Research Note

A Method for the Positive Selection of Spontaneous *Rhizobium* Mutants Showing Transcriptional Activation of Nodulation Genes in the Absence of *nod*-Inducers

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A plasmid (pMUS248) containing a promoterless tetracycline resistance gene under the control of the *Rhizobium leguminosarum* bv. *viciae nodA* gene was constructed. Selection of tetracycline resistance in the absence of naringenin allowed the selection of spontaneous *Rhizobium* mutants showing transcriptional activation of nodulation genes in the absence of *nod*-inducers. These mutants were effective on *Glycine max*.

Additional keywords: regulatory *nod* mutants, *Rhizobium*, symbiotic plasmid transfer.

The NodD protein acts as the positive transcriptional regulator of *Rhizobium* nodulation genes upon activation by flavonoid inducers released by the host-plant root (Mulligan and Long 1985). Promoter regions of nodulation genes contain a conserved DNA sequence known as *nod*-box. The NodD protein binds to the *nod*-box for the transcriptional regulation of nodulation genes (Kondorosi *et al.* 1989). Burn *et al.* (1987) isolated *R. leguminosarum* bv. *viciae nodD* mutants that showed activation of the transcription of *nod* genes in the absence of inducer molecules. However, these *nodD* mutants were Fix$^-$ on *Pisum sativum*. Similar *R. l.* bv. *trifolii nodD* mutants showed a decrease in nodulation efficiency (McIver *et al.* 1989). Spaink *et al.* (1989) constructed by *in vivo* homologous recombination a hybrid *nodD* gene, consisting of 75% of the *nodD*1 of *R. meliloti* and 27% of the *nodD* of *R. meliloti* bv. *trifolii* that showed flavonoid-independent transcription activation (FITA phenotype). Interestingly, one of these mutants shows increased symbiotic nitrogen fixation capacity with clovers. These results suggest that *nodD* might be somehow linked to the proccess of nitrogen fixation.

The aim of this work was to develop a genetic system for the selection of spontaneous *Rhizobium* mutants showing transcription of the nodulation genes irrespective of the presence of *nod* inducers. For this purpose, we constructed a plasmid (pMUS248) in which transcription of the tetracycline resistance gene is controlled by the *nodA* promoter (*nodAp*) of *R. l.* bv. *viciae* (Fig. 1). The promoter of *nodA* was also cloned on the opposite direction (pMUS247). To make it possible to use plasmid pMUS248 in bacteria containing transposon Tn*5*, the *Pst*I fragment containing the km^r marker of pMUS248 was replaced by a 2.4-kb *Pst*I fragment carrying the gentamicin resistance marker from plasmid pWRK329B; this new plasmid was designated pMUS262.

R. fredii strains HH103-1 and AB-145 (Table 1) carrying plasmid pMUS248 grew on complete TY medium supple-

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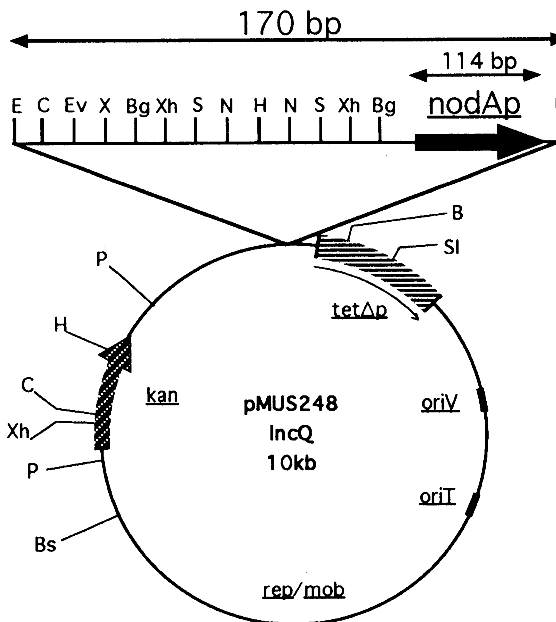

Fig. 1. To construct plasmid pMUS248, we cloned a DNA fragment containing *nodAp* (114 bp) from plasmid pMP1070 into the unique *Eco*RI site of plasmid pGL3. This site is located upstream of the promoterless tetracycline resistance gene (*tet*Δ*p*). To achieve this purpose, several subcloning steps were done in plasmid pIC20R to obtain the *nodAp* flanked by *Eco*RI restriction sites. All recombinant DNA techniques were as described in Maniatis *et al.* (1982). Restriction sites: E, *Eco*RI; C, *Cla*I; Ev, *Eco*RV; X, *Xba*I; Bg, *Bgl*II; Xh, *Xho*I; S, *Sac*I; N, *Nru*I; H, *Hin*dIII; Sl, *Sal*I; P, *Pst*I; B, *Bam*HI; Bs, *Bst*II. Restriction sites indicated in the figure may not be unique. *nodAp*, promoter of the *Rhizobium leguminosarum* bv. *viciae nodA* gene; *tet*Δ*p*, promoterless tetracycline-resistance gene.

mented with tetracycline (20 µg/ml) and the *nod*-inducer naringenin (3.6 µM). As expected, *R. fredii* strains HH103-1 and AB-145 carrying plasmid pMUS247 and strain AB-359 (a pSym-cured derivative of AB-145) containing pMUS248 failed to grow in media containing tetracycline, and there was no response to the presence of naringenin. These results show that the expression of tetracycline resistance encoded by plasmid pMUS248 is dependent on the presence of a Sym plasmid (or *nodD*) and a *nod* inducer (such as naringenin).

Although the presence of naringenin is necessary for growth of *R. fredii* strain HH103-1 (pMUS248) on TY medium containing tetracycline, a few tetracycline-resistant colonies arose in the absence of the inducer. There are three possible explanations for the appearance of these Tc^r colonies: 1) spontaneous tetracycline-resistant mutants, 2) mutations in the promoter of *nodA* or rearrangements in pMUS248, producing transcription of the promoterless *tet* gene, and 3) mutations in the regulatory *nodD* gene or in another gene involved in *nod* gene regulation.

The first possibility is very unlikely since spontaneous tetracycline-resistant mutants of *Rhizobium* have never been reported. We made the following studies on 19 Tc^r of *R. fredii* strain HH103-1 (pMUS248): 1) restriction analysis of plasmid pMUS248 using endonucleases *Cla*I and *Sal*I, 2) transfer of plasmid pMUS248 from the Tc^r *Rhizobium* clones to their parental strain HH103-1 and to *Escherichia coli*, 3) curing of plasmid pMUS248 from the Tc^r *Rhizobium* clones and introduction of plasmid pMP240 (a plasmid carrying a transcriptional fusion between the *nodA* promoter and the promoterless *lacZ* gene) to investigate whether the Tc^r clones had higher levels of β-galactosidase activity in the absence of naringenin than that shown by the parental strain *R. fredii* HH103-1 (pMP240).

These studies allowed us to classify the 19 Tc^r clones in three different classes (Table 2). Class I clones did not show alterations in the *Cla*I-*Sal*I restriction pattern of plasmid pMUS248 and had higher levels of β-galactosidase activity in the absence of naringenin than the parental strain (Table 3). It is likely that class I mutants originate by mutations in the *nodD* gene. Alternatively, the mutation could be located in other genes involved in *nod* gene regulation (for instance, a repressor). These mutations would produce transcription of

Table 1. Bacterial strains and plasmids used in this study[a]

Strain	Derivation and relevant properties	Source or reference
Rhizobium fredii		
HH103-1	HH103 Str^r	Buendia-Claveria *et al.* (1989a)
SVQ-205	Class I Tc^r clones of HH103-1	This work
SVQ-217		
SVQ-220		
AB-54	USDA 192 Str^r	Buendia-Claveria and Ruiz-Sainz (1985)
SVQ-244	pSym cured derivative of USDA 192, Rif^r	R. Romero, Seville
AB-145	USDA 193 Str^r	This work
AB-359	pSym-cured derivative of USDA 193, Str^r, Spc^r	Buendia-Claveria *et al.* (1989b)
Escherichia coli		
S17-1	294 Rec^-, chromosomally integrated RP4 derivative, Tp^r Sm^r	Simon *et al.* (1983)
Plasmids		
pRK2013	Used for mobilizing plasmids	Figurski and Helinski (1979)
pMH1701	Suicide vector derived from pSUP5011, carries Tn*5*-B12S (Tn*5*-Mob-*sac*)	Hynes *et al.* (1989)
pGL3	IncQ, containing the promoterless tetracycline resistance gene *tet*Δ*p* of pBR327, Km^r	Labes and Simon (1990)
pMUS248	pGL3 in which the *tet* gene is under the control of the pRL1JI *nodAp*	This work
pWKR329B	pACYC177 Km^r Gm^r Mob	W. Klipp, Bielefeld
pMUS262	Km^s Gm^r derivative of pMUS248	This work
pMP240	IncP, containing the promoterless *lacZ* gene under the control of the pRL1JI *nodAp*	de Maagd *et al.* (1988)

[a] *Rhizobium* strains were grown on complete (TY) medium. For growth of *Escherichia coli* strains, LB medium was used. When required, the media were supplemented with the appropriated antibiotics (µg/ml): rifampicin, 50; streptomycin, 400; spectinomycin, 50; kanamycin, 50 (25 for *E. coli*); neomycin, 100; gentamicin, 50 (10 for *E. coli*); tetracycline, 4 (10 for *E. coli*).

Table 2. Characteristics of Tc^r derivatives of *Rhizobium fredii* HH103-1 (pMUS248)

Characteristics	Classes of Tc^r derivatives: I	II	III
Restriction map of pMUS248 in the clones[a]	Normal	Altered[b]	Normal
Cotransfer of pMUS248 and the Tc^r marker[c]	No to *Rhizobium* No to *E. coli*	Yes to *Rhizobium* Yes to *E. coli*	Yes to *Rhizobium* No to *E. coli*
Induction level of β-galactosidase activity of pMP240 in the absence of naringenin[d]	2.5–6	1.0	1.0
Proportion of clones	3/19	5/19	11/19

[a] Endonucleases *Sal*I and *Cla*I were used.
[b] The *Cla*I-*Sal*I fragment that contains the *nodAp* is 0.8 kb larger than that of plasmid pMUS248.
[c] Conjugal transfer of plasmid pMUS248 from *Escherichia coli* S17-1 to *Rhizobium* was done by selection of the transfer of the kanamycin resistance marker. Transfer of plasmids from *Rhizobium* to *E. coli* were done by transformation. Tetracycline was assayed at 20 µg/ml for *Rhizobium* and at 2 µg/ml for *E. coli*. Growth of *E. coli* on media containing tetracycline was scored after 16 hr of incubation.
[d] Data are fold induction over the background activity of *Rhizobium fredii* HH103-1 (pMP240).

nod genes and other genes (such as *tet* of pMUS248 or *lacZ* of pMP240) that are under the control of typical *nod* promoters that contain *nod*-boxes. Table 3 also shows that the levels of β-galactosidase activity in the absence of naringenin varied widely among the different class I *Rhizobium* clones, indicating that these mutants have originated from different mutations.

Plasmid pMUS248 contains a 1-kb *ClaI-SalI* fragment that includes the promoter of *nodA* and the 5′ end of the promoterless *tet* gene (Fig. 1). Instead, pMUS248 derivatives isolated from clones belonging to class II showed that the *ClaI-SalI* band had acquired an extra 0.8-kb fragment. It is likely that this 0.8-kb DNA fragment is an insertion sequence able to promote transcription (ISp). In fact, plasmid pGL3 (from which plasmid pMUS248 was derived) was constructed to isolate ISp elements from *R. meliloti* (Labes and Simon 1990). The length (0.75 kb) of one of the *R. meliloti* ISp elements is similar to that of the additional DNA fragment of class II mutants. Class III clones did not show alterations in plasmid pMUS248, and their β-galactosidase activity was identical to that shown by the parental strain HH103-1 (pMP240). Class III mutants could originate by mutations in the *nodAp* region that increase basal transcription of the tetracycline resistance gene. These mutations would not alter the restriction map of pMUS248.

It is generally accepted that overexpression of nodulation genes is detrimental for nodulation (Knight *et al.* 1986). However, it is not clear whether mutations in *nodD* that produce expression of nodulation genes *ex planta* are detrimental for nodule formation and symbiotic nitrogen fixation (Burn *et al.* 1987; McIver *et al.* 1989; Spaink *et al.* 1989). In our case, the *R. fredii* class I clones SVQ-205, SVQ-217, and SVQ-220 formed nitrogen-fixing nodules on *Glycine max* 'Williams' and *Macroptilium atropurpureum.* Plant-top dry weights of soybean plants inoculated with these clones were not significantly different from those shown by the parental strain HH103-1. Similar results were obtained with *M. atropurpureum*, except that strain SVQ-205 produced a lower plant-top dry weight than the parental strain (Table 4). Isolates from soybean and siratro nodules induced by class I clones were used as recipients of plasmid pMP240 and assayed for β-galactosidase activity in the absence of naringenin. All nodule isolates retained the β-galactosidase levels that were characteristic of their respective inoculant strains. It is possible that some types of mutations producing FITA-like phenotypes could be beneficial, whereas others could be detrimental for nitrogen fixation. In addition, the symbiotic properties of these mutants could be altered for some legume hosts but not for others, as was observed with clone SVQ-205.

Table 3. β-Galactosidase activity (μM) of class I mutants of *Rhizobium fredii* strain HH103-1 in the presence and absence of 3.6 μM naringenin

Strain	Naringenin	
	Absent	Present[a]
HH103-1 (pMP240)	2,122	17,563
SVQ-205 (pMP240)	10,999	20,764
SVQ-217 (pMP240)	5,264	15,189
SVQ-220 (pMP240)	11,683	22,396

[a] Assays for β-galactosidase activity using naringenin as inducer were performed in liquid media as described by Spaink *et al.* (1987). Each test was performed in triplicate and the variation of the expression levels was within 10%.

Because expression of tetracycline resistance encoded by plasmid pMUS262 is *nodD*-dependent and this regulatory gene is usually located on the symbiotic plasmid, we investigated the possibility of using plasmid pMUS262 to detect the transfer of symbiotic plasmids from *Rhizobium* donors that also contain additional cryptic plasmids. In a model experiment, we showed that transfer of the symbiotic plasmid pJB5JI (= pRL1JI *mep*::Tn*5*) from *R. leguminosarum* RBL5560 to *R. fredii* SVQ-244 (pMUS262) produced Nm^r transconjugants that were Tc^r in the presence of naringenin. In contrast, transfer of plasmid pRL1JI *nod*D::Tn*5*, using the same donor and recipient strains, produced Nm^r SVQ-244 (pMUS262) transconjugants that were Tc^s in the presence of the *nod*-inducer. This strategy was further used to illustrate an easy identification of a symbiotic plasmid in a more difficult case: *R. fredii* strain USDA 192 contains three plasmids; one of them, the symbiotic plasmid pRfr192b, is Tra^-, while the cryptic plasmid pRfr192a is Tra^+. Random transposon Tn*5*-Mob-*sac* mutagenesis (Hynes *et al.* 1989) was done on *R. fredii* strain AB-54 (= USDA 192 Str^r) by using the suicide plasmid pMH1701. One hundred and fifty Nm^r AB-54 transconjugants were individually assayed in triparental conjugations (using the helper plasmid pRK2013) for neomycin-resistance donor ability to *R. fredii* SVQ-244. Six Nm^r AB-54 clones transferred the neomycin-resistance marker at high frequency (10^{-4} per donor cell), but all Nm^r SVQ-244 transconjugants failed to grow on media containing tetracycline and naringenin and did not nodulate soybean cv. Peking. Plasmid electrophoresis (as described by Buendía-Clavería and Ruiz-Sainz 1985) showed that they had inherited the cryptic plasmid pRfr192a. In contrast, three Nm^r AB-54 clones cotransferred, at very low frequency (10^{-9} per donor cell), the neomycin-resistance marker with the ability to grow on media supplemented with tetracycline and naringenin. Two of them had acquired the capacity to nodulate effectively on soybean cv. Peking. The other transconjugant failed to nodulate soybeans; plasmid electrophoresis showed that it had inherited a plasmid that is slightly smaller than pRfr192b, probably a deletion in this plasmid.

Table 4. Plant responses to inoculation of *Glycine max* cv. Williams and *Macroptilium atropurpureum* with *R. fredii* class I clones SVQ-205, SVQ-217, and SVQ-220[a]

Inoculant	Plant top dry weight,[b] mg	
	G. max	*M. atropurpureum*
HH103-1	3,504 a	135 a
SVQ-205	3,083 a	85 b
SVQ-217	2,940 a	139 a
SVQ-220	3,493 a	153 a
Uninoculated	733 b	16 c

[a] Nodulation tests were done as described by Buendia-Claveria *et al.* (1989a). Data represent averages of eight plants for *G. max* and 15 plants for *M. atropurpureum.* Determinations were made 7 wk after inoculation.

[b] Numbers in the same column flanked by the same letter are not significantly different at the 5% level of probability of analysis of variance.

In summary, the use of plasmid pMUS248 (or pMUS262) has several advantages. It could allow a ready selection of large numbers of *nodD* mutants (or mutants in other genes involved in the regulation of *nod* genes) in bacteria containing the entire pSym plasmid or only *nodD* cloned in a plasmid. It selects spontaneous mutants; since these are not genetically engineered, can be tested as legume inoculants in field conditions. It makes possible the easy detection of transfer of symbiotic plasmids, avoiding time-consuming experiments in the primary screening, such as plasmid electrophoresis and plant tests. It could allow the detection of the loss of *nodD*-mediated transcriptional activation, which might be useful in pSym-curing experiments.

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