

The Type III Secretion System of *Bradyrhizobium japonicum* USDA122 Mediates Symbiotic Incompatibility with *Rj2* Soybean Plants

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The *rhcJ* and *ttsI* mutants of *Bradyrhizobium japonicum* USDA122 for the type III protein secretion system (T3SS) failed to secrete typical effector proteins and gained the ability to nodulate *Rj2* soybean plants (Hardee), which are symbiotically incompatible with wild-type USDA122. This suggests that effectors secreted via the T3SS trigger incompatibility between these two partners.

Leguminous plants form root nodule symbioses with nitrogen-fixing soil bacteria from the order *Rhizobiales*. Association of *Bradyrhizobium japonicum* with agronomically important soybean cultivars is widespread throughout the world, including the United States (1), Brazil (2), and Japan (3). Several soybean genotypes restrict nodulation with specific rhizobial strains; these include *Rj2*, *Rj3*, *Rj4*, and *Rfg1* (allelic to *Rj2*), which behave as single dominant genes (4, 5). In particular, the soybean cultivar Hardee (*Rj2*) is incompatible with *B. japonicum* USDA122 (6, 7). The *Rj2/Rfg1* gene encodes a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of proteins, involved in resistance to microbial pathogens (5). Different amino acid substitutions in *Rj2* and *Rfg1* restrict nodulation with *B. japonicum* USDA122 and *Sinorhizobium fredii* USDA257, respectively (5). The type III protein secretion system (T3SS) mutant of *S. fredii* USDA257, which is unable to secrete effector proteins (8), is capable of nodulating *Rfg1* soybean plants. Thus, T3SS effectors may negatively affect symbiosis with *Rfg1* soybean plants (5). Since the cell surface components of *B. japonicum* Is-1 and *S. fredii* USDA257 were suggested to be involved in the incompatibility with *Rj2* soybean plants (8, 9), the question still remains how *Rj2* prevents effective nodulation. Thus, the aim of the present work was to test whether functional T3SS causes the symbiotic incompatibility between *B. japonicum* USDA122 and *Rj2* soybean plants.

The bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. *B. japonicum* cells were grown at 30°C in HM salt medium (supplemented with 0.1% arabinose and 0.025% [wt/vol] yeast extract [Difco, Detroit, MI]) for DNA isolation and plant inoculation (see Table S2 in the supplementary material) or at 28°C in arabinose gluconate (AG) medium (1) for analysis of extracellular proteins (10). *Escherichia coli* cells were grown at 37°C in Luria-Bertani medium (11). Isolation of plasmids, DNA ligation, and transformation of *E. coli* were performed as described by Sambrook et al. (11). Total DNA of *B. japonicum* USDA122 was prepared as described previously (12). The draft genomic sequences were obtained by using a 454 GS-FLX Titanium pyrosequencer (454 Life Sciences, Branford, CT) (13).

A 3.7-kb DNA fragment carrying *rhcJ* was excised from prb01211 (14) with EcoRI and inserted into the EcoRI site of

pK18mob, resulting in pSE11 (see Table S1 in the supplemental material). A 2.0-kb Ω cassette derived from pHP45 (15) was inserted into a MluI site within *rhcJ* in pSE11 (Fig. 1B). A 3.5-kb DNA fragment carrying *ttsI* was excised from brp13612 (14) with HindIII and inserted into the HindIII site of pK18mob, resulting in pSE12 (see Table S1). The Ω cassette was then inserted into a BstXI site within *ttsI* in pSE12 (Fig. 1C). Triparental mating with USDA122 and pRK2013 was conducted on HM agar plates using pSE11 and pSE12, resulting in 122 Δ rhcJ and 122 Δ ttsI, respectively (see Table S1). Double-crossover events were verified by PCR.

B. japonicum USDA122 and the mutant 122 Δ rhcJ were grown at 28°C for 48 h in 500 ml AG medium in the absence or in the presence of genistein (10 μ M). Extracellular proteins were prepared as described previously (10). The protein samples were subjected to SDS-PAGE (5 to 20% gradient gel in Tris-glycine buffer). Small pieces of the Coomassie-stained gel corresponding to bands of interest were digested with trypsin. Peptides were analyzed by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS/MS) (TOF/TOF 5800 system; AB Sciex, Foster City, CA). Proteins were identified by the MS-Fit software program (<http://prospector.ucsf.edu/prospector/mshome.htm>) with the USDA110 database as a reference (14). The mass fragments were further identified by MS/MS analysis.

Surface-sterilized soybean seeds (*Glycine max* cultivars Hardee [*Rj2*] and Lee [*rj2*]) were germinated in sterile vermiculite for 4 days at 25°C. The seedlings were then transplanted into Leonard jar pots (one plant per pot) with sterile vermiculite and nitrogen-free nutrient solution (16) and inoculated with *B. japonicum* (1×10^7 cells per seedling). Plants were grown in a phytotron (Koito Industries, Tokyo, Japan) for 30 to 40 days at 25/20°C under a 16-h light/8-h dark cycle (16).

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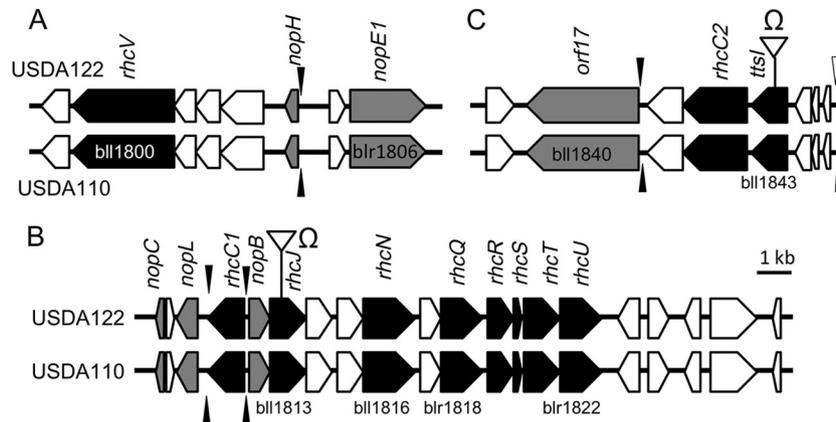


FIG 1 Comparison of gene clusters for the type III secretion system (T3SS) in the USDA122 and USDA110 strains of *Bradyrhizobium japonicum*. Three major loci within the *tts* clusters are shown (panels A, B, and C). The open reading frames encoding the T3SS structural components and putative effector proteins are shown in black and gray, respectively. The positions of *nod* and *tts* boxes are indicated by open and black arrowheads, respectively. The insertion positions of Ω cassettes (see Table S1 in the supplemental material) in USDA122 are also shown.

Genes implicated in T3SS were organized similarly in the genomes of *B. japonicum* USDA122 and USDA110 (Fig. 1) (17). Their DNA sequences were also highly conserved. In particular, the amino acid sequences of the regulatory (TtsI) and structural (RhcV, RhcC1, RhcC2, RhcJ, RhcN, RhcQ, RhcR, RhcT, RhcU, and RhcS) components of T3SS were identical between USDA122 and USDA110, although synonymous nucleotide substitutions occurred in genes encoding four T3SS components (RhcV, RhcC2, RhcR, and RhcT) (see Table S3 in the supplemental material). In addition, a *nod* box upstream of *ttsI* and 5 *tts* boxes were highly conserved between USDA122 and USDA110 (Fig. 1) (17, 18). In USDA110, the T3SS gene cluster can be induced by flavonoids and is controlled via the following regulatory cascade: flavonoid signal \rightarrow NodD1 \rightarrow *nod* box \rightarrow TtsI \rightarrow *tts* box (17–19).

To examine whether T3SS is active in USDA122, we compared extracellular proteins in wild-type USDA122 and in 122 Δ rhcJ cultures (since *rhcJ* is required for a functional T3SS in USDA110 [17, 20]). Since flavonoids induce T3SS, we also analyzed extracellular proteins in cell cultures treated or not treated with the isoflavone genistein. Several bands were unique to genistein-induced wild-type USDA122 (Fig. 2).

The excised pieces of gel corresponding to individual bands (a to e in lanes 1 to 3 in Fig. 2) were subjected to MALDI-TOF-MS/MS analysis (see Fig. S1 to S5 in the supplemental material). The peptides derived from ORF17 (blr1840) were detected in genistein-induced wild-type USDA122 (Fig. 2, WT+) and to a lesser extent in untreated wild-type USDA122 (WT–) but not in the genistein-induced 122 Δ rhcJ mutant (Δ rhcJ+) (band a in Fig. 2). Band “a” also contained the blr4853 protein, but the intensities of its peptide signals were similar in all 3 samples (see Fig. S1). Interestingly, a typical *tts* box was present upstream of *orf17* in USDA122 and *blr1840* in USDA110 (Fig. 1C). These results suggested that ORF17 is a T3SS-dependent secreted protein in USDA122.

Based on similar analyses, GunA2 (blr1656), NopE1 (blr1806), NopE2 (blr1649), NopF (blr18201), and NopM (blr1904) were identified as T3SS-dependent secreted proteins in USDA122 (Fig. 2; see also Fig. S2 to S5 in the supplemental material). Most of these proteins (GunA2, NopE1, NopE2, and NopF) were previously identified in USDA110 (20). NopE1 is a true USDA110 ef-

factor, because it translocates into the host cells and affects nodulation efficiency on *Vigna radiata* (21). The peptides of band “d” (approximately 28 kDa) were derived from the C-terminal half of the NopE1 protein (51 kDa) (see Fig. S4), consistent with a self-cleavage activity of NopE1 (21). Overall, these results showed that 122 Δ rhcJ failed to secrete typical effector proteins, indicating that the T3SS is active in *B. japonicum* USDA122 and is inactivated by the loss of RhcJ.

Next, we examined the symbiotic phenotypes of Hardee with 122 Δ rhcJ and 122 Δ ttsI. Both T3SS knockout mutants induced

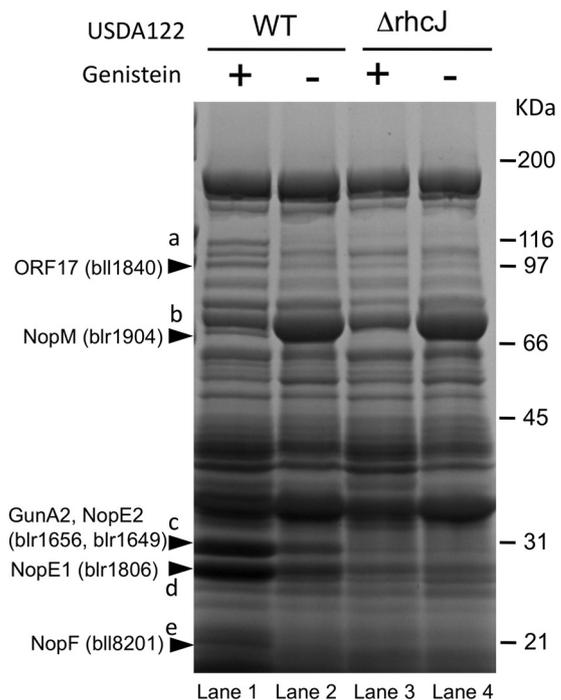


FIG 2 Secreted proteins of the wild-type strain (WT) and the *rhcJ* mutant (Δ rhcJ) of *B. japonicum* USDA122 in the presence (+) or absence (–) of 10 μ M genistein. Proteins induced by genistein in wild-type USDA122 but not in the *rhcJ* mutant (bands a to e) were identified by MALDI-TOF-MS/MS analysis (see Fig. S1 to S5 in the supplemental material).

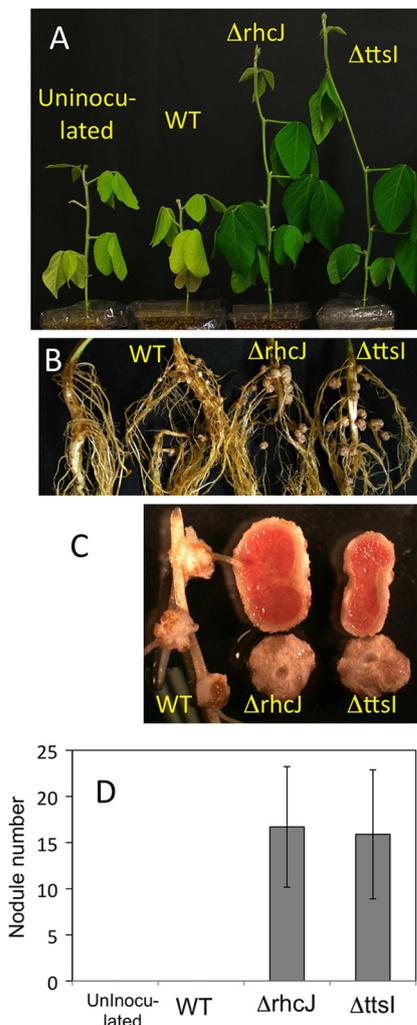


FIG 3 Symbiotic phenotypes of *Rj2* soybean cultivar Hardee inoculated with the wild-type strain (WT) and the *rhcJ* ($\Delta rhcJ$) and *ttsI* ($\Delta ttsI$) mutants of *B. japonicum* USDA122. Panels A, B, C, and D show plant growth, root systems, nodules, and nodule numbers, respectively. Error bars indicate standard deviations ($n = 7$ to 10).

normal nodules with red leghemoglobin in nodule sections, and the host plants had normal green coloring (Fig. 3). In contrast, wild-type USDA122 failed to induce normal nodules, and plants had yellow shoots (Fig. 3). Wild-type USDA122 occasionally induced host cell division and formation of bump structures without bradyrhizobial cells at the lateral root junctions (WT in Fig. 3C) (5). Wild-type USDA122 and both T3SS knockout mutants induced normal nodules on Lee (*rj2*) soybean plants (see Fig. S6 in the supplemental material).

Symbiotic nitrogen fixation in Hardee was evaluated by the acetylene reduction assay. Acetylene-reducing activity (ARA) was detected only in the root systems inoculated with the T3SS knockout mutants. ARA levels were similar in root systems inoculated with USDA110 and both USDA122 mutants (see Fig. S7 in the supplemental material). No ARA was detected upon inoculation with wild-type USDA122. Therefore, the loss of T3SS in USDA122 restored nitrogen fixation and nodulation during its interaction with Hardee.

The present work demonstrated that T3SS mediates symbiotic incompatibility between *Rj2* soybean Hardee plants and *B. japonicum* USDA122. Since T3SS functions to secrete effectors during early stages of rhizobial infection (19), it is critical to identify proteins that induce symbiotic incompatibility between *Rj2* soybean plants and USDA122. Such effectors can be anticipated to directly or indirectly interact with the *Rj2* variants of the *Rj2/Rfg1* gene product in soybean plants to restrict nodulation with *B. japonicum* USDA122 (5). A preliminary survey of putative effectors around the T3SS gene cluster showed that the amino acid substitutions of three gene (*nopE1*, *nopP1*, and *orf17*) products occurred between *B. japonicum* USDA122 and USDA110 (see Table S4 in the supplemental material).

Little is known about the ecological and evolutionary implications of exploitation of pathogenic secretion systems and effectors by rhizobia for symbiotic purposes (5, 19). Modern *rj2* soybean cultivars often show lower nitrogen-fixing activity when inoculated with USDA122 than with USDA110 (3). Thus, it would be fascinating to speculate that *Rj2* soybean plants may have evolved incompatibility with *B. japonicum* USDA122 to prevent its inferior symbionts from using the pathogenic systems. In this regard, the “zigzag” model of pathogen-plant interactions (22) might be adopted to describe rhizobium-plant interactions, because only two amino acid substitutions in and around the NBS domain of the *Rj2/Rfg1* gene product define the genetic basis of *Rj2* soybean plant incompatibility with *B. japonicum* USDA122 (5).

Nucleotide sequence accession numbers. The contig sequences relevant to T3SS gene clusters were identified by BLASTN searches and deposited in the DDBJ/EMBL/GenBank database under accession numbers AB758605 to AB758607.

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