Roles of flavonoids and the transcriptional regulator TtsI in the activation of the type III secretion system of \textit{Bradyrhizobium elkanii} SEMIA587

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\textit{Bradyrhizobium elkanii} SEMIA587 is a symbiotic nitrogen-fixing bacterium of the group commonly called rhizobia, which induce nodule formation in legumes, and is widely used in Brazilian commercial inoculants of soybean. In response to flavonoid compounds released by plant roots, besides Nod factors, other molecular signals are secreted by rhizobia, such as proteins secreted by type III secretion systems (T3SSs). Rhizobial T3SSs are activated by the transcription regulator Ttts, which binds to sequences present in the promoter regions of T3SS genes via a conserved sequence called the \textit{tts} box. To study the role of the T3SS of \textit{B. elkanii} SEMIA587, \textit{ttsI} was mutated. Protein secretion and flavonoid induction analysis, as well as nodulation tests, were performed with the wild-type and mutant strains. The results obtained showed that \textit{B. elkanii} SEMIA587 secretes at least two proteins (NopA and NopL, known rhizobial T3SS substrates) after genistein induction, whilst supernatants of the \textit{ttsI} mutant did not contain these Nops. Unusually for rhizobia, the promoter region of the \textit{B. elkanii} SEMIA587 \textit{ttsI} gene contains a \textit{tts} box, which is responsive to flavonoid induction and to which TtsI can bind. Nodulation tests performed with three different leguminous plants showed that the \textit{B. elkanii} SEMIA587 \textit{ttsI} mutant displays host-dependent characteristics; in particular, nodulation of two soybean cultivars, Peking and EMBRAPA 48, was more efficient when Tts of \textit{B. elkanii} was functional.

INTRODUCTION

Type III secretion systems (T3SSs) are specialized machines for protein secretion used by many Gram-negative bacteria. The secretion machinery is formed by about 20 proteins, many of which are homologous to those of the apparatus for flagella assembly. When not used for flagella assembly, T3SSs function to deliver proteins (called effectors) directly into the cytosol of eukaryotic cells (Pallen et al., 2003). T3SSs were first thought to be exclusive to pathogenic bacteria (Hueck, 1998), but after sequencing the symbiotic plasmid of \textit{Rhizobium} sp. NGR234 (hereafter NGR234), sequences homologous to all the genes encoding a T3SS and to possible effectors were found (Freiberg et al., 1997). The NGR234 T3SS is functional and involved in the determination of the host range of nodulation by NGR234 (Viprey et al., 1998). Proteins secreted by rhizobial T3SSs are termed nodulation outer proteins (Nops) (Marie et al., 2001), and can have different roles, depending on the host plant. Some effector Nops can enhance nodulation in specific leguminous plants, whilst in others Nops may be recognized as virulence factors, decreasing nodulation formation (Viprey et al., 1998; Krishnan et al., 2007; Bartsev et al., 2003, 2004; Skorpil et al., 2005).

In rhizobia, T3SSs are activated by NodD1 in a flavonoid-dependent manner. NodD1 activates the expression of the transcriptional activator Ttts by binding to the \textit{nod} box in the promoter region of its gene (\textit{ttsI}). A \textit{nod} box element has been identified in the promoters of all \textit{ttsI} genes studied (Viprey et al., 1998; Krause et al., 2002; Marie et al., 2003, 2004; López-Baena et al., 2008; Wassem et al., 2008). No \textit{nod} box sequences are found in the promoter regions of other genes encoding T3SSs; instead, a different \textit{cis} element has been identified and named the \textit{tts} box by Krause et al.
TtsI has homology to the DNA-binding response regulators of two-component regulatory systems (Viprey et al., 1998; Marie et al., 2004; Wassem et al., 2008). Such regulators are usually activated by their cognate sensor kinase, which transfers a phosphoryl group to an aspartate residue in the regulator, leading to a conformational change that activates the latter protein. Once phosphorylated, the regulator protein binds to cis elements located in the promoter region of genes that are required to process the environmental signal detected by the sensor partner. TtsI, however, has a glutamate residue instead of the usual conserved aspartate. It has already been shown that this type of substitution leads to a constitutive activation of the regulator protein, without the need of its partner sensor kinase (La & Igo, 1998). It has been proposed that TtsI acts as a transcriptional activator independently of phosphorylation by the sensor kinase (Marie et al., 2004; Wassem et al., 2008). Thus, transcription of T3SS genes would be dependent on the NodD1 activation of the ttsI gene, in the presence of the specific flavonoid compound released by the host plant (Kobayashi et al., 2004; Marie et al., 2004; Wassem et al., 2008).

Nodulation of soybean can be induced by various rhizobial strains that possess a T3SS. Mutation of the T3SS of B. japonicum USDA110 leads to a delay in nodule formation by Glycine max cultivar Williams (Krause et al., 2002). Similarly, a functional T3SS in S. fredii HH103 enhances nodulation of various soybean cultivars (López-Baena et al., 2008). The T3SS of S. fredii USDA257 has varying effects on the nodulation of soybean cultivars. With cultivar Peking, an active T3SS improves nodulation, whereas the T3SS must be mutated to allow nodulation of cultivar McCall (Meinhardt et al., 1993).

In Brazil, Bradyrhizobium elkanii is successfully used in the formulation of commercial inoculants to increase soybean yields and, together with B. japonicum, fully supplies plant nitrogen demands. At present, only four B. japonicum and B. elkanii strains are routinely used in the formulation of Brazilian commercial inoculants, and they have resulted in an established rhizobial population in most soils cropped with soybean (Ferreira et al., 2000). Of the recommended strains, SEMIA587 and SEMIA5019 belong to the species B. elkanii, whilst SEMIA5080 and SEMIA5079 belong to the species B. japonicum (Rumjanek et al., 1993).

Genomic hybridization performed by Viprey et al. (1998) has shown that T3SS homologues are present in B. elkanii USDA61. A 47.1 kb DNA sequence containing a T3SS cluster from B. elkanii USDA61 was recently obtained by Okazaki et al. (2009). The creation of T3SS mutants of this strain has shown that it has a host-dependent nodulation phenotype: a functional T3SS improves nodulation of cultivar Clark but blocks nodulation of cultivar Hill (Okazaki et al., 2009). Characterization of the DNA sequence in the T3SS locus of B. elkanii USDA61 has shown that it possesses ttsI (preceded by a nod box) as well as numerous tts boxes upstream of key genes encoding T3SS components. Despite containing all the elements required for flavonoid-based induction of protein secretion, this has not been observed and the presence of flavonoids in the growth medium has no effect on protein secretion (Okazaki et al., 2009).

In this work we investigated the role of the T3SS in the agronomically important Brazilian (soybean) inoculant B. elkanii SEMIA587 (hereafter SEMIA587). Identification of T3SS-related sequences permitted the creation of a ttsI mutant and thus an inactive T3SS. The effects on the nodulation of soybean cultivars (and other legumes) known to differentially respond to rhizobial T3SSs, as well as on a Brazilian soybean cultivar, were assessed. Characterization of the SEMIA587 TtsI showed it (and hence the T3SS) to be induced by flavonoids produced by soybean.

**METHODS**

**Bacterial cultures and growth conditions.** SEMIA587 and its derivatives were grown in YEM (yeast extract mannitol) (Vincent, 1970) and RMS (Rhizobium minimal medium containing succinate as the carbon source; Broughton et al., 1986) media at 28 °C. Escherichia coli strains DH5a, BL21 (Rosetta) and XL1-Blue were grown in Luria–Bertani (LB) broth at 37 °C (Sambrook & Russell, 2001). Antibiotics were added to the media at the following final concentrations: tetracycline, 50 μg ml⁻¹; kanamycin, 30 μg ml⁻¹; spectinomycin, 100 μg ml⁻¹. Where indicated, cultures were induced with genistein or daidzein at a final concentration of 10⁻⁶ M. Control cultures were treated with the corresponding amount of methanol, which was used as the solvent for these flavonoids.

**SEMIAS87 ttsI mutant construction.** Comparison of rhizobial ttsI sequences available in GenBank allowed primers to be designed corresponding to conserved regions of the gene (ttsI-FOR, 5′-TCTGCCCAGTGGACGGCTT-3′, and ttsI-REV, 5′-TGCGTAGCCGATCCCGA-3′). These primers were used to amplify an internal fragment of ttsI using SEMIA587 genomic DNA as template. PCRs were performed in a 25 μl volume, containing 50 ng genomic DNA, 1 U Taq DNA polymerase (Life Technologies), 1 × Taq DNA polymerase reaction buffer, 1.5 mM MgCl₂, 200 mM dNTPs (Life Technologies) and 10 μmol each primer. Amplification was carried out as follows: one initial denaturation step at 94 °C for 5 min, 30 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 52 °C and extension for 45 s at 72 °C, and one final extension step for 5 min at 72 °C. Reactions were performed in a PCR Express temperature cycling system (Thermo Hybaid), and the fragments were visualized after electrophoresis at 80 V for 1 h on a 1.5% agarose gel stained with ethidium bromide. A molecular size marker (1 kb Plus DNA ladder, Gibco-BRL) was loaded onto all gels. The amplified fragment (364 bp) was cloned into the pGEM T-Easy vector (Promega), and sequenced in both the forward and the reverse
The 364 bp amplified fragment containing the partial tts sequence was then subcloned into the EcoRI site of plasmid pK18mobsAC8 (Schäfer et al., 1994), generating pK18ttsI. This plasmid was linearized with Mdel, and then overhanging ends were filled in with Klenow fragment (Invitrogen). The Omega cassette contained in pH450 (Prentki & Kirsch, 1984) was removed with SmaI and inserted into the pK18ttsI Mdel-linearized-filled-in plasmid, generating plasmid pK18ttsIfr. This plasmid was mobilized from E. coli DH5α to strain SEMIA587 by triparental mating, using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Double recombination was selected by plating bacteria onto YEM plates containing the appropriate antibiotics (tetracycline and spectinomycin). Putative mutant strains (B. elkanii SEMIA587 ttsI) were confirmed by PCR and Southern blotting, using standard procedures (Sambrook & Russell, 2001; data not shown).

Cloning of promoter sequences into the pPROBE NT reporter plasmid. The tts box motif identified in the promoter region of the SEMIA587 ttsI was isolated using a SiteFinding-PCR approach, as described by Tan et al. (2005). The 700 bp fragment was cloned into the pGEM T-Easy vector and sequenced in both the forward and the reverse direction. The ttsI promoter region was PCR-amplified from pGEM T-Easy using pTtsI-sense (5'-GGGTACCGATTTTTTCAAT-3') and ttsI-REV primers. The PCR conditions were the same as described above. The 700 bp amplified fragment was filled in with Klenow fragment and subcloned into the SmaI site of the broad-host-range reporter vector pPROBE NT (Miller et al., 2000), generating plasmid pNTttsI.

The tts box motif from the nopC promoter region of B. elkanii SEMIA587 was amplified with primers pNopC-FOR (5'-TGACGAGGAGTCCGTCTG-3') and pNopC-REV (5'-CAGGGATCTTTCCAGTTTCAATT-3'), which were designed according to the B. elkanii USDA61 nopC sequence (GenBank accession no. FM162234). The amplified fragment (~1.0 kb) was cloned into pGEM T-Easy and sequenced in both the forward and the reverse direction. The fragment was excised from pGEM T-Easy with HindIII and Sall and subcloned into pPROBE NT (also digested with HindIII and Sall), generating plasmid pNTnopC.

The pPROBE NT constructs were mobilized into SEMIA587 and SEMIA587 ttsI by triparental matings using pRK2013 as the helper plasmid. Flavonoid induction assays were performed as described previously (Kobayashi et al., 2004; Wassem et al., 2008).

Measurements of GFP activity. OD955 and fluorescence (excitation filter at 485 nm and emission filter at 535 nm) were measured on 100 µl volumes of cultures 24 h post-induction using a Plate CHAMELEON multilabel detection platform (Hidex Oy). Optical densities and fluorescence were corrected to background levels using uninoculated media, and the results represent the means of at least three independent experiments. For the analysis, the fluorescence values obtained were normalized to the mean optical density at each time point. Data were statistically analysed using Student’s t test with a P <0.05 considered as significant.

Isolation and sequencing of SEMIA587 ttsI coding and promoter regions. To isolate a DNA fragment containing the complete tts ORF and upstream region from SEMIA587, primers were designed based on the sequence of the B. elkanii USDA61 tts cluster (GenBank accession no. FM162234). The primers NB-up (5'-CGTTTTGGAAGACCAGTAAG-3'), NB-down (5'-ATTGCCGAATGTTGAGC-3'), pTtsI-sense, NB-down (5'-GATCATACGGCCGAAACCT-3') and TtsI-down (5'-TGTGTTCTTTCTGAGTTCGATT-3') were used in several combinations (NB-up/NB-down, pTtsI-sense/TB-down, NB-up/TB-down, pTtsI-sense/TsI-down). PCR conditions were as described above. All amplified fragments were cloned in pGEM-T Easy and sequenced in both the forward and the reverse direction with M13 universal primers.

Electrophoretic mobility shift assay (EMSA). The ttsI coding sequence (of SEMIA587) was PCR-amplified using primers TtsI-up (5'-GTCATCGCATTTTCACTC-3') and TtsI-down, using the proof-reading Platinum Pfx DNA Polymerase (Invitrogen) and the PCR conditions described above. The 900 bp amplified DNA fragment was cloned into the vector pENTR/TR/D-TOPO (Invitrogen), and sequenced to verify PCR fidelity. The insert was recombined into pGEX KG Gateway, using Gateway LR Clonase enzyme mix (Invitrogen), in E. coli DH5α competent cells, generating pGEttsI. The recombinant plasmid was confirmed by PCR. pGEttsI was used to transform E. coli BL21 (Rosetta), and E. coli BL21 (Rosetta) carrying pGEttsI was induced with 0.5 mM IPTG for 6 h at 28 °C. Purification of the glutathione S-transferase (GST)–TtsI fusion was carried out using Glutathione Sepharose 4B from GE Healthcare Life Sciences. Protein concentration was assayed with Bradford reagent (Bioagency; Bradford, 1976).

A 55 bp double-stranded oligonucleotide corresponding to the putative tts box from upstream of ttsI was obtained by annealing the sequences TBup (5'-AAGCCGAGCAGTCTGACTCCGACCGTCCACCGTTGCAACCGGCTTAAA-3') and TBdo (5'-TAAACGCCCCGTCAGTTGACCAAAGAGGTCGACGAAATGCAGACAGCTCCGCTT-3'). The same procedure, using sequences NBup (5'-AAGCCGACTCGCCTTTGGAATGGTTCAACCGGCTTAAA-3') and NBdo (5'-TCGAGGACGGCAGAATTTGTTTGTAGGGAATGGTTCAACCGGCTTAAA-3'), was used to obtain a 58 bp double-stranded oligonucleotide, which contained the nod box cis element. This fragment was used as a negative control. A second negative control used in this work was a 56 bp double-stranded oligonucleotide containing the tts box upstream of the ttsI gene nucleotide modifications (TBlmuUp, 5'-AGCCGACGTGCTTTGGAATGGATGGAATGGTTCAACCGGCTTAAA-3', and TBlmuDo, 5'-TAAACGCCCCGTCAGTTGACCAAAGAGGTCGACGAAATGCAGACAGCTCCGCTT-3').

Binding conditions were assayed with the EMSA kit from Invitrogen as specified by the supplier. The double-stranded oligonucleotides were used at a concentration of 50 ng, and the purified TtsI–GST protein was tested at 50, 200 and 400 ng, for the tts box double-stranded oligonucleotide, and 200 ng for the controls.

Analysis of secreted proteins. Extracellular proteins from SEMIA587 and its ttsI mutant strains, with or without flavonoid induction, were recovered from 50 ml of RMS cultures grown in an orbital shaker (180 r.p.m.) for 72 h. Secreted proteins were extracted as described by Viprey et al. (1998). Aliquots of purified proteins were separated on 12 % SDS-PAGE gels, using BenchMark Protein Ladder (Invitrogen) as a molecular mass standard, and stained with silver (Ausubel et al., 1994). For immuno detection, SDS-PAGE-separated proteins were transferred to PVDF Immobilon-P membranes (Millipore) and probed with antibodies raised against Rhizobium sp. NGR234 NopA and NopL proteins (1:2000 dilution). Horseradish peroxidase-labelled goat anti-rabbit immunoglobulin antibodies from the ECL kit (GE Life Sciences Amersham Pharmacia Biotech) were used as secondary antibodies.

Nodulation tests. Nodulation tests were performed in Magenta jars as described by Skorpil et al. (2005). All plants were grown at
RESULTS

Cloning of DNA sequences from the T3SS locus of SEMIA587

A PCR-based approach was used to amplify the coding sequence of the *tsl* gene from SEMIA587 genomic DNA. The DNA sequences of all rhizobial *tsl* genes were aligned to design specific primers from conserved regions for PCR. A product of the expected size (~350 bp) was generated, cloned and verified to be highly homologous to *tsl* by DNA sequencing (data not shown). The *tsl* fragment was used to create a *tsl* mutant by disrupting the gene with an omega (Ω) cassette (see Methods for more details). The phenotype of the mutant was subsequently characterized (see below).

The *tsl* promoter region was accessed by chromosome walking, using the SiteFinding-PCR approach (Tan et al., 2005). Analysis of the sequence obtained identified a potential *tsl* box *cis* element 45 bp upstream of the *tsl* coding region (Fig. 1). No *nod* box was detected, but as the length of the sequence obtained was only 200 bp, a *nod* box motif could still be present further upstream. Nevertheless, the presence of the *tsl* box motif in the promoter region of the *B. elkanii* SEMIA587 *tsl* gene was unexpected, since this element has not been found in other *tsl* promoter regions already sequenced.

Whilst this work was carried out, Okazaki *et al.* (2009) published the entire sequence of the T3SS cluster from another strain of *B. elkanii*, USDA61. Using the available data (GenBank accession no. FM162234), new primers were designed and used to amplify fragments of *ttsI* and its regulatory regions from SEMIA587 genomic DNA. The amplified fragments covered a total of 2409 bp of the *tsl* region, including 1697 nt upstream of the *tsl* start codon. With this extra sequence of the *ttsI* regulatory region, a *nod* box motif was found at a distance of 1621 bp from the beginning of the *ttsI* coding region. The presence of the *tsl* box was confirmed 45 bp from the *ttsI* coding region. The *ttsI* coding region was identified between nucleotides 1697 and 2386 of the sequenced region.

A second *tsl* box was also isolated, from the promoter of a gene shown in other rhizobia to be under TtsI/ftsI box control (Wassem *et al.*, 2008). The *nopC* *tsl* box (59 bp upstream of the beginning of the *nopC* coding region) was obtained by PCR, using primers designed from the corresponding gene sequence of *B. elkanii* USDA61 (see Methods for more details). DNA sequence analysis confirmed the correct amplification of a *tsl* box (Fig. 1).

The flavonoids genistein and daidzein induce both the *nopC* and the *ttsI* promoter

The promoter sequences of *nopC* and *ttsI* were subcloned into the pPROBE NT vector (Miller *et al.*, 2000), and conjugated into the SEMIA587 wild-type and *tsl* mutant, in order to test their transcription activation potential in the presence or absence of flavonoids. The flavonoids used were genistein and daidzein, which are known to be released by soybean. After 24 h of culture, SEMIA587 showed an elevated GFP fluorescence in the presence of the flavonoids only with the two plasmids containing the *tsl* box promoter sequences upstream of GFP. The SEMIA587 *tsl* mutant containing the same plasmids, however, did not display any significant fluorescence augmentation in 24 h-grown cultures containing the same flavonoids (Fig. 2).

EMSAs

The presence of a *tsl* box upstream of *tsl* in SEMIA587 was unusual. We decided to verify its functionality by demonstrating the binding of TtsI to this sequence with an EMSA. A TtsI–GST fusion protein was purified using Glutathione Sepharose 4B (GE Heathcare Life Sciences). The purification procedure was carried out according to the manufacturer’s procedures. The TtsI–GST fusion was used in an EMSA, and Fig. 3 shows that the migration rate of the DNA fragment containing the *tsl* box from the promoter region of *tsl* was delayed when it was incubated with two different amounts of the purified TtsI protein (200 and 400 ng; Fig. 3, lanes 5 and 6, respectively). Fifty nanograms of protein was not sufficient to delay the DNA fragment containing the *tsl* box, however. The addition of 200 ng of the same protein to control DNA sequences (i.e.

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**Fig. 1.** Comparison of type III secretion (*tsl*) box motifs found in the promoter regions of *B. elkanii* SEMIA587 *tsl* and *nopC* genes with the *tsl* box consensus sequence described for *B. elkanii* USDA61 T3SS genes (Okazaki *et al.*, 2009). In the consensus sequence, all invariant nucleotides are shown in upper-case type. Lower-case type is used for nucleotides conserved in at least 50% of the sequences. Bold type in the *tsl* box sequences of SEMIA587 indicates nucleotides that are identical to the consensus sequence. Numbers indicate distances in bp between the *tsl* box and the assumed translational start site of the corresponding ORF.
containing the nod box upstream of ttsI or with a mutated version of the tts box; Fig. 3, lanes 8 and 10, respectively) caused no delay.

**Two Nops were not detected in culture supernatants of the ttsI mutant**

The results of the GFP activity experiments showed that both daidzein and genistein could activate T3SS genes and that TtsI was required, since no induction was seen in the ttsI mutant. We thus tested whether these flavonoids could cause the appearance of extra proteins in culture supernatants of SEMIA587 and not in the SEMIA587 ttsI mutant. Cultures of both strains were induced with genistein and secreted proteins were isolated (see Methods). The supernatant protein extracts were separated by SDS-PAGE, but silver staining of these gels gave inconclusive results. Identical gels were then blotted onto membranes and tested with antibodies raised against known T3SS-secreted proteins, NopA and NopL, of *Rhizobium* sp. NGR234. The presence of these two Nops in culture supernatants of the wild-type strain was observed only in the presence of genistein. In the ttsI mutant, these proteins were not detected, even after flavonoid induction (Fig. 4).

**Nodulation tests show a *B. elkanii* T3SS host-dependent phenotype**

As previously demonstrated, the T3SSs of several rhizobial strains display a host-dependent phenotype (Viprey et al., 1998; Krause et al., 2002; Okazaki et al., 2009). Since the ttsI mutant of SEMIA587 was blocked for T3SS activity, comparison of this mutant with the wild-type in nodulation tests permitted the analysis of the role of the SEMIA587 T3SS in symbiosis. Three soybean cultivars, McCall, Peking and EMBRAPA 48, as well as *Macroptilium atropurpureum* (siratro) and *Vigna unguiculata* (cowpea), were tested. Soybean cultivars McCall and Peking were used, since it was shown that they have different responses to the *S. fredii* USDA257 T3SS (Meinhardt et al., 1993).
EMBRAPA 48 is a modern commercial soybean cultivar extensively used by Brazilian soybean producers. Siratro and cowpea were used because of the observed effects of the *B. japonicum* USDA110 T3SS on the nodulation of these legumes (Krause et al., 2002).

Nodule numbers formed were assessed 14 and 28 days after plant inoculation with the wild-type and *ttsI* mutant strains (Fig. 5). For the soybean cultivars Peking and EMBRAPA 48, higher nodule numbers were observed when these plants were inoculated with the wild-type strain relative to the *ttsI* mutant. No significant differences in nodule number were observed with the soybean cultivar McCall or siratro. With cowpea, however, although no differences were observed 14 days post-inoculation, by 28 days the wild-type had induced significantly more nodules (Fig. 5).

DISCUSSION

T3SSs have been characterized from several rhizobia and shown to be important determinants of host range. These T3SSs are functional and capable of secreting numerous Nops (Deakin & Broughton, 2009). The identification of T3SSs within *B. elkanii* has led to the investigation of their effects in the interaction of this key inoculant with soybean and other leguminous plants. Sequencing of the *tts* cluster of *B. elkanii* USDA61 has shown that it possesses many of the characteristics of other rhizobial T3SSs (Okazaki et al., 2009).

Generally, rhizobial T3SSs are controlled by NodD1 and activated when flavonoids are perceived (Kobayashi et al., 2004; Marie et al., 2004; Wassem et al., 2008). NodD1 induces the T3SS-specific regulator *ttsI* via a *nod* box in the promoter of *ttsI* (Viprey et al., 1998; Krause et al., 2002; Marie et al., 2004; López-Baena et al., 2008; Wassem et al., 2008). TtsI is able to activate other T3SS-related genes, as the promoters of *tts* genes share a common *cis* element, the *tts* box, to which TtsI binds (Krause et al., 2002). This element is conserved amongst all the T3SS-containing rhizobia (Marie et al., 2004; López-Baena et al., 2008; Wassem et al., 2008; Okazaki et al., 2009; Zehner et al., 2008). Analysis of the DNA sequence of the T3SS locus of *B. elkanii* USDA61 suggests a similar pattern of regulation, as *ttsI* is preceded by a *nod* box, and other T3SS genes/operons contain *tts* boxes in their promoters. No flavonoid activation was observed, however (Okazaki et al., 2009).

In this work we investigated the regulation of the T3SS of another *B. elkanii* strain, SEMIA587. Sequences corresponding to *ttsI* and *tts* box-containing promoters were identified and used to create a *ttsI* mutant and reporter gene fusions, respectively. Two *tts* boxes, in the *ttsI* and *nopC* promoter regions, were both activated after 24 h of induction with genistein and daidzein, flavonoids known...
to be released by soybean. Furthermore, this activation required a functional ttsI gene, since no induction was seen with a ttsI mutant. Thus, the T3SS of SEMIA587 can be induced by soybean-derived flavonoids, and appears to follow the standard rhizobial mechanism, i.e. Tsl controlling tts box-containing promoters.

The promoter region of ttsI from SEMIA587 is unusual, however, as it contains a tts box. A fragment of the promoter sequence carrying this tts box (fused to GFP) was shown to be flavonoid-inducible (see above), and thus we characterized this tts box further. An EMSA was performed, and showed that Tsl was able to bind to this tts box. One interpretation of the presence of a functional tts box upstream of ttsI would be to reinforce the activation of ttsI transcription after flavonoid induction. Such a potential positive-feedback loop implies that in SEMIA587, the regulation of T3SS genes is markedly different from that of other T3SS-possessing rhizobia. The coding and regulatory regions of ttsI from SEMIA587 were almost identical to those from B. elkanii USDA61 described by Okazaki et al. (2009). Those authors identified nine tts box elements in the sequenced region of B. elkanii USDA61, including one upstream of ttsI but even more distal than the nod box identified 1627 bp from ttsI. The tts box identified and characterized in the present work is located only 45 bp from ttsI (see Fig. 1).

We performed a protein secretion assay with cultures of SEMIA587 wild-type and ttsI mutant strains in the presence of genistein. Two Nops (NopA and NopL) were not detectable in culture supernatants of the ttsI mutant strain, whilst the wild-type strain was able to secrete both proteins, although only in the presence of the flavonoid.

Similar results were obtained when an S. fredii HH103 ttsI mutant strain was tested for Nop secretion (López-Baena et al., 2008). Therefore, inactivation of the ttsI gene blocks Nop secretion and/or production in these bacteria.

Okazaki et al. (2009) did not find any difference in protein secretion when they analysed the USDA61 strain of B. elkanii in the presence or absence of the same flavonoid used in our work (genistein). Nops in culture supernatants could only be identified from B. elkanii USDA61 when comparisons were made between the wild-type strain and a mutant in a T3SS gene (rhcJ) which encodes an essential structural component of the machinery. The discrepancy between the results obtained by Okazaki et al. (2009) and those reported in this work could be due to the culture medium used in the two experiments. Whilst in the present work RMS medium was used, Okazaki et al. (2009) used arabinose gluconate medium (Sadowsky et al., 1987). It has been shown that Nop secretion can be influenced by some compounds of the culture medium. For example, Krishnan et al. (2007) have shown that calcium prevents the accumulation of NopB and NopA, and drastically reduces that of NopX and NopL in S. fredii USDA257. It would be interesting to determine whether B. elkanii USDA61 secretes NopA and NopL when cultured in RMS medium, and whether the addition of flavonoids has any effect.

Nodulation tests were performed with SEMIA587 and a derivative ttsI mutant strain on three soybean cultivars (EMBRAPA 48, McCall and Peking), as well as M. atropurpureum and V. unguiculata. Characterization of the resulting plants showed that the number of nodules formed by soybean cultivars EMBRAPA 48 and Peking was affected when ttsI was inactive, particularly at 14 days post-inoculation. Nodule formation by cultivar Peking is thus enhanced by the presence of an active T3SS in SEMIA587 as well as in S. fredii strains USDA257 and HH103. The number of nodules formed by cowpea was significantly higher when plants were inoculated with the wild-type strain only after 28 days; there was no difference after 14 days. Nodulation tests performed with a B. japonicum USDA110 ttsI mutant and soybean have shown that there is a statistically significant difference in the number of nodules 10 days after inoculation, although this phenotype is no longer significant after 20 days of inoculation (Krause et al., 2002). Although there were no obvious effects of the SEMIA587 T3SS on McCall (unlike S. fredii USDA257) and siratro (as for other T3SS-utilizing rhizobia), on some legumes an active T3SS of SEMIA587 appeared to increase the number (and the speed of formation) of nodules. A T3SS host-dependent phenotype has also been observed with B. elkanii USDA61 and derivative rhcC2 and rhcJ mutants (Okazaki et al., 2009), where nodule formation differs according to the cultivar of soybean (Hill or Clark in that case) or Vigna radiata (CN36 or KP31). With M. atropurpureum, the T3SS mutant strains induced fewer but larger nodules than the wild-type strain (Okazaki et al., 2009).

The B. elkanii USDA61 rhcC2 and rhcJ mutants blocked Nop secretion alone. In the case of SEMIA587, although the ttsI mutant could not secrete Nops, an alternative possibility for the plant phenotypes that we cannot rule out is that Tsl in SEMIA587 might regulate other symbiotically relevant genes besides the T3SS. As well as the T3SS, Tsl of Rhizobium sp. NGR234 also activates the production of a new lipopolysaccharide species which is a key signal for nodule formation by some legumes (Marie et al., 2004). As no further information for the genome of SEMIA 587 is available, this hypothesis remains to be verified.

In conclusion, our work demonstrates the importance of the T3SS of SEMIA587 in the establishment of symbiosis with legumes. As for B. elkanii USDA61, the transcriptional mechanisms for the activation of the T3SS differ from the rhizobial paradigm. In USDA61 no flavonoid induction is observed, whereas for SEMIA587 the potential for a flavonoid-induced positive feedback activation of the T3SS exists. SEMIA587 is an important inoculant for the Brazilian soybean crop and we demonstrate that functions of the SEMIA587 TtsI have a positive role in the formation of nodules on one crop cultivar of soybean, probably by activating the T3SS. Future work will determine whether nodulation of other Brazilian soybean varieties is similarly improved, which rhizobial effectors are responsible for the improvement, and whether there is continual production of these effectors during the SEMIA587–soybean interaction.
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