Journal of Microbiological Methods 117 (2015) 155–163

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of molecular tools to monitor conjugative transfer in rhizobia

Gonzalo Torres Tejerizo [a,*], Luis Alfredo Bañuelos [b], Laura Cervantes [b], Paul Gaytán [c], Mariano Pistorio [a], David Romero [b], Susana Brom [b]

[a] Instituto de Biotecnología y Biología Molecular, CCT-La Plata-CONICET, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[b] Programa de Ingeniería Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad 1001, Col. Chamilpa, 62210 Cuernavaca, Morelos, Mexico
[c] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 1001, Col. Chamilpa, 62210 Cuernavaca, Morelos, Mexico

ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 5 August 2015
Accepted 6 August 2015
Available online 10 August 2015

Keywords:
Rhizobia
Plasmid
Conjugative transfer
Fluorescent cassettes

ABSTRACT

Evolution of bacterial populations has been extensively driven by horizontal transfer events. Conjugative plasmid transfer is considered the principal contributor to gene exchange among bacteria. Several conjugative and mobilizable plasmids have been identified in rhizobia, and two major molecular mechanisms that regulate their transfer have been described, under laboratory conditions. The knowledge of rhizobial plasmid transfer regulation in natural environments is very poor. In this work we developed molecular tools to easily monitor the conjugative plasmid transfer in rhizobia by flow cytometry (FC) or microscopy. 24 cassettes were constructed by combining a variety of promotors, fluorescent proteins and antibiotic resistance genes, and used to tag plasmids and chromosome of donor strains. We were able to detect plasmid transfer after conversion of non-fluorescent recipients into fluorescent transconjugants. Flow cytometry (FC) was optimized to count donor, recipient and transconjugant strains to determine conjugative transfer frequencies. Results were similar, when determined either by FC or by viable counts. Our constructions also allowed the visualization of transconjugants in crosses performed on bean roots. The tools presented here may also be used for other purposes, such as analysis of transcriptional fusions or single-cell tagging. Application of the system will allow the survey of how different environmental conditions or other regulators modulate plasmid transfer in rhizobia.

1. Introduction

Rhizobia are Gram-negative bacteria able to grow in the soil under free-living conditions and in symbiosis associated with the roots of legumes, as nitrogen-fixing organisms. A general feature of rhizobia is the presence of a large amount of plasmid DNA in their genomes. Among the major evolutionary forces that have shaped microbial diversity is horizontal gene transfer (HGT); conjugative transfer (CT) has been proposed as one of the crucial mechanisms for HGT. CT of rhizobial plasmids has been well documented since decades ago, and a variety of regulatory mechanisms affecting this function have been described, which include *quorum-sensing* (Danino et al., 2003; Tun-Garrido et al., 2003), repression by RctA (Nogales et al., 2013), and other regulators or mechanisms (Ding et al., 2013; Lopez-Fuentes et al., 2015; Pistorio et al., 2013; Torres Tejerizo et al., 2014). Specifically in *Rhizobium etli* CFN42, we have previously described that plasmid pRet42a (194 kbp) carries a cluster of transfer genes, whose expression is regulated by *quorum-sensing* elements (Tun-Garrido et al., 2003), while transfer of the symbiotic plasmid pRet42d (371 kbp) depends on its cointegration with pRet42a (Brom et al., 2004). Additionally, transfer of pRet42d has been shown to occur independently of pRet42a when the RctA repressor is inactivated (Pérez-Mendoza et al., 2005). However, the experiments for evaluating rhizobial CT have been performed under laboratory conditions, where plasmid transfer can be easily detected by growing bacteria in selective media (after tagging the plasmids with antibiotic resistance genes). Nevertheless, there are only a few reports that analyze CT in soils, resembling natural conditions (Klumper et al., 2015; Musovic et al., 2010; Shintani et al., 2014; van Elsas et al., 1988, 1989), but they are focused mainly on transfer of antibiotic resistance markers by *Pseudomonas* spp. or *Escherichia* spp. strains. In order to evaluate if the frequency and regulatory mechanisms affecting CT in rhizobia present in their natural environments are the same as those described in laboratory conditions, we designed a system to specifically detect the transfer of tagged plasmids from known-donor rhizobia, avoiding the need for lethal selections. The system was modified from a report originally set up for *Pseudomonas* (Nancharaiah et al., 2003). A schematic figure of how the system works is shown in Fig. 1. The donor strain was tagged in the chromosome with a Red Fluorescent Protein (RFP) gene and the plasmid was tagged with a Green Fluorescent Protein (GFP) gene. Donor strains show both fluorescent tags while transconjugants that inherit the plasmid display only the green tag.

* Corresponding author.
E-mail address: gatt@biol.unlp.edu.ar (G. Torres Tejerizo).

http://dx.doi.org/10.1016/j.mimet.2015.08.005
0167-7012/

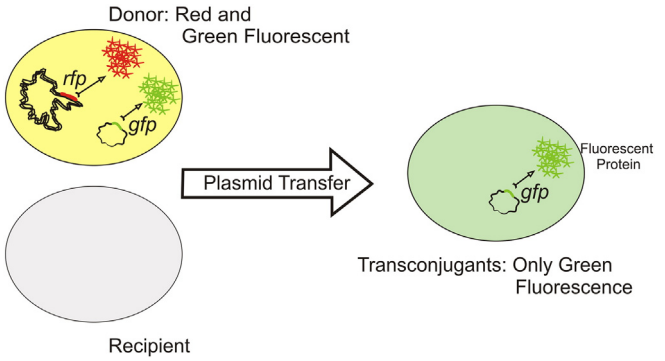

Fig. 1. Schematic representation of the plasmid transfer detection-system. A donor strain (red fluorescence in the chromosome and green fluorescence in the plasmid) showing both tags simultaneously is mixed with a non-fluorescent recipient strain. Conjugative transfer of the plasmid converts the non-fluorescent recipient into a green fluorescent transconjugant; thus discerning donors, recipients and transconjugants by their fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Tagging bacteria with fluorescent protein is a widely used technique, but sometimes the overproduction of a fluorescent protein can be toxic for the bacteria. To avoid this, we examined the usefulness of different promoters by constructing several cassettes harboring fluorescent proteins (*mCherry* or *gfp*mut3*) under diverse promoters and containing different combinations of antibiotic resistance genes (Gentamicin, Spectinomycin and Tetracycline). The combination of promotors, fluorescent proteins and antibiotic resistance enables the users to select the one appropriate for their purpose. To apply this approach in rhizobia, we developed derivatives of *R. etli* CFN42 containing different promoters and fluorescent proteins, evaluated them using microscopy and optimized cell counting by flow cytometry to detect plasmid transfer.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* was grown on Luria-Bertani (LB; Miller, 1972) medium at 37 °C. *Rhizobium* spp. and *Agrobacterium tumefaciens* strains were grown on PY (Noel et al., 1984) at 30 °C. For solid media 15 g of agar per liter of medium were added. The final concentration of antibiotics used was (in μg ml^{-1}): gentamicin (Gm) 10, kanamycin (Km) 25, and tetracycline (Tc) 10 for *E. coli*. For *Rhizobia* and *A. tumefaciens*: streptomycin (Sm) 400, nalidixic acid (Nal) 20, neomycin (Nm) 60, rifampicin (Rif) 100, spectinomycin (Sp) 100, Gm 30, and Tc 5.

2.2. Bacterial matings

Conjugation experiments were performed as described by Cervantes et al. (2011). Briefly, overnight cultures were grown to stationary phase. Donor and recipient strains were mixed in a 1:2 volume ratio and incubated overnight on PY plates at 30 °C. The mixtures were resuspended in 1 ml of 10 mM $MgSO_4$–0.01% Tween 40 (vol/vol). Serial dilutions were plated on selective PY medium supplemented with the corresponding antibiotics to quantify the number of donor, recipient, and transconjugant cells. The conjugation frequencies are expressed as the number of transconjugants per donor cell. The visualization of plasmids in the transconjugants (plasmid profiles) was evaluated by Eckhardt-gels (Eckhardt, 1978) as modified by Hynes & McGregor (Hynes and McGregor, 1990). Donor and recipient strains were included as controls.

2.3. DNA manipulation and genetic constructs

Procedures to obtain total DNA, plasmid purification, restriction-enzyme analysis, cloning, and *E. coli* transformation, were performed according to previously established techniques (Sambrook et al., 1989). When necessary, plasmids were isolated with a High Pure Plasmid Isolation kit (Roche) and DNA from agarose gels was obtained with a GeneJet extraction kit (Fermentas).

Oligonucleotides (Supplementary Table 1) were purchased from "Unidad de Síntesis y Secuenciación de DNA. IBT-UNAM". PCR amplification was carried out with recombinant *Taq* DNA polymerase or *Pfu* DNA polymerase as specified by the manufacturers in a Mastercycler 5330 (Eppendorf) or in an iCycler (Bio-Rad) thermocycler.

2.3.1. Construction of a new vector harboring Gm resistance

Vector pGX90 allows the release of the Gm resistance gene as a blunt *SspI* fragment with few restriction sites. For the construction of pGX90, pGX82 was digested with *XbaI* and ligated with a 1.5 kbp *NheI*-fragment containing the gentamicin (*accCI*) resistance gene from pBSL142 (Alexeyev et al., 1995). pGX82 was constructed by excising the polylinker of pK18mob (Schäfer et al., 1994) with *EcoRI* and *HindIII*; and substituting it by a new linker containing the following restriction sites "*HindIII*, *EcoRI*, *SmaI*, *BamHI*, *SspI*, *XbaI*, *SspI*, *BamHI*, *SmaI*, *EcoRI*, and *HindIII*", using oligonucleotides "linkerEXba" and "linkerHXba".

2.3.2. Construction of a new vector harboring the mCherry gene

The *mCherry* gene was assembled with 24 oligonucleotides of 60 bases via the Simplified Gene Synthesis approach (Wu et al., 2006) and was cloned in the non-commercial plasmid vector pJOQ kindly donated by Dr. Joel Osuna-Quintero (IBT-UNAM) yielding pJOQ_mCherry. This vector (2726 bp) was constructed from a fragment of plasmid pET-28a and a fragment from the plasmid pTRC99a. This plasmid contains the kanamycin-resistance gene, the pBR322 origin of replication and all genes cloned as NdeI/XhoI inserts are transcribed from the *trc* promoter.

2.4. Microscopy set up for evanescence field illumination

For the analyses of free-living cells, Epi-fluorescence and total internal reflection fluorescence (TIRF) measurements were performed on an Olympus IX-81 inverted microscope (Olympus, cell^TIRF™ Illuminator). For TIRF measurements the critical angle was set up such that the evanescence field had a penetration depth of ~100 nm (xcellence software v1.2, Olympus soft imaging solution GMBH). The samples were continuously illuminated using excitation sources depending of the fluorophore used. Green- and Red-absorbing fluorescent proteins were either excited with a 488-nm or a 561-nm diode-pumped solid-state laser. Beam selection and modulation of laser intensities were controlled via xcellence software v.1.2. A full multiband laser cube set was used to discriminate the selected light sources (LF 405/488/561/635 A-OMF, Bright Line; Semrock). Fluorescence was collected using an Olympus UApo N 100×/1.49 numerical aperture oil-immersion objective lens with an extra 1.6× intermediate magnification lens. All images were recorded with an electron-multiplying charge couple device (EMCCD) camera (iXon 897, Model No: DU-897E-CS0-#BV; Andor) at 100 nm per pixel. Fifteen images were collected at ~100 ms for each imaging field, which were averaged to reduce noise.

The analysis of bacteria on plant roots was performed using a Zeiss LSM 510 Meta confocal microscope attached to an Axiovert 200 M. GFP excitation was performed at 488 nm, using an Ar/2 laser and a HFT UV 488/543/633 nm dual dichroic excitation mirror with a BP 500–530 IR emission filter for detection. DsRed was excited at 543 nm with a He/Ne1 laser, with the same dual dichroic excitation mirror and a BP 565–615 IR emission filter. We used Objective EC "Plan-Neofluar" 10×/0.30 Ph1 with DarkField and C-Apochromat 63×/1.2 W Korr, Ph3, DIC. The Brightfield images were obtained with differential

Table 1
Bacterial strains and plasmids.

Name	Description	Resistance	Reference
Strains			
Rhizobium CFN42	Wild type	Nal	Quinto et al. (1982)
Rhizobium CFN42-RFP	Derivative of CFN42 containing MiniTn7-RFP-Gm in the chromosome	Nal, Gm	This work
Agrobacterium tumefaciens GMI9023	*Agrobacterium tumefaciens* plasmid free	Rif	Rosenberg and Huguet (1984)
E. coli DH5α	*supE44 ΔlacU169 ϕ80dlacZΔM15 hsdR171 recA1 endA1 gyrA96 thi-1 relA1*		Bethesda Res. Lab.
E. coli S17-1	*E. coli 294*::[RP4-2 (Tc::Mu) (Km::Tn7)] *thi pro hsdR hsdM ΔrecA*		Simon et al. (1983)
Plasmids			
MiniTn7(Gm)-dsred-a	$P_{A1/04/03}$-*dsred* cloned into *NotI* site of pBK-miniTn7-ΩGm	Gm	Lambertsen et al. (2004)
pBSL142	Vector with Gm cassette flanked by transcription and translation terminators.	Gm	Alexeyev et al. (1995)
pFAJ1708	Low copy number vector harboring *NptII* promoter	Amp, Tc	Dombrecht et al. (2001)
pGX143	Derivative of pGX166, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX144	Derivative of pGX166, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Tc	This work
pGX166	Derivative of pGX30, carrying pR of lambda phage as *EcoRI-XbaI* fragment from pSJ5 instead of $P_{A1/04/03}$	Km	This work
pGX173	Derivative of pGX30, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Tc	This work
pGX185	Derivative of pGX194, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Tc	This work
pGX186	Derivative of pGX166, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Sp	This work
pGX194	Derivative of pGX30, carrying *mCherry* as *SphI–HindIII* fragment instead of *gfpmut3**	Km	This work
pGX195	Derivative of pGX166, carrying *mCherry* as *SphI–HindIII* fragment instead of *gfpmut3**	Km	This work
pGX207	Derivative of pGX78 harboring the *NotI*-cassette from pGX64	Km, Sp	This work
pGX210	Derivative of pGX78 harboring the *NotI*-cassette from pGX144	Km, Sp,	This work
pGX212	Derivative of pGX194, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Sp	This work
pGX241	Derivative of pGX78 harboring the *NotI*-cassette from pGX212	Km, Sp	This work
pGX245	Derivative of pGX195, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Tc	This work
pGX251	Derivative of pGX195, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX252	Derivative of pGX194, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX298	Derivative from pRet42a with pRet42a-$p_{A1/04/03}$::*mCherry*::Sp, obtained by double crossover with pGX241	Sp	This work
pGX30	Derivative from pGX84 carrying a 2 Kbp-*NotI* cassette ($P_{A1/04/03}$-RBSII-*gfpmut3**-T0-T1) from pJBA28.	Km	This work
pGX384	Derivative of pGX78 harboring the *NotI*-cassette from pGX533	Km, Sp	This work
pGX412	Derivative from pRet42a with pRet42a-pR:: *gfpmut3**::Sp obtained by double crossover with pGX210	Sp	This work
pGX421	Derivative from pRet42a with pRet42a-pNptII::*mCherry*::Sp obtained by double crossover with pGX384	Sp	This work
pGX474	Derivative of pGX30, carrying promoter *NptII* wild-type as *EcoRI–XbaI* fragment substituting $P_{A1/04/03}$	Km	This work
pGX475	Derivative of pGX78 harboring the *NotI*-cassette from pGX529	Km, Sp	This work
pGX477	Derivative of pGX78 harboring the *NotI*-cassette from pGX534	Km, Sp	This work
pGX485	Derivative of pGX30, carrying promoter Lac wild-type as *EcoRI–XbaI* fragment substituting $P_{A1/04/03}$	Km	This work
pGX493	Derivative of pGX474, carrying *mCherry* as *SphI–HindIII* fragment substituting *gfpmut3**	Km	This work
pGX494	Derivative of pGX485, carrying *mCherry* as *SphI–HindIII* fragment substituting *gfpmut3**	Km	This work
pGX507	Derivative from pRet42a with $pLAC_{WT}$:: *gfpmut3**::Sp obtained by double crossover with pGX477	Sp	This work
pGX508	Derivative from pRet42a with $pLAC_{WT}$::*mCherry*::Sp obtained by double crossover with pGX475	Sp	This work
pGX527	Derivative of pGX493, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Tc	This work
pGX528	Derivative of pGX493, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Sp	This work
pGX529	Derivative of pGX494, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Gm	This work
pGX530	Derivative of pGX195, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Sp	This work
pGX531	Derivative of pGX485, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX533	Derivative of pGX493, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Gm	This work
pGX534	Derivative of pGX485, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Tc	This work
pGX535	Derivative of pGX494, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Tc	This work
pGX536	Derivative of pGX485, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Sp	This work
pGX537	Derivative of pGX474, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Tc	This work
pGX538	Derivative of pGX474, carrying ΩTc-*SmaI* fragment from pHP45-Tc in the *SspI* site	Km, Sp	This work
pGX539	Derivative of pGX494, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Sp	This work
pGX540	Derivative of pGX474, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX541	Derivative of pGX78 harboring the *NotI*-cassette from pGX537	Km, Sp	This work
pGX557	Derivative from pRet42a with pRet42a-pNptII:: *gfpmut3**::Sp obtained by double crossover with pGX541	Sp	This work
pGX580	Derivative from pRet42a with pRet42a-$p_{A1/04/03}$:: *gfpmut3**::Sp obtained by double crossover with pGX207	Sp	This work
pGX64	Derivative of pGX30, carrying ΩSp-*SmaI* fragment from pHP45-Sp in the *SspI* site	Km, Sp	This work
pGX66	Derivative of pGX30, carrying Gm resistance-*SspI* fragment from pGX90 in the *SspI* site	Km, Gm	This work
pGX78	Derivative of pK18mob-SacB with a fragment from pRet42a in the *SmaI* site	Km	This work
pGX82	Derivative of pK18mob with a new polylinker *HindIII*, *EcoRI*, *SmaI*, *BamHI*, *SspI*, *XbaI*, *SspI*, *BamHI*, *SmaI*, *EcoRI*, *HindIII* substituting the *EcoRI–HindIII* original linker	Km	This work
pGX84	Derivative from pK18mob with a polylinker with a single *NotI* site instead of the *EcoRI–HindIII* original linker	Km	This work
pGX90	Derivative of pGX82 carrying the Gm resistance gene from pBSL142, as a *NheI* fragment, cloned in the *XbaI* site	Km, Gm	This work
pHP45-Sp	Vector carrying a DNA cassette for Sp^R flanked by transcription and translation terminators	Amp, Sp	Fellay et al. (1987)
pHP45-Tc	Vector carrying a DNA cassette for Tc^R flanked by transcription and translation terminators	Amp, Tc	Fellay et al. (1987)
pJBA28	Delivery plasmid for mini-Tn5-Km-$P_{A1/04/03}$-RBSII-*gfpmut3**-T0–T1	Km, Amp	Andersen et al. (1998)
pJOQ_mCherry	High copy number cloning vector, harboring *mCherry* gene	Km	This work
pK18mob	High copy number cloning vector	Km	Schäfer et al. (1994)

(continued on next page)

Table 1 (*continued*)

Name	Description	Resistance	Reference
pK18mob-sacB	Cloning vector, mobilizable	Km	Schäfer et al. (1994)
pRK600	ori ColE1 RK2-Mob+ RK2-Tra+; helper plasmid in triparental matings	Cm	Kessler et al. (1992)
pSJ5	Derivative of pUJ9 vector, with lambda promoter Right (pR) promoter and 5′-leader region of *tnp*	Amp	Jaenecke et al. (1996)
pUX-BF13	Helper plasmid, providing the Tn7 transposase proteins	Amp	Bao et al. (1991)

Nal, Rif, Nm, Sp, Tc, Gm, Km, Amp, and Cm = nalidixic acid, rifampicin, neomycin, spectinomycin, tetracycline, gentamicin, kanamycin, ampicillin, and chloramphenicol resistance, respectively.

interference contrast (DIC). Images were processed using ImageJ 1.47v (Wayne Rasband National Institutes of Health, USA) and Adobe Photoshop 7.0 software (Adobe Systems Inc., Mountain View, CA, USA).

2.5. Flow cytometry

In preparation for FC analysis, aliquots of overnight cultures (donor and recipient strains) were incubated overnight on PY plates at 30 °C. Samples (controls and matings) were resuspended in 1 ml of 10 mM $MgSO_4$–0.01% Tween 40 (vol/vol). Serial dilutions were performed in NaCl 0.9%. All the solutions were filtered by 0.2 μm pore filters. Flow cytometric detection of cells was carried out using a FACS CantoII (Becton Dickinson Biosciences, San Jose, CA). The forward (FSC) and side scatter (SSC) photomultiplier voltages were set at 951 V and 720 V, respectively. Threshold levels were set at 1000 in FSC and 3000 in SSC were set up. The detectors for green (GFP) and red fluorescence (Texas-Red) were set at 868 V and 819 V, respectively. For optimal count, a flow rate of 1 μl/s was chosen, where 500 to 3000 events/s were counted.

Bacterial populations were gated according GFP vs. Texas-Red plots. The high voltages used detect background events. A buffer solution was used as a negative control in order to exclude non-bacterial particles (non fluorescence events). In each case, the noise produced by the buffer was subtracted from samples. The BD FACS Diva software was used for data acquisition, and FlowJo v10 software was used for subsequent analysis.

2.6. Plant assays

To prepare Negro Jamapa *Phaseolus vulgaris* seeds, they were first washed thrice with sterile water, incubated for 1 min in ethanol, for 5 min in 20% sodium hypochlorite, and washed 5 times with sterile water. The seeds were then germinated as previously described (Cardenas et al., 1995), in agar plates. Two-day germinated plantlets were introduced into tubes with Fåhraeus medium (Fåhraeus, 1957), inoculated with donor and/or recipient strains adjusted at 0.05 OD at 600 nm. After 20 days, the roots were subjected to confocal microscopy.

3. Results

3.1. Construction of adaptable NotI-cassettes and fluorescence evaluation in E. coli

We developed a set of versatile cassettes carrying different promoters, fluorescent proteins and antibiotic resistance genes, to use them according to the requirements. The first step for the constructions was to remove the polylinker of pK18mob (Schäfer et al., 1994) with *EcoRI* and *HindIII*, and substitute it with a new linker with a single *NotI* site using oligonucleotides "linkerENot" and "linkerHNot", the resulting plasmid was designated pGX84.

Next, plasmid pJBA28 (Andersen et al., 1998) was digested with *NotI* and a 2 kbp cassette containing the synthetic lac promoter $P_{A1/04/03}$, a synthetic ribosome binding site (RBSII), a fluorescent protein (*gfpmut3**) and two transcriptional terminators, T0 and T1, was cloned into the single *NotI* site of pGX84, yielding plasmid pGX30. This plasmid harbors the backbone of the cassettes employed for the next steps. To exchange them, the synthetic lac promoter was replaced with an *EcoRI–SphI* fragment from pSJ5 (Jaenecke et al., 1996) containing the *Pr* promoter of bacteriophage lambda, yielding pGX166. Similarly, a wild-type copy of the lac promoter was obtained by PCR with oligonucleotides "plac-wt-fw-eco" and "plac-wt-rv-xba", digested with *EcoRI* and *XbaI* and used to replace the synthetic lac promoter of pGX30, for the construction of pGX485. Finally, the *NptII* promoter was obtained by PCR with primers "pNPT-Fw-*EcoRI*" and "pNPT-Rv-*XbaI*", digested with *EcoRI* and *XbaI*, and cloned to replace the synthetic lac promoter, for the construction of pGX474.

With the goal of using a different fluorescent protein for dual tagging, in each vector containing the different promoters and the *gfp*mut3* (pGX30, pGX166, pGX485 and pGX474), the fluorescent protein was substituted with a *SphI–HindIII* fragment containing a copy of the *mCherry* protein, obtained by PCR with oligos "cherryNSphI" and "cherryCHind" using pJOQ_mCherry as template, yielding plasmids pGX194, pGX195, pGX494 and pGX493, respectively (Supplementary Fig. 1). The eight combinations of promoters and Fluorescent Proteins were digested in the single *SspI* site, and the following antibiotic resistance genes were introduced: ΩTc as a *SmaI* fragment from pHP45Ω-Tc (Fellay et al., 1987), ΩSp as a *SmaI* fragment from pHP45Ω (Prentki and Krisch, 1984) and Gm as a *SspI* fragment from pGX90. The constructions are listed in Table 1. All the cassettes were restricted with *NotI* to corroborate their correct excision.

To evaluate the expression of GFP and *mCherry* proteins, we visualized the *E. coli* strains carrying the cassettes by microscopy. The cassettes were harbored in multi-copy based-vectors, and fluorescence was observed (Supplementary Fig. 2). The analysis showed that the Lac promoters, the wild type as well as the synthetic one, and the *NptII* promoter exhibit a similar intensity, while expression from the *Pr* promoter was very weak. It is noteworthy that these evaluations were performed in *E. coli*, thus the expression in rhizobia could be different.

3.2. Tagging pRet42a of R. etli CFN42 with the cassettes

Aiming to evaluate our cassettes in *R. etli* we tagged plasmid pRet42a as follows: first we amplified a fragment of 602 bp (primers "p42a_left_in" and "p42a_rigth_in") and cloned it in the *SmaI* site of the pK18mob-sacB vector, generating plasmid pGX78. The criteria for the selection of the fragment to be amplified were that it should contain a region with two genes arranged tail to tail and a *NotI* site in the middle, to avoid effects on CT properties of pRet42a. Next, we introduced the different cassettes with promoter-*gfp*mut3*-Sp into the *NotI* site of pGX78. The resulting vectors were introduced into *R. etli* and substitution events were selected, thus generating CFN42 derivatives containing the cassettes integrated in pRet42a. The correctness of the integration was evaluated by PCR, using primers internal to the cassette (see Supplementary Fig. 1) and external primers from pRet42a for each

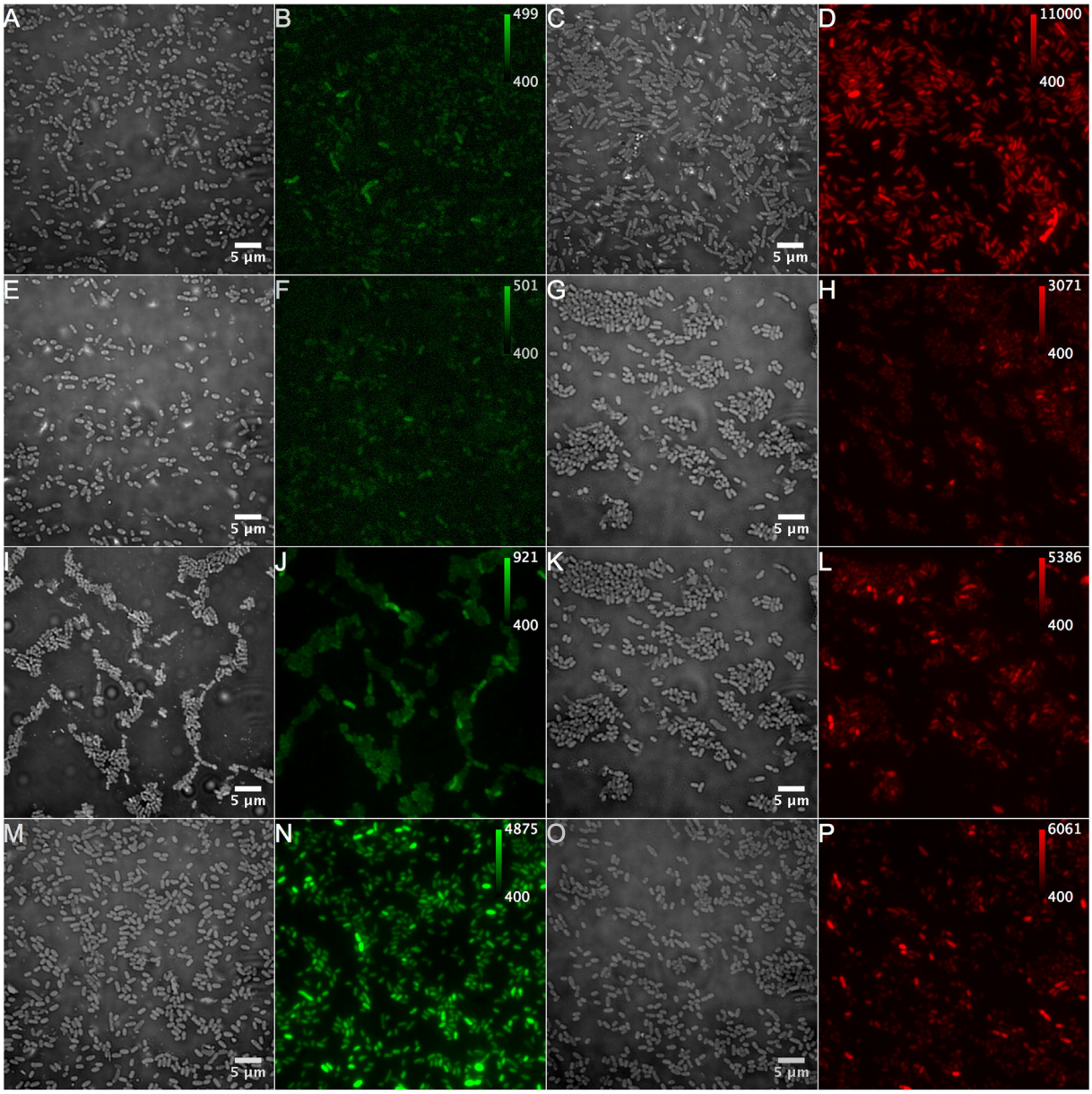

Fig. 2. Confocal microscopic observations of cassette expressions in *R. etli*. *R. etli* CFN42 derivatives expressing fluorescent proteins under control of different promoters were observed. As some proteins displayed very weak fluorescence, different settings were chosen; to compare them, the scale of intensity of the fluorescence is included in each photo. For *Visible Field-Green fluorescence*: A–B, *Pr* promoter-GFP; E–F, synthetic lac promoter $P_{A1/04/03}$-GFP; I–J, wild-type lac promoter-GFP; M–N, *NptII* promoter-GFP. For *Visible Field-Red fluorescence*: C–D, dsRED; G–H synthetic lac promoter $P_{A1/04/03}$-mCherry; K–L, wild-type lac promoter-mCherry; O–P, *NptII* promoter-mCherry.

side, "p42a_left_out" and "p42a_rigth_out". Fluorescence expression was evaluated by confocal microscopy. We observed that expression of GFP was almost undetectable with the *Pr* promoter. The *NptII* promoter was the strongest promoter, while the Lac promoters were weaker than *NptII*. The same procedure was performed to introduce the promoter-*mCherry*-Sp cassettes (with the exception of *Pr* that was very weak) into pRet42a. Analysis of their fluorescence showed similar results, with *NptII* being the strongest promoter (Fig. 2).

Subsequently we evaluated the fluorescence by flow cytometry. We were able to detect GFP fluorescence with three out of four promoters; expression induced by the Lac promoters was not strong enough to separate the tagged derivatives from the wild type, while those under control of *NptII* promoter showed a satisfactory separation (Supplementary Fig. 3A). Also, we transferred each plasmid to an *Agrobacterium* plasmid free strain by conjugation, and observed similar results (Supplementary Fig. 3B).

3.3. Tagging of the chromosome of *R. etli* CFN42 with RFP cassettes and construction of donor strains

Unfortunately, the *mCherry* was not detected with the cytometer we used (data not shown) and thus we tagged *R. etli* in the chromosome by means of a Mini-Tn*7*-dsRED (Lambertsen et al., 2004). Expression from this insertion was detected by microscopic observation (Fig. 2D) and in the FC, and the derivative carrying it showed a separate fluorescence peak from the wild type *R. etli* (Supplementary Fig. 3C). Mini-Tn*7* integration has been described to occur downstream of the gene encoding glucosamine-6-phosphate synthetase (*glmS*) (Lambertsen et al., 2004). To check the correctness of the integration, PCR amplification was performed with primers Tn7-glms and Tn7R109 as described in Lambertsen et al. (2004).

For the construction of a derivative containing markers in the chromosome and in pRet42a, plasmid pGX557 (labeled with

Table 2
Comparison of CFU and FC count of rhizobial strains and evaluation of conjugative transfer frequencies.

Experiment	Donor Strain ($\times 10^{08}$) CFN42-RFP/pGX557			Recipient Strain ($\times 10^{09}$) GMI9023			Transconjugants ($\times 10^{06}$)			Conjugation Frequency (Transconjugants per donor cell) ($\times 10^{-01}$)			Conjugation Frequency (Transconjugants per recipient cell) ($\times 10^{-03}$)		
	CFU	FC	W-test	CFU	FC	W-test	CFU	FC	W-test	CFU	FC	W-test	CFU	FC	W-test
1	5.63 ± 0.95	3.95 ± 0.73	ns	11.93 ± 1.50	4.67 ± 0.96	ns	7.93 ± 0.78	6.00 ± 3.61	ns	0.14 ± 0.02	0.15 ± 0.06	ns	0.67 ± 0.06	1.22 ± 0.49	ns
2	1.26 ± 0.13	2.48 ± 1.06	ns	9.27 ± 1.59	3.61 ± 2.22	ns	80.67 ± 10.07	19.67 ± 4.04	ns	6.40 ± 0.33	0.89 ± 0.34	ns	8.76 ± 0.79	7.32 ± 4.84	ns
3	5.73 ± 0.60	5.17 ± 1.36	ns	11.90 ± 1.30	8.97 ± 2.15	ns	13.07 ± 4.00	16.00 ± 7.00	ns	0.23 ± 0.05	0.30 ± 0.05	ns	1.09 ± 0.25	1.74 ± 0.36	ns

Statistics test performed: Wilcoxon rank sum test (non-parametric).
ns, non-significant (p-value > 0.055).

promoter *NptII*-GFP-Sp) was introduced by conjugation into the strain tagged with dsRED in the chromosome, CFN42-RFP. This construct was also analyzed by fluorescence under the microscope and by flow cytometry.

3.4. Optimization of parameters for FC, evaluation in rhizobia and detection of transconjugants by FC

Counting bacteria by FC is not an easy task, especially because of their size. Ferrari et al. (2004) have recommended that the cell flow and the voltage setting should be optimized for each species. To establish the parameters to obtain similar cell numbers when counted by the FC or by colony forming units (CFU) in plates, we tested different conditions until a satisfactory count was reached. The final settings were FSC = 951, SSC = 720, GFP = 868 and Texas-Red = 819, with threshold levels of 1000 in FSC and 3000 in SSC. Table 2 shows the comparison of the data obtained by CFU and FC counts for different strains and experiments, where similar results were obtained.

After the adequate settings were established, we performed mating experiments to determine if we could detect transconjugants. Matings were performed, and cells were counted by CFU and by FC. An example of the dot plots obtained (Fig. 3) clearly shows the separation of the donor, recipient and transconjugant populations. Dot plots of controls utilizing similar gates are also presented. In a donor population the red intensity was lower than in the mating experiment, but this did not perturb our results. To validate our approach, from the conjugation experiments we also counted bacteria by CFU. Table 2 shows that similar transfer frequencies were observed with both methods, FC or CFU.

3.5. In vivo evaluation of the system on plant surfaces

To visualize the *in vivo* appearance of transconjugants, we performed matings on plant roots. Germinated *P. vulgaris* seeds were inoculated with donor and recipient strains. As controls, roots were also inoculated separately with the mentioned strains. After 20 days, fluorescence microscopy was performed and transconjugants were detected on the plant roots inoculated simultaneously with the donors and recipients (Fig. 4).

4. Discussion

HGT has largely been studied using two approaches, evaluating directly how bacteria acquire genes or by analysis of genomic sequences, looking for incongruent phylogenetic relationships (Sorensen et al., 2005). CT has been studied mainly by tagging plasmids with antibiotics to assess their transfer under laboratory conditions. Attempts to visualize plasmid transfer by GFP expression in cultivable bacteria were first performed by Christensen et al. (1996). Jaenecke et al. (1996) described a system to analyze gene transfer between bacteria, using a *lacZ* reporter gene under the control of a synthetic promoter, constructed by fusing the *Pr* promoter of phage Lambda and an antisense RNA of the *tnp* gene from IS*10*. This promoter is repressed by chromosomally encoded regulators in the donor strain. Repression is relieved upon plasmid transfer to a recipient, allowing expression of the *lacZ* reporter in the transconjugants. However, the detection of plasmid transfer still needed cultivable bacteria. To get a deeper insight into the *in situ* evaluation of CT, Sorensen et al. (2003) proposed a genetic system to detect plasmid transfer events by tagging plasmids with a GFP marker whose expression is regulated by a repressible Lac promoter, and inserting the gene encoding the LacI repressor in the bacterial chromosome, resulting in cells that are non-fluorescent. When plasmid transfer is achieved, the recipient strain becomes fluorescent, as it inherits the plasmid carrying the GFP, but not the chromosome encoded repressor. More recently, Nancharaiah et al. (2003) adapted the system with a two fluorescent protein set; in this case, red fluorescence is encoded in the chromosome

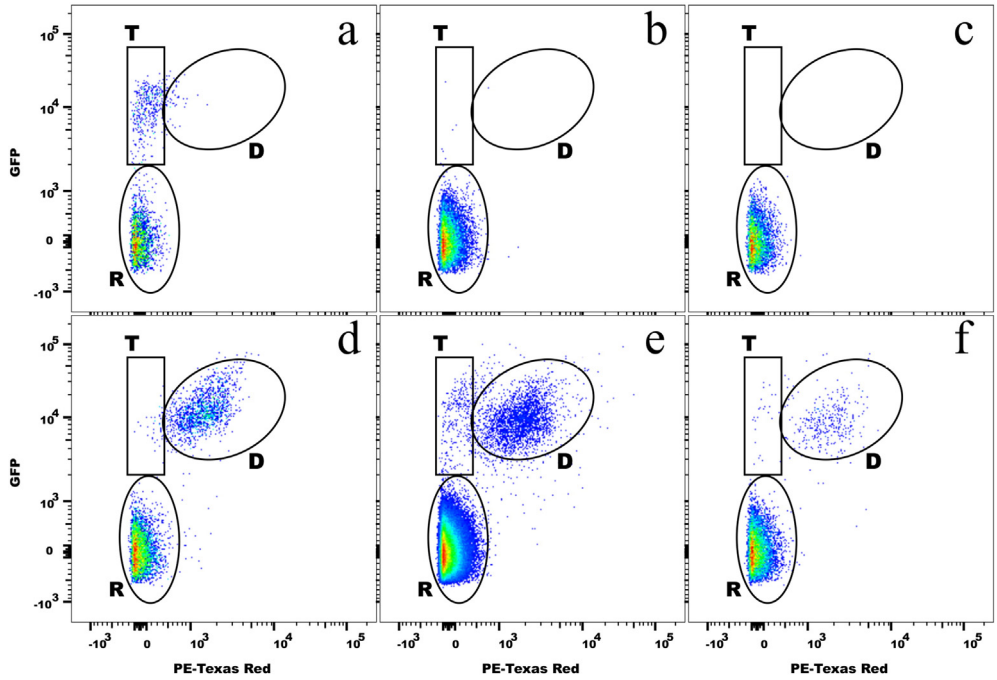

Fig. 3. Evaluation of rhizobial matings by FC. *Dot plot* of matings evaluated in the flow cytometer by PE-Texas Red and GFP parameters. Gates set up were T for transconjugants, D for donors and R for recipients and background events. The events counted were a) GMI (pRetA-p*NptII*-GFP) 2508, b) GMI 11,001, c) Blank solution 4047, d) CE3-RFP (pRetA-p*NptII*-GFP) 5985, e) 100 μl of Mating among GMI and CE3-RFP (pRetA-p*NptII*-GFP), and f) 10 μl of mating among GMI and CE3-RFP (pRetA-p*NptII*-GFP). Two dilutions of a mating are shown. Donor, recipient and transconjugant populations can be distinguished.

of the donor strain while GFP is expressed from the plasmid. After plasmid transfer, transconjugants express only the GFP, while donors express both fluorescent tags. A few studies appeared in the last year, which use FISH or FC to detect plasmid transfer (Klumper et al., 2015; Musovic et al., 2006, 2010; Shintani et al., 2014). All these studies have been performed using *Pseudomonas*, *Escherichia* or *Enterobacter* strains as donors.

In our laboratory, we have been interested in studying plasmid transfer in rhizobia. In order to test the effect of environmental conditions on rhizobial conjugation, we constructed a set of cassettes that contain a fluorescent protein gene under the control of different promoters, in addition to diverse antibiotic resistance genes. This allows the combination of different tags, maximizing the versatility of the system. The constructions were based on the previously described systems, but adapted for rhizobia. The cassettes developed here could also be used with other purposes as analyses of transcriptional fusions or single-cell tagging.

Our results show that in *E. coli* the Lac promoters and the *NptII* promoter presented a similar intensity, while the *Pr* promoter was very weak. Here, a dose-effect could be present, as fluorescent proteins are expressed from multi-copy plasmids. In contrast, in *R. etli* background only the *NptII* promoter showed a strong enough intensity to separate the GFP bacteria from the control by FC (Supplementary Fig. 3A). Hence, we discarded the system based on a repressible promoter for rhizobia because of the impossibility to separate donors from transconjugants, and focused on the dual tag system. Regarding the *mCherry*, even if its red fluorescence was detectable by microscopy, we could not detect it with the FC, and thus we used a MiniTn*7*-dsRED to tag the bacterial chromosome. The construction containing a MiniTn7-dsRED in the chromosome and promoter *NptII*-GFP in pRet42 was finally chosen and employed to check transfer in rhizobia.

Ferrari et al. (2004) previously reported that for cell counts in the FC special settings and a low flow has to be used. Also, recent studies (Klumper et al., 2015; Loftie-Eaton et al., 2014) used FC to count bacteria, but neither of them employed rhizobial strains. Rhizobia are smaller than *E. coli* and *Pseudomonas*, and so, the optimization of FC settings became harder; high voltages have to be set, which implies a higher noise by the buffers employed. Nevertheless, after iteration of optimization cycles, settings allowing us to obtain similar cell counts by FC and by CFU were established, validating the system for counting single cell cultures of rhizobia. Also, analysis of mating experiments using this approach revealed that conjugation frequencies obtained by counting CFU in selective media or through FC were similar, validating the system for CT analysis by FC. This approach allowed us to measure CT in a few hours, instead of the usual 2–3 days required to grow bacteria in selective media. Finally, we evaluated our system *in situ*. We inoculated *P. vulgaris* plant roots with the donor and recipient strains, 20 days after inoculation fluorescence microscopy of the roots was performed, revealing the presence of transconjugants and a potential use to assess these events with different plants or under different conditions.

Two recent publications have used FC to detect and characterize CT in different environments (Klumper et al., 2015; Shintani et al., 2014). Shintani et al. (2014) used broad and narrow host range plasmids from incompatibility group IncP. They were able to detect non-cultivable transconjugants, and showed that the host range was broader than previously thought. New analysis by cell sorting and 16S rRNA gene pyrosequencing indicated that broad host range plasmids are able to transfer to very diverse bacteria from soils, reinforcing the importance of plasmids as mobile genetic pools (Klumper et al., 2015). Our future aim focuses on the evaluation of the natural host range of rhizobial plasmids, which have usually been described as being of narrow host range. The system presented here will also allow us to analyze different environments (e.g., plants, soil conditions) that may affect CT, under diverse laboratory or natural conditions, easily and in a faster way. This system is a valuable tool for the study of the mechanisms involved in the evolution and diversification of rhizobia, by measuring the impact of CT in natural environments, and detecting the factors that influence CT of the plasmids.

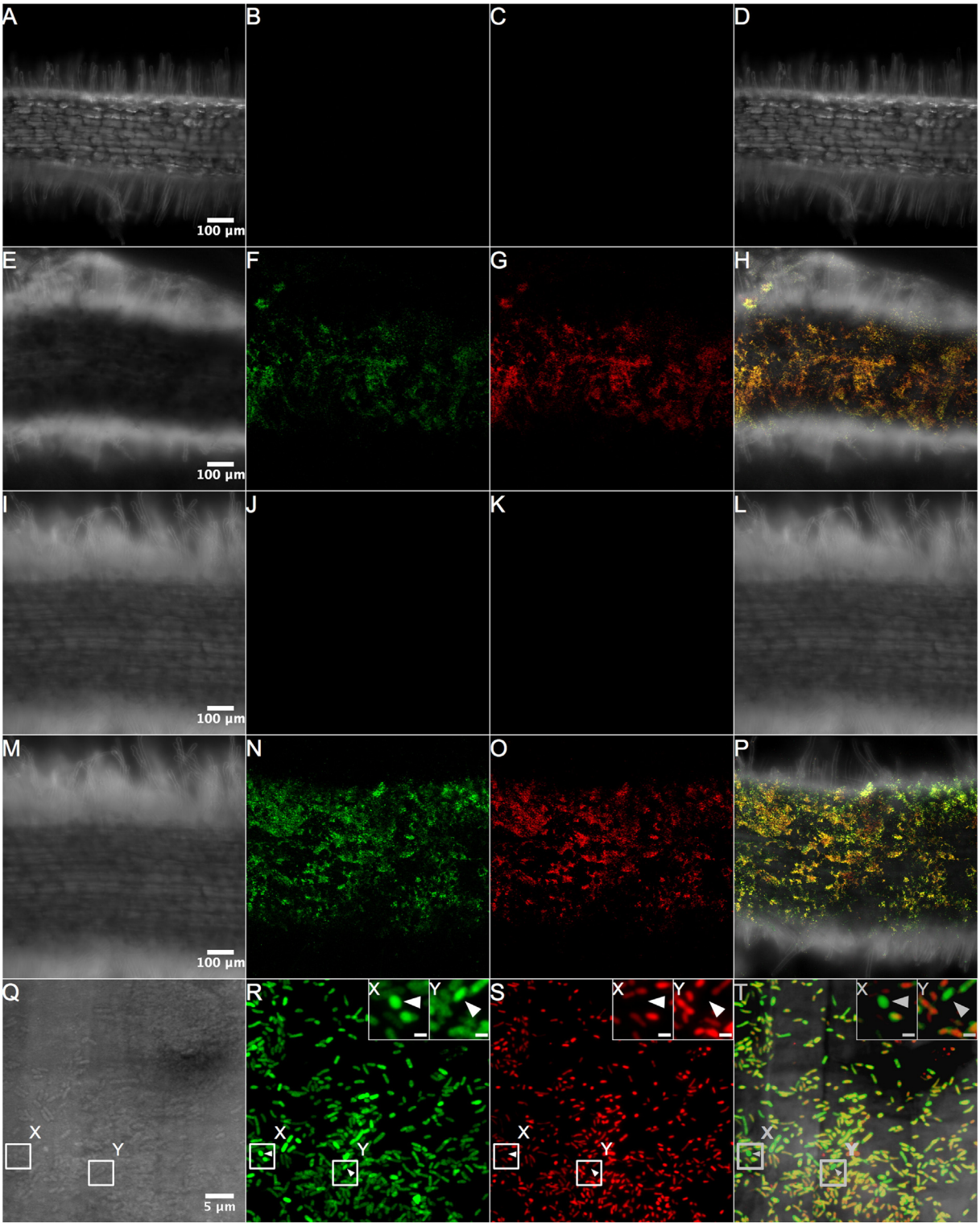

Fig. 4. Evaluation of rhizobial plasmid transfer on roots of *Phaseolus vulgaris*. Roots were inoculated with buffer (A–D), donor strain (E–H), recipient strain (I–L) and co-inoculated with donor and recipient (M–P). A zoom from image P is shown in Q–T and transconjugants are marked (X–Y). The columns are: first: visible field, second: green fluorescence, third: red fluorescence and fourth: merge. Bars indicate the size, except for X–Y that is 1 μm.

Acknowledgments

To Yvonne Rosenstein and Erika Melchy for use of the flow cytometer, to the Laboratorio Nacional de Microscopía Avanzada, UNAM and Xóchitl Alvarado for the confocal microscopy images, and to Fabiola Miranda-Sánchez for help with the statistical analysis. This work was partially supported by PICT-2013-0113 to G. T. T., PICT-2012-0518 to M. P., grant IN203515 from DGAPA, UNAM to S. B. and by grant 188967 of a Bilateral Mexico–Argentina Cooperation, from CONACYT–CONICET. G. T. T. and M. P. are members of the Research Career of CONICET, Argentina.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.08.005.

References

Alexeyev, M.F., Shokolenko, I.N., Croughan, T.P., 1995. Improved antibiotic-resistance gene cassettes and omega elements for *Escherichia coli* vector construction and in vitro deletion/insertion mutagenesis. Gene 160, 63–67.

Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjorn, S.P., Givskov, M., Molin, S., 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. 64, 2240–2246.

Bao, Y., Lies, D.P., Fu, H., Roberts, G.P., 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109, 167–168.

Brom, S., Girard, L., Tun-Garrido, C., García-de los Santos, A., Bustos, P., González, V., Romero, D., 2004. Transfer of the symbiotic plasmid of *Rhizobium etli* CFN42 requires cointegration with p42a, which may be mediated by site-specific recombination. J. Bacteriol. 186, 7538–7548.

Cardenas, L., Dominguez, J., Quinto, C., Lopez-Lara, I.M., Lugtenberg, B.J., Spaink, H.P., Rademaker, G.J., Haverkamp, J., Thomas-Oates, J.E., 1995. Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Mol. Biol. 29, 453–464.

Cervantes, L., Bustos, P., Girard, L., Santamaría, R.I., Dávila, G., Vinuesa, P., Romero, D., Brom, S., 2011. The conjugative plasmid of a bean-nodulating *Sinorhizobium fredii* strain is assembled from sequences of two *Rhizobium* plasmids and the chromosome of a *Sinorhizobium* strain. BMC Microbiol. 11, 149.

Christensen, B.B., Sternberg, C., Molin, S., 1996. Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequorea victoria* as a marker. Gene 173, 59–65.

Danino, V.E., Wilkinson, A., Edwards, A., Downie, J.A., 2003. Recipient-induced transfer of the symbiotic plasmid pRL1JI in *Rhizobium leguminosarum* bv. *viciae* is regulated by a quorum-sensing relay. Mol. Microbiol. 50, 511–525.

Ding, H., Yip, C.B., Hynes, M.F., 2013. Genetic characterization of a novel rhizobial plasmid conjugation system in *R. leguminosarum* bv. *viciae* strain VF39SM. J. Bacteriol. 195, 328–339.

Dombrecht, B., Vanderleyden, J., Michiels, J., 2001. Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in Gram-negative bacteria. Mol. Plant Microbe Interact. 14, 426–430.

Eckhardt, T., 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid 1, 584–588.

Fåhraeus, G., 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16, 374–381.

Fellay, R., Frey, J., Krisch, H., 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene 52, 147–154.

Ferrari, B.C., Oregaard, G., Sorensen, S.J., 2004. Recovery of GFP-labeled bacteria for culturing and molecular analysis after cell sorting using a benchtop flow cytometer. Microb. Ecol. 48, 239–245.

Hynes, M.F., McGregor, N.F., 1990. Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. 4, 567–574.

Jaenecke, S., de Lorenzo, V., Timmis, K.N., Diaz, E., 1996. A stringently controlled expression system for analysing lateral gene transfer between bacteria. Mol. Microbiol. 21, 293–300.

Kessler, B., de Lorenzo, V., Timmis, K.N., 1992. A general system to integrate lacZ fusions into the chromosomes of Gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. 233, 293–301.

Klumper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sorensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945.

Lambertsen, L., Sternberg, C., Molin, S., 2004. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environ. Microbiol. 6, 726–732.

Loftie-Eaton, W., Tucker, A., Norton, A., Top, E.M., 2014. Flow cytometry and real-time quantitative PCR as tools for assessing plasmid persistence. Appl. Environ. Microbiol. 80, 5439–5446.

Lopez-Fuentes, E., Torres-Tejerizo, G., Cervantes, L., Brom, S., 2015. Genes encoding conserved hypothetical proteins localized in the conjugative transfer region of plasmid pRet42a from *Rhizobium etli* CFN42 participate in modulating transfer and affect conjugation from different donors. Front. Microbiol. 5, 793.

Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Musovic, S., Oregaard, G., Kroer, N., Sorensen, S.J., 2006. Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-positive and Gram-negative bacteria indigenous to the barley rhizosphere. Appl. Environ. Microbiol. 72, 6687–6692.

Musovic, S., Dechesne, A., Sorensen, J., Smets, B.F., 2010. Novel assay to assess permissiveness of a soil microbial community toward receipt of mobile genetic elements. Appl. Environ. Microbiol. 76, 4813–4818.

Nancharaiah, Y.V., Wattiau, P., Wuertz, S., Bathe, S., Mohan, S.V., Wilderer, P.A., Hausner, M., 2003. Dual labeling of *Pseudomonas putida* with fluorescent proteins for *in situ* monitoring of conjugal transfer of the TOL plasmid. Appl. Environ. Microbiol. 69, 4846–4852.

Noel, K.D., Sánchez, A., Fernández, L., Leemans, J., Cevallos, M.A., 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn5 insertions. J. Bacteriol. 158, 148–155.

Nogales, J., Blanca-Ordóñez, H., Olivares, J., Sanjuán, J., 2013. Conjugal transfer of the *Sinorhizobium meliloti* 1021 symbiotic plasmid is governed through the concerted action of one- and two-component signal transduction regulators. Environ. Microbiol. 15, 811–821.

Pérez-Mendoza, D., Sepúlveda, E., Pando, V., Muñoz, S., Nogales, J., Olivares, J., Soto, M.J., Herrera-Cervera, J.A., Romero, D., Brom, S., Sanjuán, J., 2005. Identification of the *rctA* gene, which is required for repression of conjugative transfer of rhizobial symbiotic megaplasmids. J. Bacteriol. 187, 7341–7350.

Pistorio, M., Torres Tejerizo, G.A., Del Papa, M.F., de Los Angeles Giusti, M., Lozano, M., Lagares, A., 2013. *rptA*, a novel gene from *Ensifer* (*Sinorhizobium*) *meliloti* involved in conjugal transfer. FEMS Microbiol. Lett. 345, 22–30.

Prentki, P., Krisch, H.M., 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29, 303–313.

Quinto, C., de la Vega, H., Flores, M., Fernandez, L., Ballado, T., Soberon, G., Palacios, R., 1982. Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. Nature 299, 724–726.

Rosenberg, C., Huguet, T., 1984. The pAtC58 plasmid of *Agrobacterium tumefaciens* is not essential for tumour induction. Mol. Gen. Genet. MGG 196, 533–536.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., Pühler, A., 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145, 69–73.

Shintani, M., Matsui, K., Inoue, J., Hosoyama, A., Ohji, S., Yamazoe, A., Nojiri, H., Kimbara, K., Ohkuma, M., 2014. Single-cell analyses revealed transfer ranges of IncP-1, IncP-7, and IncP-9 plasmids in a soil bacterial community. Appl. Environ. Microbiol. 80, 138–145.

Simon, R., Priefer, U., Pühler, A., 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology 1, 784–791.

Sorensen, S.J., Sorensen, A.H., Hansen, L.H., Oregaard, G., Veal, D., 2003. Direct detection and quantification of horizontal gene transfer by using flow cytometry and GFP as a reporter gene. Curr. Microbiol. 47, 129–133.

Sorensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer *in situ*: a critical review. Nat. Rev. Microbiol. 3, 700–710.

Torres Tejerizo, G., Pistorio, M., Althabegoiti, M.J., Cervantes, L., Wibberg, D., Schluter, A., Puhler, A., Lagares, A., Romero, D., Brom, S., 2014. Rhizobial plasmid pLPU83a is able to switch between different transfer machineries depending on its genomic background. FEMS Microbiol. Ecol. 88, 565–578.

Tun-Garrido, C., Bustos, P., González, V., Brom, S., 2003. Conjugative transfer of p42a from *Rhizobium etli* CFN42, which is required for mobilization of the symbiotic plasmid, is regulated by quorum sensing. J. Bacteriol. 185, 1681–1692.

van Elsas, J.D., Trevors, J.T., Starodub, M.E., 1988. Bacterial conjugation between pseudomonads in the rhizosphere of wheat. FEMS Microbiol. Lett. 53, 299–306.

van Elsas, J.D., Nikkel, M., Van Overbeek, L.S., 1989. Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil bacteria. Curr. Microbiol. 19, 375–381.

Wu, G., Wolf, J.B., Ibrahim, A.F., Vadasz, S., Gunasinghe, M., Freeland, S.J., 2006. Simplified gene synthesis: a one-step approach to PCR-based gene construction. J. Biotechnol. 124, 496–503.