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The *Sinorhizobium fredii* HH103 Type III secretion system effector NopC blocks nodulation with *Lotus japonicus* Gifu.

Running title: NopC blocks nodulation with *Lotus japonicus*.

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Highlight: The *Sinorhizobium fredii* HH103 Rif^R T3SS effector NopC blocks nodulation with *Lotus japonicus* Gifu. The infection-blocking event occurs at early stages of the infection process.

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Abstract

The broad host-range bacterium *Sinorhizobium fredii* HH103 cannot nodulate the model legume *Lotus japonicus* Gifu. This bacterium possesses a Type III secretion system (T3SS), a specialized secretion apparatus used to deliver effector proteins (T3E) into the host cell cytosol to alter host signaling and/or suppress host defence responses to promote infection. However, some of these T3E are recognized by specific plant receptors and hence trigger a strong defence response to block infection. In rhizobia, T3E are involved in nodulation efficiency, host-range determination and, in some cases, directly activate host symbiosis signalling in a Nod factor-independent manner.

In this work, we show that HH103 Rif^R T3SS mutants, unable to secrete T3E, gain nodulation with *L. japonicus* Gifu through infection threads (IT), suggesting that plant recognition of a T3E could block the infection process. To identify the T3E involved, we performed nodulation assays with a collection of mutants affected in secretion of each T3E identified in HH103 Rif^R so far. The *nopC* mutant could infect *L. japonicus* Gifu by IT invasion and switch the infection mechanism in *L. burttii* from intercellular infection to IT formation. *L. japonicus* gene expression analysis indicated that the infection-blocking event occurs at early stages of the symbiosis.

Keywords: Type III secretion system (T3SS); effector; *Lotus japonicus*; nodulation; *Sinorhizobium fredii*; *Rhizobium*-legume symbiosis; infection thread; plant defense.

Introduction

Soil bacteria, commonly known as rhizobia, can establish symbiosis with leguminous plants, developing new plant root organs called nodules. Within these nodules, rhizobia differentiate into bacteroids able to reduce atmospheric nitrogen to ammonia, which is assimilated by the plant (Oldroyd *et al.*, 2011). The symbiosis is highly regulated and needs the interchange of symbiotic signals between both partners. At very early stages of the symbiotic process, the bacterial transcriptional regulator NodD recognises some phenolic compounds, called flavonoids, exuded by legume roots (Cooper, 2007). NodD, once activated by flavonoids, binds to specific promoter sequences called *nod* boxes (NB), inducing the expression of the nodulation genes (*nod* genes), which code for proteins necessary for Nod factors (NF) production and export (Gage, 2004). NF are specifically recognized by susceptible legumes, triggering both root hair curling and cortical cell division by activation of specific plant nodulation genes (Oldroyd, 2013). In fully compatible rhizobium-legume interactions, root hair deformation entraps rhizobia that, in most cases, enter into the root tissue through infection threads (IT). Then, rhizobia reach the cortex, multiply and colonize the intracellular spaces in root nodules (Oldroyd, 2013). However, there are other factors/molecules affecting symbiotic efficiency and nodulation host-range, such as surface polysaccharides or proteins secreted through the Type III secretion system (T3SS) (Downie, 2010).

Due to its sessile lifestyle, plants have developed a basal defence response that is induced upon recognition of conserved microbe-associated molecular patterns (MAMP) by specific plant extracellular receptors. This recognition results in MAMP-triggered immunity or MTI (Alfano and Collmer, 2004). The T3SS is used by pathogenic bacteria to deliver effector proteins (T3E) directly into the host cytoplasm to promote virulence through alteration of the plant cell metabolism and/or suppression of the MTI response

(Feng and Zhou, 2012; Macho and Zipfel, 2015). Plants, in turn, have evolved to recognize some T3E by specific resistance (R) proteins (Jones and Dangl, 2006; Duxbury *et al.*, 2016). Upon T3E recognition, R proteins elicit a plethora of defence responses collectively known as effector-triggered immunity (ETI). Elicitation of the ETI response is responsible of the incapacity of some rhizobial strains with a functional T3SS to nodulate some legume plants (Yasuda *et al*., 2016; Sugawara *et al*., 2018).

In rhizobia, proteins secreted through the T3SS are collectively known as nodulation outer proteins (Nops). Synthesis and secretion of Nops are controlled by the transcriptional regulator TtsI, whose expression is induced by NodD and flavonoids. TtsI binds to specific promoter sequences, called *tts* boxes (TB), located upstream of T3SS-related genes, activating their transcription. Hence, TtsI is the intermediary in the regulatory cascade between flavonoid-activated NodD and T3SS-related genes (Krause *et al*., 2002; López-Baena *et al*., 2008; Wassem *et al*., 2008). Nops are involved in nodulation efficiency and host-range determination but can also hijack nodulation signalling induced by NF and directly activate host symbiosis signalling (Okazaki *et al*., 2013; López-Baena *et al.*, 2016; Teulet *et al*., 2019).

Sinorhizobium fredii HH103 Rif^R, hereafter HH103 Rif^R, is a broad-host range bacterium able to nodulate dozens of legumes, including soybean, its natural host plant (Margaret *et al.*, 2011). Previous reports indicated that HH103 Rif^R only forms ineffective nodules in the model legume *L. japonicus* Gifu but nitrogen-fixing nodules in *L. burttii* (Sandal *et al.*, 2012). HH103 Rif^R invades *L. burttii* by a less evolved mechanism termed intercellular infection in contrast to its natural symbiont *Mesorhizobium loti* R7A (Madsen *et al*., 2010), recently reclassified as *M. japonicum* (Martínez-Hidalgo *et al*., 2016), which nodulates both *L. japonicus* Gifu and *L. burttii* by IT formation (Acosta Jurado *et al*., 2016). However, *L. japonicus* Gifu plants

inoculated with HH103 Rif^R mutants affected in the regulatory genes *nolR*, *nodD2*, and *syrM* are effectively nodulated through IT and, in *L. burttii*, the mechanism of infection changes from intercellular infection to IT formation (Acosta-Jurado *et al.*, 2019, 2020). Interestingly, in the presence of inducer flavonoids, these mutants synthetize higher concentrations of NF in comparison to the parental strain. This increase is due to higher levels of *nod* gene expression. Moreover, a double *nodD1*/*nodD2* mutant, which does not synthetize NF, fails to nodulate *L. japonicus* Gifu. All together, these findings suggest that the capacity of the HH103 Rif^R *nodD2*, *nolR* and *syrM* mutants to infect *L. burttii* and *L. japonicus* Gifu by IT infection is related to NF overproduction. With respect to T3SS-related genes, the expression of some but not all of these genes slightly increased in these mutants (López-Baena *et al.*, 2008; Acosta-Jurado *et al.*, 2019, 2020).

To date, nine putative T3E have been identified in HH103 Rif^R by genomic, transcriptomic, and proteomic studies: *gunA*, *nopC*, *nopD*, *nopI*, *nopL*, *nopM1*, *nopM2*, *nopP*, and *nopT* (Rodrigues *et al.*, 2007; Vinardell *et al.*, 2015; López-Baena *et al.*, 2016; Pérez-Montaño *et al.*, 2016). As expected, T3SS-dependent secretion of most of these proteins has been already confirmed (Rodrigues *et al.*, 2007; Jiménez-Guerrero *et al.*, 2015a, b, 2017).

In this work, we have investigated the symbiotic capacity of a collection of HH103 Rif^R T3E mutants with the model legume *L. japonicus* Gifu and *L. burtii*. We determined that T3SS mutants unable to secrete Nops induce the formation of nitrogen-fixing nodules in *L. japonicus* Gifu. These mutants infected both *Lotus* species through IT. Among all the HH103 Rif^R effectors, NopC was identified as the main responsible for the nodulation-blocking phenotype in *L. japonicus* Gifu.

Materials and methods

Microbial and molecular techniques

Bacterial strains and plasmids used in this work are listed in Table S1. *Sinorhizobium* and *Mesorhizobium* strains were grown at 28 ºC on tryptone yeast (TY) medium (Beringer, 1974) or yeast extract mannitol (YM) medium (Vincent, 1970). *Escherichia coli* strains were cultured on LB medium (Sambrook *et al*., 1989) at 37 ºC. When required, the media were supplemented with the antibiotics ampicillin (Ap, 100 µg ml^{-1}), rifampicine (Rif, 50 µg ml^{-1}), spectinomycin (Spc, 50 µg ml^{-1} for *Sinorhizobium* and 100 µg ml^{-1} for *E. coli*), kanamycine (Km, 60 µg ml^{-1} for *Sinorhizobium* and 30 µg ml^{-1} for *E. coli*), tetracycline (Tc, 2 µg ml^{-1} for *Sinorhizobium* and 10 µg ml^{-1} for *E. coli*), and gentamycine (Gm, 5 µg ml^{-1} for *Sinorhizobium* and 10 µg ml^{-1} for *E. coli*). Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by triparental conjugation as described by Simon (1984) using plasmid pRK2013 (Figurski and Helinski, 1979) as helper.

Recombinant DNA techniques were performed according to the general protocols of Sambrook *et al*. (1989). For hybridization, DNA was blotted to Hybond-N nylon membranes (Amersham, United Kingdom) and the DigDNA method of Roche (Switzerland) was employed following the manufacturer's instructions. PCR amplifications were performed as previously described (Pérez-Montaño *et al*., 2014). Primer pairs used for the amplification of the HH103 Rif^R genes are summarized in Table S2.

Construction of the *Sinorhizobium fredii* HH103 nodulation outer protein mutants and complemented strains

158 Mutants in the *nopC*, *nopI*, *nopL*, *nopP*, *gunA*, *ttsI*, and *rhcJ* genes, as well as their
159 respective complemented strains, were obtained previously (de Lyra *et al*., 2006; López-
160 Baena *et al*., 2008; Jiménez-Guerrero *et al*., 2015a, b, 2017, 2019).

161 To inactivate the HH103 Rif^{R} *nopD* gene, an internal fragment (1.8-kb) of *nopD* was
162 amplified using specific primers (Table S2). The resulting DNA fragment was cloned
163 into pGEM-T Easy (Promega, USA), yielding plasmid pGEM-T Easy::*nopD*. Plasmid
164 pHP45Ω was digested with *Bam*HI and the resulting 2-kb fragment carrying the
165 interposon was cloned into an unique *Bam*HI restriction site of pGEM-T Easy::*nopD*
166 located in the *nopD* gene, yielding plasmid pGEM-T Easy::*nopD*::Ω. The fragment
167 containing *nopD*::Ω was then subcloned into plasmid pK18*mob* (Schäfer *et al*., 1994)
168 using suitable enzymes, obtaining plasmid pK18*mob*::*nopD*::Ω. Finally, this plasmid
169 was used for the homogenotization of the mutated version of the *nopD* gene in HH103
170 Rif^{R}. The double recombination event was confirmed by hybridization (Fig. S1).

171 To construct the mutant in *nopT*, a DNA fragment containing the HH103 Rif^{R} *nopT*
172 gene and its upstream *tts* box was amplified using specific primers (Table S2). The
173 amplified DNA fragment was cloned into pBlueScriptII SK (Stratagene, USA)
174 obtaining plasmid pBlueScriptII SK::*nopT*. Plasmid pHP45Ω was digested with *Sma*I
175 and the resulting 2-kb fragment carrying the interposon was cloned into an unique *Sph*I
176 restriction site of pBlueScriptII SK::*nopT* located in the *nopT* gene, yielding plasmid
177 pBlueScriptII SK::*nopT*::Ω. The fragment containing *nopT*::Ω was subcloned into
178 plasmid pK18*mob* using the appropriate restriction enzymes, obtaining plasmid
179 pK18*mob*::*nopT*::Ω. Finally, this plasmid was used for the homogenotization of the
180 mutated version of the *nopT* gene in HH103 Rif^{R}. The double recombination event was
181 confirmed by hybridization (Fig. S1).

The nucleotide sequences of the HH103 Rif^R *nopM1* and *nopM2* genes and about 3-kb upstream and downstream the two copies of *nopM* are exactly the same. Therefore, for the construction of the double *nopM1/nopM2* mutant, first a random deletion of one of the copies of *nopM* was carried out. This deletion was constructed by overlap extension polymerase chain reaction (Griffitts and Long, 2008) using two pairs of primers (Table S2). The final DNA fragment obtained of about 1.7-kb, with the HH103 Rif^R *nopM* gene deleted, was cloned into plasmid pK18*mobsacB* (Schäfer *et al*., 1994), obtaining plasmid pK18*mobsacB*::Δ*nopM*. This plasmid was then used for the homogenotization of the mutated version of one of the *nopM* genes in HH103 Rif^R. The deletion event was confirmed by PCR and DNA sequencing. On the other hand, a second plasmid was constructed by insertion of the *lacZ*-Gm^R cassette from plasmid pAB2001 (Becker *et al*., 1995) into the unique *Eco*RV restriction site of *nopM* gene and further cloning in pK18*mob* (pK18*mob*::*nopM*::*lacZ*-Gm^R) following the methodology described above. This plasmid was used for the homogenotization of the mutated version of the second copy of the *nopM* gene in the HH103 Rif^R Δ*nopM* mutant background. The double recombination event was confirmed by hybridization (Fig. S1).

For complementation *in trans* of the mutations in *nopD*, *nopT*, and *nopM1/nopM2*, DNA fragments containing these genes and their respective promoter sequences (*tts* boxes) were cloned into the high-copy plasmids pBBR1MCS-5 or pMP92 (Spaink *et al.*, 1987; Kovach *et al.*, 1995). For this purpose, pGEM-T Easy and pBlueScriptII SK plasmids harbouring these genes and their respective *tts* boxes were digested with the appropriate restriction enzymes and the DNA fragments containing *nopD*, *nopT* and *nopM* were cloned into plasmids pBBR1MCS-5 or pMP92, previously digested with the same enzymes, obtaining plasmids pBBR1MCS-5::*nopD*, pMP92::*nopT* and

pMP92::*nopM*. These plasmids were transferred by conjugation to their respective mutant strains to obtain the *in trans* complemented strains.

Plant assays

Nodulation tests on *Lotus japonicus* (Regel) K. Larsen ecotype Gifu and *L. burttii* Borsos were carried out as described by Acosta-Jurado *et al.* (2016). Briefly, *Lotus* seeds were surface sterilized as previously described (Heckmann *et al.*, 2011). Four germinated seeds were transferred to each Leonard jar, which were composed of an upper vessel containing 220 ml of washed and sterilized vermiculite supplemented with Rigaud and Puppo's nutrient solution (Rigaud and Puppo, 1976) and a lower recipient filled with 180 ml (pH 7.0) of the plant nutrients solution. Each Leonard jar was inoculated with about 10^8 bacteria. Inoculated *Lotus* plants were grown for 6-9 weeks in a plant-growth chamber with a 16 hour-photoperiod at 21 ºC in the light and 18 ºC in the dark. Detection of nitrogenase activity in *Lotus* nodules was carried out by acetylene reduction assays (ARA) as previously described (Buendía-Clavería *et al.*, 1986). At least two independent experiments were carried out for each inoculant/*Lotus* species combination tested in this work. Fig. S2 and S3 show representative jars, roots and nodules from *L. japonicus* Gifu and *L. burttii* nodulation tests.

Microscopy analysis

L. japonicus Gifu and *L. burttii* plants were grown on ¼ B&D medium (Kelly *et al.*, 2018) in 12 cm square plates at 21 °C with a 16 hour-photoperiod at 21 ºC in the light and 18 ºC in the dark. To observe plant IT, *Lotus* seedlings grown in square plastic dishes were inoculated with *M. loti* R7A and the *S. fredii* strains HH103 Rif^R, HH103 Rif^R *nolR*::*lacZ*-Gm^R, HH103 Rif^R *rhcJ*::Ω, HH103 Rif^R *ttsI*::Ω and HH103 Rif^R Δ*nopC*

carrying the DsRed fluorescent marker at an OD_{600} = 0.5 (about 10^8 bacteria) six days after germination. Twenty-one days after inoculation, the IT were observed using a Zeiss fluorescence ApoTome.2 microscope (Germany). Image pictures were processed and merged using Zen 2.6 blue edition and Image J software. Twenty one-day old *L. japonicus* Gifu and *L. burttii* nodules formed in plants inoculated with HH103 Rif^R, HH103 Rif^R *rhcJ*::Ω, HH103 Rif^R *ttsI*::Ω, and HH103 Rif^R Δ*nopC* carrying the DsRed fluorescent marker were embedded in 6% agarose in water and sliced in thick layer sections (30 µm) using a Leica VT 1000S vibratome. Sections of nodules were stained with 0.04% calcofluor and observed using Zeiss ApoTome.2 fluorescence microscope as previously described (Kawaharada *et al.*, 2017).

Identification of Nod factors

Purification and analyses of NF produced by HH103 Rif^R and HH103 Rif^R *ttsI*::Ω grown in B^- minimal medium supplemented with 3.6 µM genistein were performed as described previously (Vinardell *et al.*, 2004b; Acosta-Jurado *et al.*, 2016, 2019). HPLC and High Resolution Mass Spectrometry (HPLC-HRMS) were performed using a Thermo Scientific liquid chromatography system consisting of a quaternary UHPLC Dionex Ultimate 3000 SD, connected to a quadrupole-orbitrap QExactive hybrid mass spectrometer (Thermo-Fisher Scientific, USA) with HESI ionization probe. Separation was performed on a C18 column Tracer Excel 120 ODSB C18 (2.1 x 200 mm, 5 µm) (Teknokroma, Spain). Injection volume was 10 µL and flow rate was 0.3 ml min-1. Two different solvents were used as a mobile phase: Solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The elution profile was 30% B (30 min), linear up to 30% B (15 min), 100% B (2 min), linear up to 30% B (3 min), and isocratic for 5 min (30% B). The method was acquired in positive mode by full

range acquisition covering the m/z 800-1,700 and 70,000 of resolution. AGC 106 and maximum IT 10 ms. HESI source parameters were: spray voltage 3,500 V, capillary temperature 256 ºC, sheath and auxiliary gas flow (N_2) 47.5 and 11.25 (arbitrary units) and probe heater temperature 412 ºC. Xcalibur software was used for instrument control and data acquisition TraceFinder 3.3 software was used for data analysis. In a recent work it has been stablished that HPLC-HRMS signal areas could actually reflect the relative abundance of each NF (Acosta-Jurado *et al.*, 2019). Establishing correlations between signal areas of any particular NF and its relative abundance in wild-type cultures is perfectly valid, so that quantitative variations for any individual NF can be estimated. NF samples used to construct Table 3 were analysed at the same time to minimize variations due to experimental conditions.

RNA isolation, cDNA synthesis and quantitative RT-PCR

L. japonicus Gifu plants were inoculated with *M. loti* R7A and with the *S. fredii* strains HH103 Rif^R, HH103 Rif^R *ttsI*::**Ω**, HH103 Rif^R Δ*nopC*, and HH103 Rif^R *nolR*::**Ω** at an OD_{600} = 0.5. RNA was extracted at 10 days after inoculation (dai) using TRI reagent (Sigma-Aldrich, USA) plus Directzol RNA miniprep kit (Zymo Research, USA) and RNAase Free DNA Set (Qiagen, USA) according to manufacturer's instructions. Two independent RNA extractions were performed. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and the integrity of the RNA was assayed on 2% agarose gels. cDNA was synthesized using the PrimeScript RT Reagent kit (Takara, Japan) according to the manufacturer's instructions.

The expression of the *L. japonicus LjNSP1*, *LjNSP2*, *LjNIN*, *LjENOD40*, *LjLb3*, and *LjPR1* genes was quantified using quantitative RT-PCR (*q*RT-PCR) as previously

281 described (López-Baena *et al*., 2009). Primers used are listed on Table S2. Briefly, the
282 reactions were performed in a 10 µl final volume containing 25 ng of cDNA, 0.6 pmol
283 of each primer and 5 µl of FastStart SYBR Green Master Mix (Roche, Switzerland).
284 PCR was conducted on a Light Cycler 480 II (Roche, Switzerland) with the following
285 conditions: 95 ºC, 10 min; 95 ºC, 30 sec; 50 ºC, 30 sec; 72 ºC, 15 sec; 45 cycles,
286 followed by the melting curve profile from 65 to 95 ºC to verify the specificity of the
287 reaction. The threshold cycles (Ct) were determined with the Light Cycler 480 II
288 software and the individual values for each sample were generated by averaging three
289 technical replicates that varied less than 0.5 cycles. Expression was calculated relative
290 to not inoculated plants. The *L. japonicus LjUBI2* gene was used as an internal control
291 to normalize gene expression. At least two independent *q*RT-PCR reactions were
292 performed in triplicates for each RNA extraction.
293

Results

Sinorhizobium fredii HH103 Rif^R *ttsI* and *rhcJ* mutants gain the capacity to induce the formation of nitrogen-fixing nodules with *Lotus japonicus* Gifu

As previously mentioned, HH103 Rif^R effectively nodulates *L. burttii* but only induces the formation of ineffective nodules or macroscopic root outgrowths (MRO) on *L. japonicus* Gifu (Sandal *et al*., 2012; Fig. S2A). The symbiotic capacity of two HH103 Rif^R mutants unable to assemble a functional secretion system (*ttsI*::Ω, a mutant in the gene that codes for the transcriptional regulator of the T3SS, and *rhcJ*::Ω, a mutant in a gene encoding an essential component of the T3SS apparatus) with *L. japonicus* Gifu was investigated to assess the role of the HH103 Rif^R T3SS in this symbiotic interaction. Both HH103 Rif^R *ttsI*::Ω and HH103 Rif^R *rhcJ*::Ω mutants developed nitrogen-fixing nodules (Nod$^+$/Fix$^+$) and formed pink nodules not only with *L. burttii* but also with all *L. japonicus* Gifu plants tested (Tables 1 and 2; Fig. S3). Complementation of the mutation in *ttsI* (the mutation in *rhcJ* cannot be complemented by unknown reasons; de Lyra *et al.*, 2006; López-Baena *et al.*, 2008) partially restored the symbiotic phenotype with *L. japonicus* Gifu, with intermediated values in the number of nodules (11.1 ± 1.2 nodules/plant), shoot fresh weight (60.5 ± 10.2 mg/plant), and ARA (12.43 ± 1.47 nmoles ethylene/plant hour) in comparison to values obtained with both the parental strain (0 ± 0 nodules/plant, 12.8 ± 0.8 mg/plant and 0 ± 0 nmoles ethylene/plant, respectively) and the *ttsI* mutant (25.1 ± 1.4 nodules/plant, 106.8 ± 9.9 mg/plant, and 30.47 ± 3.45 nmoles ethylene/plant hour, respectively) (Table 1). These findings suggest a correlation between the absence of a functional T3SS and the acquired capacity to develop effective symbiosis with *L. japonicus* Gifu. On the other hand, nodulation assays with *L. burttii* showed a slight increase in the symbiotic parameters analysed (number of nodules and shoot fresh weight) when plants were

inoculated with HH103 Rif^R *ttsI*::Ω (17 ± 4.7 nodules/plant and 81.7 ± 14.9 mg/plant, respectively) but not with HH103 Rif^R *rhcJ*::Ω (12.5 ± 2.1 nodules/plant and 55.6 ± 14.6 mg/plant, respectively) in comparison with the wild-type strain (10.6 ± 3.1 nodules/plant and 41.1 ± 5.2 mg/plant, respectively). Complementation of the mutation in *ttsI* restored the symbiotic phenotype (12.6 ± 3.9 nodules/plant and 67.4 ± 26.2 mg/plant, respectively) (Table 2; Fig. S3).

The *Sinorhizobium fredii* HH103 Rif^R *ttsI* and *rhcJ* mutants infect *Lotus japonicus* Gifu through infection threads

Mutants affected in the regulatory genes *nolR*, *nodD2* or *syrM* gain nodulation with *L. japonicus* Gifu through epidermal IT formation. Besides, in the symbiotic interaction with *L. burttii*, the mechanism of infection switches from intercellular infection to IT formation (Acosta-Jurado *et al.*, 2016, 2019, 2020). These results prompted us to investigate how both T3SS defective mutants (HH103 Rif^R *ttsI*::Ω and HH103 Rif^R *rhcJ*::Ω) infect *L. japonicus* Gifu and *L. burttii* roots. *Lotus* plants inoculated with the *ttsI* or *rhcJ* mutants carrying plasmid pFAJDsRed (Kelly *et al*., 2013) were analysed by epifluorescence microscopy. Both *M. loti* R7A (pFAJDsRed) and HH103 Rif^R *nolR*::*lacZ*-Gm^R (pFAJDsRed) were used as positive controls for bacterial invasion through IT. HH103 Rif^R (pFAJDsRed) was employed as a negative control of infection mediated by IT (Acosta-Jurado *et al.*, 2019). As shown in Fig. 1A, epidermal IT were observed in *L. japonicus* Gifu roots inoculated with both HH103 Rif^R *ttsI*::Ω and *rhcJ*::Ω 21 dai as well as in plants inoculated with controls *M. loti* R7A and the HH103 Rif^R *nolR* mutant. Despite IT were not detected in *L. japonicus* Gifu roots in the presence of the parental strain HH103 Rif^R, cells were detected in curled root hairs forming microcolonies (Fig. 1A). Similarly, at 21 dai, all *L. burttii* plants inoculated

with both T3SS mutants showed epidermal bacterial infection through IT (Fig. 1A). Moreover, at 21 dai, central nodule tissues of both *Lotus* species were extensively infected by both HH103 Rif^{R} *ttsI*::Ω and HH103 Rif^{R} *rhcJ*::Ω, connecting this area with the outer nodule cortex through IT (Fig. 1B). These results indicate that the presence of a functional T3SS in HH103 Rif^{R} prevents the formation of IT as well as further nodule invasion in *L. japonicus* Gifu (Fig. 1, Fig. S2B).

The *Sinorhizobium fredii* HH103 Rif^{R} *ttsI* mutant does not show a general overproduction of Nod factors

In the presence of genistein, an isoflavone that strongly activates the transcription of the HH103 Rif^{R} *nod* genes (Vinardell *et al*., 2004a), the HH103 Rif^{R} *nolR*, *nodD2*, and *syrM* mutants synthetize higher amounts of NF than the parental strain by increasing *nod* gene expression (Acosta-Jurado *et al.*, 2019, 2020). Despite the fact that the expression of genes related to NF production does not change in the HH103 Rif^{R} *ttsI*::Ω mutant upon flavonoids induction (Pérez-Montaño *et al*., 2016), we investigated whether the NF produced by the mutant strain were quantitatively and/or qualitatively different from the NF synthesized by the parental strain when bacterial cultures were induced with genistein. HPLC followed by High Resolution Mass Spectrometry (HPLC-HRMS/MS) detected 44 different NF in HH103 Rif^{R} cultures in the presence of genistein (Table 3). Values of signal areas scored by the mass spectrometer for the same particular NF can be used to compare the production of these molecules among tested strains upon the same treatment (Acosta-Jurado *et al.*, 2019). Interestingly, 53 different NF were produced by HH103 Rif^{R} *ttsI*::Ω in the presence of genistein, 35 of them also detected in cultures of the parental strain HH103 Rif^{R} (Table 3). Although some common NF were detected at higher or lower concentrations (9 and 7 NF, respectively)

in the *ttsI* mutant with respect to those produced by HH103 Rif^R, most of them were detected at similar quantities in both bacterial cultures induced with this isoflavone. Interestingly, the HH103 Rif^R *ttsI, nolR, nodD2,* and *syrM* mutant strains only shared the overproduction of four NF (Table S3), suggesting that the gained capacity to nodulate *L. japonicus* Gifu shown by the T3SS mutant and the mutants in the other regulatory genes could be determined by different biological processes.

The *Sinorhizobium fredii* HH103 Rif^R effector NopC is the main determinant for the nodulation-blocking phenotype in *Lotus japonicus* Gifu

We have previously shown that HH103 Rif^R T3SS defected mutants gain the capacity to effectively nodulate *L. japonicus* Gifu through IT in a NF overproduction-independent manner. This suggests that the recognition of a T3E by an R plant protein might be responsible for the infection-blocking phenotype. To identify possible T3E involved in this recognition, we carried out nodulation assays in both *Lotus* species with a collection of mutants affected in the secretion of every putative effector already identified in HH103 Rif^R. Mutants HH103 Rif^R *nopD*::Ω, HH103 Rif^R Δ*nopI*, HH103 Rif^R *nopL*::Ω, HH103 Rif^R Δ*nopM1-nopM2*::*lacZ*-Gm^R, and HH103 Rif^R *nopT*::Ω, did not induce the formation of nodules or induced ineffective nodules in *L. japonicus* Gifu (Nod$^-$/Fix$^-$ or Nod$^{-/+}$/Fix$^-$) but established nitrogen-fixing symbiosis with *L. burttii* (Nod$^+$/Fix$^+$). In both *Lotus* species, no significant differences in the symbiotic parameters analysed were observed with respect to the parental strain, with the exception of the *nopI* mutant in *L. burttii*, which showed lower shoot fresh weight (19.6 ± 7.8 vs 41.1 ± 5.2 mg/plant, respectively) (Tables 1 and 2). In contrast, HH103 Rif^R mutants affected in *nopC*, *nopP*, and *gunA* could develop nitrogen-fixing nodules not only with *L. burttii* but also with *L. japonicus* Gifu (Tables 1 and 2; Fig. S3). However,

394 the number of nodules formed in *L. japonicus* Gifu by the HH103 Rif^R *nopP* and *gunA*
395 mutants was very low (0.61 ± 0.1 and 0.38 ± 0.09 nodules/plant, respectively) and the
396 number of nodulated plants was scarce. These plants poorly developed and reduced
397 levels of nitrogenase activity were detected (1.5 ± 0.48 nmoles ethylene/plant hour in
398 plants inoculated with HH103 Rif^R *nopP*::*lacZ*-Gm and 2.41 ± 0.59 nmoles
399 ethylene/plant hour in plants inoculated with HH103 Rif^R Δ*gunA*) (Table 1). In contrast,
400 the nitrogenase activity detected in nodules of *L. japonicus* Gifu inoculated with HH103
401 Rif^R Δ*nopC* (17.02 ± 1.69 nmoles ethylene/plant hour) was considerably higher (Table
402 1). Furthermore, shoot dry weight of *L. japonicus* Gifu plants inoculated with the *nopC*
403 mutant was 51.6 ± 5.4 mg, while that of plants inoculated with the *nopP* and the *gunA*
404 mutants was only 25.7 ± 2.2, and 18.4 ± 1.6 mg, respectively. Remarkably, all *L.*
405 *japonicus* Gifu plants inoculated with HH103 Rif^R Δ*nopC* developed nitrogen-fixing
406 nodules (8.13 ± 0.49 nodules/plant). Complementation of all mutations restored the
407 symbiotic phenotype with *L. japonicus* Gifu to that of the parental strain (Table 1).
408 These results clearly indicate that NopC is the main responsible for the nodulation-
409 blocking phenotype in *L. japonicus* Gifu, whereas NopP and GunA appear to play a
410 secondary or complementary role in the incompatibility of this symbiotic interaction.
411 On the other hand, *L. burttii* response to inoculation with mutants in *nopC*, *nopP*, and
412 *gunA* and their respective complemented derivatives was similar to that observed with
413 HH103 Rif^R (Table 2).

414 Finally, to investigate the infection mechanism of the *nopC* mutant, *L. japonicus*
415 Gifu and *L. burttii* plants inoculated with HH103 Rif^R Δ*nopC* (pFAJDsRed) were also
416 analysed by epifluorescence and confocal microscopy. As previously observed with the
417 *ttsI* and *rhcJ* mutants, IT were found both in epidermal root hairs and connecting the
418 outer nodule cortex with the infected zone in all *L. japonicus* Gifu and *L. burttii* plants

analysed (Fig. 1), confirming that in the absence of NopC, HH103 Rif^R invades both legumes through IT.

Sinorhizobium fredii HH103 Rif^R activates early but not late nodulation genes in *Lotus japonicus* Gifu

The expression of the *NSP1*, *NSP2*, *NIN*, and *ENOD40* plant genes is sequentially induced early after NF recognition and is associated with the formation of IT and nodules. Besides, the expression of *Lb3*, which codes for leghemoglobin, is strongly up-regulated when nitrogen is fixed within the nodule, in the late stages of the symbiotic process (Geurts *et al.*, 2005; Marsh *et al.*, 2007; Roy *et al.*, 2020). Results previously shown indicate that the HH103 Rif^R mutants in *ttsI*, *rhcJ* or *nopC* gain nodulation with *L. japonicus* Gifu and infect through IT. To determine whether inactivation of these genes correlated with higher expressions of early and late *L. japonicus* Gifu nodulation genes, the relative expression of *LjNSP1*, *LjNSP2*, *LjNIN*, *LjENOD40* and *LjLb3* was quantified at 10 dai by *q*RT-PCR. Strains HH103 Rif^R, HH103 Rif^R *ttsI*::Ω, HH103 Rif^R Δ*nopC*, HH103 Rif^R *nolR*::*lacZ*-Gm^R, and *M. loti* R7A were used as inoculants (Fig. 2). Very early nodulation genes (*LjNSP1* and *LjNSP2*) were activated in all treatments with respect to uninoculated plants. Whereas highest values were detected in plants inoculated with the *ttsI* mutant (about 13-fold for *LjNSP1* and 8-fold for *LjNSP2*), lowest values were observed when plants were inoculated with HH103 Rif^R or R7A (gene expression in a range from 2.3 to 5-fold) (Fig. 2A, B). Gene expression patterns among treatments changed when the expression of *LjNIN* and *LjENOD40* was analysed. Thus, in the case of *LjNIN*, the strongest up-regulation was detected when plants were inoculated with the *nolR* mutant (about 680-fold) and the *nopC* mutant (about 220-fold) (Fig. 2C). With respect to *LjENOD40*, gene expression was similar to uninoculated

444 controls when plants were inoculated with HH103 Rif^R. In the rest of treatments,
445 differences were significant with respect to uninoculated plants, with highest gene
446 expression levels in plants inoculated with the *nolR* mutant and R7A (7.5- and 5-fold,
447 respectively). Overall, these results suggest that HH103 Rif^R is inducing early
448 nodulation genes (*LjNSP1*, *LjNSP2*, and *LjNIN*) in *L. japonicus* Gifu, but the gene
449 activation regulatory cascade is later blocked and, as a consequence, the symbiotic
450 process progress stops. In fact, transcriptional levels of *LjENOD40* and *LjLb3* in plants
451 inoculated with HH103 Rif^R were indistinguishable to those detected in uninoculated
452 plants (Fig. 2D, E). By contrast, *LjENOD40* and *LjLb3* expression in plants inoculated
453 with the different bacterial genetic backgrounds able to induce the formation of
454 nitrogen-fixing nodules were strongly up-regulated (Fig. 2D, E).

455 Our previous results showed that the inactivation of the HH103 Rif^R T3SS negatively
456 affects *Glycine max* (soybean) nodulation in the early stages of the symbiotic process,
457 which correlates with a lower expression of *GmNIN* and *GmENOD40* genes. This
458 symbiotic phenotype seems to be the consequence of the impairment of the T3SS
459 mutant to suppress soybean defence responses triggered by HH103 Rif^R, which are
460 associated to the synthesis of the pathogenesis-related protein PR1 and the plant
461 hormone salicylic acid (Jiménez-Guerrero *et al.* 2015b). To determine whether the
462 inactivation of the T3SS affects the activity of the *L. japonicus* Gifu defence genes, the
463 expression of *LjPR1* was analysed in plants inoculated with all the rhizobial strains
464 previously tested. Interestingly, all plants inoculated with HH103 Rif^R or any of its
465 mutants activated the expression of the *LjPR1* gene with respect to uninoculated plants.
466 Highest fold-change values were detected in plants inoculated with HH103 Rif^R Δ*nopC*
467 and, especially, with HH103 Rif^R *ttsI*::Ω (about 8- and 12-fold, respectively). No
468 differences were observed among plants inoculated with HH103 Rif^R or HH103 Rif^R

469 *nolR*::*lacZ*-Gm^{R}. Finally, *L. japonicus* Gifu plants inoculated with the *M. loti* R7A
470 behaved as uninoculated controls (Fig. 2F). Overall, these results indicate that the T3SS
471 is suppressing defence responses associated to the pathogenesis-related protein PR1
472 elicited by HH103 Rif^{R} in *L. japonicus* Gifu. This defence response, however, is only
473 partially associated to secretion of NopC (Fig. 2F).

474 To better determine the expression of early nodulation genes at very early stages of
475 the symbiotic process, the relative expression of *LjNSP1, LjNSP2* and *LjNIN* was also
476 quantified at 12 hours after inoculation (hai) by *q*RT-PCR. Strains HH103 Rif^{R}, HH103
477 Rif^{R} *ttsI*::Ω, and HH103 Rif^{R} Δ*nopC* were used as inoculants (Fig. 3). At this early time
478 point, no significant differences were observed in the expression values of *LjNSP1*
479 among the different treatments. However, the expression of *LjNSP2* was significantly
480 higher in plants inoculated with HH103 Rif^{R} Δ*nopC* (about 2-fold) and, specially, with
481 the HH103 Rif^{R} *ttsI*::Ω mutant (about 5-fold), both compared with plants inoculated
482 with the parental strain. Likewise, plants inoculated with either HH103 Rif^{R} *ttsI*::Ω or
483 HH103 Rif^{R} Δ*nopC* showed a higher induction of the *LjNIN* gene (about 125- and 80-
484 fold, respectively) when compared with plants inoculated with HH103 Rif^{R} (about 15-
485 fold).
486

Discussion

HH103 Rif^R is a broad host-range rhizobial strain that forms ineffective nodules with *L. japonicus* Gifu (Sandal *et al*., 2012). As demonstrated by Acosta-Jurado *et al*. (2016), neither NF nor surface polysaccharides are the molecular signals involved in this symbiotic impairment. However, three HH103 Rif^R mutants, affected in the transcriptional regulators *nolR*, *nodD2*, and *syrM*, which overproduce NF, gain the capacity to nodulate through IT and develop effective symbioses with *L. japonicus* Gifu. In all these cases, the overproduction of NF correlates with a higher expression of the *nod* genes. Moreover, these mutations can also modify the way in which HH103 Rif^R infects *L. burttii* and hence switch from a primitive intercellular infection to a more evolved pathway using IT (Madsen *et al*., 2010; Ibañez *et al*., 2017; Acosta-Jurado *et al*., 2019, 2020). Overall, these results would suggest that higher amounts of NF are required for nodule formation and invasion through IT in these two *Lotus* species (Acosta-Jurado *et al*., 2016, 2019, 2020).

To identify new HH103 Rif^R mutations that could extend the nodulation range to *L. japonicus* Gifu, mutants affected in the T3SS (*ttsI* and *rhcJ*) were tested for their symbiotic capacities. Both HH103 Rif^R mutants were able to induce nitrogen-fixing nodules in *L. japonicus* Gifu and, in the case of the *ttsI* mutant, also improve the symbiotic performance with *L. burttii* (Tables 1 and 2; Fig. S2 and S3). The symbiotic phenotypes observed together with results obtained in microscopy studies (Fig. 1 and S2), indicate that, in the absence of a functional T3SS, HH103 Rif^R gains nodulation with *L. japonicus* Gifu through the formation of IT and the mechanism of infection in *L. burttii* switches from intercellular infection to IT invasion.

In the presence of *L. japonicus* Gifu root exudates, the HH103 Rif^R transcriptional regulators NolR and NodD2 not only repress the expression of the *nod* genes, but also

512 modulate the expression of many other symbiotic and non-symbiotic genes (Acosta-
513 Jurado *et al*., 2019). A similar situation is observed with the HH103 Rif^{R} SyrM
514 transcriptional regulator upon induction with genistein (Acosta-Jurado *et al*., 2020).
515 However, these studies also indicate that the expression of the T3SS genes does not
516 substantially change in the HH103 Rif^{R} *nolR*, *nodD2*, and *syrM* mutants (Acosta-Jurado
517 *et al*., 2019, 2020). Interestingly, the picture that emerges from previous transcriptomic
518 studies with the HH103 Rif^{R} *ttsI* mutant is quite different. Thus, TtsI does not control
519 the expression of the *nod* genes neither in the presence nor the absence of genistein
520 (Pérez-Montaño *et al*., 2016). In agreement with these results, the amount of NF
521 produced by the HH103 Rif^{R} *ttsI* mutant did not undergo a general increase regarding to
522 NF synthesized by the parental strain, as indicated by HPLC-HRMS/MS analyses
523 (Table 3). Therefore, the gained capacity to nodulate *L. japonicus* Gifu shown by the
524 HH103 Rif^{R} *ttsI* mutant does not seem to be associated with a general over-production
525 of NF as occurred with the *nolR*, *nodD2*, or *syrM* mutants. In fact, the comparison of
526 fold values of the HPLC-HRMS/MS signals obtained with the 23 NF shared by the
527 HH103 Rif^{R} *nolR*, *nodD2*, *syrM* (Acosta-Jurado *et al*., 2019 and 2020) and *ttsI* mutants
528 (Table S3) in response to genistein showed a very different situation. Whereas the *nolR*,
529 *nodD2* and *syrM* mutants show an over-production of 18-20 out of these 23 different
530 NF, in the *ttsI* mutant, 10 out of the 23 NF were found in lower amounts than in the
531 wild-type strain and only 6 NF were overproduced. However, the possibility that any of
532 the four different NF detected in higher amounts in all these mutants (*ttsI*, *nolR*, *nodD2*,
533 and *syrM*) could be to some extent responsible for the new capacity to nodulate *L.*
534 *japonicus* Gifu cannot be fully discarded.

535 For this reason, it was expected that the capacity to effectively nodulate *L. japonicus*
536 Gifu of both HH103 Rif^{R} *ttsI* and *rhcJ* mutants was likely due to the lack of secretion of

537 at least one effector. The T3SS is used by phytopathogenic and symbiotic bacteria to
538 deliver T3E directly into the host cell cytoplasm to promote infection through alteration
539 of cell metabolism and/or suppression of plant defence responses (Feng and Zhou,
540 2012; Macho and Zipfel, 2015). However, plants recognize some T3E by the
541 corresponding R proteins, eliciting the ETI response and blocking bacterial infection
542 (Duxbury *et al*., 2016; Jones and Dangl, 2006). Thus, the recognition of at least one
543 HH103 Rif^{R} T3E during the nodulation process must be responsible for the infection-
544 blocking phenotype with *L. japonicus* Gifu.

545 In this work, the systematic analysis of a collection of mutants affected in the
546 secretion of each individual T3E from the set of bacterial effectors already identified in
547 HH103 Rif^{R}, resulted in the identification of NopC as the main T3E involved in the
548 incapacity of HH103 Rif^{R} to nodulate *L. japonicus* Gifu (Table 1; Fig. S2).
549 Interestingly, the *nopC* mutant was able to nodulate both *L. japonicus* Gifu and *L.*
550 *burttii* through IT, as the T3SS mutants did (Fig. 1). Furthermore, the HH103 Rif^{R}
551 NopP and GunA effector proteins also seemed to be somehow involved in nodulation of
552 *L. japonicus* Gifu, but at a very much lesser extent (Table 1). Overall, these results
553 would suggest that *L. japonicus* Gifu detects HH103 Rif^{R} as a incompatible bacterium
554 mainly due to secretion of NopC and, consequently, blocks nodulation at early stages of
555 the infection process (Fig. 1A). In fact, expression studies of early and late nodulation
556 genes in *L. japonicus* Gifu support this hypothesis, since HH103 Rif^{R} induced the
557 expression of early nodulation genes (*LjNSP1*/*NSP2* and *LjNIN*). However, the
558 activation cascade stopped before expression of *LjENOD40*. By contrast, the common
559 nodulation pathway was fully activated when plants were inoculated with the *ttsI* and
560 *nopC* mutants (Fig. 2). Very recently, Kusakabe *et al*. (2020) have shown that the *B.*
561 *elkanii* USDA61 NopF blocks nodule infection in *L. japonicus* Gifu and that NopM

induces a nodule early senescence-like response in *L. burttii* and *L. japonicus* MG-20. However, NopF is not present in the genome of HH103 Rif^R suggesting that alternative mechanisms, probably T3E recognition by different R proteins, are used by *L. japonicus* Gifu to block infection.

The LjPR1 gene is not expressed in *L. japonicus* Gifu plants inoculated with *M. loti* R7A suggesting that the host plant identifies this rhizobial partner as a fully compatible strain. However, the identification of HH103 Rif^R triggers a defence response, since the expression of the *LjPR1* gene was induced by all the HH103 Rif^R strains analysed at 10 dai (Fig. 2). Mutation of *nolR* caused a slight reduction in *LjPR1* expression compared with the HH103 Rif^R parental strain, indicating that overexpression of NF could somehow suppress *L. japonicus* Gifu defence responses. In fact, a correlation between NF and suppression of plant defence responses has been already reported (Martínez-Abarca *et al*., 1998; Liang *et al*., 2013). Interestingly, despite nodulation is not blocked with the T3SS mutants, the highest activation of *LjPR1* was detected with the *ttsI* mutant, suggesting that the complete cocktail of T3E secreted by HH103 Rif^R could be suppressing initial *L. japonicus* Gifu defence responses. In the case of the *nopC* mutant, the expression of *LjPR1* was just slightly higher than in plants inoculated with HH103 Rif^R, indicating that function of this T3E is associated to elicitation of *L. japonicus* Gifu defence responses. These results suggest that either directly inducing defence responses or using an alternative and still unknown pathway, *L. japonicus* Gifu blocks HH103 Rif^R infection upon recognition of NopC. We cannot discard that an ETI response could have been elicited by NopC at a time preceding the time-point analysed. However, Kelly *et al*. (2018) could not detect defence responses in *L. japonicus* Gifu plants inoculated with HH103 Rif^R at 1 and 3 dpi.

586 Overall, results obtained in this manuscript suggest that in the symbiotic interaction
587 between *S. fredii* HH103 Rif^{R}-*L. japonicus* Gifu, the T3SS would play a double role: it
588 would suppress early defence responses but also later block the nodulation process.
589 Interestingly, mutations in *nolR*, *nodD2*, *syrM*, *ttsI*, *rhcJ*, and *nopC*, which confer
590 HH103 Rif^{R} the capacity to effectively nodulate *L. japonicus* Gifu are detrimental to
591 some extent for symbiosis with soybean (Vinardell *et al*., 2004b; de Lyra *et al.*, 2006;
592 López-Baena *et al.*, 2008; Jiménez-Guerrero *et al*., 2015a; Acosta-Jurado *et al*., 2019,
593 2020), the primary host plant of HH103 Rif^{R}. The present work reinforces the idea that
594 the set of symbiotic signals produced by HH103 Rif^{R} is the most suitable for soybean
595 nodulation but can difficult the potential capacity of this strain to establish a successful
596 symbiotic interaction with other legumes.
597

598 **Supplementary data**

599 **Table S1.** Bacterial strains and plasmids used in this study.

600 **Table S2.** Primers used in this study.

601 **Table S3.** Fold-change values of HPLC-HRMS signal areas from shared Nod Factors
602 produced by the *Sinorhizobium fredii* HH103 Rif^R *ttsI* and *nolR* mutants with respect to
603 the parental strain.

604 **Figure S1**. Southern blot analysis of the *Sinorhizobium fredii* HH103 Rif^R derivate
605 mutants in the *nopD*, *nopM1*/*nopM2*, and *nopT* genes. **A.** *S. fredii* HH103 Rif^R
606 *nopD*::**Ω**. Total DNA from the parental and the mutant strains was digested with both
607 *Sac*I and *Eco*RV restriction enzymes. A specific probe was used from a *nopD* internal
608 fragment. Single ~5.4 kb or ~3.4 kb bands were expected in the parental (lane 1) and the
609 mutant (lane 2) strains, respectively. **B**. *S. fredii* HH103 Rif^R Δ*nopM*/*nopM*::*lacZ*-Gm^R.
610 Total DNA from the parental and the mutant strains was digested with the *Bgl*I
611 restriction enzyme. A specific probe was used from a *nopM* internal fragment. Single
612 ~3.1 kb, or double ~3.5 kb + ~1 kb bands were expected in the parental (lane 1) and the
613 mutant (lane 2) strains, respectively. **C**. *S. fredii* HH103 Rif^R *nopT*::**Ω**. Total DNA from
614 the parental and the mutant strains was digested with the *Eco*RV restriction enzyme. A
615 specific probe was used from a *nopT* internal fragment. Single ~6.7 kb or ~8.7 kb bands
616 were expected in the parental (lane 1) and the mutant (lane 2) strains, respectively.
617 Asterisks indicate specific bands detected by each probe. Sizes (kb) from the λ
618 DNA/*Hind*III marker are indicated on the left.

619 **Figure S2**. Nodule occupancy studies of *Lotus japonicus* Gifu plants inoculated with
620 *Sinorhizobium fredii* HH103 Rif^R (wt), HH103 Rif^R *ttsI*::**Ω** (*ttsI*), HH103 Rif^R *rhcJ*::**Ω**
621 (*rhcJ*), and HH103 Rif^R Δ*nopC* (*nopC*). Light (**A**) and epifluorescence (**B**) microscopy
622 of nodules of *L. japonicus* Gifu plants inoculated with selected strains carrying the

623 *DsRed* reporter gene. Panel A, bars correspond to 1 mm. Panel B, bars correspond to
624 100 μm. Images were captured at 68 dai (**A**) and 21 dai (**B**).
625 **Figure S3**. *Lotus* responses to inoculation with *Mesorhizobium loti* R7A (R7A),
626 *Sinorhizobim fredii* HH103 Rif^R (wt), HH103 Rif^R *ttsI*::**Ω** (*ttsI*), HH103 Rif^R *rhcJ*::**Ω**
627 (*rhcJ*), HH103 Rif^R Δ*nopC* (*nopC*), HH103 Rif^R *nopP*::*lacZ*-Gm^R (*nopP*), HH103 Rif^R
628 Δ*gunA* (*gunA*) and their respective complemented strains. *L. japonicus* Gifu (**A**) and *L.*
629 *burttii* (**B**) plants grown for 6-9 weeks in Leonard jars. Numbers indicate the mean ±
630 standard deviation of the mean of shoot fresh weight values expressed in mg.
631

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Author contributions:

I. J-G and S. A-C. designed and performed the experiments and analyzed the data; C. M. performed microscopy analysis and provided technical assistance to I. J-G. and S. A-C.; F. P-M. peformed *q*RT-PCR experiments and contributed to write the manuscript; C. A-V. performed nodulation assays; J. M. V. and F. J. O. supervised and completed the writing; F. J. L-B. supervised the experiments and analyzed the data, conceived the project and wrote the article with contributions of all authors.

Competing interests

The authors declare no competing interests.

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Table 1. *Lotus japonicus* Gifu response to inoculation with *Sinorhizobium fredii* HH103 Rif^{R}, T3SS and T3-effector mutants, and their respective complemented derivatives.

Treatment	**n**	**Number of nodules** [#]	**Shoot fresh weight (mg)**[#]	**ARA (nmoles ET/plant h)**[#]	**Nod/Fix**
Uninoculated	29	0 ± 0	14.7 ± 1	0 ± 0	Nod^{-}/Fix^{-}
Mesorhizobium loti R7A	25	40.7 ± 3.3*	196.4 ± 15.3*	181.41 ± 8.71*	Nod^{+}/Fix^{+}
Sinorhizobium fredii HH103 Rif^{R}	29	0 ± 0	12.8 ± 0.8	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *ttsI*::Ω	27	25.1 ± 1.4*	106.8 ± 9.9*	30.47 ± 3.45*	Nod^{+}/Fix^{+}
HH103 Rif^{R} *ttsI*::Ω comp. *trans*	29	11.1 ± 1.2*	60.5 ± 10.2*	12.43 ± 1.47*	Nod^{+}/Fix^{+}
HH103 Rif^{R} *rhcJ*::Ω	21	17.2 ± 1.1*	99 ± 7.5*	38.01 ± 4.63*	Nod^{+}/Fix^{+}
HH103 Rif^{R} Δ*nopC*	25	8.1 ± 0.5*	51.6 ± 5.4*	17.02 ± 1.69*	Nod^{+}/Fix^{+}
HH103 Rif^{R} Δ*nopC* comp. *cis*	19	0 ± 0	14.5 ± 0.9	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} Δ*nopC* comp. *trans*	32	0 ± 0	13.2 ± 0.6	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *nopD*::Ω	22	0 ± 0	13.6 ± 1.1	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *nopD*::Ω comp. trans	24	0 ± 0	12.9 ± 0.9	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} Δ*nopI*	27	0 ± 0	14.1 ± 0.9	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} Δ*nopI* comp. *cis*	23	0 ± 0	15.7 ± 1.3	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *nopL*::Ω	31	0.07 ± 0.02*	12.8 ± 0.4	0 ± 0	Nod$^{-/+}$/Fix^{-}
HH103 Rif^{R} *nopL*::Ω comp. *trans*	27	0.05 ± 0.02*	14.8 ± 0.9	0 ± 0	Nod$^{-/+}$/Fix^{-}
HH103 Rif^{R} Δ*nopM1-nopM2*::*lacZ*-Gm	16	0 ± 0	16.5 ± 3	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} Δ*nopM1-nopM2*::*lacZ*-Gm comp. *trans*	19	0 ± 0	14.1 ± 1.45	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *nopP*::*lacZ*-Gm	27	0.6 ± 0.1*	25.7 ± 2.2*	1.5 ± 0.48*	Nod$^{-/+}$/Fix$^{-/+}$
HH103 Rif^{R} *nopP*::*lacZ*-Gm comp. *trans*	30	0.13 ± 0.02*	22.5 ± 1.1*	0.72 ± 0.28*	Nod$^{-/+}$/Fix$^{-/+}$
HH103 Rif^{R} *nopT*::Ω	28	0 ± 0	9.85 ± 0.29	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} *nopT*::Ω comp. *trans*	30	0 ± 0	10.8 ± 0.26	0 ± 0	Nod^{-}/Fix^{-}
HH103 Rif^{R} Δ*gunA*	24	0.38 ± 0.09*	18.4 ± 1.6*	2.41 ± 0.59*	Nod$^{-/+}$/Fix$^{-/+}$
HH103 Rif^{R} Δ*gunA* comp. *cis*	25	0 ± 0	12.48 ± 0.3	0 ± 0	Nod^{-}/Fix^{-}

n: number of plants evaluated.
ARA: acetylene reduction activity per plant expressed in nmoles ethylene/plant hour.
Numbers indicate the mean ± standard error of the mean (SEM) per plant.
Nod^{-}: absence of nodules or macroscopic root outgrowths. $Nod^{-/+}$: macroscopic root outgrowths and/or ineffective nodules in a variable number of plants. Fix^{-}: No nitrogen fixation detected by ARA. $Fix^{-/+}$: very low nitrogen fixation detected by ARA.
Symbiotic parameters were individually compared with the parental strain *Sinorhizobium fredii* HH103 Rif^{R} by using the Mann-Whitney non-parametric test. Values followed by an asterisk are significantly different at the level $\alpha = 5\%$.

Table 2. *Lotus burttii* responses to inoculation with *Sinorhizobium fredii* HH103 Rif^R, T3SS and T3E mutants, and their respective complemented derivatives.

Treatment	**n**	**Number of nodules** [#]	**Shoot fresh weight (mg)** [#]
Uninoculated	35	0 ± 0*	11.5 ± 4.9*
Mesorhizobium loti R7A	40	26.6 ± 2.3*	149.4 ± 38.5*
Sinorhizobium fredii HH103 Rif^R	36	10.6 ± 3.1	41.1 ± 5.2
HH103 Rif^R *ttsI*::Ω	35	16.9 ± 4.7	81.7 ± 14.9*
HH103 Rif^R *ttsI*::Ω comp. *trans*	34	12.6 ± 3.9	67.4 ± 26.2
HH103 Rif^R *rhcJ*::Ω	34	12.5 ± 2.1	55.6 ± 14.6
HH103 Rif^R Δ*nopC*	36	9.2 ± 2.2	41.7 ± 13.5
HH103 Rif^R Δ*nopC* comp. *trans*	35	10.1 ± 2	39.8 ± 11.4
HH103 Rif^R *nopD*::Ω	37	15.1 ± 4.3	53.6 ± 20.8
HH103 Rif^R *nopD*::Ω comp. *trans*	38	14 ± 3.6	47.1 ± 14.9
HH103 Rif^R Δ*nopI*	36	7.5 ± 3.2	19.6 ± 7.8*
HH103 Rif^R Δ*nopI* comp. *cis*	35	10.9 ± 1.9	36.4 ± 11.3
HH103 Rif^R *nopL*::Ω	36	13.9 ± 3.4	37.4 ± 16.6
HH103 Rif^R *nopL*::Ω comp. *trans*	33	12.5 ± 3.7	38.6 ± 18.4
HH103 Rif^R Δ*nopM1-nopM2*::*lacZ*-Gm	33	10 ± 3.9	39.4 ± 16.8
HH103 Rif^R Δ*nopM1-nopM2*::*lacZ*-Gm comp. *trans*	34	11.8 ± 2.8	47.3 ± 18.8
HH103 Rif^R *nopP*::*lacZ*-Gm	32	9.4 ± 3.7	28.6 ± 13.3
HH103 Rif^R *nopP*::*lacZ*-Gm comp. *trans*	34	11.1 ± 2.6	33.4 ± 10.5
HH103 Rif^R *nopT*::Ω	32	10.2 ± 3.7	36.8 ± 19.5
HH103 Rif^R *nopT*::Ω comp. *trans*	35	11.6 ± 4.3	42.9 ± 16.8
HH103 Rif^R Δ*gunA*	36	8.8 ± 3.4	40.1 ± 14.8
HH103 Rif^R Δ*gunA* comp. *cis*	34	10.7 ± 3.5	40.1 ± 17.3

n: number of plants evaluated.
ARA: acetylene reduction activity per plant expressed in nmoles ethylene/plant hour.
Numbers indicate the mean ± standard error of the mean (SEM) per plant.
Symbiotic parameters were individually compared with the parental strain *Sinorhizobium fredii* HH103 Rif^R by using the Mann-Whitney non-parametric test. Values followed by an asterisk are significantly different at the level $\alpha = 5\%$.

Table 3. Nod Factors produced by *Sinorhizobium fredii* HH103 Rif^{R} and HH103 Rif^{R} *ttsI*::Ω grown in the presence of genistein.

Chemical structures detected	Areas of NF signals in HH103 Rif^{R} (× 10^{7})^{A}	Areas of NF signals in HH103 Rif^{R} *ttsI*::Ω (× 10^{7})^{A}	Fold values of HPLC-HRMS signal areas in the *ttsI* mutant with respect to HH103 Rif^{R} ^{B}
II-Hex-GlcNac-GlcNAc (C18:1, MeFuc)	4.65	3.57	-1.3
III (C14:0, MeFuc)	N.D.	3.02	-
III (C16:0)	2.06	15.5	7.54
III (C16:0, Fuc)	1.88	2.66	1.42
III (C16:0, MeFuc)	28.4	104	3.66
III (C16:1)	8.27	N.D.	-
III (C16:1, Fuc)	11.19	2.16	-5.26
III (C16:1, MeFuc)	80.45	25.1	-3.22
III (C16:1-OH, MeFuc)	N.D.	3.89	-
III (C16:2)	N.D.	5.08	-
III (C16:2, MeFuc)	N.D.	10.3	-
III (C18:0)	N.D.	6.74	-
III (C18:0, MeFuc)	26.84	22.2	-1.2
III (C18:1)	22.53	4.91	-4.54
III-Hex (C18:1)	1.18	N.D.	-
III (C18:1, Fuc)	27.99	4.92	-5.55
III (C18:1, MeFuc)	264.5	48.5	-5.55
III (C18:1-OH, MeFuc)	N.D.	7.12	-
III-Fuc-GlcNAc (C18:1, MeFuc)	4.08	N.D.	-
III (C18:2)	2.44	18.8	7.72
III (C18:2, MeFuc)	N.D.	15.7	-
III (C18:2-OH, MeFuc)	N.D.	11.2	-
III (C20:1, MeFuc)	1.13	N.D.	-
IV (C14:0, MeFuc)	1.19	7.49	6.32

IV (C16:0)	2.53	15	5.94
IV (C16:0, Fuc)	4.43	10.4	2.35
IV (C16:0, MeFuc)	46.2	241	5.22
IV (C16:0, NMe, MeFuc)	3.04	4.89	1.61
IV (C16:1)	3.42	N.D.	-
IV (C16:1, Fuc)	12.41	N.D.	-
IV (C16:1, MeFuc)	36.85	16.8	-2.17
IV (C16:1, NMe, MeFuc)	1.39	N.D.	-
IV (C16:2, MeFuc)	N.D.	3.76	-
IV (C18:0)	7.46	10.2	1.37
IV (C18:0, Fuc)	5.36	3.92	-1.37
IV (C18:0, MeFuc)	76.75	109	1.42
IV (C18:1)	68.4	25.8	-2.63
IV (C18:1, Fuc)	103.3	29.6	-3.45
IV (C18:1, MeFuc)	623	278	-2.22
IV-Hex (C18:1)	4.16	1.13	-3.7
IV (C18:1-OH)	N.D.	2.38	-
IV (C18:1-OH, Fuc)	N.D.	7.75	-
IV (C18:1-OH, MeFuc)	2.74	65.1	23.76
IV (C18:2)	1.33	15	11.28
IV (C18:2-OH)	N.D.	5.15	-
IV (C18:2, MeFuc)	4.09	59.2	14.47
IV (C18:2-OH, NMe, MeFuc)	N.D.	1.38	-
V (C14:0, MeFuc)	N.D.	2.27	-
V (C16:0, Fuc)	N.D.	1.21	-
V (C16:0, MeFuc)	30.85	33.3	1.08
V (C16:1, Fuc)	2.68	N.D.	-
V (C16:1, MeFuc)	22.7	7.22	-3.12
V (C16:1-OH, MeFuc)	N.D.	2	-

V (C16:2, MeFuc)	N.D.	1.12	-
V (C18:0)	N.D.	1.09	-
V (C18:0, MeFuc)	40.55	28.1	-1.45
V (C18:1)	14.5	2.12	-6.67
V (C18:1, Fuc)	18.6	3.61	-5.26
V (C18:1, MeFuc)	280.34	57.7	-4.76
V (C18:1-OH, MeFuc)	2.54	14.1	5.55
V (C18:2, MeFuc)	3.8	9.62	2.53
V (C20:1, MeFuc)	1.92	N.D.	-

N.D.: Not detected.

[A] Area values ($\times 10^7$) of HPLC-HRMS signals obtained for each NF produced by, at least, two independent cultures grown in the presence of genistein.

[B] Comparisons made between the mutant and the wild-type strains:

In red: NF highly overproduced by the mutant with respect to the parental strain (fold-change value > 5)

In light red: NF moderately overproduced by the mutant with respect to the parental strain (2 < fold-change value < 5)

In blue: NF production highly reduced in the mutant with respect to the parental strain (fold-change value < -5)

In light blue: NF production moderately reduced by the mutant with respect to the parental strain (-2 > fold-change value > -5)

Figure legends

Fig. 1. *Sinorhizobium fredii* HH103 Rif^R T3SS mutants infect *Lotus japonicus* Gifu and *L. burttii* through infection threads. A. Epifluorescence microscopy of root hairs of *L. japonicus* Gifu and *L. burttii* inoculated with *S. fredii* HH103 Rif^R (wt), HH103 Rif^R *ttsI*::Ω (*ttsI*), HH103 Rif^R *rhcJ*::Ω (*rhcJ*), HH103 Rif^R Δ*nopC* (*nopC*), HH103 Rif^R *nolR*::*lacZ*-Gm^R (*nolR*), and *Mesorhizobium loti* R7A (R7A) carrying a red fluorescent marker in plasmid pFAJDsRed. Bars correspond to 100 µM. Images were captured at 21 dai. Blue arrows indicate curled root hairs entrapping rhizobia. **B.** Epifluorescence microscopy of *L. japonicus* Gifu (*L.j.*) and *L. burttii* (*L.b.*) 21 dai nodules infected by strains HH103 Rif^R (wt), HH103 Rif^R *ttsI*::Ω (*ttsI*), HH103 Rif^R *rhcJ*::Ω (*rhcJ*), and HH103 Rif^R Δ*nopC* (*nopC*) carrying a red fluorescent marker in plasmid pFAJDsRed. Bars correspond to 50 µM. Yellow arrows indicate outer cortical infection threads and yellow asterisks mark intercellular bacterial invasion.

Fig. 2. *Sinorhizobium fredii* HH103 Rif^R T3SS mutants differentially induce the expression of nodulation and defence genes in *Lotus japonicus* Gifu. Quantitative RT-PCR (*q*RT-PCR) analyses of the expression of selected *Lotus japonicus* Gifu genes in plants inoculated with *Sinorhizobium fredii* HH103 Rif^R (wt), HH103 Rif^R *ttsI*::Ω (*ttsI*), HH103 Rif^R Δ*nopC* (*nopC*), HH103 Rif^R *nolR*::*lacZ*-Gm (*nolR*), and *Mesorhizobium loti* R7A (R7A). Bars correspond to fold-change values of several *L. japonicus* genes differentially expressed in plants inoculated with the above mentioned strains with respect to their expression in uninoculated plants. Expression data shown are the mean (± standard deviation of the mean) for three biological replicates performed at least in triplicates. Represented points correspond to each biological replicate from one representative experiment. Treatments with the same letter were not

significantly different (One-Way ANOVA, α = 5%.). n: results were not significantly different with respect to those obtained for uninoculated plants. **A.** *LjNSP1*; **B.** *LjNSP2*; **C.** *LjNIN*; **D.** *LjENOD40*; **E.** *LjLb3*; **F.** *LjPR1*.

Fig. 3. *q*RT-PCR analyses of the expression of *Lotus japonicus* Gifu genes at early stages of the symbiosis. The expression of the *LjNSP1* (**A**), *LjNSP2* (**B**) and *LjNIN* (**C**) genes was quantified in plants inoculated with *S. fredii* HH103 Rif^R (wt), HH103 Rif^R *ttsI*::**Ω** (*ttsI*) and HH103 Rif^R Δ*nopC* (*nopC*) and collected at 12 hai. Bars correspond to fold-change values of several *L. japonicus* genes differentially expressed in plants inoculated with the above mentioned strains with respect to their expression in uninoculated plants. Expression data shown are the mean (± standard deviation of the mean) for three biological replicates performed at least in triplicates. Represented points correspond to each biological replicates from one representative experiment. Treatments with the same letter were not significantly different (One-Way ANOVA, α = 5%.).