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Development of new positive-selection RIVET tools: Detection of induced promoters by the excision-based transcriptional activation of an *aacCI* (Gm^R)–*gfp* fusion

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ABSTRACT

RIVET (*R*ecombination *B*ased *in vivo E*xpression *T*echnology) is a powerful genetic tool originally conceived for the identification of genes induced in complex biological niches where conventional transcriptomics is difficult to use. With a broader application, genetic recombination-based technologies have also been used, in combination with regulatory proteins and specific transcriptional regulators, for the development of highly sensitive biosensor systems. RIVET systems generally comprise two modules: a promoter-trap cassette generating genomic transcriptional fusions to the *tnpR* gene encoding the *Tn*-γδ TnpR resolvase, and a reporter cassette carrying *res*-flanked selection markers that are excised upon expression of *tnpR* to produce an irreversible, inheritable phenotypic change.

We report here the construction and validation of a new set of positive-selection RIVET systems that, upon induction of the promoter-trap module, generate the transcriptional activation of an antibiotic-resistant and a green-fluorescent phenotype. Two classes of promoter-trap tools were constructed to generate transcriptional fusions to *tnpR*: one based on the use of a narrow-host-range plasmid (pRIVET-I), integrative in several Gram-negative bacteria, and the other based on the use of a broad-host-range plasmid (pRIVET-R). The system was evaluated in the model soil bacterium *Sinorhizobium meliloti*, where a clear-cut phenotypic transition from Nm^R-Gm^S-GFP^- to Nm^S-Gm^R-GFP^+ occurred upon expression of *tnpR*. A *S. meliloti* integrative RIVET library was constructed in pRIVET-I and, as expected, changes in the extracellular conditions (*e.g.*, salt stress) triggered a significant increase in the appearance of Gm^R-GFP^+ (excised) clones. The *sacB*-independent positive-selection RIVET systems here described provide suitable basic tools both for the construction of new recombination-based biosensors and for the search of bacterial markers induced when microorganisms colonize and invade complex environments and eukaryotic hosts.

1. Introduction

The availability of the complete genomic sequence of a large number of microorganisms has made possible the application of different high-throughput methods to investigate gene and protein expression at an omic scale and under different physiological conditions (Pandey and Mann, 2000; Sturdevant et al., 2010; Zhu et al., 2003). Although these approaches are usually highly informative, they are not always suitable for the identification of transiently expressed markers. In addition, the application of conventional transcriptomics and proteomics is severely restricted when the collection of samples from poorly accessible biological environments is limiting (*e.g.*, in the case of intracellular bacteria). Alternative methods to explore gene induction in complex natural environments have included DFI (Differential Fluorescence Induction) (Allaway et al., 2001); STM (Signature-Tagged Mutagenesis) (Pobigaylo et al., 2008); and several variants of IVET (*In Vivo* Expression Technology) (Mahan et al., 1993) including RIVET (Recombination-based *In Vivo* Expression Technology) (Osorio et al., 2005). RIVET was first introduced by Camilli et al. (1994) as a promoter-trap method where inducible promoters trigger the expression of the *Tn*-γδ site-specific recombinase TnpR with the subsequent excision of a DNA reporter cassette that is flanked by the TnpR target sequences (i.e., *res* sequences). Thus, a DNA library upstream from the promoterless *tnpR* has to be created in order to investigate those inserts that trigger promoter activities under specific conditions or environments. After excision of the reporter cassette, a permanent selectable phenotype appears (genetic

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memory), thus allowing for the identification of even those clones that had only transiently expressed *tnpR*. The efficiency with which a given promoter activity is finally sensed by the RIVET tool (i.e., the degree of responsiveness of the system) can be potentially adjusted by modifying the nucleotide sequence at the *res* sites, and/or the ribosome binding site of *tnpR* (Camilli et al., 1994; Newman and Grindley, 1984; Osorio et al., 2005).

With respect to the general configuration of the RIVET systems, different formats have been used to support the promoter-trap module, including narrow (Castillo et al., 2008; Gao and Teplitski, 2008; Osorio et al., 2005) and broad-host-range plasmids (Ballering et al., 2009; Bron et al., 2004; Zhang and Cheng, 2006). The most commonly used selection strategy in RIVET systems has been based on the loss of *res*-flanked antibiotic-resistance cassettes (negative-selection approach). In contrast, second-generation RIVET systems introduced the possibility of a positive selection for those clones that, after cassette excision, either trigger the translational activation of an antibiotic-resistance marker (Livny and Friedman, 2004) or eliminate a *sacB*-containing reporter module (Osorio et al., 2005; Saviola et al., 2003). Unfortunately, since sucrose is a ubiquitous compound in plants, the use of *sacB* has to be avoided in plant-associated bacteria (mainly in endophytes and endosymbionts) as such an expression could interfere with the normal colonization of plant tissues. A third mechanism for the positive-selection of the excised clones has been reported in recombination-based biosensors for arabinose and toluene (Casavant et al., 2002, 2003). In these cases, the activation of a transcriptional regulator by specific compounds induced the deletion of the *cI* repressor with the consequent transcription of a reporter green fluorescent protein (GFP) from a P_L promoter.

In this report we describe the construction and validation of new and versatile RIVET tools that include two different configurations for the promoter-trap module, and a reporter cassette based on the transcriptional activation of both an antibiotic-resistance marker (facilitating a positive selection) and a GFP. The configuration and markers used in the new RIVET system make it suitable for its application in different Gram negative bacteria to explore gene induction in complex environments.

2. Materials and methods

2.1. Bacterial strains and plasmids

Table 1 lists the strains and plasmids used in this work. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37 °C. *Sinorhizobium meliloti* strains were grown either in TY (Beringer, 1974) or in YEM (Vincent, 1970) at 28 °C. For solid media 15 g of agar per liter of medium was added. The final concentration of antibiotics per milliliter of medium was 10 μg gentamicin (Gm), 10 μg tetracycline (Tc), 200 μg ampicillin (Ap), and 25 μg of kanamycin (Km) for *E. coli*; and 400 μg streptomycin (Str), 20–50 μg Gm, 120 μg neomycin (Nm) and 2 μg Tc for *S. meliloti*. For white-blue screening in cloning experiments 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 40 μg/ml.

2.2. DNA manipulation and genetic constructs

Plasmid-DNA preparation, restriction-enzyme analysis, cloning procedures, and *E. coli* transformations were performed according to previously established techniques (Sambrook et al., 1989).

2.3. Bacterial mating

Transfer of plasmids between *E. coli* S17-1 or *E. coli* SM10-λ-*pir* and *S. meliloti* were performed as previously described by Simon et al. (1983).

2.4. Construction of RIVET tools

2.4.1. The positive-selection TnpR target modules: construction of the reporter cassettes RΩNGG and R1ΩNGG

The *res* (or *res1*) sequences were PCR-amplified through the use of either plasmid pAC20 (for *res*) or pAC21 (for *resI*) (Table 1) as template, and primers E-res-f and S-res-r (Table 2) along with the primers X-res-f and H-res-r (Table 2). The resulting *res* amplicons were cloned into pUC18 and pG18mob2 to create plasmids pMJL31 and pMJL32, respectively (Fig. 2A). Likewise, a similar procedure was used to generate the plasmids pMJL41 and pMJL42 carrying the *res1* sequences.

The Km/Nm resistance cassette (*nptII*) from pIVET6 (Camilli et al., 1994) was subsequently cloned as a *Sac*I fragment into plasmid pMJL31 (or pMJL41 for *res1*) to create pMJL33 (or pMJL43 for *res1*). Then, plasmid pMJL33 (or pMJL43 for *res1*) was *Eco*RI–*Bam*HI-digested and the resulting fragments cloned into pMJL32 (or pMJL42 for *res1*) to produce plasmid pMJL34 (or pMJL44 for *res1*) (Fig. 2A).

In parallel, plasmid pMJL23 containing a promoterless *aacC1–gfp* transcriptional fusion was created. To do so, a promoterless Gm-resistance gene *aacC1* containing the TIR region from pGreenTir (Miller and Lindow, 1997) was PCR-amplified by means of the primers aacC1-f and aacC1-r (Table 2). The amplification product was cloned into plasmid pSM10 (Selbitschka et al., 1995) to generate plasmid pMJL21. Subsequently, the promoterless *gfp*-F64L/S65T gene was PCR-amplified from pMP6 (Pistorio et al., 2002) by means of the primers gfp-f and gfp-r (Table 2) and cloned into pK18mob to give rise to the plasmid pMJL22. Finally, the *gfp*-F64L/S65T was transferred as a *Bam*HI fragment into plasmid pMJL21 to produce plasmid pMJL23 (Fig. 2B).

In the last cloning step the [*res-nptII-res*] and [*res1-nptII-res1*] cassettes were transferred from plasmids pMJL34 and pMJL44 to plasmid pMJL23 to generate plasmids pMJL24 and pMJL25, respectively (Fig. 2C). In both of these vectors, as indicated in Fig. 2, the *nptII* marker was exchanged for the ΩNm/Km cassette from pHP45Ω-Km, containing transcriptional terminators in both directions (Fellay et al., 1987), to generate plasmids pRΩNGG and pR1ΩNGG as carriers of the reporter modules promoter^{nptII}-[*res*-ΩNm/Km-*res*]-*aacC1-gfp* and promoter^{nptII}-[*res1*-ΩNm/Km-*res1*]-*aacC1-gfp*, respectively.

2.4.2. The promoter-trap vectors: construction of pRIVET-I and pRIVET-R

Two promoter-trap vectors, pRIVET-I and pRIVET-R, were constructed based on the use of either a narrow- or a broad-host-range-plasmid replicon. For pRIVET-I, the Tc-resistance cassette from pHP45Ω-Tc (Fellay et al., 1987) was first cloned into plasmid pIVET5n (Osorio et al., 2005) and the resulting plasmid pMJL11 was then digested with *Xba*I and *Bgl*II and religated in the presence of the oligonucleotide adapters Xb-c Xb-d (Table 2). The resulting plasmid pMJL12 was finally digested with *Xba*I and ligated with the *Xba*I–*Nhe*I fragment from plasmid pSM10 (Selbitschka et al., 1995) that carries the replication region from plasmid p15A. Fig. 3A details the cloning steps used for the construction of pRIVET-I.

For the construction of the pRIVET-R plasmid, pRK442 was first digested with *Lgu*I and religated in the presence of the oligonucleotide adapter Ad-f/Ad-r (Table 2), to give plasmid pMJL51. Since this plasmid was not stably maintained in the absence of a selective pressure, the stabilization module *parABCDE* from plasmid RK2 was PCR-amplified using pFAJ1708 (Dombrecht et al., 2001) and then cloned as an *Nco*I-released cassette into pMJL51 to generate plasmid pMJL54. Finally, the *tnpR–lacZ* transcriptional fusion from pIVET5n was PCR-amplified with primers tnpR–lacZ f/r (Table 2),

Table 1
Bacterial strains and plasmids used in this work.

Strains	Main characteristics	Source
Escherichia coli DH5α	*recA*, vlacU169, F80d*lac*ZDM15	Bethesda Res. Lab.
E. coli S 17-1	*E. coli* 294 RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Simon et al. (1983)
E. coli DH5α-λ-*pir*	F-φ8Od*lac*ZAM15 A (lacZYA-argF)U169 *recA1 endA1 hsdR17 supE44* λ*-thi-1 gyrA96 relAI,* λ*pir*	Osorio et al. (2005)
E. coli SM10-λ*pir*	*thi recA thr leu tonA lacY supE* RP4-2-Tc::Mu λ::*pir* Km^R	Osorio et al. (2005)
S. meliloti 2011	Str^R, Nod^+ Fix^+ in alfalfa, derived from strain SU47	J. Dénarié, France
S. meliloti 2011 RNGG	Str^R, Km^R, derivative of *S. meliloti* 2011 gfp-F64L/S65T	This work
S. meliloti 2011 R1NGG	Str^R, Km^R, derivative of *S. meliloti* 2011 gfp-F64L/S65T	This work
S. meliloti 2011 RΩNGG	Str^R, Km^R, derivative of *S. meliloti* 2011 gfp-F64L/S65T	This work
S. meliloti 2011 R1ΩNGG	Str^R, Km^R, derivative of *S. meliloti* 2011 gfp-F64L/S65T	This work

Plasmids	Main characteristics	Source
pAC20	Ap^R, pACYC 184::*lacZ*:: *res-tet*-res	Camilli and Mekalanos (1995)
pAC21	Ap^R, pACYC 184::*lacZ*:: *res1-tet*-res1	Camilli and Mekalanos (1995)
pIVET6	Ap^R oriR6K mobRP4, *tnpR res1-tet-res1 neo*	Camilli et al. (1994)
pHP45Ω-Tc	Ap^R, carrying a DNA cassette for Tc^R flanked by transcription and translation terminators	Fellay et al. (1987)
pIVET5n	Ap^R, *oriR6K*, *mobRP4*, *tnpR–lacZ*	Osorio et al. (2005)
pMJL11	Tc^R, Ap^R, pIVET5n derivative carrying Tc^R cassette from pHP45Ω-Tc	This work
pMJL12	Tc^R, pMJL11::Δ*bla*	This work
pRIVET-I	Tc^R, pMJL12 derivative carrying replication origin from pSM10 plasmid	This work
pUC18	Ap^R	Messing (1983)
pUC19	Ap^R	Messing (1983)
pG18mob2	Gm^R, $oriV_{E.c}$, *oriT*	Kirchner and Tauch (2003)
pK18mob	Km^R, $oriV_{E.c}$, *oriT*	Schafer et al. (1994)
pMP6	Gm^R, Tc^R, gfp-F64L/S65T (from pGreenTir)	Miller and Lindow (1997) and Pistorio et al. (2002)
pSM10	Gm^R, partial sequences of the *S. meliloti alaS* and *recA* genes	Selbitschka et al. (1995)
pMJL21	Gm^R, pSM10 derivative carrying *aac*C1 cassette	This work
pMJL22	Km^R, pK18mob2 derivative carrying *gfp*-F64L/S65T PCR amplified from pMP6	This work
pMJL23	Gm^R, pSM10-*aacC1* derivative carrying gfp-F64L/S65T cassette from pMJL22	This work
pMJL31	Ap^R, pUC18 derivative, carrying *res* sequence as a *Eco*RI–*Sac*I cassette	This work
pMJL41	Ap^R, pUC18 derivative, carrying *res1* sequence as a *Eco*RI–*Sac*I cassette	This work
pMJL32	Gm^R, pG18mob2 derivative, carrying *res* sequence as a *Xba*I–*Hind*III cassette	This work
pMJL42	Gm^R, pG18mob2 derivative, carrying *res1* sequence as a *Xba*I–*Hind*III cassette	This work
pMJL33	Ap^R, Km^R, pMJL31 derivative carrying *nptII* (Km^R) cassette from pIVET6	This work
pMJL43	Ap^R, Km^R, pMJL41 derivative carrying *nptII* (Km^R) cassette from pIVET6	This work
pMJL34	Gm^R, Km^R, pMJL32 derivative carrying *res-nptII* cassette from pMJL33	This work
pMJL44	Gm^R, Km^R, pMJL42 derivative carrying *res1-nptII* cassette from pMJL43	This work
pMJL24	Gm^R, Km^R, pSM10-*aacC1-gfp* derivative carrying *res-nptII-res* cassette from pMJL34	This work
pMJL25	Gm^R, Km^R, pSM10-*aacC1-gfp* derivative carrying *res1-nptII- res1* cassette from pMJL44	This work
pMJL26	Gm^R, pMJL24 Δ*nptII*	This work
pMJL27	Gm^R, pMJL25 Δ*nptII*	This work
pHP45Ω-Km	Ap^R, Km^R, carrying a DNA Km^R cassette flanked by transcription and translation terminators	Fellay et al. (1987)
pMJL28	Km^R, pK18mob derivative with an alternative MCS	This work
pMJL29	Km^R, pMJL28 derivative carrying Km^R from pHP45Ω-Km	This work
pR1Ω*N*GG	Gm^R, Km^R, pMJL27 derivative carrying Km^R from pHP45Ω-Km	This work
pRΩ*N*GG	Gm^R, Km^R, pMJL26 derivative carrying Km^R from pHP45Ω-Km	This work
pRK442	Tc^R, versatile broad-host-range vector of the RK2 family	Scott et al. (2003)
pMJL51	Tc^R, pRK442 Δ*LguI*. Contains an alternative MCS	This work
pFAJ1708	Tc^R, Stable RK2-derived cloning vectors	Dombrecht et al. (2001)
pG18mob2::H1	Gm^R, pG18mob2 derivative	Not published
pMJL52	Gm^R, pG18mob2::H1 derivative containing *tnpR–lacZ* transcriptional fusion from pIVET5n	This work
pMJL53	Ap^R, pUC19:: *parABCDE*. Contains stabilization genes from RK2 plasmid	This work
pMJL54	Tc^R, pMJL51: *parABCDE*	This work
pRIVET-R	Tc^R, pMJL54: *tnpR–lacZ*	This work
pRIVET-R-*pnptII*	Tc^R, pMJL54: *tnpR–lacZ. p-nptII* promoter cloned upstream of *tnpR*	This work

and was then cloned as a *Kpn*I–*Xba*I insert into plasmid pMJL54 to produce pRIVET-R. Fig. 3B provides a summary of the cloning steps used for the construction of pRIVET-R.

2.4.3. Construction of the RIVET reporter strains S. meliloti 2011 RΩNGG and S. meliloti 2011-R1ΩNGG

Plasmids pRΩNGG and pR1ΩNGG were introduced into the conjugative helper strain *E. coli* S17-1 and were then used as shuttle vectors to homogenotize the RIVET reporter modules RΩNGG and R1ΩNGG into the *recA–alaS* region of the wild-type strain *S. meliloti* 2011 in order to generate the respective strains *S. meliloti* 2011 RΩNGG and *S. meliloti* 2011 R1ΩNGG.

2.5. Construction of a S. meliloti 2011 R1ΩNGG RIVET library using vector pRIVET-I

2.5.1. Library construction in E. coli and transfer to S. meliloti 2011 R1ΩNGG. Elimination of clones bearing tnpR fusions to constitutive rhizobial promoters

For the construction of the RIVET library, plasmid pRIVET-I was first digested with the endonuclease *Bgl*II (Promega) and then treated with shrimp alkaline phosphatase (Promega) following the protocol provided by the manufacturer. In parallel, total DNA from *S. meliloti* 2011 was prepared by a standard alkaline-phenol extraction (Sambrook et al., 1989) and partially digested with the endonuclease *Sau*3AI. The resulting DNA products were

Table 2
Oligonucleotides used in this work.

PCR primers	Sequence (5′ → 3′)	Description	Source
E-res-f	GAATTCCCGTCCGAAATA	Primer for the amplification of *res*/*res1* sequences	This work
S-res-r	GAGCTCTGTATCCTAAATCAAAT	Primer for the amplification of *res*/*res1* sequences	This work
X-res-f	TCTAGACCGTCCGAAATA	Primer for the amplification of *res*/*res1* sequences	This work
H-res-r	AAGCTTTGTATCCTAAATCAAAT	Primer for the amplification of *res*/*res1* sequences	This work
aacC1-f	GAATTCAAGCTTAGGAGGAAAAACATATGTTACGCAGCAGC	Primer for the amplification of promoterless *aac*C1 Gm resistance gene	This work
aacC1-r	GGATCCTTAGGTGGCGGTACT	Primer for the amplification of promotorless *aac*C1 Gm resistance gene	This work
gfp-f	GGATCCGTTAACTTTATAAGGAGGAAAAAC	Primer for the amplification of promotorless *gfp* gene	This work
gfp-r	GGATCCTTATTTGTATAGTTCATCCATGCC	Primer for the amplification of promotorless *gfp* gene	This work
Lk1	AATTGGGTACCGAATTCCCCGGGGATATCGGTACCT	Oligonucleotide for polylinker replacement	This work
Lk2	AGCTAGGTACCGATATCCCCGGGGAATTCGGTACCC	Oligonucleotide for polylinker replacement	This work
XB-d	CTAGAgggcccA	Used to remove *bla* from pIVET5n	This work
XB-c	GATCTgggcccT	Used to remove *bla* from pIVET5n	This work
alaS-A	TTGCGGAAATAGTCGAGGAA	Used to check the resolution of res flanked cassette	This work
recA-C	AGAATGGCGGGCCGGAAAGC	Used to check the resolution of res flanked cassette	This work
tnpR–lacZ-f	GGTACCTGGTAAATCGTACGCCGACTAGAATGTCGGCTTTTTCTC	Primer for the amplification of the *TnpR–LacZ* transcriptional fusion	This work
tnpR–lacZ-r	TCTAGAGGGGCTAGAGCGGCCGCCACCGCGGG	Primer for the amplification of the *TnpR–LacZ* transcriptional fusion	This work
ad-f	GCTGGTACCAGATCTTCTAGA	Adapter for *ΔLgu*I deletion on pRK442	This work
ad-r	GCTTCTAGAAGATCTGGTACC	Adapter for *ΔLgu*I deletion on pRK442	This work
parABCDE-f	CCATGGTCAGCCCTTGAGCCTGTC	Primer for the amplification of the RP4 stability genes from pFAJ1708	This work
parABCDE-r	CCATGGTTATTTTTGCTGCTGC	Primer for the amplification of the RP4 stability genes from pFAJ1708	This work

then ligated into the previously *Bgl*II-digested and dephosphorylated pRIVET-I plasmid, and electroporated into competent *E. coli* DH5α-λ-pir cells. Approximately 1.2×10^4 clones were obtained, pooled, and stored at −80 °C. Random clone analysis revealed that, on the average, 80% of the clones had a DNA fragment of size ranging between 200 bp and 1 kb. Plasmid DNA from the library was prepared *en masse*, transformed into competent cells of *E. coli* SM10-λ-pir (with approximately 3×10^4 clones being obtained), and finally conjugated into *S.meliloti* 2011 R1ΩNGG. Transconjugants were plated on TY Tc-Str solid medium supplemented with Nm to select for those clones that did not express the TnpR (unresolved clones). Those clones that expressed *tnpR* from a rhizobial promoter in TY medium had excised the *nptII* marker and were thus eliminated in the plates with Nm. More than 5×10^4 clones were pooled and stored at −80 °C.

2.5.2. Library refinement to eliminate clones with high rate of spurious excisions

In order to eliminate those *S. meliloti* RIVET clones that spontaneously generate more than 1% excisions after 6–8 growth generations, more than twenty-five thousand clones were individually screened for their rate of excision. To that end, clones were grown in liquid TY medium in 96-well plastic plates and then individually plated by dropping 5 μl of an appropriate dilution onto agar TY medium containing Str-Tc-Gm. A total of approx. 6400 clones that had excised at a rate lower than 1% were isolated, pooled, and cryopreserved at −80 °C (Library S-6000). The rates of excision ($E\% = [(\text{Tc-Gm})\ \text{clones}/(\text{Str-Tc})\ \text{clones}] \times 100$) were determined for the original and the refined library and found to be 2.5% and 0.22%, respectively.

2.6. Response of the S. meliloti 2011 R1ΩNGG RIVET library to 0.38 M NaCl

The response of the Library S-6000 to a saline stress was evaluated by growing the bacteria in TY medium with 0.38 M NaCl through eight generations. The excision rates (*E*%) were calculated as indicated in the previous section for both the NaCl-treated and control (TY) cultures. The relative increase in the rate of excision resulting from the presence of NaCl was calculated as $R = E\%_{(\text{NaCl})}/E\%_{(\text{TY})}$.

3. Results

3.1. Design and construction of a new positive-selection RIVET system

Most of the previously reported RIVET systems lacked the advantage of a positive-selection strategy (Camilli et al., 1994). To facilitate the screening step, Saviola et al. (2003) recently introduced a modified positive-selection system based on the use of the *sacB* gene from *Bacillus subtilis*. Unfortunately, since sucrose is the main energy-transport molecule in plants, *sacB* would not be a good positive-selection marker for either an endophytic or a symbiotic plant-associated bacterium.

We therefore designed and constructed a new promoter-trap positive-selection RIVET tool based on the coordinate use of two antibiotic-resistance cassettes, one of which becomes lost (ΩNm/Km) while the other becomes expressed (*aacCI*, for gentamicin resistance) upon induction of the *tnpR* at the promoter-trap site (Fig. 1). Bacteria that do not transcribe *tnpR* bear a functional ΩNm/Km resistance gene which disrupts transcription of the *aacCI* and *gfp-F64L/S65T* genes from an upstream *nptII* promoter (Fig. 1B). When *tnpR* is expressed, the *res*-flanked ΩNm/Km cassette is lost with the concomitant appearance of gentamicin resistance (Gm^R) and green fluorescence (GFP^+) (Fig. 1C). A step-by-step description of the strategy used for the construction of the positive-selection module is summarized in Fig. 2. As already described by Newman and Grindley (1984), a useful strategy for modulating DNA resolution by TnpR is the sequence modification at the *res* sites. As shown in Fig. 2, we constructed two different versions of the selection module; one based on the use of wild-type *res* sequences (module *RΩNGG*), and the other containing the *res1* sequences (module *R1ΩNGG*) bearing a T → C transition that results in lower resolution rates (Camilli et al., 1994; Newman and Grindley, 1984).

The RIVET system was completed with the construction of two promoter-trap vectors, the narrow host-range pRIVET-I derived from pIVET5n (Osorio et al., 2005) and the broad host-range pRIVET-R derived from the mini-RP4 plasmid pRK442 (Scott et al., 2003). Both new vectors carry a promoterless *tnpR* with an upstream *Bgl*II site to clone and trap DNA promoter activities. pRIVET-I was designed to be used as shuttle vector for creating integrative (site-directed) transcriptional fusions to *tnpR* in different Gram-negative bacteria. In contrast, pRIVET-R can be used to generate plasmid-borne (replicative) promoter-fusions to *tnpR*.

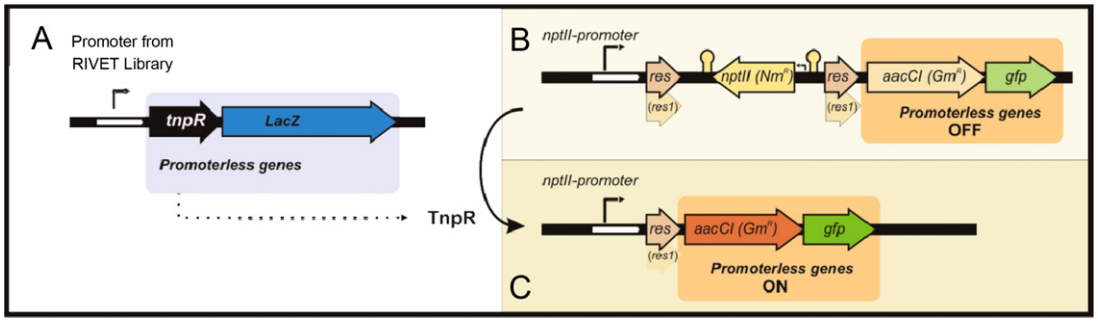

Fig. 1. General scheme of the new positive-selection RIVET system. (A) Promoter-trap module that contains the promoterless *tnpR–lacZ* transcriptional fusion under the control of promoter-containing DNA fragments from the RIVET library. (B) Reporter cassette before excision by TnpR: The *res/res1*-flanked cassette interrupts transcription from the *nptII*-promoter (phenotype: Nm^R-Gm^S-GFP^-). Hairpin structures correspond to transcriptional terminators. (C) Reporter cassette after excision by TnpR (phenotype: Nm^S-Gm^R-GFP^+). *res/res1*: TnpR target sequences; *nptII*: Km/Nm-resistance cassette; *gfp*: promoterless GFP structural gene; *aacCI*: promoterless gentamicin-resistance gene.

Fig. 3 shows a summary of the main cloning steps used to construct the promoter-probe plasmids. Since many experiments have to be performed in the absence of antibiotic selection for the pRIVET plasmids, stability of pRIVET-R is crucial for maintaining the promoter-trap within the sensor bacteria during long-lasting assays. As shown in Fig. 3B, a *parABCDE* stabilization module from RK2 was introduced into pRIVET-R (Section 2.4.2) that greatly improved the stability of the plasmid (not shown).

3.2. Functional evaluation of the new RIVET tool

In order to evaluate the new RIVET modules, and since we work in the biology of plant-associated bacteria, we introduced the selection cassettes into the model legume symbiont *S. meliloti* 2011 (Galibert et al., 2001). To that end, the modules RΩNGG and R1ΩNGG were cloned into the shuttle vector pSM10 (Selbitschka et al., 1995) and homogenotized within the *S. meliloti* chromosomal genes *recA* and *alaS* to produce the strains *S. meliloti* 2011 RΩNGG and *S. meliloti* 2011 R1ΩNGG (Section 2.4.3), respectively (hereafter designed as the reporter strains).

To test the functionality of the positive-selection system, the constitutive promoter of the *nptII* gene was cloned in the pRIVET-R vector, and the resulting plasmid was transferred by conjugation to the recipient strains *S. meliloti* 2011 RΩNGG, and *S. meliloti* 2011 R1ΩNGG. Transconjugants resistant to streptomycin and gentamicin showed up as fluorescent under blue-light illumination. The phenotypic change from Nm^R-Gm^S-GFP^- to Nm^S-Gm^R-GFP^+ was consistent with the expected resolution events at the reporter modules (Fig. 4). Before cassette excision the fluorescent background of the reporter strains was low compared to the fluorescence resulting after resolution (Fig. 4C). No phenotypic changes were observed in control experiments with the reporter strains devoid of the plasmid that carried the *nptII–tnpR* fusion.

3.3. Evaluation of the response of a S. meliloti 2011 R1ΩNGG RIVET library to modifications in the extracellular medium

In order to evaluate the new RIVET system's capability of responding to changes in the extracellular environment, a *S. meliloti* DNA–*tnpR* fusion library was first created in *E. coli* through the use of vector pRIVET-I, the library was then integrated into the reporter strain *S. meliloti* 2011 R1ΩNGG, and the excision rate was finally determined under a strong saline stress (0.38 M NaCl) (Sections 2.5 and 2.6). The collection of *S. meliloti* 2011 R1ΩNGG::pRIVET-I (RIVET library) consisted of *ca.* 10^4 independent clones, with *tnpR* fused at different genome locations. Clones that expressed *tnpR* in the control medium at the beginning of the experiment excised the *res1*-flanked *nptII* marker and thus could be eliminated by sequential cultivation in the presence of neomycin (Section 2.5.1). The remaining clones mainly corresponded to those where *tnpR* was fused either in antisense to rhizobial genes, or in the sense orientation to still untranscribed genomic regions. Since a spontaneous background of excision (i.e., excisional noise) usually occurs when cells are propagated over time under control conditions in the absence of any further stimuli (Osorio et al., 2005), we applied a refinement procedure to eliminate those clones that displayed resolution at a rate ≥1% after 5–8 culture-growth generations (Section 2.5.2). More than six thousand clones that fulfilled this criterion were selected out, on the basis of excision rates analyzed in more than 2×10^4 individually tested clones. At the end of the refinement procedure the overall spontaneous rate of excision of the RIVET library proved to be 0.22 ± 0.08%, a value that was tenfold lower than the one corresponding to the original library.

In order to evaluate the response of the library to a previously studied stress condition (Dominguez-Ferreras et al., 2006; Ruberg et al., 2003; Shamseldin et al., 2006; Talibart et al., 1997), the collection of clones was grown under saline stress (0.38 M NaCl) overnight, and the percent of excised clones that resulted was calculated based on the proportion of Gm^R bacteria. The saline-stressed culture exhibited an excision level of 0.91 ± 0.22%, compared to the control value of 0.21 ± 0.05% for the cultures that were grown in the control TY medium (significant difference, Student's *t*-test $p = 0.005$). Thus, we observed an approximate fourfold average increase in the percent of excised clones under the stress condition of 0.38 M NaCl relative to the value in the control TY medium. This result indicates that about 75% of the Gm^R clones that appear during bacterial growth under the high-salt condition correspond to genotypes with *tnpR* fusions to *S. meliloti* genes specifically induced by the saline stress. To characterize the rhizobial DNA that controlled *tnpR* expression in clones that excised under high salt, integrated pRIVET-I plasmids were recovered from selected Gm^R-GFP^+ and then sequenced. We observed that 3 out of 15 sequences corresponded to a *tnpR* fusion to DNA located immediately downstream of the chromosomal ORF *Smc04182*. Sequence redundancy has also been reported in other RIVET studies (Castillo et al., 2008), and it is indicative of promoter activities that likely respond to the challenging condition used. For a final confirmation that the rescued DNA was associated to the rhizobial response to salt stress, two independent assays were carried out: (a) the intact reporter cassette R1ΩNGG was first re-introduced into the excised rhizobia that carried the *tnpR* downstream *Smc04182*, and the excision under high-salt analyzed again, and (b) the recovered plasmid pRIVET-I with cloned DNA from downstream *Smc04182* was re-introduced into the indicator rhizobia 2011 R1ΩNGG, and the excision under high salt also evaluated again. As expected, both strategies showed excision rates under high salt that were nearly 2 times the excision rates observed in the control TY medium without salt (i.e. 13.5 ± 1.8% vs. 6.1 ± 1.1%, respectively). These results

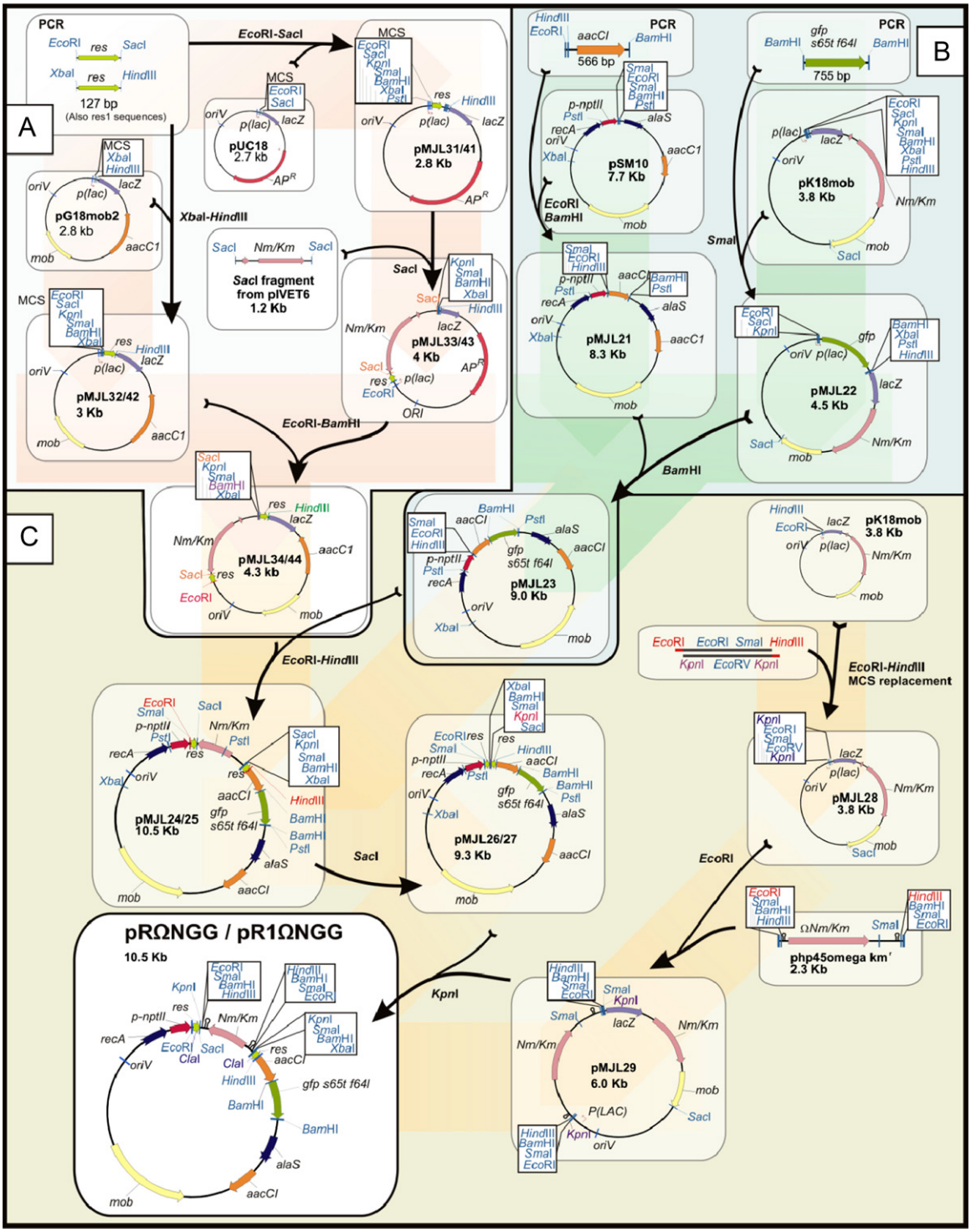

Fig. 2. Cloning strategy used for the construction of the positive selection reporter cassettes RΩNGG and R1ΩNGG. (A) Construction of the *res* (or *res1*) flanked Km/Nm-resistance (*nptII*) cassette. (B) Construction of the cassette (promoter^{nptII}-*aacC1*-*gfp*) within the *recA*–*alaS* region of plasmid pSM10. (C) Assembly of the complete positive-selection module (promoter^{nptII}-[*res*-ΩNm/Km-*res*]-*aacC1*-*gfp*) and its homolog containing *res1* sequences within plasmid pSM10 to generate plasmids pRΩNGG and pR1ΩNGG, respectively (Section 2.4.1).

demonstrate that the system presented here is fully functional to the identification of responsive promoters to specific conditions. Irrespective of the results shown here, in the general situation, the absolute number of specific excisions as well as their relative proportion to the (nonspecific) spurious resolutions will result from both the particular condition under analysis and the quality of the RIVET library.

4. Discussion

Despite the development of several high-throughput technologies capable of identifying differentially expressed genes under diverse conditions, the study of complex biological systems still remains in many cases elusive. The inaccessibility of occult niches in intricate natural environments as well as the limited amount of

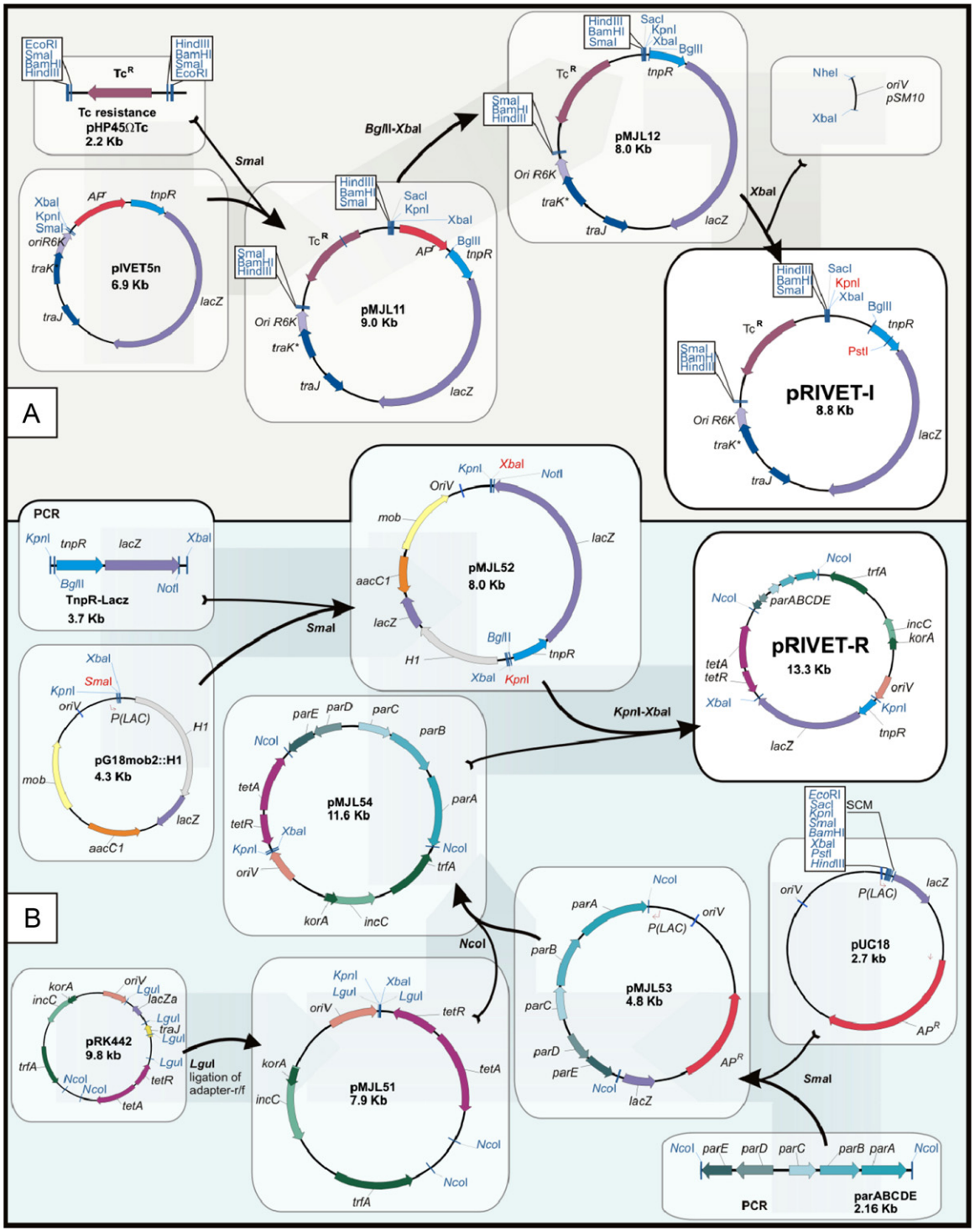

Fig. 3. Cloning strategy used for the construction of the promoter-probe plasmids pRIVET-I and pRIVET-R. (A) Construction of the narrow-host range plasmid pRIVET-I bearing the replication origin from plasmid pSM10. (B) Construction of the broad-host range plasmid pRIVET-R bearing the replication origin from plasmid RP4 and the partitioning functions *parABCDE* from plasmid RK2. Both plasmids carry a promoterless *tnpR–lacZ* transcriptional fusion, with a unique *Bgl*II restriction site for the construction of promoter libraries (Section 2.4.2).

sample material available, severely restrict the possibility of characterizing bacterial behavior in nature at an omic scale by means of conventional analytical platforms. In addition, classical transcriptomics and proteomics are useful to characterize either repressed or induced markers, but usually fail in identifying transiently expressed genes. As stated in the introduction, RIVET provides a suitable technical alternative to circumvent the above-mentioned limitations in the characterization of differentially induced genes in bacteria.

In this report, we describe the construction and functional validation of a new RIVET positive-selection cassette as well as two associated promoter-trap vectors. Replicative vectors, such as pRIVET-R, were constructed to search for promoter activities that reside within the plasmid inserts. In contrast, inserts from inte-

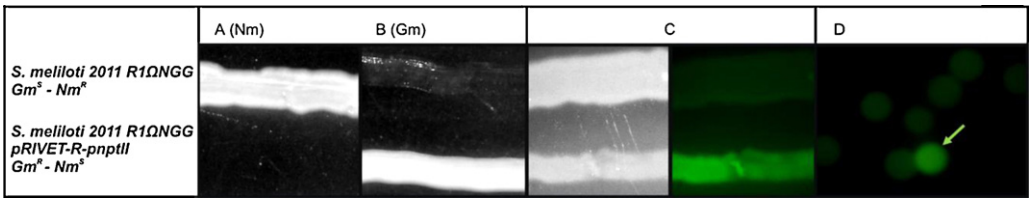

Fig. 4. Agar plates showing the modification of antibiotic resistance and green-fluorescent phenotype (Nm^R-Gm^S-GFP^- → Nm^S-Gm^R-GFP^+) in a model reporter bacterium after excision at the *res1* site by TnpR. (A) *S. meliloti* 2011 R1NΩGG streaked before (top) and after (bottom) excision in agar TY containing Nm. (B) *S. meliloti* 2011 R1NΩGG streaked before (top) and after (bottom) excision in agar TY containing Gm. (C) *S. meliloti* 2011 R1NΩGG streaked before (top) and after (bottom) excision in TY with Tc, and observed under white- (left) and blue-light (right) illumination. (D) A single colony of *S. meliloti* 2011 R1NΩGG in TY plates with Tc showing the green fluorescence of an excised clone (arrow).

grative RIVET plasmids mainly serve to mediate the site-specific integration of *tnpR* into the host genome. While the use of pRIVET-I as promoter-trap vehicle requires the recovery of the integrated plasmids with their associated genomic inserts, the use of the replicative pRIVET-R facilitates plasmid's recovery. Nevertheless, because of copy-number effects, the use of pRIVET-R might increase the spurious (nonspecific) excision of the reporter module. The integrative systems have at least two practical advantages: (a) The screening of all promoters will be formally satisfied with the integration of one RIVET plasmid per gene or operon; (b) the quality of the RIVET library will not depend on the insert size, but instead on the insert's diversity. While a replicative RIVET library may contain many promoterless (nonfunctional) inserts, all sense-oriented inserts from integrative libraries would be expected to contribute to the screening of promoter activities upon integration into the host genome.

With respect to the selection module, the markers and promoters used (*nptII*, *aacCI*, *gfp*) make the system suitable for the construction of RIVET libraries in diverse Gram-negative bacteria. Furthermore, considering the ubiquitous distribution of sucrose in plants, we avoided the use of *sacB* in order to make the system usable in studies with plant-associated and/or plant-colonizing bacteria. This new positive-selection system was based on the use of two antibiotic-resistance cassettes. Upon induction of TnpR, one resistance marker is lost (*nptII*), while the other is expressed (*aacCI*). As a result, clones that transcribe *tnpR*-fused promoters under a condition of interest can be selected by plating the RIVET library in the presence of gentamicin. In contrast with the transcriptional activation of the system constructed here, other authors have reported a translational activation of an antibiotic-resistance gene upon the action of TnpR (Livny and Friedman, 2004). The activation system implemented here can be redesigned to replace or exchange the antibiotic-selection markers to modify or expand the range of bacteria where the RIVET tool could be used.

Once the inducible genes are identified, a central question concerns the characterization of the precise physical location and circumstance where the induction of each marker occurs. In our system this issue is expected to be facilitated by the concomitant appearance of GFP when the gentamicin resistance is activated. Such a feature will be very valuable for identifying the place and timing of the induction of specific markers in the colonization of abiotic environments as well as throughout complex biological niches. In previous works aimed at the biosensing of arabinose and toluene in root environments, Casavant et al. (2002, 2003) reported the use of the site-specific recombination machinery of bacteriophage P22 to trigger the expression of a reporter *gfp*. In the RIVET system here described the expression of *gfp* is accompanied by the appearance of an antibiotic resistance. Irrespective of the characteristics of the reporter module the development of new and highly specific recombination-based biosensors will depend on the availability of specific (and low background) signal-responsive promoters (Casavant et al., 2002, 2003). The positive-selection RIVET systems constructed in this work provide the basic tools for the design of new recombination-based biosensors.

The new RIVET system presented here was tested through the use of the model soil bacterium *S. meliloti*, and a clear phenotypic change was observed in the excised clones that converted from Gm^S to Gm^R phenotype and displayed a strong green fluorescence under blue-light illumination. As expected, a statistically significant ($p = 0.005$) increase in the rate of excisions was also observed (fourfold) when the extracellular conditions in the culture medium were altered by mean of a salt stress, an indication of the responsiveness of the system to changes in the environment. Cloning and sequencing of several recovered RIVET plasmids allowed for the identification of a salt responsive *tnpR* transcriptional fusion located downstream *Smc04182*.

Though RIVET tools have been used to characterize the expression *in planta* of specific and well-known rhizobial promoters (i.e. *nodC*, *sinI*, *ftsZ2*, and *expG*) (Gao and Teplitski, 2008), the methodology has never been applied to the discovery of new (inducible) symbiotic markers in a general screening. Such a screening could be approached with a *sacB*-independent system using the tools designed, constructed, and validated here. From a general point of view, the RIVET configurations presented in this work provide a strong positive-selection tool for the systematic search of bacterial markers induced when microorganisms colonize and invade complex environments and their eukaryotic hosts.

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