Mutational and Transcriptional Analysis of the Type III Secretion System of *Bradyrhizobium japonicum*

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Sequencing the symbiotic region of *Bradyrhizobium japonicum* revealed a gene cluster (*tts*) encoding a type III secretion system (TTSS) that is similar to those found in *Mesorhizobium loti* MAFF303099 and *Rhizobium* strain NGR234. In addition to genes that are likely to encode structural core components of the TTSS, the cluster contains several open reading frames that are found exclusively in rhizobia or that are specific to *B. japonicum*. Depending on the host, mutations within this cluster affected nodulation capacity to different extents. One of the genes likely encodes a transcriptional activator (*TtsI*) of the two-component regulatory family. Upstream of *ttsI*, a *nod* box promoter was identified. Expression of *ttsI* could be induced by genistein. This induction depended on the transcriptional activator protein NodW as well as the *nodD1nodD2nolA* gene region. *TtsI* was found to be involved in transcriptional regulation of the *tts* gene cluster. Sequence comparison revealed a conserved *tts* box element within putative promoter regions of several genes. Here, we propose a model of the regulatory cascade leading to the induction of the *tts* gene cluster.

Additional keywords: nitrogenase activity, symbiosis.

Type III secretion systems (TTSS), highly conserved multi-protein complexes spanning the entire bacterial cell envelope, play an essential role in the pathogenicity of many gram-negative bacteria infecting humans, animals, and plants (Hueck 1998). In response to multiple environmental and host-derived signals, TTSS transport a wide variety of effector proteins either into the extracellular medium or into the cytoplasm of eukaryotic host cells, where they often provoke disease symptoms (Galán and Collmer 1999; Hueck 1998).

TTSS had previously been thought to be unique to pathogenic bacteria, but recently, these systems have been identified in rhizobia (Marie et al. 2001). TTSS have been found in *Rhizobium* strain NGR234 (Freiberg et al. 1997), in *Mesorhizobium loti* MAFF303099 (Kaneko et al. 2000), and in *Bradyrhizobium japonicum* 110spc4 (Göttfert et al. 2001). Additionally, limited sequence data suggest the presence of TTSS in *Sinorhizobium fredii* USDA257 (Meinhardt et al. 1993), USDA191 (Bellato et al. 1997), and HH103 (Bellato et al. 1997; Marie et al. 2001; GenBank: AY034152). In contrast, *Sinorhizobium meliloti* (Galibert et al. 2001) and the symbiosis island of *M. loti* strain R7A (Sullivan et al. 2002) do not contain such a system. Thus, TTSS are present in some but not in all rhizobia.

In response to host-derived flavonoids, rhizobia secrete proteins into the medium via the TTSS (Krishnan et al. 1995;

Marie et al. 2001; Viprey et al. 1998). Until now, *nopX* and *nopL* from *Rhizobium* strain NGR234 (previously named *nolX* and *y4xL*) are the only identified genes that are known to encode such secreted proteins (Viprey et al. 1998). However, the function of the secreted proteins in the symbiotic interaction is not yet determined. Mutations in the type III gene clusters (*tts*) of *Rhizobium* strain NGR234 and *S. fredii* affect nodule development. Depending on the host, symbiotic capacity of mutant strains is impaired, improved, or unaltered (Bellato et al. 1997; Meinhardt et al. 1993; Viprey et al. 1998).

Rhizobia have the unique capability to induce development of root nodules on legume plants. Within these new plant organs, the bacteria are hosted intracellularly as symbionts, finding ideal environmental conditions to reduce atmospheric nitrogen to ammonia. The development of nodules depends on the presence of plant root-derived flavonoids, which initiate a signal cascade in rhizobia (Long 2001). Perception of these compounds by rhizobia activates the transcriptional activator NodD. NodD binds to the *nod* box, a conserved element in the promoter region of *nod* genes, and triggers the expression of genes located downstream. Additionally, in *B. japonicum* the two-component regulatory system NodVW is required for efficient *nod* gene expression, which also depends on the presence of flavonoids (Göttfert et al. 1990; Loh et al. 1997). Nod proteins, in turn, carry out the synthesis of Nod factors, bacterial signals that provoke the first steps of nodule development (Long 2001).

Here, we report the analysis of the *tts* gene cluster from *B. japonicum* and show that the same transcriptional activators that are involved in *nod* gene regulation are required for expression of genes within this cluster.

RESULTS

The *tts* gene cluster of *B. japonicum* contains conserved, as well as unique, open reading frames (ORFs).

Within a 47-kb region of the *B. japonicum* chromosome, ten ORFs show similarity to genes encoding TTSS in rhizobia and pathogenic bacteria (Fig. 1) (Göttfert et al. 2001). Following the nomenclature suggested by Viprey and associates (1998), these genes are named *rhc* (*Rhizobium* conserved). For the corresponding gene cluster, we propose the designation *tts* (type three [III] secretion).

Apart from a few rearrangements and the presence of several IS-related elements within the *B. japonicum* cluster, the gene order within the *tts* clusters of different rhizobia is relatively well conserved (Fig. 1). Besides *rhc* genes, several of the ORFs are conserved in all rhizobial *tts* clusters but are missing in pathogenic bacteria (e.g., *nolB* and *nolU*). Other ORFs are present only in some rhizobial strains. For example, *nopL* exists in *Rhizobium* strain NGR234 and *B. japonicum*, whereas *nopX* is found in *Rhizobium* strain NGR234 and *M. loti*

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MAFF303099. Additionally, some ORFs, like *id199* and *id274* (tentative designations), seem to be specific for *B. japonicum* (Fig. 1).

One of several putative operons of the *tts* gene cluster is preceded by a *nod* box promoter (Fig. 1). The deduced amino acid sequence of the first conserved ORFs downstream of this *nod* box has similarity to transcriptional activators of the two-component regulatory system. We suggest the designation *ttsI* for this gene.

Nodulation properties and nitrogenase activities of deletion mutants.

To elucidate the role of the TTSS in the interaction of *B. japonicum* with its host plants, deletion mutants were constructed (Fig. 1). In mutant strains $\Delta 139$ and $\Delta 171$, only one gene—*ttsI* or *rhcC1*, respectively—is deleted. The other mutants carry deletions of several genes. The symbiotic properties of the mutant strains were determined in plant infection tests with *Glycine max*, *Vigna unguiculata*, and *Macroptilium atropurpureum* and were compared with the phenotype of the wild-type strain.

With respect to nodule numbers, mutations do not seem to greatly influence the interaction with *V. unguiculata* (Fig. 2). *G. max* developed similar nodule numbers with all strains within 3 weeks but exhibited a delay in nodule development, especially with strains $\Delta 136$ and $\Delta 139$, which lack a functional *ttsI* gene and also, in the case of strain $\Delta 136$, several neighboring ORFs. Nodule numbers were more reduced with *M. atropurpureum*. However, most of the nodules on *M. atropurpureum* developed to an unusually large size. Nitrogenase activity per nodule increased about twofold, with the exception of nodules from plants inoculated with strain $\Delta 139$ (Table 1). With *V. unguiculata* but not with *G. max*, nitrogenase activity per nodule also seems to be slightly increased in combination with some mutant strains.

Expression of the *tts* gene cluster is inducible by genistein.

To study the expression of the *tts* gene cluster, translational fusions of *lacZ* with *id205*, *nolU*, *nopL*, *rhcC1*, *rhcV*, and *ttsI* (Fig. 1) were constructed and were integrated into the chromo-

Fig. 2. Nodulation properties of *Bradyrhizobium japonicum* wild type and deletion derivatives. Nodule numbers (average $\pm$ standard deviation) were determined from at least 20 plants per strain.

Fig. 1. Comparison of the *tts* gene clusters of *Bradyrhizobium japonicum*, *Mesorhizobium loti* MAFF303099, and *Rhizobium* strain NGR234. Open reading frames (ORFs) unique to *B. japonicum* are shown in black. ORFs present in *Rhizobium* strain NGR234 only are marked by stripes. Gray ORFs are conserved in at least two of the three strains. ORFs with similarity to IS-related elements are displayed in white. Positions of *nod* boxes are indicated by gray arrowheads. Black arrowheads depict positions of *tts* boxes. Deletions are marked by horizontal arrows. The direction of the arrows shows the orientation of the inserted *Km^r* cassette, and the corresponding strain designations are given. Rectangular arrows indicate positions of the translational *lacZ* fusions.

some of the wild type as well as mutant strains 613 and $\Delta 1267$. Because *ttsI* is part of the *tts* gene cluster and has similarity to transcriptional activators of the two-component regulatory family, the *id205*-, *nolU*-, *nopL*-, *rhcC1*-, and *rhcV-lacZ* fusion constructs were also integrated into a *ttsI* mutant background (strain $\Delta 139$). Strain 613 is a *nodW* mutant. In mutant $\Delta 1267$ *nodD1*, *nodD2*, and *nolA* are deleted. NodD1 and NodW are both required for efficient transcription of the *nod* box-regulated *nodABC* gene cluster (Göttfert et al. 1990, 1992; Loh et al. 1997, 2001).

Addition of genistein to the culture medium led to an induction of *id205*, *nolU*, *nopL*, *rhcV*, and *ttsI* in the wild-type back-

ground (Fig. 3). None of the genes was induced in the *nodW* and *nodD1nodD2nolA* mutants. Furthermore, expression of *id205*, *nolU*, *nopL*, and *rhcV* is strictly dependent on TtsI. In contrast, the expression level of *rhcC1* was not enhanced by genistein and was independent of the transcriptional activators NodW and TtsI as well as the *nodD1nodD2nolA* gene region.

It was demonstrated previously that NodD1 activates *nod* box-controlled nodulation genes (Banfalvi et al. 1988; Göttfert et al. 1992). In order to verify that NodD1 is also required for flavonoid-dependent induction of *ttsI* and *nolU*, mutant strains containing the corresponding *lacZ* fusions in the *nodD1nodD2nolA* background were complemented with a

Table 1. Acetylene reduction per nodule 28 days after inoculation, shown in percent of wild-type activity^a

Plant/Strain	Wild type	$\Delta 121$	$\Delta 122$	$\Delta 132$	$\Delta 136$	$\Delta 139$	$\Delta 171$
<i>Glycine max</i>	100 $\pm$ 31.0	59.0 $\pm$ 6.9	64.1 $\pm$ 18.8	55.2 $\pm$ 12.3	63.0 $\pm$ 19.9	71.9 $\pm$ 17.7	102.1 $\pm$ 21.8
<i>Macroptilium atropurpureum</i>	100 $\pm$ 35.8	232.7 $\pm$ 96.3	265.7 $\pm$ 95.1	209.6 $\pm$ 50.9	243.0 $\pm$ 51.0	153.8 $\pm$ 59.0	269.1 $\pm$ 88.8
<i>Vigna unguiculata</i>	100 $\pm$ 34.9	106.5 $\pm$ 25.7	106.7 $\pm$ 66.3	70.6 $\pm$ 59.2	76.8 $\pm$ 13.9	59.9 $\pm$ 21.5	145.7 $\pm$ 48.6

^a Values (average $\pm$ standard deviation) are calculated from five plants per strain. The actual ethylene peak areas for the wild-type strain on *G. max*, *M. atropurpureum*, and *V. unguiculata* are 9682.9, 6097.9, and 2725.2, respectively.

Fig. 3. Expression analyses of the *tts* gene cluster. β -Galactosidase activity was determined 24 h after induction for strains carrying the *ttsI*- and *rhcC1-lacZ* fusions and 48 h after induction for the other strains. Activities are given in pmol MU per μ g of protein per min of reaction time. Values (average $\pm$ standard deviation) are determined from at least five independent cultures measured in duplicate.

broad host range plasmid carrying *nodD1* (pRJ420). Figure 4 shows that *nodD1* is sufficient for restoring inducibility.

The putative promoter regions within the *tts* gene cluster contain a conserved element.

To identify potential promoter elements, we aligned putative promoter regions with corresponding sequences of the *tts* gene clusters of NGR234, *M. loti*, and *S. fredii* (Fig. 5). This analysis revealed a conserved sequence motif, for which we suggest the designation *tts* box. In the case of the *S. fredii* *nolB* and *nolX* genes, the conserved region is located 10 and 11 bp upstream of the transcriptional start site (Kovács et al. 1995). Thus, it is highly likely that the *tts* box is part of the promoter. Based on the sequence analysis and the results obtained from expression analyses (Figs. 3 and 4), we propose a regulatory cascade for the activation of the *tts* gene cluster that is depicted in Figure 6.

DISCUSSION

Here, we have shown that the expression of *ttsI*, which is preceded by a *nod* box, is inducible by genistein. NodW and

Fig. 4. Activation of *ttsI* and *nolU* gene expression by NodD1. β -Galactosidase activity was determined 48 h after induction. Activities are given in pmol MU per μ g of protein per min of reaction time. Values (average $\pm$ standard deviation) are determined from four independent cultures measured in duplicate.

one or more proteins encoded by the *nodD1nodD2nolA* region are required for induction. Our data indicate that the likely regulatory candidate of the *nodD* region is NodD1, which is already known to activate *nod* box-controlled nodulation genes.

Surprisingly, *id205*, *nolU*, *nopL*, and *rhcV*, which have no *nod* box promoter, are also regulated by *nodW* and the *nodD* gene region in a flavonoid-dependent manner (Fig. 3). In addition, *ttsI* is essential for the activation of all of these genes. Therefore, we postulate that TtsI, which—based on sequence similarity—belongs to the two-component regulatory family, is the direct activator of the *tts* gene cluster (Fig. 6). Common to the investigated operons is the presence of a *tts* box sequence within the putative promoter regions (Fig. 5). The same element is also conserved in *tts* clusters of other rhizobia. In the case of *nolB* and *nolX* of *S. fredii*, the *tts* box is located 10 and 11 bp upstream of the transcriptional start site (Kovács et al. 1995). Moreover, a deletion that extends into the *tts* box of *nolB* from *S. fredii* abolishes promoter activity (Gu et al. 1997). Thus, it is highly likely that the *tts* box is a central component of the promoter. Further analyses of the *tts* box are in progress to confirm this.

Interestingly, *rhcC1*, which is transcribed divergently from *nolU*, seems to be constitutively expressed (Fig. 3). Constitutive expression was also reported for the homologue in *S. fredii* (Kovács et al. 1995, there called *nolW*). According to Viprey and associates (1998), the protein is part of the transport complex of the TTSS and is located in the outer membrane. The different expression pattern suggests that the protein might have an additional, still unknown, function.

Mutations within the *tts* gene cluster of *B. japonicum* affected symbiosis in a host-dependent manner. Similar observations have been reported for *S. fredii* (Bellato et al. 1997; Meinhardt et al. 1993) and *Rhizobium* strain NGR234 (Viprey et al. 1998). This host-specific effect may be due to differences in *tts* gene expression in the various hosts or due to differences in reaction of the hosts to the putatively secreted proteins.

One of the most important questions is about the nature and function of the secreted proteins. *Rhizobium* strain NGR234 transports at least eight proteins in a TTSS-dependent manner (Marie et al. 2001). The only two identified proteins are NopX and NopL; however, their function is unknown (Viprey et al. 1998). *S. fredii* appears to be similar (Krishnan et al. 1995). Based on Southern hybridization, *B. japonicum* does not seem to contain *nopX* (data not shown). However, it contains an ORF with similarity to *nopL* and several ORFs outside the *tts* gene

Bjap	<i>rhcC1</i>	90 bp	-	.TCCGTCAGGTTTTTCGTCAGCTCGGCAGCCTA	-	61 bp	<i>nolB</i>
<i>M. loti</i>	<i>mll6338</i>	120 bp	-	.TCAGTCAGCTTGTCTGTCAGCTCGGCCACCTA	-	71 bp	<i>mll8763</i>
NGR234	<i>rhcC1</i>	85 bp	-	.GTAGTCAGCGTGTCTGTCAGCTCGCCTCGCTA	-	39 bp	<i>nolB</i>
Sf257	<i>nolW</i>	85 bp	-	.GTAGTCAGCGTGTCTGTCAGCTCGCCTCGCTA	-	39 bp	<i>nolB</i> 11 bp to +1
Bjap				ATCATCGTCAGCTTTTTCGACAGGTGTTTCGGGCTA	-	221 bp	<i>id205</i>
NGR234				ACAATTGTCAGCTTTTTCGAAAGCTGGAGCTCATA	-	410 bp	<i>orf</i>
Bjap				AGGCTCGTCAGCTTTTTCGAAAGCTAGCGCCCTA	-	415 bp	<i>nopA</i>
Bjap				CCGATCGTCAGCTTTTTCGAAAGCTAAAGCCCCCA	-	208 bp	<i>nopL</i>
NGR234				AGCCTCGTCAGTTTCTCGAAAGCTAAACCGCTCA	-	188 bp	<i>nopX</i>
Sf257				AGCCTCGTCAGTTTCTCGAAAGCTAAACCGCTCA	-	189 bp	<i>nolX</i> 10 bp to +1
<i>M. loti</i>				GAAGTCGTCAGTTTACCGAAAGCTAAACCGCTCA	-	100 bp	<i>mll6337</i>
<i>M. loti</i>				AGACTCGTCAGGTTCTCGAAAGCTCCTGCTCGTA	-	446 bp	<i>orf</i>
NGR234				CTGATTGTCAGCTTCTCGAAAGGTATGTCCTCTTA	-	82 bp	<i>nopL</i>
Bjap				GCGAT-GTCAGGTTTTGGAAAGCAAACGTGAGTA	-	36 bp	<i>id274</i>
<i>tts</i> box consensus				tcGTCAGctT.tcGaaAGct...cc.cctA			

Fig. 5. Comparison of putative promoter regions within *tts* gene clusters. Numbers indicate distances in bp between the *tts* box and the assumed translational start site of the corresponding open reading frame. For *nolX* and *nolB* from *Sinorhizobium fredii*, the distance between the conserved sequence and the transcriptional start site is indicated in bp at the end of the lines. In the consensus sequence, all invariant nucleotides are shown in capital letters. Lower case letters are used for nucleotides conserved in at least 50% of the sequences. Bjap = *Bradyrhizobium japonicum*; *M. loti* = *Mesorhizobium loti* MAFF303099; NGR234 = *Rhizobium* strain NGR234; and Sf257 = *S. fredii* USDA257.

cluster whose products are similar to type III secreted proteins from pathogens (Göttfert et al. 2001). Moreover, there are several ORFs located within the *tts* gene cluster that currently do not match database entries. All these ORFs are candidates for encoding proteins transported by the TTSS. Our current effort is directed towards the identification of secreted proteins as the next step for the analysis of protein function.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used in this study are listed in Table 2. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) with appropriate antibiotics added.

B. japonicum strains were cultivated at 28°C in peptone-salts-yeast extract (PSY) medium (Regensburger and Hennecke 1983), AG (arabinose-gluconate) medium (Sadowsky et al. 1987) or yeast extract-mannitol (YEM) medium (Daniel and Appleby 1972). PSY was used for bacterial crosses with *E. coli*, AG medium for routine cultures, and YEM medium for β -galactosidase activity assays. Antibiotics were used at a final concentration of 100 μ g per ml.

Bacterial crosses.

E. coli S17-1 containing recombinant pSUPPOL2SCA derivatives and *B. japonicum* strains were grown to stationary phase in LB or PSY, respectively, with appropriate antibiotics

Fig. 6. Model of the regulatory cascade controlling expression of the *tts* gene cluster. Open reading frames (ORFs) unique to *Bradyrhizobium japonicum* are in black, and *nod* genes are shown in white. ORFs with homologues in *tts* gene clusters of other rhizobia are gray. ORFs related to IS elements are not shown. The gray arrowhead indicates the position of the *nod* box. Positions of *tts* boxes are marked by black arrowheads. Cm = cytoplasmic membrane.

Table 2. Bacterial strains used in this study

Strain	Relevant characteristics ^a	Source or reference
<i>Bradyrhizobium japonicum</i>		
110spc4	referred to as wild type, Sp ^r	Regensburger and Hennecke 1983
613	<i>nodW::Ω</i> , Sm ^r Sp ^r	Göttfert et al. 1990
Δ1267	Δ (ORF3 ORF2 <i>nolA nodD2 nodD1</i>), Km ^r Sp ^r	Göttfert et al. 1992
Δ121	Δ (<i>rhcN^b y4yJ rhcQ rhcR^b</i>), Km ^r Sp ^r	This work
Δ122	Δ (<i>rhcV^b id192 id193 y4yQ nopA id202^b</i>), Km ^r Sp ^r	This work
Δ132	Δ (<i>nolB rhcJ nolU nolV rhcN^b</i>), Km ^r Sp ^r	This work
Δ136	Δ (<i>id274^b y4xK rhcC2 ttsI id287 id289 id293</i>), Km ^r Sp ^r	This work
Δ139	Δ <i>ttsI</i> , Km ^r Sp ^r	This work
Δ171	Δ <i>rhcC1</i> , Km ^r Sp ^r	This work
wt-167	<i>nolU-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
Δ139-167	<i>nolU-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ139, Km ^r Sp ^r Tc ^r	This work
613-167	<i>nolU-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-167	<i>nolU-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
wt-179	<i>rhcV-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
Δ139-179	<i>rhcV-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ139, Km ^r Sp ^r Tc ^r	This work
613-179	<i>rhcV-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-179	<i>rhcV-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
wt-180	<i>rhcC1-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
Δ139-180	<i>rhcC1-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ139, Km ^r Sp ^r Tc ^r	This work
613-180	<i>rhcC1-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-180	<i>rhcC1-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
wt-182	<i>nopL-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
Δ139-182	<i>nopL-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ139, Km ^r Sp ^r Tc ^r	This work
613-182	<i>nopL-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-182	<i>nopL-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
wt-185	<i>id205-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
Δ139-185	<i>id205-lacZ</i> integrated into the <i>ttsI</i> deletion derivative Δ139, Km ^r Sp ^r Tc ^r	This work
613-185	<i>id205-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-185	<i>id205-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
wt-516	<i>ttsI-lacZ</i> integrated into the wild-type background, Sp ^r Tc ^r	This work
613-516	<i>ttsI-lacZ</i> integrated into the <i>nodW</i> mutant strain 613, Sm ^r Sp ^r Tc ^r	This work
Δ1267-516	<i>ttsI-lacZ</i> integrated into the <i>nodD2nodD1nolA</i> mutant derivative Δ1267, Km ^r Sp ^r Tc ^r	This work
<i>Escherichia coli</i>		
XL1-Blue	<i>endA1 gyrA96 hsdR17 recA1 relA1 supE44 thi-1 lac[F' proAB lacI^qΔM15 Tn10 (Tc^r)]</i>	Bullock et al. 1987
RR28	<i>hsdR hsdM recA pheS12 thi leu pro lac gal ara ml1 xyl5 supE44 endA</i> , Sm ^r	Hennecke et al. 1982
S17-1	<i>hsdR pro thi</i> (RP4-2 <i>km::Tn7 tc::Mu</i> , integrated into the chromosome), Sm ^r Sp ^r	Simon et al. 1983

^a Ap = ampicillin; Km = kanamycin; Sm = streptomycin; Sp = spectinomycin; and Tc = tetracycline.

^b The corresponding open reading frame (ORF) is not completely deleted from the genome.

added. Bacteria from 1 ml of each culture were pelleted, were resuspended in 1 ml (*E. coli*) or 0.1 ml (*B. japonicum*) 0.9% NaCl, and were mixed at a volume ratio of 1:5. The mixture (80 µl) was placed as a drop on PSY solid medium and was incubated for 3 days at 30°C. The cells were harvested, were resuspended in 1 ml of 0.9% NaCl, and were plated on selective PSY solid media. Broad host range plasmids pRJ420 and pRJ536 were mobilized into *B. japonicum* by triparental mating. Strains were grown, pelleted, and resuspended as described above. Donor, helper, and recipient were mixed at a volume ratio of 1:1.5:1.5. The mixture was treated as described.

DNA protocols and sequence data analysis.

Recombinant DNA techniques were performed following established protocols as compiled by Sambrook and associates (1989). Blast similarity searches were done at the National Center for Biotechnology Information website. The program FastA of the GCG software package (Accelrys Inc., Cambridge, U.K.) was used for the identification of the *tts* box sequences. Alignments were done with MultAlin at the INRA website. (Published research using this software should cite: "Multiple sequence alignment with hierarchical clustering" F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890)

Generation of mutations in the *tts* gene cluster.

Detailed information about strains and plasmids is given in Tables 2 and 3. In summary, gene regions were cloned into suitable vectors. DNA segments to be deleted were replaced by a Km^r cassette. The modified fragments were recombined into the mobilizable vector pSUPPOL2SCA. The resulting plasmids were transformed into *E. coli* S17-1 and were mobilized into *B. japonicum* wild type. Double-crossover events at homologous sites resulted in kanamycin-resistant strains. Correct recombinants were confirmed by polymerase chain reaction (PCR) and Southern blot analyses.

Construction and chromosomal integration of translational *lacZ* fusions.

Strains and vectors are listed in Tables 2 and 3. Appropriate parts of *id205*, *nolU*, *nopL*, *rhcC1*, *rhcV*, and *ttsI* were cloned into the *lacZ* fusion vectors pNM480X, pNM481X, or pNM482N. Because RhcC1 and RhcV are predicted to be membrane proteins, *lacZ* was fused directly to the first triplet.

Similarly, *lacZ* was fused to the first codon of *id205* and to the second codon of *nopL*, because both genes might encode secreted proteins. The correct fusion sites were confirmed by DNA sequencing. The fusions were recombined into the mobilizable vector pSUPPOL2SCA. The recombinant plasmids were transformed into *E. coli* strain S17-1 and were mobilized into *B. japonicum* wild type as well as mutant derivatives. The vector-encoded Tc^r marker allowed the selection of strains with plasmids integrated into the chromosome. The correct integration event was confirmed by PCR and Southern blot analysis.

Plant infection tests.

Plants used in this study are listed in Table 4. Seeds were surface-sterilized as described by Krause and Broughton (1992) and were germinated at room temperature for 2 to 3 days on water agar. *B. japonicum* strains were grown in PSY at 28°C to late logarithmic phase, were pelleted, and were resuspended in H₂O to an optical density at 600 nm of 0.4. Germinated seeds were transferred to Magenta GA7 vessels (Invitrogen Corp., San Diego, CA, U.S.A.) filled with vermiculite (2- to 3-mm particle size), and 200 ml 0.5× nitrogen-free Jensen medium (Vincent, 1970). Each seedling was inoculated with 0.4 ml of the bacterial suspension. Plants were cultivated in a growth chamber (Sanyo MRL-350; Sanyo Electric Co., Moriguchi City, Osaka, Japan) with a daytime of 16 h, 26°C, and light intensity of 370 µmol/sec/m², followed by a nighttime of 8 h and 22°C. To determine the number of root nodules, plants were harvested 10 and 20 days after inoculation.

Acetylene reduction assays.

Acetylene reduction of whole root systems was measured 28 days after inoculation, essentially using the method of Somasegaran and Hoben (1994). Each root was placed in a 100-ml rubber-stopped flask for *G. max* and *V. unguiculata* or a

Table 4. Legume species used in this study

Plant	Cultivar	Seed source
<i>Glycine max</i> (L.) Merr.	Williams	P. Gresshoff, University of Queensland, Australia
<i>Macroptilium atropurpureum</i> (Moç & Sessé ex DC.) Urb.	Siratiro	Wrightson Seeds (Seven Hills, Australia)
<i>Vigna unguiculata</i> (L.) Walp.	Red Caloona	Wrightson Seeds (Seven Hills, Australia)

Table 3. Plasmids and cosmids used in this study

Cosmid/plasmid	Relevant characteristics ^a	Source or reference
Cosmid		
46-B6	Chromosomal fragment of <i>Bradyrhizobium japonicum</i> in Loris6, Km ^r	Kündig 1994
57-D12	Chromosomal fragment of <i>B. japonicum</i> in Loris6, Km ^r	Kündig 1994
Plasmid		
pBluescript II SK (+)	Cloning vector, Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pNM480X	Derivative of pNM480 (Minton 1984) with a <i>Xba</i> I restriction site instead of a <i>Stu</i> I site, Ap ^r	Göttfert et al. 1992
pNM481X	Derivative of pNM481 (Minton 1984) with a <i>Xba</i> I restriction site instead of a <i>Stu</i> I site, Ap ^r	Göttfert et al. 1992
pNM482N	Derivative of pNM481 (Minton 1984) with a <i>Nhe</i> I restriction site instead of a <i>Stu</i> I site, Ap ^r	A. Krause, unpublished data
pRJ420	Broad host range plasmid containing <i>nodD1</i> and a streptomycin/spectinomycin resistance marker, Sm ^r Sp ^r Tc ^r	M. Göttfert, unpublished data
pRJ536	Broad host range plasmid containing a streptomycin/spectinomycin resistance marker, Sm ^r Sp ^r Tc ^r	M. Göttfert, unpublished data
pRK2013	<i>tra</i> ⁺ , Km ^r	Figurski and Helinski 1979
pSUPPOL2SCA	Derivative of pSUP202 (Simon et al. 1983) with a polylinker in the <i>Pst</i> I site that reads <i>Sac</i> II- <i>Xma</i> III- <i>Not</i> I- <i>Xba</i> I- <i>Spe</i> I- <i>Bam</i> HI- <i>Sma</i> I- <i>Pst</i> I- <i>Eco</i> RI- <i>Eco</i> RV- <i>Hind</i> III- <i>Clal</i> - <i>Sal</i> I- <i>Xho</i> I- <i>Dra</i> II [derived from pBluescript II SK (+)], deletion of the 1.3-kb fragment encompassing the chloramphenicol resistance gene, <i>oriT</i> of RP4, Tc ^r	A. Krause, unpublished data
pUC-4-KIXX	Source of the Km ^r cassette, Ap ^r Km ^r	Pharmacia, Uppsala, Sweden
pUC9	Cloning vector, Ap ^r	Norlander et al. 1983
pUC18	Cloning vector, Ap ^r	Norlander et al. 1983

^a Ap = ampicillin; Km = kanamycin; Sm = streptomycin; Sp = spectinomycin; and Tc = tetracycline.

15-ml rubber-stopped glass tube for *M. atropurpureum*. Ten percent of the air was replaced by acetylene 2.6 (Messer-Griesheim, Ottendorf-Okrilla, Germany). After 1.5 h incubation at 30°C, 0.5 ml of the gas phase was injected into a gas chromatograph (GC 17A; Shimadzu Europe, Duisburg, Germany) stainless steel column 6 ft × 1/8 in. × 2 mm packed with Porapak N, 100/200 mesh size (Machery-Nagel, Düren, Germany). Acetylene reduction per nodule was calculated as peak area ethylene per nodule. The peak area of ethylene was corrected by the area of the ethylene peak found in the acetylene control. The values of the wild-type strain on *G. max*, *M. atropurpureum*, and *V. unguiculata* were set to 100%.

Determination of β-galactosidase activity.

Cultures were grown in selective YEM medium for 4 days at 28°C. Cells were collected by centrifugation and were resuspended in half volume fresh selective YEM medium. Genistein dissolved in methanol was added to a final concentration of 1 μM. Controls received methanol only. After an additional incubation for 24 to 48 h at 28°C, two 1-ml samples were taken. Cells were pelleted, were washed with RC buffer (25 mM Tris HCl pH 7.5, 125 mM NaCl, 2 mM MgCl₂ × 6 H₂O, 12 mM β-mercaptoethanol), and were resuspended in 200 μl RC buffer. To permeabilize the cells, 20 μl chloroform and 20 μl sodium dodecyl sulfate (0.1%) were added. This cell suspension (40 μl) was mixed with 160 μl of RC buffer containing 4-methylumbelliferyl-β-D-galactopyranosid (300 μM). Reactions were incubated for 2 h at 30°C and were terminated by the addition of 50 μl of trichloroacetic acid (25%). Reaction mixture (100 μl) was added to 1.9 ml GC buffer (133 mM glycine, 83 mM Na₂CO₃, pH 10.7). Fluorescence of the reaction product 4-methylumbelliferone (MU) was measured with a fluorometer (VersaFluor Fluorometer, Bio-Rad Laboratories, Hercules, CA, U.S.A.) by excitation at 365 nm and by emission at 455 nm. β-Galactosidase activity was calculated as pmol MU per μg of protein per min reaction time.

Permeabilized cells 40 (μl) were used to determine protein concentrations using the Bio-Rad protein assay (Bio-Rad Laboratories).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/BLAST/>
- INRA Multiline multiple sequence alignment page: <http://prodes.toulouse.inra.fr/multalin/multalin.html>