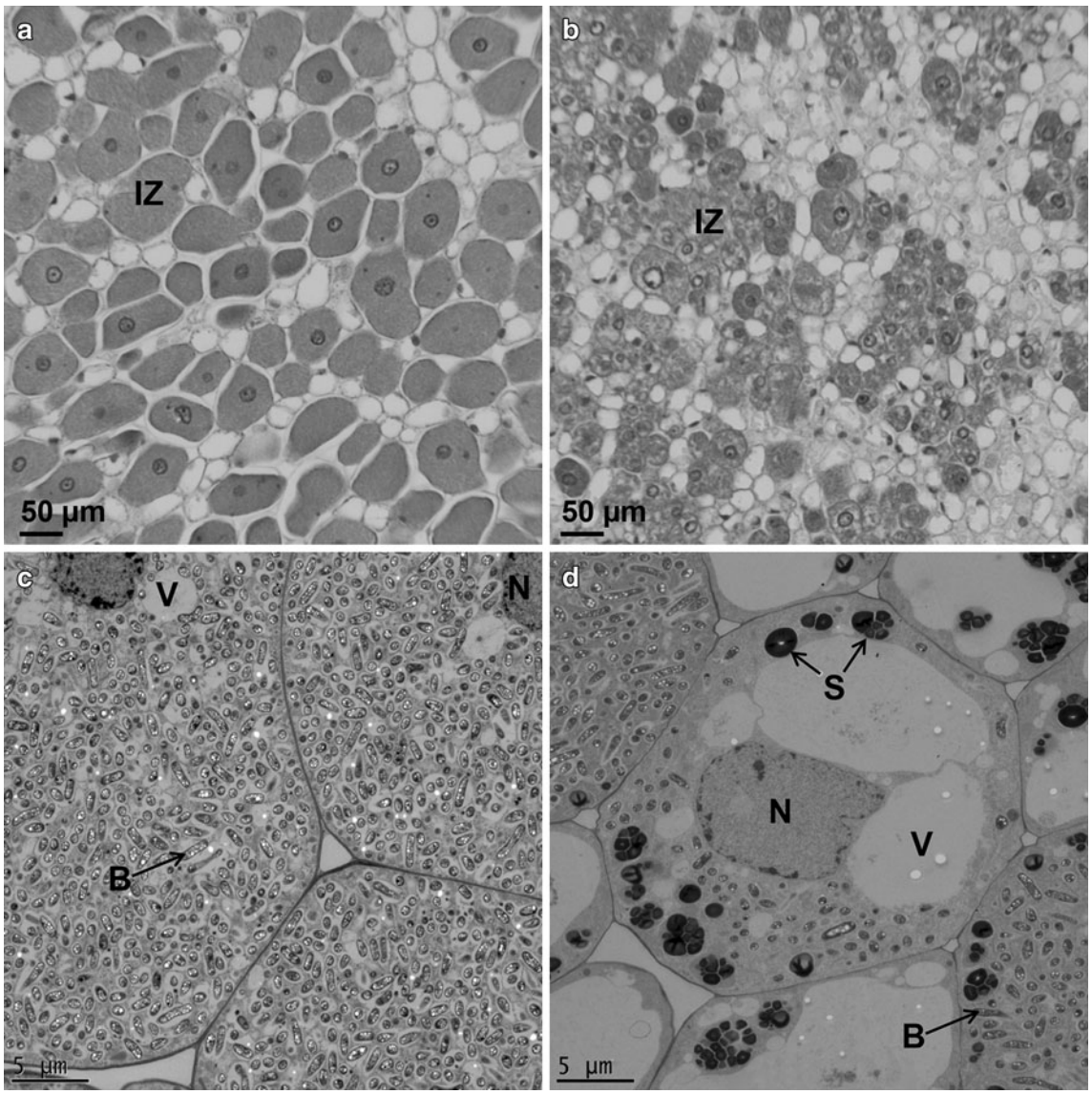

Fig. 3 Light and electron microscopic analysis of *Glycine max* cv. Williams 82 nodules. Nodules at 20 days after inoculation were embedded in Spur's resin. Thick sections of USDA191 initiated nodules (**a**) and 257Δ*nopA* initiated nodules stained with toluidine blue (**b**). Thin sections of USDA191 initiated nodules are packed with bacteroids (**c**) while 257Δ*nopA* initiated nodules contain prominent vacuoles and starch grains (**d**). *B* bacteroids; *IZ* infected zone; *N* nucleus; *S* starch grain; *V* vacuole

has also been proposed [4, 19]. It was reported that the presence or absence of HH103 Nops could alter the expression of pathogenesis-related (PR) genes GmPR1, GmPR2, and GmPR3 in soybean roots and shoots. Inactivation of *nopP* decreased PR1 expression while the presence of a complete set of Nops promoted the expression of PR genes [19]. This observation suggest that the elicitation of soybean defense response can either be up or down regulated presumably in a host-dependent manner. Thus, inoculation of USDA257 *nopA* mutant on the roots of soybean cultivar McCall and Williams 82 could lead to the elicitation of defense response thereby restricting the number of nodules formed on these two soybean cultivars.

The role of type III-secreted proteins in regulating nodulation either positively or negatively at both the genus and species level has been well documented [14, 16, 22, 23, 27]. Broughton and his associates have enumerated numerous examples of the positive and negative effect of type III-secreted proteins on nodulation of diverse legumes utilizing T3SS-negative mutants of rhizobia [5]. Inactivation of T3SS of *M. loti* MAFF303099 has been shown to affect nodulation either positively or negatively at a species level within the genus *Lotus* [23]. For example, T3SS mutant strains positively impacted the nodulation of *L. corniculatus* subsp. *frondosus* and *L. filicaulis* or negatively on *L. halophilus* [23]. An effector protein Mir6361, which contains a

shikimate kinase-like domain at the carboxyl terminus, was shown to be responsible for nodulation reduction on *L. halophilus*. Our results demonstrate that type III-secreted proteins can regulate nodulation not only at the species level but also at cultivar level. Nops produced by soybean cultivar specific strains like USDA257 and non-specific strains such as USDA191 and HH103 elaborate, in addition to common proteins, some unique proteins [7, 17]. However, the identity of the effector proteins which are responsible for the nodulation restriction on North American soybean cultivars remains yet to be determined.

Earlier we have characterized several USDA257 T3SS mutants and reported that these mutants have acquired the ability to nodulate and fix nitrogen with agronomically improved North American soybean cultivars unlike the parental strain [16, 20, 21]. Though USDA257 *nopA* mutant can initiate nodules on agronomically improved North American soybean cultivars the number of nodules per plant (2.5 ± 0.71) is drastically lower than that of USDA191 (29.7 ± 10.3). We have previously shown that USDA191 also produces several Nops including those that are elaborated by USDA257 [17]. Thus, the ability of these two strains to nodulate different legumes may be controlled by the presence or absence or abundance of Nops. Depending on the legume host the presence of Nops can either elicit or prevent plant defense against the invading rhizobia [4, 19]. Since USDA257 *nopA* mutant is unable to secrete any of these Nops, the host plant may perceive the invading rhizobia as a pathogen and mount a defense response. This possibility is strengthened by our anatomical observation of USDA257 *nopA* mutant induced nodules, which show signs of defense reaction.

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