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Construction of a *Sinorhizobium meliloti* strain carrying a stable and non-transmissible chromosomal single copy of the green fluorescent protein GFP-P64L/S65T

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Abstract

A single copy of the green fluorescent protein (GFP)-encoding gene *gfp*-P64L/S65T under the control of the constitutive *nptII* promoter was introduced in a neutral region of the *Sinorhizobium meliloti* chromosome, between the genes *recA* and *alaS*. Within the same chromosomal region downstream of *gfp*-P64L/S65T a tetracycline (Tc) resistant cassette was also inserted. Both markers were very stable during at least 40 bacterial generations without any selective pressure. Similarly, the *gfp*-Tc cassette was stable and functional in all rhizobia that were recovered from alfalfa nodules. The GFP-associated fluorescence derived from the (single copy) chromosomal *gfp*-P64L/S65T allowed detection of rhizobia during the colonisation of the root, infection thread formation, and nodule development. The *gfp*-Tc rhizobia showed indistinguishable phenotypes for nodulation, competitiveness, and nitrogen-fixation from the parental strain. The labelling system described here can be used for the stable fluorescent tagging of *S. meliloti* strains allowing their detection in biologically complex soil environments.

Keywords: Green fluorescent protein; Nodulation; Soil; *Sinorhizobium meliloti*

1. Introduction

A significant amount of practical and fundamental knowledge on the biology of the rhizobium–legume symbioses has been gathered over the years [1,2], allowing this association to become a model system for the study of other plant–microbe interactions. However, owing to the difficulty of monitoring specific bacterial genotypes in the background of large numbers of heterogeneous microbial populations, most studies on the molecular ecology of rhizobia have been carried out principally in the last decade. The study of the establishment of rhizobia inoculated into natural soils, their rhizosphere colonisation, nodule occupancy, and putative exchange of genetic traits with other soil bacteria all require the use of properly tagged strains for their unequivocal recognition in a biodiverse environment.

The green fluorescent protein (GFP) from *Aequorea victoria* is being used increasingly as a reporter for gene expression and protein localisation in bacterium–host interactions, as well as for in vivo studies in microbe, plant, insect, and mammalian systems [3]. The ability of GFP to fluoresce in the absence of any added cofactor makes it particularly useful for in situ studies. The use of GFPs and other autofluorescence proteins has already been reported in rhizobia. First, GFP-S65T was cloned in a replicative plasmid and was successfully expressed in *Sinorhizobium meliloti* cells [4]. However, 40% of the rhizobia recovered from the root nodules had lost the fluorescent marker due to plasmid instability. To ensure that every bacterial cell remained labelled with GFP throughout nodulation without selective pressure, Cheng and Walker [5] constructed a GFP-expressing vector that is stable and replicates in *S. meliloti* cells in planta. Using a different approach,

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the stability of the fluorescent marker in *S. meliloti* was recently achieved by the genomic integration of a GFP-S65T-encoding non-replicative plasmid [6]. The system was successfully used to investigate the expression of exudate-inducible *S. meliloti* genes in the presence of different plant species. Color variants of fluorescent proteins were also cloned in a stable broad-host-range vector to visualise rhizobia interacting with plants [7]. Although these constructions are useful for studies under controlled laboratory conditions, they should not be used in non-sterile soil assays since the recombinant plasmids (either integrated or not) could be easily dispersed by conjugal transfer from the reference strain to other rhizobia or even to another species [8,9]. The conjugal-transfer origin present in the plasmids might eventually promote genetic mobilisation if compatible helper functions are provided by other replicons acquired from a second strain (i.e. present in the soil).

In this work we present the construction and characterisation of a *S. meliloti* strain that carries a chromosomal single copy of the *gfp*-P64L/S65T which proved to be stable, symbiotically neutral, and useful to detect single fluorescent rhizobial cells.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used are listed in Table 1. *Escherichia coli* and *S. meliloti* strains were grown on Luria–Bertani medium [10] at 37°C, and on TY [11] or YEM [12] at 28°C, respectively. For solid media 15 g of agar per litre of medium was added. The final concentration of antibiotics per millitro of medium was 5 μg gentamycin (Gm) and 6 μg tetracycline (Tc) for *E. coli*, and 400 μg streptomycin (Sm), 50 μg Gm and 6 μg Tc for *S. meliloti*.

2.2. DNA manipulation and genetic constructs

Plasmid DNA preparation, restriction enzyme analysis, cloning procedures, and *E. coli* transformation were performed according to previously established techniques [13]. Southern hybridisation was carried out using DNA probes labelled with digoxigenin. The probes were synthesised by PCR using digoxigenin–dUTP (Boehringer Mannheim) and appropriate primers to amplify the region of interest. For hybridisation, DNA extracted from bacteria was digested and transferred to nitrocellulose membranes (Hybond N; Amersham) as described by Chomczynski [14]. The digoxigenin-labelled DNA probes were hybridised to the membranes at 65°C overnight after the blocking of non-specific binding sites for 1 h at 68°C, using the solutions and experimental conditions specified by Boehringer Mannheim (Catalogue No. 1093 657). For the visualisation of positive bands, the membranes were incubated with an antibody against the digoxigenin ligand and washed, and a final colour reaction was initiated at alkaline pH by the addition of X-phosphate plus nitroblue tetrazolium chloride as specified by the manufacturer.

2.3. Bacterial matting

Transfer of plasmids between *E. coli* S17-1 and *S. meliloti* were performed according to Simon et al. [15].

2.4. Plant nodulation assays

Medicago sativa seeds (alfalfa, cv CUF101 obtained from Instituto Nacional de Tecnología Agropecuaria, La Pampa, Argentina) were surface-sterilised for 10 min with commercial bleach 20% v/v (NaClO concentration equivalent to 55 g active Cl_2 l^{-1}) followed by six washes with sterile distilled water. Surface-sterilised seeds were germinated on water agar (1.5%, w/v). For infection and competition studies 2-day-old seedlings were transferred to

Table 1
Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source
Strains		
E. coli DH5α	*recA*, Δ*lacU169*, F80d*lac*ZDM15	Bethesda Res. Lab.
E. coli S 17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	[15]
S. meliloti 2011	Sm^r, Nod^+ Fix^+ in alfalfa, derived from strain SU47	J. Dénarié, France
S. meliloti 20MP6	Derivative of *S. meliloti* 2011, Sm^r, Tc^r, GFP	This work
Plasmids		
pGreenTIR	Ap^r,*gfp*-P64L/S65T cassette	[22]
pHP45Ω-Tc	Ap^r, carrying a DNA cassette for Tc^r flanked by transcription and translation terminators	[19]
pSM10	Gm^r, *S. meliloti* partial sequences of the *S. meliloti alaS* and *recA* genes	[18]
pMP5	Gm^r, Tc^r, pSM10 carrying in *Bam*HI (blunt ended by Klenow enzyme) a 2-kb Tc^r DNA cassette from pHP45Ω-Tc cloned as *Sma*I	This work
pMP6	Gm^r, Tc^r, pMP5 carrying in *Eco*RI a 0.75-kb DNA fragment containing the *gfp*-P64L/S65T cassette from pGreenTIR cloned as *Eco*RI	This work

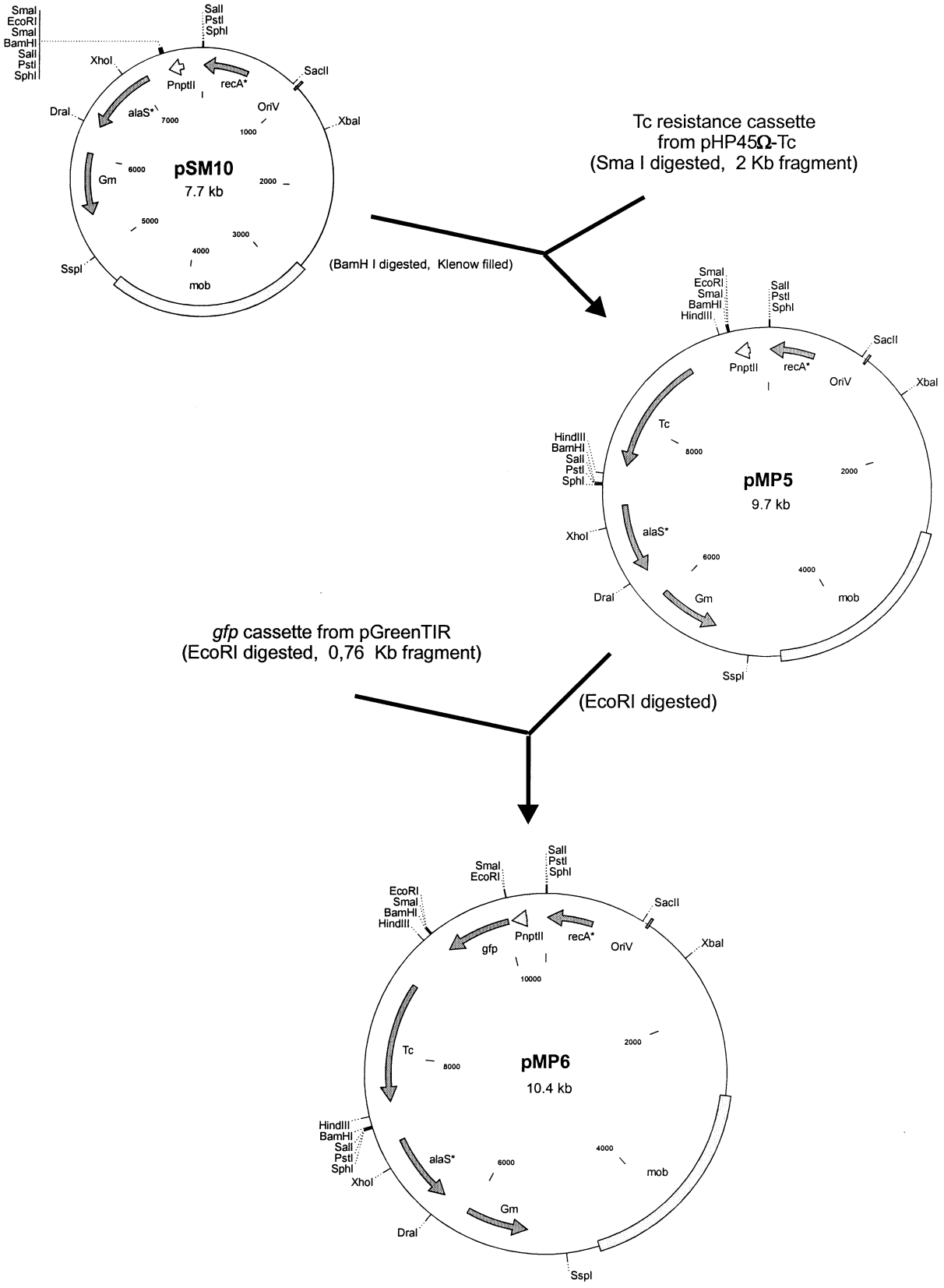

Fig. 1. Construction of the delivery vector pMP6. Restriction sites relevant for the construction of the vector are shown. Each step in the construction is indicated by an arrow. *PntpII*, promoter of the neomycin-resistance gene, Tc and Gm, genes encoding Tc and Gm resistance respectively. The C-terminal coding region of the *S. meliloti recA* gene (*recA**) as well as the N-terminal coding region of the downstream located *alaS* gene (*alaS**) are indicated. Mobilisation functions of plasmid RP4 are also indicated (*mob*). The intermediate Tc^r construct was designated pMPS.

gamma-irradiated sterilised plastic growth pouches (Mega Minneapolis International, Minneapolis, MN, USA) containing 10 ml of nitrogen-free Jensen mineral solution, pH 6.7 [16]. Three days later, primary roots were inoculated with 10^6 rhizobia in Jensen medium by dripping 100 μl of a bacterial suspension onto the root from the tip toward the base. The rhizobia were obtained from exponential-phase YEM cultures. The plants were cultured in a growth chamber at 22°C for a 16-h photoperiod. When two different strains were co-inoculated for competition experiments both rhizobia were mixed prior to plant inoculation (10^6 CFU/100 μl inoculant each). The CFU contained in the inocula were estimated by plate counts.

Root hair curling, infection threads, and nodules were examined under a Carl Zeiss Jena fluorescent microscope either with visible light or UV with a filter set for fluorescence. In this last case a 470–490-nm excitation filter and a 520–560-nm barrier filter were used. Nodule occupancy was scored 4 weeks after inoculation by counting fluorescent nodules. In addition random-selected nodules from the same experiments were removed from the roots, surface-sterilised with 30 vol. H_2O_2 for 10 min followed by washing with abundant sterile water, and crushed in 10 μl of sterile isotonic solution. Appropriate dilutions were plated on YEM Petri dishes with or without Tc, and the proportion of the antibiotic resistant CFUs was compared to the proportion of fluorescent nodules scored by eye under the microscope.

Nitrogen fixation was estimated [12] through the dry matter of the aerial part of inoculated alfalfa plants cultivated in vermiculite with N-free Jensen medium. The average dry weight for each treatment was obtained using 10 individual plants (mean ± S.D. are shown). Statistical analysis was performed using analysis of variance as previously described [17].

3. Results and discussion

3.1. Construction of the pMP6, a GFP-Tc derivative pSM10, and its integration into the S. meliloti chromosome

Our first goal was the construction of a shuttle vector for the stable integration of a *gfp* marker into a neutral site on the *S. meliloti* chromosome. The vector pMP6 was constructed as outlined in Fig. 1. Using the basic vector pSM10 [18] which carries partial sequences of the chromosomal *S. meliloti recA* (3′-end) and *alaS* genes (5′-end), we cloned a variant of *gfp* and a DNA cassette for Tc resistance (Tc^r) [19]. The modified *gfp*-referred to as *gfp*P64L/S65T-contains the mutations F64L and S65T associated with an increased GFP solubility and a red-shift in the fluorescence [20,21]. As indicated in Fig. 1 the integration vector pSM10 was first digested with *Bam*HI and the cohesive ends were filled-in with Klenow fragment enzyme. The digested vector was then ligated to an approximately 2.0-kb *Sma*I DNA fragment carrying the Tc^r gene from vector pHP45Ω-Tc [19]. The resultant plasmid designated pMP5 was finally digested with *Eco*RI and ligated to *gfp*-P64L/S65T (760 bp) from pGreenTIR [22] to yield plasmid pMP6. For integration of the transgenic markers into the *S. meliloti* chromosome plasmid pMP6 was transferred from *E. coli* S17-1 to *S. meliloti* 2011 by conjugation, and transconjugants that had presumable undergone the marker exchange (non-repetitive sequences bordering the transgenes) were identified by their expected Tc^r (from the omegon-cassette), Sm^r (marker of the recipient bacteria), and Nm^s (after plasmid segregation) phenotype. The genomic structure was confirmed by Southern transfer analysis.

3.2. Stability of the fluorescent phenotype and Tc^r of S. meliloti 20MP6

The stability of the markers was analysed after extensive cultivation of strain *S. meliloti* 20MP6 in TY medium without any selective pressure (no antibiotics were added). The Tc^r and the green fluorescence under UV illumination were analysed over the course of four serial batch cultures that spanned approximately 40 rhizobial generations. The frequency of Tc-resistant CFU was assessed by plating appropriate dilutions of the cultures in TY Petri dishes with and without the antibiotic. As shown in Fig. 2, the Tc-resistance phenotype was maintained during the whole period of analysis showing a very high stability of the marker. All colonies were also fluorescent under UV light both in the presence and absence of the antibiotic. The results showed a remarkable stability for the chromosomal construct under the experimental conditions used.

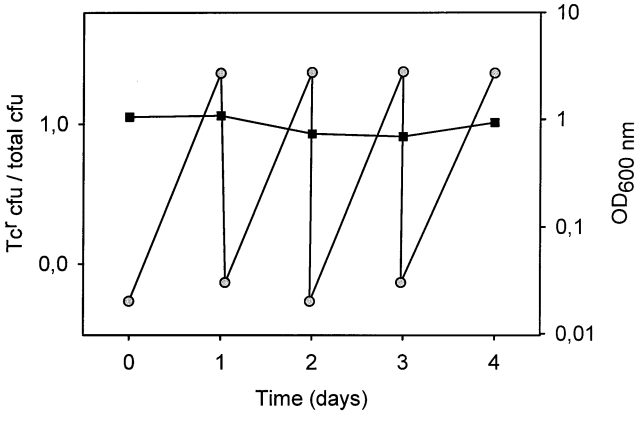

Fig. 2. Maintenance of the Tc^r marker in strain *S. meliloti* 20MP6 in the absence of selection. *S. meliloti* 20MP6 was grown overnight in TY medium with Tc, and then diluted the next day into TY medium without the antibiotic. The culture was allowed to grow to saturation and then diluted. This was done four consecutive times over the course of the experiment. The proportion of Tc resistant bacterial cells was determined by plating at the indicated times. OD_{600}, optical density at 600 nm.

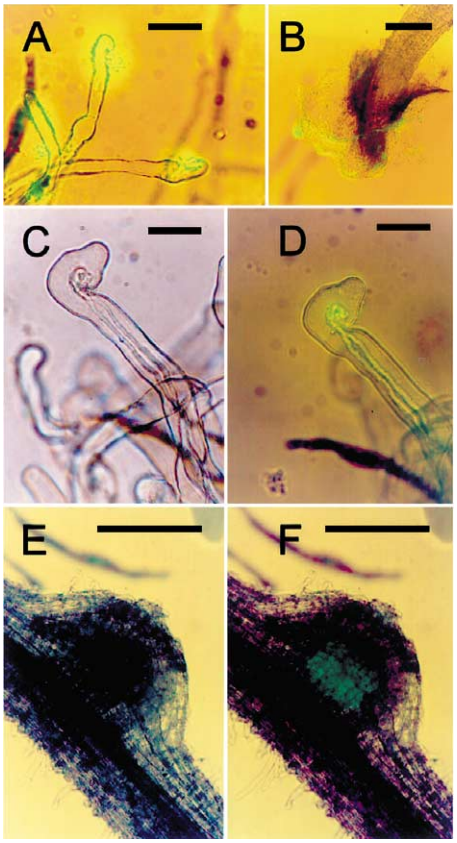

Fig. 3. Microscopic observations of *S. meliloti* 20PM6 expressing GFP-P64L/S65T at different stages of symbiosis in alfalfa plants. Combined fluorescence and bright-field image of adsorbed bacteria on root hair tips (A), and on the mucigel of the root tip of a young plant (B). Bright-field image of a root hair containing a double infection thread (C). Combined fluorescence and bright-field image of infection threads in panels C,D. Bright-field image of an emerging root nodule (E). Combined fluorescence and bright-field image of the root nodule in panels E,F. Bars: 100 μm (A), 50 μm (C,D), and 1 mm (B,E,F)

3.3. Phenotypic expression of fluorescence by S. meliloti 20MP6 at difference stages of symbiosis

The early colonisation of alfalfa roots and their further infection by *S. meliloti* 20MP6 were analysed using fluorescence and phase contrast microscopy (Fig. 3). Bacteria could be seen adsorbed and embedded in the mucigel surrounding the root tip (Fig. 3A,B). Infection threads could also be easily detected (Fig. 3C,D). No loss of fluorescence was detected during root invasion by *S. meliloti* 20MP6 as infection threads or within the developing nodules (Fig. 3E,F). Results showed a continuous phenotypic expression of the GFP, from outside the root to the infected plant tissues at different stages of symbiosis. Clearly, the single chromosomal copy of the *gfp*P64L/S65T under the control of the strong *nptII* promoter was sufficient for the detection of free-living or symbiotic rhizobia. Single fluorescent free-living rhizobia could be detected when a bacterial suspension of strain 20MP6 was observed under UV light with 1000× magnification (not shown).

3.4. Symbiotic properties of S. meliloti 20MP6: nodulation, competitiveness and nitrogen fixation

Previous reports indicated that the DNA at the intergenic region between the *S. meliloti* genes *recA* and *alaS* can be modified in different ways without any detectable changes in the symbiotic properties of the resultant recombinant rhizobia [18]. Here, we evaluated if the *gfp*-P64L/S65T and the Tc-resistant marker had any effects on the symbiotic behaviour of strain 20MP6. Results from Fig. 4A showed that a similar number of root nodules on alfalfa plants was induced by strain 20MP6 and by the parental wild-type 2011. Furthermore, as is shown in Fig. 4B the aerial dry weight of alfalfa plants inoculated with *S. meliloti* 20MP6 was statistically comparable to the dry weight of alfalfa plants inoculated with strain 2011. Because in the growing conditions used no N-sources other than atmospheric N_2 were present, dry matter accumulation was directly related to N_2-fixation. The results indicated that the chromosomal insertion of the new markers did not significantly affect either nodulation or nitrogen fixation in strain 20MP6. To compare in more detail the nodulation capabilities of parental and recombinant rhizobia we performed a co-inoculation assay (10^6 CFU of each rhizobia/root 1:1 inoculant ratio of strains 20MP6:2011) and we evaluated root nodule occupancy. From 67 nodules analysed the ratio of fluorescent nodules: non-fluorescent nodules was 0.49:0.51 showing a similar level of competitiveness for strains 20MP6 and 2011. Fluorescent bacteria recovered from nodules were all resistant to Tc. This result suggests that *gfp*-P64L/S65T and the Tc^r cassette are both stable in symbiosis in agreement with their observed stability in culture medium (Fig. 2).

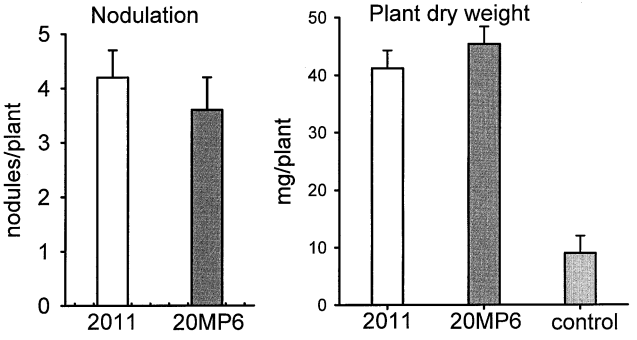

Fig. 4. Nodulation and dry weight of the aerial part of alfalfa plants inoculated with strain *S. meliloti* 20MP6. Five-day-old alfalfa plants were inoculated with approx. 10^6 CFU/root of either *S. meliloti* 20MP6, or the wild-type strain *S. meliloti* 2011. The number of root nodules/plant (A) and the dry weight of the plants (B) were obtained 4 weeks after inoculation (number of plants = 10). The control corresponds to the dry weight of the aerial part of uninoculated alfalfa plants. Bars correspond to mean values ± S.D.

3.5. Concluding remarks

Current studies on molecular bacterial ecology have an increasing demand for the specific detection of particular genotypes in complex and biodiverse environments. We developed in this work a *gfp*-P64L/S65T-Tc-based labelling system for the specific tagging of the soil bacteria *S. meliloti*. Although previous work used plasmid-borne versions of the *gfp* marker in *S. meliloti*, two main problems arise from the use of broad host range and transmissible replicons to track strains in the soil: the loss of the markers due to plasmid instability, and vector dispersion via conjugal transfer mechanisms. Unfortunately, the extent to which such events occur in a given soil sample are difficult to predict. For this reasons the use of chromosomally integrated markers represent a more suitable choice for those strains that have to be studied in non-sterile and biologically complex systems. In this work, the *gfp*-P64L/S65T marker was stable over time with appropriate phenotypic expression at different stages of the life cycle of the bacteria. Interestingly, although *gfp*-P64L/S65T was introduced as a single copy within the rhizobial chromosome, the expression level of GFP supported the detection of single fluorescent bacteria. It was also shown that the recombinant rhizobia did not show any detectable change in symbiosis when compared to parental rhizobia. The delivery plasmid pMP6 constructed here represents a suitable and reliable tool for the fluorescent labelling and detection of specific *S. meliloti* genotypes in soil.

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