Expression of the *Bradyrhizobium japonicum* Type III Secretion System in Legume Nodules and Analysis of the Associated *tts* box Promoter

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In *Bradyrhizobium japonicum*, as in some other rhizobia, symbiotic efficiency is influenced by a type III secretion system (T3SS). Most genes encoding the transport machinery and secreted proteins are preceded by a conserved 30-bp motif, the type-three secretion (*tts*) box. In this study, we found that regions downstream of 34 *tts* boxes are transcribed. For *nopB*, *nopL*, and *gunA2*, the transcriptional start sites were found to be 12, 11, and 10 bp downstream of their *tts* boxes, respectively. The deletion of this motif or modification of two or more conserved residues strongly reduced expression of *nopB*. This indicates that the *tts* box is an essential promoter element. Data obtained with *lacZ* reporter gene fusions of five genes preceded by a *tts* box (*gunA2*, *nopB*, *rhcV*, *nopL*, and *blr1806*) revealed that they are expressed in 4-week-old nodules of *Macroptilium atropurpureum*. These data suggest that the T3SS is active in mature nitrogen-fixing nodules. The two-component response regulator TtsI is required for the expression of *rhcV*, *nopL*, and *blr1806* in bacteroids. Staining of inoculated roots showed that *nopB* is also expressed in early infection stages.

The symbiotic interaction between rhizobia and legumes is initiated by a highly specific recognition of the two partners (Broughton et al. 2000). Plant flavonoids induce the expression of *nod* genes in rhizobia. The Nod proteins are responsible for the biosynthesis of lipo-chito-oligosaccharides, the Nod factors. These compounds trigger the formation of root nodules by the host plants (Long 2001; Spaink 2000). Flavonoids also induce the expression of a type III secretion system (T3SS), which is present in several rhizobia (Marie et al. 2001). Depending on the host plant, the T3SS can be detrimental or beneficial for symbiosis or may have no obvious influence (Marie et al. 2001; Viprey et al. 1998). In *Bradyrhizobium japonicum*, a type III secretion mutant forms fewer nodules on *Macroptilium atropurpureum* than the wild type and has a delayed nodulation phenotype on soybean. The mutation does not strongly influence the interaction with cowpea (Krause et al. 2002). Genes that probably encode core components of the secretion apparatus (*rhizobia*-conserved proteins [Rhc]) are clustered. This type-three secretion (*tts*) gene cluster also encodes secreted proteins (e.g., nodulation outer proteins NopB and NopL). *nopB* is conserved in all rhizobia encoding a T3SS. It is the first gene of a highly conserved operon containing several *rhc*

genes. NopB was found to be secreted by *Sinorhizobium fredii* USDA 257 and *Rhizobium* sp. strain NGR234, and it is associated with extracellular structures of the secretion apparatus (Lorio et al. 2004; Saad et al. 2005). *nopL* is present in NGR234, *S. fredii*, and *B. japonicum* (Göttfert et al. 2001; Krishnan et al. 2003; Viprey et al. 1998). NopL probably acts as an effector protein in plants (Bartsev et al. 2003; Marie et al. 2003). In *B. japonicum*, type III-secreted proteins are also encoded outside of the *tts* gene cluster. An example is GunA2 (Süß et al. 2006), which exhibits cellulase activity in vitro (Caldelari Baumberger et al. 2003).

One of the conserved genes in the *tts* cluster is *ttsI*. The deduced protein has high similarity to regulators belonging to the two-component regulatory systems. It is essential for induction of other genes in this cluster and for type III secretion (Krause et al. 2002; Marie et al. 2004; Süß et al. 2006). Upstream of TtsI-regulated genes, a conserved *tts* box motif was identified (Krause et al. 2002; Marie et al. 2004). We were interested in the role of the *tts* box for expression of the downstream genes. Here, we give experimental evidence that the *tts* box is an essential promoter element. Our data also suggest that the T3SS is expressed at early stages of plant infection and in mature nodules.

RESULTS

Distribution and transcription of *tts* box regions in the *B. japonicum* genome.

A genome survey revealed 39 conserved *tts* box motifs in *B. japonicum* (Süß et al. 2006) (Fig. 1). The *tts* box-like sequences were found upstream of i) *rhc* and *nop* genes, ii) genes that encode proteins with similarity to virulence factors, iii) a *flgB*-like gene, and iv) genes encoding proteins of hypothetical or unknown functions. Seven *tts* box motifs were found in regions without annotated genes in close vicinity. Most *tts* boxes ($n = 31$) are located in the symbiotic gene region of the *B. japonicum* genome. To examine the expression of downstream regions, reverse-transcription polymerase chain reaction (PCR) was applied. RNA was isolated from the wild-type strain grown in arabinose gluconate salts (AG) medium supplemented with genistein (discussed below). Primers were selected to amplify fragments located 20 to 250 bp downstream of the predicted *tts* boxes. For three regions (upstream of *blr1798*, *blr1848*, and *blr8244*), PCR with two different primer pairs failed to amplify fragments from cDNA and also from chromosomal DNA. In 34 cases, PCR fragments were obtained with cDNA templates, indicating transcriptional activity. Two genomic regions downstream of *tts* box-like motifs (9101960f and 1798864r) do not seem to be transcribed.

The *tts* box is an essential promoter element of *tts* genes.

To find out whether the *tts* box was located within the promoter region, the transcriptional start sites of *nopB*, *nopL*, and *gunA2* were determined by 5' rapid amplification of cDNA ends (RACE). This revealed that transcription starts 10 (*gunA2*), 11 (*nopL*), or 12 (*nopB*) nucleotides downstream of the assigned

tts box motifs (Fig. 2). To determine whether all three highly conserved regions (Fig. 1) are required for promoter activity, the corresponding regions upstream of a *nopB-lacZ* fusion were modified (Table 1). The promoter region of *nopB* was selected, because *nopB* is the first gene of an operon encoding several conserved components of the T3SS. The variants as

Downstream gene	-35	-24	-11	Characteristics of encoded proteins
blr1649	TTGTC GTCAGC TTTT CGACAG GTGCTGCAGGT AGAGTC			secreted protein
bsl1652	TCTTAG GTCAGC TTTT GCAAG CTGCTCCTGCT AGCATG			hypothetical
gunA2	AGCTC GTCAGC TTCTC GAAAG CCAACTTCCT AGCATT			secreted protein
blr1676	CGCTC GTCAGC TCGAC GTAAG CTAAATGTCTT AGAGGC			hypothetical
blr1704	CCTAC GTCAGC TACTC GTCAG CCAGCCAGTCT ATGCAG			unknown
nopP	CTTAG GTCAGC TTGTG GTCAG TTAGGTTGTAT ATTATG			secreted protein
blr1787	ACTGC GTCAGC TTATC GACAG GTGGCGCTCGT ATCATG			hypothetical
bl11798	TTAGC GTCAGC TTGCC GACAG CTAGGCTTACT ATGAGC			similar to NopP
bl11804 ²	GGCTC GTCAGC TTTT GAAAG CTAGCGCCCT AGCATG			unknown
blr1806	TCATC GTCAGC TTTT CGACAG GTGTTCCGGCT ACCGTA			secreted protein
bsl1808	TCTTC GTCAGC TTCTT GCAAG CTGCCCCTGCT AGCACG			unknown
nopL	CGATC GTCAGC TTTT GAAAG CTAAAGCCCC AGCATG			put. secreted
nopB	TCTCC GTCAGC TTTT GTCAG CTCGGCAGCCT ATGAAC			secreted protein ¹
bsr1831	GTCTC GTCAGC TTTCAG TAAAG CCAGCCCGGATA ACAGAG			unknown
bl11840	GCGAT GTCAGC TTTT GAAAG CAAACGTGAGT AGCGAA			unknown
bl11848	GGTCT GTCAGC TGTT CGACAG CTAGGCTTTCT AGCATA			unknown
blr1854	TCTTG GTCAGC TAGTC GTCAG CCAGTCGGCCT ATCCAG			unknown
bl11862a	GCTTT GTCAGC TTTCT GTCAG CTCGTCCAAAT AGTAGC			secreted protein
bl11862b	TCGGC GTCAGC TTAC GACAG CTAAGCTGCTT ATCTGC			secreted protein
blr1869	CCTTT GTCAGC TTCTC GAAAG CCAGCCTCTCT AACT			unknown
bl11877	AGCAC GTCAGC TTGT GACAG CTGATCCTAAT AGAGTG			unknown
blr1904	CATT GTCAGC TTATC GACAG GTAGGCCTGCT AAGGGC			secreted protein ^{1,3}
pgl	GCCTC GTCAGC TTCTC GAAAG CTTTCCCGAATA ACTCAC			secreted protein ¹
blr2052	GCTT GTCAGC TTCTC GAAAG CTAGCTTTTACATA ACG			hypothetical
blr2058	TGGTG GCCAGC TCCTC GTCAG TTTGCCCGGAT ATCGGT			similar to Blr2140
blr2140	TGTCC GTCAGC TCGCCATC AGC TTGTCCGGGT ATCCTC			secreted protein ^{1,4}
bsr3674	CTTT GTCAGC TTT CACGAC ATCTCTTT TCGAGA ACAGA			unknown
blr4695	GGTCT GTCAGC TT CGGTCAG GTAAGCCTT CGAGATCG			put. K ⁺ uptake protein
flgB	CGCCCAA CAGC TTGAC GAAAG CTTAACGCCCT AGGCTT			put. flagellar protein
blr7131	TTGA AGTCAG TTTGT CTGAG TTGTCCCGGGT AGCGCT			hypothetical
bl18201	GCTTT GTCAGC TTTCT GTCAG CTCGTCCAAAT AGTAGC			secreted protein
bl18244	TCGT CATCAG TTCTC GACAG CTTCCCATTT AAGTTT			put. SUMO protease ⁵
1798864r	GTCTC GTCAGC TTT CAGTAAG CTAGTCCCAATA AATTGG			
1806041r	GACTC GTCAGC TTCTC GAAAG GTAGCGCGCT AGCGTG			
2000571r	ATCTC GTCAGC TTCTC GAAAG CTGCGCCTGCAA ACAAT			
2154581f	ATCTC GTCAGC TTCTT GAAAG GTGCGGCTACA ACAAT			
2220599r	GGTTC GTCAGC TTTT GACAG CTATTCCGGTGT ACAATG			
6329127f	GGCTT GTCAGC GTGCT GTAAG CTCAGCCCGCT AGGGTC			
9101960f	TCTTT GTCAGC TTTT GAAAG CTAGCCCGAATA ACTCAG			
consensus	nnntc GTCAGC Ttntc GacAG ctagnccnnnt Annnnn			
	region 1	region 2	region 3	

¹ unpublished results, this lab

² *rhcV* is located downstream in the same operon

³ similar to proteins of the IpaH family of *Shigella flexneri*

⁴ YopT-like protein, put. protease

⁵ XopD-like protein

Fig. 1. Type-three secretion (*tts*) box motifs in the genome of *Bradyrhizobium japonicum*. Sequence analysis was performed as described (Süß et al. 2006). The *tts* boxes without downstream annotated genes are referred to by their position in the genome (accession NC_004463). Highly conserved nucleotide positions (≥90%) are shown in bold letters. In the consensus sequence, lowercase letters are used for nucleotides conserved in at least 50% of the sequences; n means any nucleotide. Conserved regions in the consensus are underlined.

well as the *nopB-lacZ* fusion preceded by the wild-type motif were integrated into the chromosome of *B. japonicum* (Tables 1 and 2) and β -galactosidase activity was measured (Fig. 3). With the wild-type *tts* box, β -galactosidase activity was inducible by genistein. If the *tts* box was deleted (strain BJD322), β -galactosidase activity dropped to background level. Transcriptional activity was also strongly impaired if two conserved nucleotides of region 1, 2, or 3 were modified (strains BJD328, BJD327, and BJD326, respectively) or if three nucleotides of region 1 were deleted (BJD324). Reporter activity was not inducible by genistein in any of the mutants. In strains BJD323 and BJD325, the distances between the conserved regions of the *tts* box were changed. In both strains, the *nopB-lacZ* fusion was still inducible, albeit at a low level. A substitution of just one conserved nucleotide in region 1 (BJD329) reduced reporter

activity only slightly. A similar result was obtained for strain BJD330, in which three modifications were introduced, making the *tts* box almost identical to the consensus sequence.

nopB is expressed in early and late symbiotic stages.

The expression of conserved genes probably involved in formation of the type III secretion apparatus (*rhcV*) and of genes encoding secreted proteins (*gunA2*, *blr1806*, *nopL*, and *nopB*) was analyzed in nodules of *M. atropurpureum* using *lacZ* reporter gene fusions (Table 2). Sections of 4-week-old nodules from *M. atropurpureum* stained with 5-bromo-4-chloro-3-

Table 1. Generated type-three secretion (*tts*) box modifications

Strain	<i>tts</i> Box motif/mutation ^a		
	Region 1	Region 2	Region 3
BJD321	TCCGTCAGGTTTTTCGTCAGCTCGGCAGCCTATGAAC		
BJD322	TCC TGAAC		
BJD323	TCCGTCAGGTTTTTCGTCAGCTCGG . . . CCTATGCAC		
BJD324	TCCGT . . GTTTTCGTCAGCTCGGCAGCCTATGAAC		
BJD325	TCCGTCAGGTT GAGT TCGTCAGCTCGGCAGCCTATG		
BJD326	TCCGTCAGGTTTTTCGTCAGCTCGGCAGC CA TTGAAC		
BJD327	TCCGTCAGGTTTTTCGTC GACT CGGCAGCCTATGAAC		
BJD328	TCC ACC AGGTTTTTCGTCAGCTCGGCAGCCTATGAAC		
BJD329	TCCGT AAG TTTTTCGTCAGCTCGGCAGCCTATGAAC		
BJD330	TCCGTCAG ATT TTTCG AC AGCT AGG CAGCCTATGAAC		
Consensus ^b	ntc GTCAGcT tntc GacAG ctagncnnn ctAnnnnn		

^a Nucleotide substitutions are in bold italics and nucleotide deletions are indicated by dots.

^b Highly conserved nucleotides ($\geq 90\%$) are in bold, lowercase characters are used for conserved nucleotides ($\geq 50\%$), and n = any nucleotide

Fig. 3. β -Galactosidase activity of chromosomally integrated *nopB-lacZ* fusions with wild-type (BJD321) or modified type-three secretion (*tts*) box regions. The enzymatic activity was calculated as pmol of 4-methyl umbelliferone formed per minute and cell density (optical density at 600 nm). Mean values result from measurements of at least four independent cultures. Standard deviations are indicated. Filled bars = β -galactosidase activity 24 h after induction with genistein, Open bars = β -galactosidase activity without induction, and wt = wild type without reporter gene.

tts-box

CGTCAGGTTTTTCGTCAGCTCGGCAGCCTATGAACTTGATCGGTTTCAT... 38 bp to *nopB*

CGTCAGCTTTTCGAAAGCTAAAGCCCCAGCATGAGCCTGCGAGGTC... 198 bp to *nopL*

CGTCAGCTTCTCGAAAGCCAACCTTCCTAGCATTCAAGCCCGGTCTC... 91 bp to *gunA2*

Fig. 2. Transcriptional start sites determined by 5' rapid amplification of cDNA ends. Transcriptional start sites are marked in bold. Conserved regions of the type-three secretion (*tts*) box are underlined. Numbers at the end of the lines indicate distances in base pairs between the transcriptional start site and the assumed translational start site of the corresponding open reading frame.

Table 2. *Bradyrhizobium japonicum* strains used in this study

Strain	Relevant characteristics ^a	Source or reference
110spe4	Referred to as wild type, Sp ^R	Regensburger and Hennecke 1983
1599	<i>gunA2-lacZ</i> integrated into wild type, Sp ^R Tc ^R	Caldelari Baumberger et al. 2003
wt-179	<i>rhcV-lacZ</i> integrated into wild type, Sp ^R Tc ^R	Krause et al. 2002
Δ 139-179	<i>rhcV-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ 139, Km ^R Sp ^R Tc ^R	Krause et al. 2002
wt-182	<i>nopL-lacZ</i> integrated into wild type, Sp ^R Tc ^R	Krause et al. 2002
Δ 139-182	<i>nopL-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ 139, Km ^R Sp ^R Tc ^R	Krause et al. 2002
wt-185	<i>blr1806-lacZ</i> integrated into wild type, Sp ^R Tc ^R	Krause et al. 2002
Δ 139-185	<i>blr1806-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ 139, Km ^R Sp ^R Tc ^R	Krause et al. 2002
BJD321	<i>nopB-lacZ-uidA</i> integrated into wild type, Sp ^R Tc ^R	This work
BJD322	As BJD321, deleted <i>tts</i> box	This work
BJD323	As BJD321, deletion of nonconserved CAG between <i>tts</i> box region 2 and 3; additional substitution of A by C	This work
BJD324	As BJD321, deletion of conserved CAG residue in <i>tts</i> box region 1	This work
BJD325	As BJD321, insertion of a 3-bp spacer between <i>tts</i> box region 1 and 2	This work
BJD326	As BJD321, substitution of TA by AT in <i>tts</i> box region 3	This work
BJD327	As BJD321, substitution of conserved AG by GA in <i>tts</i> box region 2	This work
BJD328	As BJD321, substitution of conserved GT by AC in <i>tts</i> box region 1	This work
BJD329	As BJD321, substitution of conserved C by A in <i>tts</i> box region 1	This work
BJD330	As BJD321, several substitutions to match consensus <i>tts</i> box	This work

^a Sp^R, spectinomycin resistant; Tc^R, tetracycline resistant; Km^R, kanamycin resistant; and *tts* = type-three secretion.

indolyl-3-D-galactoside (X-Gal) are shown in Figure 4A through I. Strong staining was observed for *gunA2*- and *blr1806-lacZ* fusion strains (Fig. 4A and G, respectively). Weaker, but clearly visible staining of the infected nodule tissue was observed for *nopB*-, *rhcV*-, and *nopL-lacZ* fusion strains (Fig. 4B, C, and E, respectively). Nodules inoculated with the wild type did not show staining (Fig. 4I).

It was shown previously that induction and function of the T3SS in liquid culture requires the regulator TtsI (Krause et al. 2002; Süß et al. 2006). To test whether this is also the case for expression of *tts* genes in nodules, reporter gene fusions of *rhcV*, *blr1806*, and *nopL* were integrated in a *ttsI* mutant background (Table 2). Nodule sections of *M. atropurpureum* infected

with corresponding mutants showed no β -galactosidase activity (Fig. 4D and F), with the exception of strain Δ 139-185 (*blr1806-lacZ*), which gave very weak staining after 6 h (Fig. 4H).

To monitor the expression of *nopB* during the infection process, roots of 10-day-old plants of *M. atropurpureum*, inoculated with BJD321, were stained with 5-bromo-4-chloro-3-indolyl-3-D-glucuronide (X-Gluc). Attachment of *B. japonicum* and blue staining due to β -glucuronidase activity was visible in the infection zone, indicating that *nopB* is active early in infection (Fig. 4J).

DISCUSSION

The *tts* box motif was first identified in rhizobial gene regions encoding a T3SS (Krause et al. 2002). Compared with *Mesorhizobium loti* MAFF303099, where we identified 7 *tts* box sequences (data not shown), and NGR234, which harbors 11 *tts* box-like elements on the symbiotic plasmid (Marie et al. 2004), *B. japonicum* contains a considerably higher number ($n = 39$) of this motif. In *B. japonicum*, as in the other strains, the *tts* box is mainly associated with genes encoding the type III secretion machinery or secreted proteins (Süß et al. 2006). However, several of the motifs described here either are upstream of genes without similarity to known genes or no genes have been annotated downstream. Therefore, the possibility exists that these motifs are associated with genes not involved in type III secretion. In NGR234, the *tts* box was also found upstream of genes involved in rhamnan biosynthesis and plasmid stability (Marie et al. 2004).

We found that most regions downstream of *tts* boxes were expressed. This is corroborated by microarray data that indicate weak expression of genes downstream of 32 *tts* boxes (Lang et al. 2008) (GEO accession GSE8580). The transcription start sites of *nopL*, *nopB* and *gunA2* were determined by 5' RACE and found to be located 11, 12, and 10 bp downstream of the *tts* box, respectively. Previously, the transcription start sites of *nopB* and *nopX* (formerly *nolB* and *nolX*) of *S. fredii* were determined (Kovacs et al. 1995). Analysis of the upstream region of these genes revealed the presence of *tts* box motifs at a distance of 10 and 11 bp (Kovacs et al. 1995; Krause et al. 2002). Therefore, the *tts* box seems to be located in the transcriptional control region of these genes and its tripartite structure could represent a new promoter type. This is supported by results obtained here with modified *tts* box motifs upstream of a *nopB-lacZ* fusion. A deletion of the *tts* box reduced β -galactosidase activity to background level (strain BJD322). The modification of two or more nucleotides in any of the conserved three regions also affected reporter activity significantly. In NGR234, an exchange of 3 nucleotides in region 1 (GTCAG to GAATC) results in a loss of function of the *tts* box in vivo (Wassem et al. 2008).

Because none of the modifications led to an increased β -galactosidase activity, we exclude the possibility that the *tts* box is a repressor-binding site. The localization of the *tts* box in close vicinity to the transcription start suggests that the RNA polymerase holoenzyme interacts with at least part of the motif. It is not known if the "housekeeping" sigma factor SigA recognizes the *tts* box. For *B. japonicum*, it was shown that SigA recognizes a -35/-10 motif (TTGACA-N₁₇-TATAAC), which is more similar to the consensus sequence of *Escherichia coli* (Beck et al. 1997). MacLellan and associates (2006) described a similar motif (CTTGAC-N₁₇-CTATAT) for *S. meliloti*. The *tts* box also has no similarity to the -24/-12 promoter motif recognized by RpoN (Fischer 1994). Therefore, the *tts* box might be the recognition site for one of the uncharacterized sigma factors encoded in the *B. japonicum* genome (Kaneko et al. 2002). However, by comparison of the putative sigma factors of *B. japonicum* with

Fig. 4. Expression analysis of type-three secretion (*tts*) genes in nodules and in the root hair zone of *Macroptilium atropurpureum* by light microscopy. Plants were infected with strains containing **A** through **H**, a *lacZ*, **J**, *uidA* reporter gene fusion, or **I**, with the wild type. **A** through **I**, Staining of cryosections (25 μ m thick) of 4-week-old *M. atropurpureum* nodules was done with 5-bromo-4-chloro-3-indolyl-3-D-galactoside. **J**, Roots of *M. atropurpureum* (10 days old) were stained with 5-bromo-4-chloro-3-indolyl-3-D-glucuronide. Strains: **A**, 1599 (*gunA2-lacZ*); **B**, BJD321 (*nopB-lacZ*); **C**, wt-179 (*rhcV-lacZ*); **D**, Δ 139-179 (*rhcV-lacZ* in *ttsI* mutant background); **E**, wt-182 (*nopL-lacZ*); **F**, Δ 139-182 (*nopL-lacZ* in *ttsI* mutant background); **G**, wt-185 (*blr1806-lacZ*); **H**, Δ 139-185 (*blr1806-lacZ* in *ttsI* mutant background); **I**, wild type. Scale bars: 200 μ m. **J**, Root hairs of *M. atropurpureum*, inoculated with BJD321 (*nopB-uidA*).

those of *M. loti* MAFF303099 (Kaneko et al. 2000), which also contains the highly conserved *tts* box motif, no obvious candidate arises. To date, TtsI, which is conserved in all rhizobial strains that contain *tts* box motifs, is the only known regulator likely to interact with the upstream regions of genes preceded by a *tts* box. The gene is essential for the genistein-dependent activation of the T3SS in liquid culture (Krause et al. 2002; Süß et al. 2006) and required for expression in planta, as we showed in this study. Electromobility shift assays using purified TtsI protein confirmed the binding of the regulator to a *tts* box-containing fragment (Wassem et al. 2008).

Although it has been shown that the T3SS of rhizobia affects symbiosis, little is known about its expression in the rhizosphere and during nodule development. Krause and associates (2002) showed the induction of *tts* genes by genistein in liquid cultures. Herein we showed that a transcriptional *nopB-uidA* fusion is expressed early in infection of *Macropodium atropurpureum*. NopX (formerly NolX), a type III-secreted protein of *S. fredii*, was localized by immunocytochemical studies in infection threads of soybean and cowpea but not in mature nodules (Krishnan 2002). In a proteome analysis-based approach, proteins from bacteroids of *B. japonicum* isolated from 4-week-old soybean nodules were analyzed. No proteins known to be involved in type III secretion were reported (Sarma and Emerich 2005). With NGR234, transcripts were detected in mature nodules of different host plants (e.g., for *nopB* and *rhcN*) but not for *rhcV*, *rhcC1*, and most of the other genes belonging to the *tts* cluster (Perret et al. 1999). Recently, Chang and associates (2007) reported a strong repression of *tts* genes in *B. japonicum* bacteroids 4 weeks after inoculation of soybean compared with free-living cells grown in rich medium. They suggest that the T3SS is required only during nodule initiation. A more detailed transcriptome analysis revealed that *tts* genes are maximally expressed in bacteroids 10 and 13 days after inoculation of soybean seedlings, and expression decreases in nodules 21 and 31 days after inoculation (Pessi et al. 2007) (GEO accession GSE8478). We found that genes coding for the T3SS are well expressed in mature nodules of *M. atropurpureum*, especially genes encoding secreted proteins (*gunA2* and *blr1806*). Although the T3SS is expressed in both host plants, it affects nodule number and development with *M. atropurpureum* much more strongly than with soybean (Krause et al. 2002). This indicates that the two host plants might respond differently to effector proteins. For NGR234, it was reported that secreted proteins have a host-specific effect on symbiosis (Skorpił et al. 2005).

We conclude that genes of the T3SS are active in early infection stages and in mature nodules of *M. atropurpureum*. Therefore, it is likely that the secretion of effector proteins influences symbiosis with *M. atropurpureum* at all stages.

MATERIALS AND METHODS

Strains and growth conditions.

B. japonicum strains used in this study are listed in Table 2. For conjugation and plant assays, *B. japonicum* was grown in peptone salts yeast extract medium (Regensburger and Hennecke 1983). Cultures were harvested by centrifugation, the supernatant was removed, and the cell pellet was washed twice in 0.9% NaCl solution. The cells were suspended in 0.9% NaCl and used for plant inoculation or conjugation. For 5' RACE and β -galactosidase assays, a single colony of *B. japonicum* was inoculated in AG medium (Sadowsky et al. 1987) containing spectinomycin (100 μ g/ml) and incubated for 3 days at 28°C. From this preculture, AG medium was inoculated 1:100 and genistein was added to a final concentration of 1 μ M.

Determination of transcriptional start sites by 5' RACE.

B. japonicum cells were harvested 24 h after induction with genistein. Primers used for 5' RACE (Frohman et al. 1988) are listed in Table 3. Total RNA isolation was performed as described (Hauser et al. 2007). cDNA was synthesized at 45°C for 50 min with Superscript II (Invitrogen, Carlsbad, CA, U.S.A.) using gene-specific primers (Table 3). The cDNA was treated with RNase H (20 min, 37°C) and subsequently purified with a microcon YM-30 filter (Millipore, Schwalbach, Germany). The cDNA solution was treated with alkaline phosphatase (10 min at 37°C and 10 min at 65°C) to avoid incorporation of dNTPs in the subsequent tailing reaction. A deoxynucleotide tail was added to the 3' end of the cDNA using terminal transferase (5 U). For *nopB* and *gunA2*, a dA tail was generated; in the case of *nopL*, a dT tail was synthesized. Second-strand synthesis was performed with an oligo (dT) primer for *nopB* and *gunA2* and with an oligo (dA) primer for *nopL*. The obtained double-stranded DNA was amplified with the anchor-1 primer and a second gene-specific primer (Table 3). The resulting PCR product was eluted from an agarose gel and cloned into pCR2.1 by TA cloning (Invitrogen). The transcriptional start site was determined by sequencing.

Reverse-transcription PCR for regions downstream of *tts* boxes.

B. japonicum was inoculated in AG medium from a preculture to an optical density at 600 nm (OD₆₀₀) of 0.4. Genistein was added to a final concentration of 1 μ M and cells were grown for 8 h. Then, 40 ml of genistein-induced cultures were transferred into centrifuge tubes containing 4 ml of stop-solution (10% water-saturated phenol in ethanol, prechilled at -20°C) and mixed by inversion. After centrifugation for 4 min (5,445 $\times$ g, 4°C), the supernatant was discarded and the pellet was frozen in liquid nitrogen. Total RNA was isolated as described (Hauser et al. 2007). RNA was checked by PCR for complete removal of DNA contamination by using primers that amplify a 108-bp fragment of *nolZ*. cDNA synthesis was performed with 10 μ g of DNA-free RNA, 750 ng of random hexamer primers, 30 U of SUPERaseIn (Ambion, Huntingdon, U.K.), and 1,500 U of M-MLV reverse transcriptase (Promega, Mannheim, Germany) in supplied reaction buffer. The reaction mixture was incubated at 37°C for 60 min and at 42°C for a further 60 min. M-MLV reverse transcriptase was inactivated by heat (70°C, 10 min) and RNA was hydrolyzed by alkaline treatment (0.2 M NaOH, 65°C, 30 min). The cDNA solution was adjusted to neutral pH with 1 M HCl, and cDNA was subsequently purified with a MinElute PCR purification kit (Qiagen, Hilden, Germany). PCR amplification was performed using 5 to 10 ng of cDNA and primers that bind 20 to 200 bp downstream of the *tts* boxes (a list of the primers is available on request). The selected product length was 80 to 120 bp. Product formation was confirmed by agarose gel electrophore-

Table 3. Oligonucleotides used for 5' rapid amplification of cDNA ends

Primer	5' $\rightarrow$ 3' Sequence ^a
<i>nopL</i> -SP1 ^b	GTCGAGATAGGGTGAATAAGCCTC
<i>gunA2</i> -SP1 ^b	TCGTTGTTCCAAGAGTAGCCAT
<i>nopB</i> -SP1 ^b	CACCCCATTTACGACAGCC
dA-anchor-1	AACTGTGTAAGCTTAGGTACG(A) ₁₇ B
dT-anchor-1	AACTGTGTAAGCTTAGGTACG(T) ₁₇ V
<i>nopL</i> -SP2 ^c	TAAGCCTCGCCCTGCAGCACTGGATGG
<i>gunA2</i> -SP2 ^c	ATCGCGTGAAAAGCTTCCGTATGGAGCG
<i>nopB</i> -SP2 ^c	GATACTGCAGCGGAGAAACGGCTCGACCCCTG
anchor-1	ACTGTGTAAGCTTAGGTACG

^a Nucleotide abbreviations: B meaning C, G or T; V meaning A, C or G.

^b Gene-specific primer used for first strand cDNA synthesis.

^c Gene-specific primer used for polymerase chain reaction.

sis. If no PCR fragment was obtained, a different pair of primers annealing further downstream was used.

Construction and chromosomal integration of translational *nopB-lacZ* and transcriptional *nopB-uidA* fusions.

For fusions of *nopB* with the reporter genes *lacZ* and *uidA*, plasmid pSUP81-ZA was used. The plasmid was constructed by cloning the promoter-less *uidA* gene of pWM3 (Metcalf and Wanner 1993) and the *lacZ* gene of pNM481 (Minton 1984) in pSUPPOL2SCA (Krause et al. 2002). The sequence of this plasmid has been deposited under accession number EU294195. Approximately 500 bp of the upstream region of *nopB* was amplified by PCR and cloned into *PacI* and *StuI* sites of pSUP81-ZA. By this, the promoter and the translational start codon of *nopB* were fused to *lacZ* and a transcriptional fusion with the downstream located *uidA* was created. The fusion site with *lacZ* was confirmed by DNA sequencing. The resulting plasmid pBJD321 was transferred to *B. japonicum* by biparental conjugation as described previously (Krause et al. 2002). The plasmid integrated into the chromosome by homologous recombination generating strain BJD321. The correct integration event was confirmed by sequencing. For mutagenesis, the *tts* box-containing region upstream of *nopB* was amplified by overlap extension PCR using modified primers. These fragments were cloned into pSUP81-ZA, resulting in plasmids pBJD322 to pBJD330. The plasmids were conjugated and integrated into *B. japonicum*, resulting in strains BJD321 to BJD330 (Tables 1 and 2). The modified and chromosomally inserted fragments were confirmed by sequencing.

Determination of β -galactosidase activity.

Cells were induced with genistein for 24 h and collected by centrifugation ($4,000 \times g$, 4°C). The cell pellet was resuspended in 10 ml of 0.9% NaCl. The cell density was measured at 600 nm. Samples of 4 by 2 ml were centrifuged ($4,000 \times g$, 10 min), the supernatant was removed, and the cells were frozen in liquid nitrogen. β -Galactosidase activity was determined as described previously using 4-methylumbelliferyl- β -D-galactopyranoside as substrate (Krause et al. 2002). Fluorescence of the reaction product 4-methyl umbelliferone (MU) was measured in a 96-well plate in a fluorometer Infinite 200 (Tecan, Crailsheim, Germany) by excitation at 360 nm and by emission at 460 nm. β -Galactosidase activity was calculated as pmol 4-MU formed per minute reaction time and cell density (OD_{600}).

Staining of nodule sections and root hairs.

M. atropurpureum cv. Aztec atro seed were surface sterilized and germinated on 1% water agar at 30°C for 2 to 3 days. Germinated seed were inoculated and grown as described previously (Krause et al. 2002). Root nodules were harvested 28 days after inoculation. Histological staining was done with minor modifications as described (Teeri et al. 1989). Nodules were fixed with 1.25% glutaraldehyde in Z' buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , pH 7) for 2 h at room temperature in vacuum and then rinsed twice with Z' buffer (pH 7.2, 15 min). The fixed nodules were embedded in Leica OCT Cryocompound (Leica Microsystems, Wetzlar, Germany), frozen at -20°C , and sectioned. Sections approximately 25 μm thick were rehydrated with distilled water and stained with the substrate X-Gal by incubation in staining solution (5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, and 0.2% X-Gal in Z' buffer) at 28°C for 2 to 4 h. Sections were analyzed by bright-field microscopy.

Root hairs were stained with X-Gluc 10 days after germination. For this, plant roots were washed and submerged in staining solution (X-Gluc at 200 $\mu\text{g}/\text{ml}$; 0.1 M sodium phosphate

buffer, pH 7; 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$; 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, and 0.3% Triton X-100), subjected to vacuum for 15 min, and incubated overnight at 28°C . Roots were observed by bright-field microscopy.

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