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ORIGINAL PAPER

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The effect of FITA mutations on the symbiotic properties of *Sinorhizobium fredii* varies in a chromosomal-background-dependent manner

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Abstract *nodD1* of *Sinorhizobium fredii* HH103, which is identical to that of *S. fredii* USDA257 and USDA191, repressed its own expression. Spontaneous flavonoid-independent transcription activation (FITA) mutants of *S. fredii* HH103 M (=HH103 Rif^R pSym::Tn*5*-Mob) showing constitutive expression of *nod* genes were isolated. No differences were found among soybean cultivar Williams plants inoculated with FITA mutants SVQ250 or SVQ253 or with the parental strain HH103M. Soybean plants inoculated with mutant SVQ255 formed more nodules, and those inoculated with mutant SVQ251 had symptoms of nitrogen starvation. Sequence analyses showed that all of the FITA mutants carried a point mutation in their *nodD1* coding region. Mutants SVQ251 and SVQ253 carried the same mutation, but only the former was symbiotically impaired, which indicated the presence of an additional mutation elsewhere in the genome of mutant SVQ251. Mutants SVQ251 and SVQ255 were outcompeted by the parental strain for nodulation of soybean cultivar Williams. The symbiotic plasmids of mutants SVQ251 and SVQ255 (pSym251 and pSym255, respectively) and that (pSymHH103M) of the parental strain were transferred to pSym-cured derivatives of *S. fredii* USDA192 and USDA193 (USDA192C and USDA193C, respectively). Soybean responses to inoculation with *S. fredii* USDA192C and USDA193C transconjugants carrying pSym251 and pSymHH103M were not significantly different, whereas more nodules were formed after inoculation with transconjugants carrying pSym255. Only transconjugant USDA192C(pSym255) produced a significant increase in soybean dry weight.

Keywords *Sinorhizobium fredii* · *nodD* · FITA mutations · Soybean · Nodulation

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Introduction

Bacteria belonging to the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*, collectively termed as rhizobia, establish symbiotic interactions with legume plants, which elicits the development of nitrogen-fixing root nodules (Perret et al. 2000; Spaink 2000; Cullimore et al. 2001). The bacterial genes responsible for infection and induction of nodules are termed nodulation genes and include *nod*, *nol* and *noe*. They are organized into various transcriptional units and regulated coordinately as the *nod* regulon (reviewed by Schlaman et al. 1998; Perret et al. 2000). Most nodulation genes are induced in the presence of the host plant. Induction is mediated by flavonoids and other compounds present in root exudates and requires a constitutively expressed bacterial protein, NodD, which perceives legume inducers and activates expression of the *nod* regulon (Györgypal et al. 1991a; Schlaman et al. 1998).

NodD binds to the *nod* box, a *cis*-acting promoter that precedes inducible nodulation operons. NodD, a LysR-type transcriptional activator (Schell 1993), is thought to bind flavonoid molecules physically and then bend the DNA helix, allowing transcription to begin (Fisher and Long 1993). This basic regulation model is complex, because multiple copies of *nodD* can coexist in combination with positive or negative regulatory elements. *nodD* also interacts with the general nitrogen regulatory system, and

it can be autoregulated (Pueppke 1996; Schlaman et al. 1998). Expression of *nod* genes results in the production and secretion of signal molecules, known as Nod-factors or LCOs (lipochitooligosaccharides). LCOs are *N*-acylated oligomers of *N*-acetyl-D-glucosamine that act as morphogens to initiate the nodulation program of the host plant and allow bacterial entry (Perret et al. 2000; Spaink 2000; Cullimore et al. 2001).

Sinorhizobium fredii includes fast-growing strains that nodulate soybean and many other legumes (Pueppke and Broughton 1999). Strains such as USDA191 and HH103 are indiscriminate with soybean, nodulating all cultivars, but others, including USDA192, USDA193, and USDA257, nodulate only certain cultivars, such as Peking, and generally fail to nodulate most American soybean cultivars (Heron and Pueppke 1984; Buendía-Clavería et al. 1989a; Balatti and Pueppke 1992). *S. fredii* harbors two copies of *nodD* (Ramakrishnan et al. 1986; Appelbaum et al. 1988; Krishnan et al. 1995). Appelbaum et al. (1988) inactivated each copy of *nodD* in strain *S. fredii* USDA191 by insertional mutagenesis. Plants inoculated with a *nodD1* insertion mutant appeared to be nitrogen-starved and had a greatly reduced nodule number, while those inoculated with a *nodD2* insertion mutant had a partially nitrogen-starved appearance and normal nodule number. Only *nodD1* complemented *nodD* mutants in other rhizobia.

The inability of inoculant-quality rhizobia to occupy a significant number of the nodules formed by legumes growing in soils containing indigenous populations of root nodule bacteria is referred as to "the competition problem". Since biotic and abiotic factors play determinant roles in the successful use of superior inoculant strains (Sadowsky 2000), a variety of strategies have been implemented to increase nodule occupancy by the inoculant strain (Robleto et al. 2000). Early recognition signals, such as plant flavonoid and bacterial nodulation factors, have been one of the chosen targets in these attempts to enhance the competitive capacity of inoculant strains.

Inoculation of soybean plants with *Bradyrhizobium japonicum* cultures that had been grown in the presence of the *nod* inducer genistein increased root and shoot dry weight, as well as the number of nodules formed (Pan and Smith 2000). Addition of genistein to the *B. japonicum* inoculant or soil is also an effective way to increase nitrogen fixation and yield (Zhang and Smith 1996). Similar results have been obtained with the flavonoids naringenin and hesperitin on the symbiosis between *Rhizobium leguminosarum* bv. *viciae* and *Pisum sativum* (Begum et al. 2001). However, to our knowledge, studies aimed at determining the impact of flavonoids on nodule occupancy by inoculant strains have not been carried out.

Flavonoids that act as inhibitors of *nod* expression also have been used to significantly reduce the nodulation capacity of sensitive *B. japonicum* strains. Unfortunately, the broad variability among strains in their response to any particular inhibitor reduces the possibilities to use chemical inhibitors under field conditions to effectively manipulate the competition between strains for soybean nodulation (Cunningham et al. 1991).

Manipulation of bacterial capacity to produce nodulation factors is an alternative strategy. Although Knight et al. (1986) reported that *nodABC* overexpression produced a negative symbiotic effect, *Rhizobium* mutants showing transcriptional activation of nodulation genes in the absence of flavonoids [flavonoid-independent transcription activation (FITA) mutants] can be either impaired (Burn et al. 1987; McIver et al. 1989) or enhanced in their symbiotic capacity (Spaink et al. 1989; Zhang et al. 2002). FITA mutants also represent an important tool to study NodD function, since they are commonly altered in symbiotic properties, including host-range, nodulation, and nitrogen-fixation efficiency.

We previously described a genetic system to positively select spontaneous FITA mutants using plasmids pMUS248 and pMUS262, which contain a promoterless tetracycline resistance gene under the transcriptional control of the *nodA* promoter from *R. leguminosarum* bv. *viciae.* Tetracycline resistance (Tc^R) thus is regulated by NodD, and *S. fredii* HH103 carrying these plasmids becomes Tc^R in the presence of flavonoids. Selection of spontaneous Tc^R derivatives of *S. fredii* HH103(pMUS248) in the absence of flavonoids allows ready isolation of FITA mutants (Vinardell et al. 1993).

Here we report that *S. fredii* HH103 NodD1, which is essential for *nod* activation and soybean nodulation, represses *nodD1* transcription. We also isolated and characterized several *nodD1* FITA mutants of *S. fredii* HH103 with altered symbiotic properties on soybean. The effect of FITA mutations on bacterial symbiotic behavior was studied in several *S. fredii* genetic backgrounds, which demonstrated that these mutations act in a chromosomal-background-dependent manner.

Materials and methods

Strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Sinorhizobium* strains were cultured in TY (Beringer 1974), YMA (Vincent 1970), or B^- media (Spaink et al. 1992). *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989). When required, the media were supplemented with antibiotics (concentrations in μg ml^{-1}): rifampicin (Rif), 50; streptomycin (Str), 400; kanamycin (Km), 50 (25 for *E. coli*); neomycin (Nm), 100; tetracycline (Tc), 4 (10 for *E. coli*); gentamicin (Gm), 5 (10 for *E. coli*).

Genetic techniques

Plasmids were introduced into *Sinorhizobium* strains by conjugation as described by Simon (1984), with plasmid pRK 2013 as helper. Plasmid profiles were visualized by agarose gel electrophoresis as described previously (Espuny et al. 1987).

Molecular techniques were carried out as described by Sambrook et al. (1989). For hybridization, DNA was blotted to Amersham Hybond-N nylon membranes, and the DigDNA method of Roche was employed according to manufacturer's instructions. The complete *nodD1* from *S. fredii* HH103 contained in cosmid pMUS283 (Lamrabet et al. 1999) was subcloned as a 1.5-kb *Apa*I–*Eco*RV fragment in the broad-host-range vector pMP92 (Spaink et al. 1987), creating plasmid pMUS296.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Derivation and relevant properties	Source or reference
Sinorhizobium fredii		
HH103-1	HH103 Str^R	Buendía-Clavería et al. (1989a)
SVQ318	HH103 Rif^R *nodD1*::Ω	Lamrabet et al. (1999)
SVQ290	USDA257 Rif^R *nodD1*::Ω	This work
HH103M	HH103 Rif^R pRfrHH103d::Tn*5*-Mob (Km^R)	Romero (1993)
SVQ250, SVQ251, SVQ253 and SVQ255	FITA derivatives of HH103 M(pMUS262)	This work
USDA192C (=SVQ244)	USDA192 Rif^R $pSym^-$	Romero (1993)
USDA193C (=AB359)	USDA193 Str^R Spc^R $pSym^-$	Buendía-Clavería et al. (1989b)
SVQ502	HH103 Rif^R *nodD1*::*lacZ*Δp-Gm^R	This work
SVQ516	SVQ251 *nodD1*::Ω	This work
Rhizobium leguminosarum bv. *viciae*		
LPR5045	RCR5 Rif^R $pSym^-$	Hooykaas et al. (1982)
Escherichia coli		
803	Gal^-, Met^-, Hfr r^-_k m^-_k	Wood (1966)
DH5α	*supE44*, Δ*lacU169*, *hsdR17*, *recA1*, *endA1*, *gyrA96* , *thi-1*, *relA1*	Sambrook et al. (1989)
Plasmids		
pBluescript II SK+	Cloning and sequencing vector, Ap^R	Stratagene
pMP154	IncQ; Str^R, Cm^R; p*nodA*::*lacZ*	de Maagd et al. (1988)
pMP240	IncP; Tc^R; p*nodA*::*lacZ*	de Maagd et al. (1988)
pRK2013	Helper plasmid, Km^R	Figurski and Helinski (1979)
pMUS262	IncQ; Gm^R; p*nodA*::*tet*Δp	Vinardell et al. (1993)
pMP92	Broad-host-range cloning vector; IncP; Tc^R	Spaink et al. (1987)
pMUS283	Cosmid pLAFR1 carrying the *nodD1* of *S. fredii* HH103	Lamrabet et al. (1999)
pMUS296	pMP92 containing a 1.5-kb *Apa*I–*Eco*RV fragment containing *nodD1* of *S. fredii* HH103	This work
pMUS343	pMP92 containing genes *nptI* (Km^R) and *sacB-sacR*	Vinardell et al. (1997)
pHP45Ω	Ap^R vector containing Ω interposon (Spc^RStr^R)	Prentki and Krisch (1984)
pMUS349	pMUS343 containing *S.fredii nodD1*::Ω	This work
pMUS381	pBluescript II SK+ containing the *nodD1* gene of SVQ251	This work
pMUS382	pBluescript II SK+ containing the *nodD1* gene of SVQ255	This work
pMP1010	Vector containing transposon Tn*5*-1010, Km^R	Spaink et al. (1994)
pMUS407	pMP1010 containing *nodD1* of SVQ251 subcloned into Tn*5*-1010	This work
pMUS408	pMP1010 containing *nodD1* of SVQ255 subcloned into Tn*5*-1010	This work
pMUS409	pMP1010 containing *nodD1* of HH103–1 subcloned into Tn*5*-1010	This work
pK18mob	Cloning vector, Km^R	Schafer et al. (1994)
pAB2001	Ap^R vector containing the *lacZ*Δp-Gm^R cassette	Becker et al. (1995)
pMUS462	pMP92 carrying the *lacZ*Δp-Gm^R cassette inserted into the *Nru*I site of HH103 *nodD1*	J.M. Vinardell
pMUS534	pK18mob carrying a 6-kb *Hin*dIII fragment containing HH103 *nodD1*::*lacZ*Δp-Gm^R derived from pMUS462	This work
Symbiotic plasmids		
pSymHH103M	pRfrHH103d::Tn*5*-Mob, Km^R	Romero (1993)
pSym251	pSymHH103 M derivative of SVQ251	This work
pSym255	pSymHH103 M derivative of SVQ255	This work

S. fredii HH103M, a derivative of strain HH103 Rif^R, was used to generate FITA mutants. The symbiotic plasmid of HH103M (pSymHH103M) carries a Tn*5*-Mob insertion that does not apparently cause any symbiotic defect (our unpublished results) and renders the plasmid mobilizable. Plasmid pMUS262, which contains a transcriptional fusion between the *nodA* promoter and a promoterless Tc^R gene, was transferred to HH103M. The resulting strain is resistant to tetracycline when flavonoid inducers, such as naringenin, are present in the culture media (Vinardell et al. 1993). FITA mutants were isolated by selection, in the absence of flavonoids, of spontaneous Tc^R-colonies of HH103M(pMUS262) in TY medium supplemented with Tc (20 μg ml^{-1}).

nodD1 derivatives of *S. fredii* strains USDA257 Rif^R and SVQ251 (a FITA derivative of HH103M) were constructed as follows: the Spc^{R}Str^R omega interposon (Prentki and Krisch 1984) was subcloned as a 2-kb *Sma*I fragment into the *Nru*I site of *nodD1* of *S. fredii* HH103 (Vinardell 1997). The *nodD1*::omega fragment was excised as a 3.5-kb *Bgl*II fragment and subcloned into the *Bam*HI site of pMUS343 (Vinardell et al. 1997), a broad-host-range vector containing the *sacBR* genes from *Bacillus subtilis*. It therefore confers sucrose sensitivity to gram-negative bacteria. The new plasmid, pMUS349, was transferred to USDA257 Rif^R and SVQ251, and Spc^R sucrose^R colonies were selected. Substitution of *nodD1* for the *nodD1*::omega fusion was assessed by hybridization with *nodD1* of *S. fredii* HH103, *nodD2* of *S. fredii* USDA257 (Krishnan et al. 1995), and the omega interposon as probes (data not shown). Mutants SVQ290 (derived from USDA257 Rif^R) and SVQ516 (derived from SVQ251) were selected for further studies.

To generate strain SVQ502 (=HH103 Rif^R *nodD1*::*lacZ*), plasmid pMUS462, a pMP92 derivative carrying the *lacZ*Δp-Gm^R cassette from plasmid pAB2001 (Becker et al. 1995) inserted as a 4.5-kb *Sma*I fragment into the *Nru*I site of *nodD1* from HH103, was used (Vinardell 1997). The HH103 *nodD1*::*lacZ*Δp-Gm^R fusion contained in pMUS462 was excised as a 6-kb *Hin*dIII fragment and subcloned into pK18mob, a Km^R plasmid that is not replicable in rhizobia. The resultant plasmid, pMUS534, was transferred to strain HH103 Rif^R, and Rif^R Gm^R transconjugants were analyzed for sensitivity to kanamycin in order to isolate putative double recombinants in which wild-type *nodD1* had been substituted by the *nodD1*::*lacZ*Δp-Gm^R fusion. Homogenotization was assessed by hybridization.

A 1,415-bp fragment from FITA mutants SVQ250, SVQ251, SVQ253, and SVQ255, covering the complete *nodD1* gene and the *nod*-box-like sequence, was amplified by PCR from genomic DNA as described by Saiki (1990). The primers were pri297rII (5′-cgaagcccctgcattgttagtggca) and pri297dII (5′-ccggctgctaaagctgaagagctaa), and the High-Fidelity Taq polymerase system from Roche were used. PCR products were purified (PCR Clean Up Kit from Roche), sequenced by the dideoxy-chain termination method (Sanger et al. 1977), and analyzed with the GCG software program (Devereux et al. 1984).

PCR fragments from SVQ251 and SVQ255 were treated successively for 10 min at room temperature with Klenow fragment to eliminate the terminal adenine residues, and dNTPs to fill possible terminal unicatenary deletions. Fragments then were incubated for 30 min at 37 °C with polynucleotide kinase in the presence of 1 mM ATP. The amplified fragments were subcloned into *Eco*RV-digested pBluescript KS II+ (Stratagene).

Assays for β-galactosidase activity in liquid bacterial cultures on YM medium were as described by Miller (1972). Compounds tested as *nod* inducers were dissolved in ethanol and used at 1 μg ml^{-1}, which gave concentrations between 2.9 μM (quercetin) and 6.1 μM (umbelliferone). Bacterial cultures at OD_{660}=0.8–1.0 were diluted (about 100-fold) before the addition of flavonoids to ensure that bacterial cultures had an OD_{660} in the range of 0.15–0.30 when β-galactosidase activity was measured (16 h after induction). For time-course experiments, bacterial cultures at OD_{660}=0.1 were supplemented with genistein at a final concentration of 3.7 μM, and β-galactosidase activity was determined at 0, 2, 4, 6, 8, 10, 14, and 16 h of incubation.

LCOs from *S. fredii* strains grown on minimal B$^-$ medium were analyzed by reverse-phase thin-layer chromatography (TLC) as described by Spaink et al. (1992).

Nodulation assays

The four FITA derivatives of HH103M were assayed for nodulation on soybean cultivar Williams under greenhouse conditions as described (Rodríguez-Navarro et al. 1996). Nodulation assays of strains HH103M, SVQ251, SVQ255, and of transconjugants of USDA192C and USDA193C harboring plasmids pSymHH103M, pSym251, and pSym255 were carried out in a plant growth chamber as described by Buendía-Clavería et al. (1989a).

Plant shoots and nodules were dried at 70 °C and weighed. Soybean nodules were surface-sterilized as described previously (Buendía-Clavería et al. 1989a), and nodule isolates tested on appropriately supplemented medium to determine whether they retained the antibiotic-resistance markers of the bacteria used to inoculate the plants. Competition studies were carried out on *Glycine max* cultivar Williams as described by Romero et al. (1993); 120–150 nodules were analyzed per treatment.

Results

NodD1 is solely responsible for *nod* activation in *S. fredii* HH103

S. fredii strains and *Rhizobium* sp. strain NGR234 nodulate many different legumes (Buendía-Clavería et al. 1989a, 2003; Pueppke and Broughton 1999). The flavonoid responsiveness conditioned by *nodD1* of strain HH103 was assessed by conjugal transfer of plasmid pMP240, which contains a *R. leguminosarum* bv. *viciae nodA*p::*lacZ* fusion, into strains HH103-1 (=HH103 Str^R) and SVQ318, a *nodD1* derivative of strain HH103. Mutant SVQ318 did not nodulate soybean cultivars Williams and Peking, and it failed to produce nodulation factors in the presence of naringenin (Fig. 1, lane 1). The introduction of plasmid pMUS296, a pMP92 derivative containing HH103 *nodD1*, into SVQ318 restored soybean nodulation and the bacterial capacity to produce LCOs (Fig. 1, lane 2). Plasmid pMUS296 also was transferred by conjugation into strain LPR5045, a pSym-cured derivative of *R. leguminosarum* bv. *viciae*, carrying plasmid pMP154 with the same *nodA*p::*lacZ* fusion.

Twenty different flavonoids were tested for their capacity to induce the *nodA*p::*lacZ* fusions of pMP240 and pMP154 in homologous (*S. fredii* HH103) and heterologous (*R. leguminosarum* bv. *viciae* LPR5045) backgrounds containing *S. fredii nodD1*. The inducing capacity of each particular flavonoid tested was similar in both strains (Table 2). Coumestrol, isoliquirtigenin, and those compounds carrying a hydroxyl group at C7 induced β-galac-

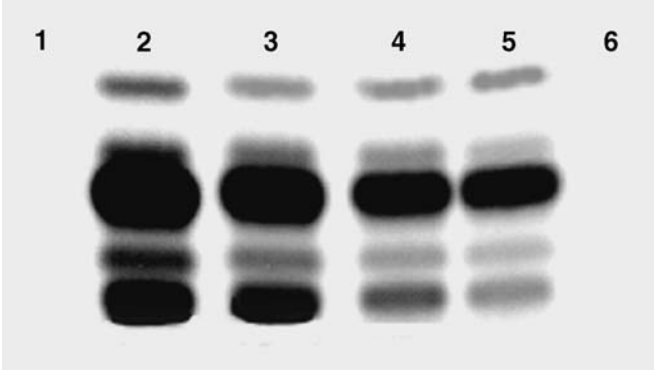

Fig. 1 TLC analysis of the lipochitooligosaccharides produced by *Sinorhizobium fredii* SVQ318 (*lane 1*), SVQ318(pMUS296) (*lane 2*), HH103 Rif^R (*lane 3*), USDA257 Rif^R (*lane 4*), SVQ290(pMUS296) (*lane 5*) and SVQ290 (*lane 6*). All strains were cultured in the presence of 3.7 μM naringenin

Table 2 Flavonoid responsiveness of *nodA* in *S. fredii* HH103, its *nodD1* derivative SVQ318 and the pSym-cured *R. leguminosarum* bv. *viciae* strain LPR5045 containing strain HH103 *nodD1*. Numbers are the mean of at least two independent experiments performed in duplicate. *n* Fold induction with respect to control without flavonoid

Flavonoids	μM	Hydroxylation pattern	HH103-1 (pMP240)		SVQ318 (pMP240)		LPR5045 (pMP154) (pMUS296)	
			Average	n	Average	n	Average	n
None			762± 34	1.0	458±52	1.0	726± 28	1.0
Flavones								
Flavone	4.5		785± 43	1.0	415±16	0.9	740± 31	1.0
5-Hydroxyflavone	4.2	5	808± 18	1.1	442±54	1.0	770± 11	1.1
7-Hydroxyflavone	4.2	7	9,903±131	13.0	489±10	1.1	7,536±440	10.4
4′,7-Dihydroxyflavone	3.9	4′, 7	10,558±329	13.9	478±60	1.0	6,745± 98	9.3
Chrysin	3.9	5, 7	9,217±378	12.1	457±44	1.0	8,363±306	11.5
Apigenin	3.7	5, 7, 4′	10,897±204	14.3	455± 8	1.0	7,216±342	9.9
Luteolin	3.5	5, 7, 3′, 4′	8,839±120	11.6	483±70	1.1	6,272±556	8.6
Kaempferol	3.5	3, 5, 7, 4′	10,515±693	13.8	457± 7	1.0	4,388±393	6.0
Quercetin	3.0	3, 5, 7, 3′, 4′	5,487±227	7.2	416±25	0.9	5,228±318	7.2
Fisetin	3.5	3, 7, 3′, 4′	6,477±310	8.5	372±98	0.8	5,489±482	7.6
Morin	3.3	3, 5, 7, 2′, 4′	6,248±356	8.2	416±25	0.9	4,080±233	5.6
Flavanones								
Flavanone	4.5		686± 11	0.9	453±90	1.0	686± 33	0.9
6-Hydroxyflavanone	4.2	6	701± 20	0.9	456±37	1.0	764± 31	1.1
Naringenin	3.7	5, 7, 4′	10,173±266	13.3	444±59	1.0	7,492±173	10.3
Hesperetin	3.3	5, 7, 3′, 4′-CH_3	11,186±495	14.7	461±71	1.0	8,231±255	11.3
Isoflavones								
Daidzein	3.9	7, 4′	10,981± 99	14.4	372±27	0.8	6,679±130	9.2
Genistein	3.7	5, 7, 4′	11,354±526	14.9	411±69	0.9	6,845±184	9.4
Others								
Coumestrol (7,12-Dihydroxycoumestan)	3.9	7, 12	11,130±293	14.6	430±99	0.9	7,376±520	10.2
Isoliquirtigenin (4, 2′, 4′-Trihydroxychalcone)	3.7	4, 2′, 4′	10,468±298	13.7	482±62	1.1	7,718±146	10.6
Umbelliferone (7-Hydroxy-2H-1-benzopyran-2-one)	6.2	7	7,971±475	10.5	429±30	0.9	8,124±576	11.2

tosidase activity in both bacteria. None of the flavonoids activated *nodA*p::*lacZ* expression in mutant SVQ318 (Table 2).

NodD1 of *S. fredii* HH103 is negatively autoregulated

To investigate whether *S. fredii* HH103 *nodD1* controls its own expression, plasmid pMUS296, carrying HH103 *nodD1*, was introduced into strain SVQ502 (a HH103 derivative whose *nodD1* was fused to *lacZ*) and bacterial β-galactosidase activity in the presence and absence of genistein was determined. The presence of plasmid pMUS296 significantly reduced (α=5%, following the Mann-Whitney non-parametric test) the level of β-galactosidase activity in strain SVQ502 in the presence (from 108.9±4.4 to 59.4±5.8 Miller units) and absence of flavonoids (from 99.3±5.8 to 64.3±8.9 Miller units). In addition, the presence of genistein in SVQ502 cultures causes a slight but significant (α=5%) increase in the expression of *lacZ* driven by the *nodD1* promoter.

The influence of pMUS296 and/or the presence of genistein on *nodD1* transcription was also investigated at different times after induction with genistein. At all time points tested, the presence of plasmid pMUS296 reduced SVQ502 *nodD1* transcription regardless of the presence or absence of genistein (Table 3). Addition of genistein to SVQ502(pMUS296) cultures did not significantly affect the β-galactosidase activity exhibited by this strain. In SVQ502 cultures, the positive effect of genistein on *lacZ* expression driven by the *nodD1* promoter was only observed 8 h after the addition of the flavonoid.

FITA mutants of *S. fredii* are symbiotically abnormal

Using the FITA-mutant selection strategy of Vinardell et al. (1993), four Tc^R derivatives of HH103M(pMUS262) were isolated and designated SVQ250, SVQ251, SVQ253, and SVQ255. Plasmid pMUS262 was removed from each Tc^R isolate by repeated subculture in the absence of the antibiotic. This allowed the subsequent conjugal introduction of plasmid pMP240, which carries a transcriptional fusion between the *nodA* promoter of *R. leguminosarum* bv. *viciae* and the promoterless *lacZ*. The four Tc^R HH103M(pMUS262) derivatives exhibited higher constitutive levels of β-galac-

Table 3 Time course experiments of β-galactosidase activity of *S. fredii* strain SVQ502 in the presence and absence of plasmid pMUS296. Data represent the mean of three independent cultures. Numbers on the same line preceded by the same capital letter are not significantly different at the level α=5% using the Mann-Whitney non-parametric test. Numbers in the same column followed by the same letter are not significantly different at the level α=5% using the Mann-Whitney non-parametric test

Time after induction (h)	Strain	β-Galactosidase activity (Miller units)	
		–Genistein	+Genistein
0	SVQ502	A 95.7±0.6 a	A 95.7±2.1 a
	SVQ502(pMUS296)	A 65.3±0.6 b	A 64.0±1.0 b
2	SVQ502	A 94.7±1.2 a	A 93.3±3.1 a
	SVQ502(pMUS296)	A 63.7±1.5 b	A 64.3±3.1 b
4	SVQ502	A 92.3±2.5 a	A 96.3±1.5 a
	SVQ502(pMUS296)	A 61.7±2.5 b	A 64.7±2.1 b
6	SVQ502	A 92.3±3.1 a	A 99.0±3.5 a
	SVQ502(pMUS296)	A 61.0±1.7 b	A 64.7±3.5 b
8	SVQ502	A 94.7±4.9 a	B 106.0±4.4 a
	SVQ502(pMUS296)	A 66.7±4.2 b	A 66.0±1.7 b
10	SVQ502	A 101.3±2.9 a	B 115.7±4.0 a
	SVQ502(pMUS296)	A 69.7±4.0 b	A 65.3±3.5 b
14	SVQ502	A 98.0±2.0 a	B 115.3±6.0 a
	SVQ502(pMUS296)	A 68.0±4.6 b	A 64.3±5.1 b
16	SVQ502	A 96.7±4.2 a	B 110.3±3.1 a
	SVQ502(pMUS296)	A 64.3±1.2 b	A 63.3±3.1 b

Table 4 β-Galactosidase activity of Tc^R derivatives of *S. fredii* strain HH103M carrying plasmid pMP240 in the presence and absence of 3.7 μM naringenin. Data represent the mean of three replicates. Each FITA mutant was individually compared to the parental strain HH103M using the Mann-Whitney non-parametrical test. Numbers in the same column followed by an *asterisk* are significantly different at the level α=5%

Strain	β-Galactosidase activity (Miller units)	
	–Naringenin	+Naringenin
HH103M	748± 59	9,048±233
SVQ250	5,615±422*	9,728±548
SVQ251	4,773±156*	11,426±152*
SVQ253	4,863±147*	9,227±451
SVQ255	4,084±277*	9,928±873

tosidase activity than that of parental strain HH103M (Table 4). Thus, strains SVQ250, SVQ251, SVQ253, and SVQ255 were by definition FITA derivatives of HH103M.

The symbiotic phenotypes of these FITA mutants were investigated with *Glycine max* cv. Williams, which nodulates and fixes nitrogen with *S. fredii* strain HH103. Soybean plants were grown for 7 weeks after inoculation. Although nodules formed, those plants inoculated with FITA mutant SVQ251 showed symptoms of nitrogen starvation, suggesting poor nitrogen-fixation capacity. FITA mutant SVQ255 formed more nodules (73.3 nodules per plant) than did its parental strain HH103M (45.0 nodules/plant) although plant-shoot dry weights were not significantly different (2.05 and 1.46 g, respectively). In contrast, the symbiotic phenotypes of FITA mutants SVQ250 and SVQ253 were not significantly different from those of their parental strain HH103M.

Strains SVQ251 and SVQ255 were selected to analyze the effect of FITA mutations on competitive ability to nodulate soybean cultivar Williams. Because both mutants and the parental strain HH103M have the same resistance markers (Rif^R and Km^R), all of them were tested in pairwise competition experiments versus their parental strain HH103 Rif^R (which lacks transposon Tn*5*-Mob). Using inocula ratios of 1:1 (3×10^8 cells per competitor), soybean nodule occupancy of strains SVQ251 and SVQ255 were respectively 7.1 and 17.9%, whereas strain HH103M occupied 45.6% of the nodules when it was coinoculated with HH103 Rif^R. Since the Tn*5*-Mob insertion carried by strain HH103M does not apparently affect its competition capacity (HH103M and HH103 Rif^R are equally competitive), the FITA mutations carried by strains SVQ251 and SVQ255 impair the competitive capacity to nodulate soybean.

Mutants SVQ251 and SVQ255 also were selected to gauge the effect of strain background on symbiotic phenotype. Since plasmid rearrangements were detected in other *S. fredii* FITA mutants (Vinardell 1997), plasmids of strains SVQ251 and SVQ255 were examined. Plasmid electrophoresis showed that the plasmid profiles of SVQ251, SVQ255 and its parental strain HH103M are apparently identical (Fig. 2A). The symbiotic plasmids of these two mutant strains, designated pSym251 and pSym255, respectively, were transferred to pSym-cured derivatives of *S. fredii* strains USDA192 and USDA193 (USDA192C and USDA193C, respectively). Both wild-type strains, USDA192 and USDA193, fail to fix nitrogen with commercial soybean varieties (Keyser et al. 1982), but their pSym-cured derivatives efficiently nodulate these cultivars when they carry pSymHH103M (Romero 1993). Plasmid profiles of the different transconjugant strains generated by transfer of plasmids pSym251 or pSym255 were examined by agarose gel electrophoresis and found to be indistinguishable from those of USDA192C(pSymHH103M)

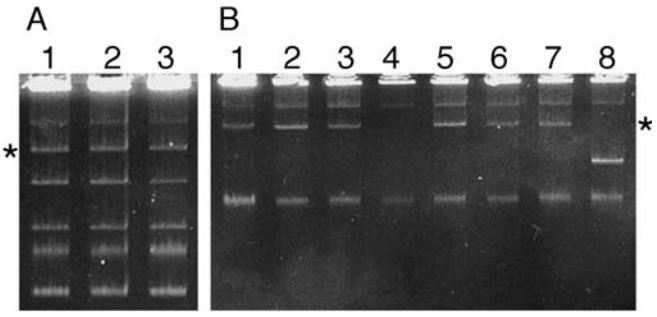

Fig. 2A, B Plasmid profiles on agarose gel electrophoresis. **A** HH103M (*lane 1*), SVQ251 (*lane 2*), SVQ255 (*lane 3*). **B** USDA193C(pSymHH103 M) (*lane 1*), USDA193C(pSym251) (*lane 2*), USDA193C(pSym255) (*lane 3*), USDA193C (*lane 4*), USDA192C(pSymHH103 M) (*lane 5*), USDA192C(pSym251) (*lane 6*), USDA192C(pSym255) (*lane 7*), and USDA192C (*lane 8*). Bands corresponding to the symbiotic plasmids are marked with an *asterisk*

or USDA193C(pSymHH103M) (Fig. 2B). However, the smallest indigenous plasmid of the recipient strain USDA192C (Fig. 2B, lane 8) was not observed in any of the three USDA192C transconjugants investigated (Fig. 2B, lanes 5–7), probably due to incompatibility with the transferred plasmids (pSymHH103 M, pSym251, or pSym255).

Plasmid pMP240 was introduced into those USDA192C transconjugants carrying pSymHH103M, pSym251, or pSym255, and β-galactosidase activity was determined in the presence or absence of naringenin. All USDA192C transconjugants showed high β-galactosidase activity in the presence of naringenin (9,627±171 to 11,751±284 Miller units). In the absence of the inducer, however, bacteria carrying plasmids pSym251 and pSym255 expressed higher constitutive β-galactosidase activity (5,078±299 and 4,241±148 Miller units, respectively) than control strain USDA192C(pSymHH103M), which produced 857±44 Miller units under these conditions. The FITA mutations thus lie within symbiotic plasmids pSym251 and pSym255.

The response of *Glycine max* to inoculation with strains HH103M, its FITA derivatives SVQ251 and SVQ255, and USDA192C and USDA193C transconjugants harboring the symbiotic plasmids pSymHH103 M, pSym251, and pSym255 is summarized in Table 5. In all cases, the presence of pSym251 and pSym255 enhanced the number of nodules formed, but differences with the corresponding strain harboring the parental plasmid pSymHH103M were significant (α=5%) only in the case of plasmid pSym255. In contrast, although nodule and plant-shoot dry weights were significantly lower with strain SVQ251 than with the parental strain HH103M, the presence of plasmid pSym251 did not significantly affect these parameters in strains USDA192C and USDA193C. The presence of plasmid pSym255 in strains SVQ255 and USDA193C(pSym255) did not influence nodule and plant-shoot dry weights, but it significantly enhanced these parameters in the case of strain USDA192C(pSym255).

The FITA mutations of pSym251 and pSym255 reside in *nodD1*

Primers pri297rII and pri297dII were used to amplify *nodD1* and its promoter region from FITA mutants SVQ251 and SVQ255. The amplified fragments were subcloned into pBluescript to generate pMUS381 and pMUS382, which contain *nodD1* from SVQ251 and SVQ255, respectively.

Transposon Tn*5*-1010 is a Tn*5* derivative containing a multiple cloning site located in the vector pMP1010, which is unable to replicate in rhizobia (Spaink et al. 1994). Tn*5*-1010 was used to introduce by transposition the *nodD1* genes of SVQ251 and SVQ255 into the genome of strain SVQ318, a *nodD1* derivative of strain HH103, carrying plasmid pMP240. The *nodD1* genes were subcloned into Tn*5*-1010 as 1.5-kb *Eco*RI–*Sal*I fragments. Wild-type *nodD1* from strain HH103 was included as a control, and the resulting plasmids were transferred to SVQ318(pMP240). Each bacterial cross was carried out in duplicate, and Km^R SVQ318(pMP240) transconjugants were selected and assayed for β-galactosidase activity in the presence and absence of the inducer naringenin. SVQ318(pMP240) transconjugants carrying *nodD1* genes from FITA mutants

Table 5 Plant responses to inoculation of *Glycine max* cv. Williams with *S. fredii* strains harboring the symbiotic plasmids pSym251 and pSym255. Data represent averages of eight plants. Determinations were made 8 weeks after inoculation. Bacteria isolated from four nodules formed by each inoculant showed the expected resistance markers. Numbers in the same column flanked by the same letter are not significantly different at the level α=5% using the non-parametric test of Kruskal-Wallis. Comparisons were made among plants inoculated with bacterial strains sharing the same chromosomal background

Inoculant	pSym	Number of nodules	Nodule dry weight (mg)	Plant-shoot dry weight (g)
HH103M	pSymHH103M	83.0±19.0 a	188.9±56.0 a	1.90±0.58 a
SVQ251	pSym251	96.7±27.3 ab	107.3±28.4 b	0.86±0.24 b
SVQ255	pSym255	111.1±19.5 b	156.6±29.3 a	1.56±0.47 a
USDA193C	pSymHH103M	90.5±28.8 a	346.3±80.8 a	3.02±0.62 a
	pSym251	125.3±22.8 ab	289.6±50.7 a	3.22±0.44 a
	pSym255	147.9±51.1 b	322.3±119.4 a	3.35±1.28 a
USDA192C	pSymHH103M	68.1±15.3 a	237.5±92.0 a	2.48±0.57 a
	pSym251	103.9±31.1 ab	238.4±70.1 a	2.54±0.78 a
	pSym255	116.4±37.0 b	346.1±62.6 b	3.43±0.45 b

SVQ251 and SVQ255 expressed constitutive β-galactosidase activities of 4,693±202 and 4,437±435 Miller units, respectively, as compared to only 1,121±185 units for SVQ318(pMP240) carrying *nodD1* of wild-type strain HH103. Addition of naringenin uniformly elevated expression to 8,449±687 to 9,970±857 units in all of these strains.

The *nodD1* genes of the four HH103M FITA derivatives were PCR-amplified and their sequences were determined and compared to that of the wild-type *nodD1* from strain HH103 (accession no. Y08938). In FITA mutants, SVQ251 (accession no. AF146795) and SVQ253, the C at position 1022 (as assigned in Y08938) is changed to an A, which causes the substitution of a leucine residue for isoleucine at position 244 in the putative FITA NodD1. The same nucleotide is also affected in mutant SVQ255 (accession no. AF147456), although in this case C is changed to G, which replaces leucine with valine at position 244 in the protein. In mutant SVQ250, the C at position 615 (as assigned in Y08938) is changed to T, leading to the substitution of threonine to methionine at position 108 of the polypeptide (accession no. AY198324).

Strain SVQ251 harbors a second mutation

FITA mutants SVQ251 and SVQ253 harbor the same mutation in their *nodD1* genes. However, the former is much less effective than the latter in nitrogen-fixation capacity on soybean cv. Williams. In addition, the FITA mutation harbored by plasmid pSym251 did not negatively affect symbiotic nitrogen fixation when it was present in strains USDA192C or USDA193C. We thus investigated whether strain SVQ251 carries an additional mutation that could be responsible for the observed decrease in nitrogen-fixing capacity. Plasmid pMUS296 (carrying wild-type *nodD1*) was transferred to strains SVQ516 and SVQ318 (*nodD1* derivatives of SVQ251 and HH103, respectively). Plant tests using soybean cultivar Williams showed that, although SVQ516(pMUS296) produced more nodules than SVQ318(pMUS296) (105.9 and 78.8 nodules per plant, respectively), plants inoculated with the former strain had shoot dry-weights that were significantly lower (α=5%) than those of plants inoculated with SVQ318(pMUS296) (1.67 and 2.80 g, respectively). These results suggest that, in addition to the *nodD1* FITA mutation, SVQ251 harbors another mutation that reduces its symbiotic nitrogen-fixation capacity.

Discussion

In this report, we have shown that *nodD1* of *S. fredii* HH103 accounts for the wide range of flavonoid responsiveness in this strain. This is consistent with the fact that NodD proteins from other broad-host-range rhizobia are responsive to a wide range of flavonoids (Györgypal et al. 1991aa, b). In fact, the NodD1 proteins from *S. fredii* HH103 and *Rhizobium* sp. NGR234 (accession numbers Y08938 and AE000065, respectively) are 98.8% identical. NodD1 of *S. fredii*, like that of *Rhizobium* sp. NGR234, only seems to require the presence of a hydroxyl group at position C7 in the flavone, flavanone, and isoflavone skeletons to activate *nod* expression. However, this structural requirement is not necessary when the inducer is a chalcone, since isoliquirtigenin lacks a hydroxyl group at C7 and activates *nod* expression.

Strains of *S. fredii* have two copies of *nodD*, *nodD1* and *nodD2* (Ramakrishnan et al. 1986; Appelbaum et al. 1988; Krishnan et al. 1995). Here we have shown that in *S. fredii* HH103 *nodD1* is necessary to nodulate soybeans. Since the previously reported *nodD1* mutant of *S. fredii* USDA191 nodulates soybean (Appelbaum et al. 1988), a *nodD1* mutant of another *S. fredii* strain was constructed. Mutant SVQ290 (a derivative of USDA257) had the same phenotype as mutant SVQ318, and it was also complemented by plasmid pMUS296 (Fig. 1).

The *nodD1* genes of *S. fredii* HH103, USDA257, and USDA191 are identical (accession numbers Y08938, L38459, and M18971, respectively). However, only the *nodD1* mutants of HH103 and USDA257 lose the ability to nodulate, suggesting that other regulatory gene(s), such as *nodD2*, could activate *nod* expression in USDA191.

nodD1 of *S. fredii* is preceded by a *nod*-box-like sequence that is oriented in the opposite direction of transcription of this gene (Appelbaum et al. 1988). Previous reports have shown that NodD proteins (NodD1 and/or NodD2) bind to this *nod*-box-like sequence, resulting in the repression of *nodD1*, and that the presence of the isoflavone genistein slightly increases the expression of this gene (Machado et al. 1998). Our results, presented in Table 3, support these observations, indicating that the NodD1 of *S. fredii* HH103 down-regulates its own expression; nonetheless, NodD2 could also play a role in the regulation of *nodD1* expression.

In this work, we combined two strategies in an attempt to isolate *S. fredii* strains with improved symbiotic properties: selection of FITA mutations and construction of hybrid strains with pSym-FITA mutations in different chromosomal backgrounds. FITA mutant SVQ251 produces less nodule mass than parental strain HH103M, resembling the symbiotically impaired *R. leguminosarum* bvs. *viciae* and *trifolii* FITA mutants previously described (Burn et al. 1987; McIver et al. 1989). However, the mutation found in *nodD1* of SVQ251 does not appear to be responsible for the impaired symbiotic capacity exhibited by this mutant, since FITA SVQ253 harbors the same mutation in *nodD1* but is not affected in its nitrogen-fixation capacity with soybean Williams. The possibility of a second mutation in SVQ251 is supported by the fact that mutant SVQ516 (SVQ251 NodD1$^-$) carrying plasmid pMUS296 (contains the wild-type *nodD1*) is significantly less effective with *G. max* cv. Williams than mutant SVQ318 (HH103 NodD1$^-$) carrying the same plasmid. This possible second mutation in SVQ251 is probably not located in the symbiotic plasmid, since dry weights of soybean plants inoculated with USDA192C (or USDA193C) transconjugants carrying pSym251 were similar to those obtained by

inoculation with USDA192C (or USDA193C) carrying the non-FITA plasmid pSymHH103M. Interestingly, the presence of plasmid pSym251 always produced an increase, although not significant, in the number of nodules formed. In contrast, FITA mutant SVQ255 forms more nodules (α = 5%) than its parental strain HH103M without any significant change in other symbiotic parameters. Similarly, USDA193C carrying plasmid pSym255 also formed more nodules than this strain carrying the non-FITA plasmid pSymHH103M, and plasmid pSym255, in combination with strain USDA192C, significantly increased (α=5%) all the analyzed parameters. Hence, SVQ255 aligns with the symbiotically improved mutants obtained by constructing a hybrid *nodD* gene between *nodD1* of *S. meliloti* and *nodD* of *R. leguminosarum* bv. *trifolii* (Spaink et al. 1989) or by UV-mutagenesis (Zhang et al. 2002).

Plant tests carried out with *B. japonicum* FITA-mutants and soybean cultivars Bayfeld and Marple Glen showed a lack of any cultivar specificity (Zhang et al. 2002). In contrast, results presented in Table 5 indicate that the bacterial genomic background also conditions the effect of FITA mutations on the symbiotic interaction.

FITA phenotypes are frequently linked to the appearance of symbiotic deficiencies (Burn et al. 1987; McIver et al. 1989). In addition to strain SVQ251, we found spontaneous derivatives of the FITA mutant SVQ250(pMP240) whose β-galactosidase expression was reduced to background levels even in the presence of flavonoids, suggesting the loss of functional *nodD1*. In another case, a HH103 FITA mutant suffered a deletion in its symbiotic plasmid that covered the entire *nodD1* gene, creating a derivative that was unable to nodulate (our own unpublished results). These facts indicate that in free living conditions some FITA mutants, if not all, appear to be under selective pressure for switching off the expression of *nod* or other genes controlled by the NodD1 regulatory circuit. As a result, the diversity of symbiotic impairments can vary from the loss of nodulation ability to retained capacity to nodulate but with poor effectiveness.

To our knowledge, this is the first report in which the competitive capacity of FITA mutants to nodulate their host legume has been investigated. The two FITA mutants described here were less competitive than their parental strain HH103M. Constitutive expression of nodulation genes might negatively affect bacterial competition to nodulate. More interesting is the possibility that FITA mutants could be altered in other symbiotically relevant functions, such as bacterial surface polysaccharides. The fact that *S. fredii* USDA191 carrying extra copies of *nodD2* forms non-mucoid colonies clearly indicates that regulators of *nod* expression can also influence polysaccharide synthesis (Appelbaum et al. 1988).

Curiously, SVQ251 and SVQ255 mutants are affected in the same leucine residue (position 244), although the substitution is different. Although the DNA-binding helix-turn-helix motif of NodD1 is located in its N-terminal part (Schell 1993), the mutations in SVQ251 and SVQ255, as well as in most other FITA mutants (Burn et al. 1989; McIver et al. 1989), are localized in the C-terminal. In contrast, the SVQ250 FITA mutation is located in a area of the NodD1 N-terminal region in which FITA mutations also have been described in *R. leguminosarum* bv. *trifolii* (McIver et al. 1989) and where the presence of a receiver protein domain has been postulated (Kofoid and Parkinson 1988).

It is remarkable that the mutation harbored by the symbiotic plasmid pSym255 increased the nodule number on soybean in all strains tested but only improved effectiveness in strain USDA192C. These results underscore the symbiotic relevance of the interaction between pSym and the rest of the genome (Chen et al. 1991; van Rhijn and Vanderleyden 1995) that has been confirmed in *S. meliloti* by using proteome analysis (Chen et al. 2000).

The potential advantage of *B. japonicum* FITA mutants for soybean cultivation under cool-season conditions has been recently reported (Zhang et al. 2002). Although our results support the possibility of isolating spontaneous FITA mutants showing improved nodulation and/or nitrogen fixation capacity, it is also clear that their competitive capacity and genetic stability should be thoroughly studied before they are used for agronomic purposes.

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