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Broad Host Range and Promoter Selection Vectors for Bacteria that Interact with Plants

Guy Van den Eede, Rolf Deblaere, Koen Goethals, Marc Van Montagu, and Marcelle Holsters

Laboratorium voor Genetica, Universiteit Gent, B-9000 Gent, Belgium.
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A plasmid vector, pGV910, and a derived cosmid, pRG930, have been constructed. Both contain the ColE1 and pVS1 origins of replication and are stably maintained in *Escherichia coli*, *Agrobacterium tumefaciens*, and *Azorhizobium caulinodans* ORS571. They are compatible with commonly used IncP cloning vectors, although pVS1 was classified as an IncP plasmid, unable to replicate in *E. coli* (Y. Itoh, J. M. Watson, D. Haas, and T. Leisinger, Plasmid 11:206-220, 1984). Promoter selection vectors were derived from both of these plasmids by using a promoterless β-glucuronidase and/or β-galactosidase gene. These vectors facilitate the study of gene expression in bacteria under particular environmental conditions. This is illustrated by the expression of the *gusA* gene under the control of a *nod* promoter in *A. caulinodans* nodulating stem-located infection sites on *Sesbania rostrata*.

Broad host range cloning vectors for gram-negative bacteria are most often derived from plasmids that belong to the incompatibility classes P, Q, or W. The RP4 (IncP), the R3000B (IncQ), and the Sa replicon (IncW) have been used extensively to construct vectors that are mobilizable but not self-transmissible (Mermod *et al.* 1986; Schmidhauser *et al.* 1988). In *Azorhizobium caulinodans* strain ORS571, a soil bacterium that nodulates the stem and root of the tropical legume *Sesbania rostrata* Brem (Dreyfus *et al.* 1988), we have been unable to introduce and maintain plasmids that belong to the IncQ or IncW incompatibility groups; only IncP plasmids could be maintained. On several occasions during our studies of *Azorhizobium* sp, it was desirable to combine two compatible plasmids that carried different inserts. This was achieved by constructing a broad host range vector that contained the origin of replication of plasmid pVS1.

Plasmid pVS1, an IncP plasmid isolated from *Pseudomonas aeruginosa* (Schroeter) Migula, has been found to replicate in a wide variety of gram-negative bacteria, including *Rhizobium* and *Agrobacterium*, but not in *E. coli* (Itoh *et al.* 1984). This 30-kb nonconjugative plasmid carries Tn*501*, which encodes mercury resistance. The regions for replication, stability, and mobilization by IncP plasmids are clustered within an 8-kb region. We have ligated this 8-kb fragment to pBR325 and studied the stability, replication properties, and copy number of the resulting plasmid, pGV910. Compatibility studies showed that this vector can be used in combination with the P-type plasmid pRK290 (Ditta *et al.* 1980) to form a novel binary vector system. This system was used to identify a regulatory nodulation (*nod*) gene in *A. caulinodans* (Goethals *et al.* 1990). The stable maintenance of pVS1 in *A. tumefaciens* already allowed the construction of derivatives that formed the basis for a binary T-DNA vector system for plant cell transformation (Deblaere *et al.* 1987).

Two promoterless *E. coli* reporter genes, *lacZ* and *gusA*, have been used to construct pGV910-based promoter selection vectors. The *lacZ* gene encodes the *E. coli* β-galactosidase (Beckwith and Zipser 1970), and *gusA* encodes the *E. coli* β-glucuronidase (Jefferson *et al.* 1986), which is a hydrolase that catalyzes the cleavage of various glucuronides. The *lacZ* gene was selected because it provides a sensitive enzyme assay for which a broad range of substrates are available. Although most higher plants show large amounts of galactosidase activity, they lack β-glucuronidase activity (GUS) (Jefferson 1987). Therefore, we included the *gusA* reporter gene to study bacterial invasion of plant tissues and *in situ* expression of bacterial genes. The *A. caulinodans-S. rostrata* interaction was used as a model system, because aerial nodules can be induced at predetermined infection sites, namely stem-located dormant root primordia, hence facilitating the analysis of the tissue that is being invaded by the symbiont.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. Growth conditions, media, and antimetabolites used were mainly as described by Goethals *et al.* (1990). Mercuric chloride was added to the medium at a final concentration of 20 μg/ml.

Triparental matings. Mobilization of pGV910, pRK290, and derivatives was performed by using pRK2013 as a helper plasmid (Ditta *et al.* 1980).

DNA biochemistry and recombinant DNA techniques. The standard techniques were as described by Sambrook *et al.* (1989). The GeneClean kit (Bio-101 Inc., La Jolla, CA) was used to purify DNA fragments from agarose gels.

Present address of G. Van den Eede: Commissione delle Comunità Europee, Centro Comune di Ricerca, Stabilimento di Ispra (TP 634), 1-21021 Ispra (Varese), Italia.
Address correspondence to: M. Van Montagu, Laboratorium voor Genetica, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium.

Enzymatic assays. β-Galactosidase assays were performed as described by Goethals *et al.* (1989).

GUS activity was measured according to Jefferson (1987). The reaction was carried out in GUS buffer (0.06 M Na_2HPO_4; 0.04 M NaH_2PO_4; 14 mM β-mercaptoethanol; 1 mM Na_2EDTA; pH 7) with para-nitrophenyl-β-D-glucuronide as a substrate and was stopped by adding 200 μl of 2.5 M 2-amino-2-methyl-1,3-propanediol. Absorbance was measured at 415 nm. Units of GUS activity were calculated by the formula of Miller (1972), in which OD_{420} was replaced by OD_{415}.

GUS activity in nodule sections. Detached stem nodules were immersed in 100 mM sodium phosphate buffer (pH 7.3) containing 0.5 mg/ml of X-gluc. Optimal results were achieved by addition of sodium dodecyl sulfate (SDS) (0.005% final concentration) and *in vacuo* incubation during 10 min. Reactions were performed in the dark at 37° C for 1–14 hr, and the *in vacuo* incubation was repeated after 1 hr.

Addition of an oxidizing substrate, such as H_2O_2, to increase oxidative dimerization of the colourless hydrolysate into blue crystals did not improve the assay. The tissues were washed several times in 100 mM sodium phosphate buffer. Nodules were dried, embedded in 5% agarose, stuck on a block, and immersed in water. Twenty- to 80-μm sections were made by using a vibro-cutter (Campden Instruments Ltd., London, U.K.).

RESULTS AND DISCUSSION

Construction of pGV910 and pRG930. To construct pGV910 (Fig. 1), a 2.3-kb *Hin*dIII-*Bam*HI fragment from

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Origin
Escherichia coli strains		
HB101	*pro, leu, thi, lacY, hsd20, endA, recA, rpsL20*(Str^r), *ara-14, galK2, xyl*-5, *mtl*-1, *supE44*	Boyer and Roulland-Dussoix 1969
MC1061	*hsdR, hsdM, hsdS, araD*139, Δ(*ara-leu*)$_{7697}$, Δ(*lac*)$_{X74}$, *galU, galK, rpsL*(Str^r)	Casadaban and Cohen 1980
DH5α	F$^-$, φ80d*lacZ*Δ*MI5*, Δ(*lacZYA-argF*)$_{U169}$, *recA1, endA1, hsdR17*(r_K^-, m_K^+), *supE44*, λ$^-$, *thi*-1 *relA1*	Hanahan 1983
CSH2110	*polA*$^-$, Nal^r	Heffron *et al.* 1977
Other strains		
ORS571	*Azorhizobium caulinodans* type strain; nodulates stems and roots of *Sesbania rostrata*, Cb^r	Dreyfus *et al.* 1988
GV3101	Rif^r derivative of *Agrobacterium tumefaciens* cured of its pTiC58 plasmid	Van Larebeke *et al.* 1974
Plasmids		
pVS1	Unclassified IncP plasmid, tra$^-$ Mob(RP4), Su^r, $HgCl_2^r$	Itoh *et al.* 1984
pRK290	IncP broad host range cloning vector tra$^-$, Mob(RP4), Tc^r, ColE1, tra^c, Mob(ColE1)	Ditta *et al.* 1980
pLAFR1	IncP broad host range cosmid cloning vector, Tc^r	Friedman *et al.* 1982
pRK2013	Mob(RP4), Km^r; used as helper plasmid in mobilizations	Figurski and Helinski 1979
pBR325	ColEl cloning vector, tra$^-$, Mob(ColEl), Cb^r Cm^r Tc^r	Bolivar 1978
pUC8	ColEl cloning vector, Cb^r	Vieira and Messing 1982
pUC19	ColEl cloning vector, Cb^r	Vieira and Messing 1982
pUCN3	1.2-kb *Bam*HI fragment containing *nodA* promoter sequence from *A. caulinodans* ORS571, cloned in pUC18	Goethals *et al.* 1989
pBI101	1.87-kb *gusA* gene ligated to pBIN19	Jefferson 1987
pTC1	*E. coli* expression vector, containing *lacZYA*	J. Botterman (personal communication)
pGV910	Unclassified IncP, 15.68-kb broad host range cloning vector, tra$^-$, Mob(ColEl), Mob(RP4), Cb^r Cm^r Sm^r/Sp^r	This work
pRG930	16.1-kb cosmid, derived from pGV910, Sm^r/Sp^r	This work
pRG960	pRG930 containing the promoterless *gusA* with start codon, Sm^r/Sp^r (17.0 kb)	This work
pRG960SD	pRG960 derivative with promoterless *gusA* start codon and Shine and Dalgarno sequence	This work
pRG970	pRG930 containing both the promoterless *lacZ* and *gusA*, Sm^r/Sp^r (23.6 kb)	This work
pMP220	10.5-kb IncP cloning vector, containing a *lacZ* reporter gene	Spaink *et al.* 1987
pMP220-31	pMP220 containing the ORS571 *nodA* promoter oriented towards *lacZ*	This work
pRG960-32	pRG960 containing the ORS571 *nodA* promoter oriented towards *gusA*	This work
pRG960SD-32	pRG960SD containing the ORS571 *nodA* promoter oriented towards *gusA*	This work
pRG970-31	pRG970 containing the ORS571 *nodA* promoter oriented towards *lacZ*	This work
pRG970-32	pRG970 containing the ORS571 *nodA* promoter oriented towards *gusA*	This work
Phage		
MudIIpR13	Defective Mu phage derivative with promoterless *lacZ* gene; Cm^r	Ratet *et al.* 1988

plasmid R702 (Leemans *et al.* 1982), encoding streptomycin (Sm) and spectinomycin (Sp) resistance, was ligated into *Hin*dIII-*Bam*HI digested pBR325 (Bolivar 1978). An 8-kb *Bam*HI-*Sal*I fragment from pVS1, which contained the regions essential for maintenance, stability, and mobilization by RP1 (Itoh *et al.* 1984), was inserted into the resulting plasmid. This plasmid, pGV910, has the following properties. It is 15.6 kb in size and contains both the ColE1 and pVS1 origins. The yield of plasmid DNA from *E. coli* MC1061(pGV910) suggests that the copy number is comparable to that of pBR325 (data not shown). pGV910 is not self-transmissible but can be mobilized by pRK2013 (Figurski and Helinski 1979), because it contains the *Mob* site of pBR325 as well as the pVS1 region essential for mobilization by RP1 (Mob[RP4]; Fig. 1). In addition to a streptomycin-spectinomycin adenyltransferase encoding gene, pGV910 carries genes for β-lactamase (*bla*) and chloramphenicol acetyltransferase (*cat*), transcribed as shown in Figure 1. We have regularly observed that the promoter of the latter gene can enhance the expression of fragments cloned in the *Eco*RI site. Insertional inactivation is achieved by cloning into the unique *Eco*RI site

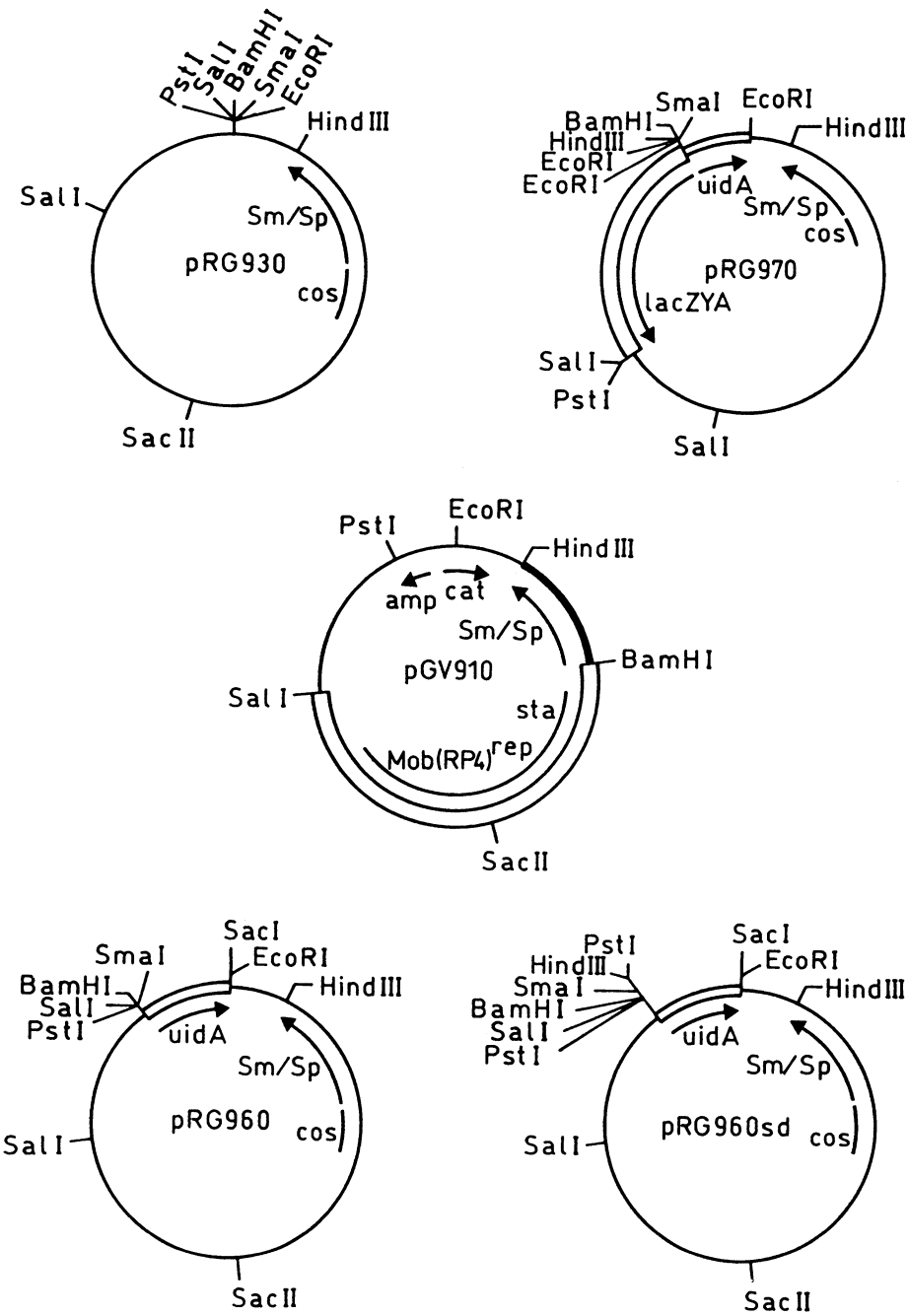

Fig. 1. Physical map of the cloning vectors, pGV910, pRG930, pRG960, pRG960SD, and pRG970. In pGV910, the pVS1 portion (with the regions required for mobilization by RP4 [Mob(RP4)], replication [rep], and plasmid stability [sta]) is represented by an open box; a broad line represents the R702 portion; and a single line represents the ColE1 portion. For construction details, see text.

within *cat* (Cm^s) or in the unique *Pst*I site within *bla* (Ap^s). Other unique restriction sites are *Bam*HI, *Sal*I, *Hin*dIII, and *Sac*II.

To construct the vector pRG930, we ligated the 1.6-kb *Bgl*II "*cos*" fragment from cosmid pLAFR1 (Friedman *et al.* 1982) into the *Bam*HI site of pGV910. Then, we inserted pUC8 into the unique *Eco*RI site. Digestion with *Pst*I followed by self-ligation yielded pRG930 (Fig. 1). The *cos* site allows packaging into phage λ particles (Collins 1979). pRG930 is 16.1 kb in size, contains unique *Eco*RI, *Bam*HI, *Pst*I, *Sma*I, *Hin*dIII, and *Sac*II restriction sites, and encodes streptomycin and spectinomycin resistance.

Host range and copy number of pGV910. pGV910 can be mobilized from *E. coli* to *A. caulinodans* at a frequency of 5×10^{-1} and to *A. tumefaciens* GV3103 at a frequency of $2\text{–}3 \times 10^{-1}$. pGV910 and pRG930 can also be introduced by mobilization as well as by transformation in the DNA polymerase A-deficient *E. coli* strain CSH2110. This is surprising because control experiments confirmed that neither pVS1, nor pBR325 can replicate in the *pol*A^- strain; thus, some form of complementation must occur to allow maintenance of the chimeric plasmid. Although the copy number of pGV910 in the *pol*A^- host is low, suggesting a pVS1 type of replication, it is equally possible that pVS1 provides a function that complements the polymerase A deficiency.

Table 2. Stability of pGV910 and pRK290 in *Escherichia coli* CSH2110, *Azorhizobium caulinodans* ORS571, and *Agrobacterium tumefaciens* GV3101

Strain (plasmids)	Number of generations	Plasmid marker loss (%)[a]	
		Initial	Final
CSH2110(pGV910)	20	3.1	13.5
ORS571(pGV910)	21	<0.6	<0.5
GV3101(pGV910)	16	<0.5	<0.5
CSH2110(pRK290)	18	1.6	2.6
ORS571(pRK290)	17	2.3	8.5
GV3101(pRK290)	15	<0.5	26.0

[a] The initial percentage is the percentage of bacteria that lost the plasmid marker after growing to saturation under selective conditions before plating on media without selection; the final percentage gives the percentage of bacteria that lost plasmid resistance after nonselective growth for a number of generations. Plasmid marker loss was measured by plating bacteria on nonselective media and subsequently screening for the plasmid marker (Sp^r for pGV910; Tc^r for pRK290) by replica plating on selective media.

Table 3. Compatibility between pRK290 and pGV910

Bacterial strains (plasmids)	Selection	Number of generations	Plasmid marker loss (%)[a]	
			Initial	Final
CSH2110(pGV910,pRK290)	Tc_{10}	20	0.5	2.0
ORS571(pGV910,pRK290)	Tc_{10}	17	<0.5	0.5
GV3101(pGV910,pRK290)	Tc_5	16	1.7	<0.5
CSH2110(pGV910,pRK290)	Sp_{100}	20	1.2	0.6
ORS571(pGV910,pRK290)	Sp_{100}	17	1.6	4.4
GV3101(pGV910,pRK290)	Sp_{100}	16	0.3	12.0

[a] Bacteria were grown for a number of generations with selection for either the pRK290 (Tc) or the pGV910 (Sp) marker and plated on agar media containing the same antibiotic. The number of bacteria that lost the other plasmid marker was determined by replica plating on selective media (final percentage). Initial loss (initial %) was measured after growth in SpTc-containing media.

The low copy number plasmid, pRK290 (Ditta *et al.* 1980), has frequently been used as a broad host range cloning vector. To compare the copy number of pGV910 to that of pRK290, total plasmid DNA was extracted from CSH2110(pRK290, pGV910), GV3101(pRK290, pGV910), and ORS571(pRK290, pGV910), digested with *Eco*RI, and separated on 0.7% agarose gels. In *E. coli* CSH2110, pRK290 has an approximately twofold higher copy number than pGV910; in *A. tumefaciens*, both plasmids have a comparable copy number; and in *A. caulinodans*, pGV910 has a threefold higher copy number than pRK290 (data not shown).

Stability and incompatibility of pGV910 and pRK290 in different hosts. Table 2 summarizes data on the stability of pGV910 and pRK290 in three different hosts: *E. coli* CSH2110, *A. caulinodans* ORS571, and *A. tumefaciens* GV3101. pGV910 replicates stably in *Agrobacterium* and *Azorhizobium*. In CSH2110, 13% of the bacteria was cured of the plasmid after growth for 19 generations in antibiotic-free medium. The P-type vector, pRK290, was rather unstable in *A. tumefaciens*, because approximately one-fourth of the bacteria lost the pRK290 marker after growth for 15 generations in the absence of antibiotics.

To study the compatibility between pRK290 and pGV910, we introduced both plasmids into *E. coli* CSH2110, *A. caulinodans* ORS571, and *A. tumefaciens* GV3101, respectively. Table 3 shows that both plasmids are stably maintained in these three different hosts. After being selected for 17 generations for the pRK290 marker only, 0.5% of the ORS571 bacteria had lost the pGV910 marker. When *A. tumefaciens* (pRK290, pGV910) was grown for 16 generations in the presence of spectinomycin only, 12% of the bacteria had lost the pRK290 marker. When we compare this with the stability of pRK290 in *Agrobacterium*, it appears that pGV910 can stabilize the replication of pRK290 in this host. We observed a related phenomenon in *E. coli* CSH2110, in which pRK290 can

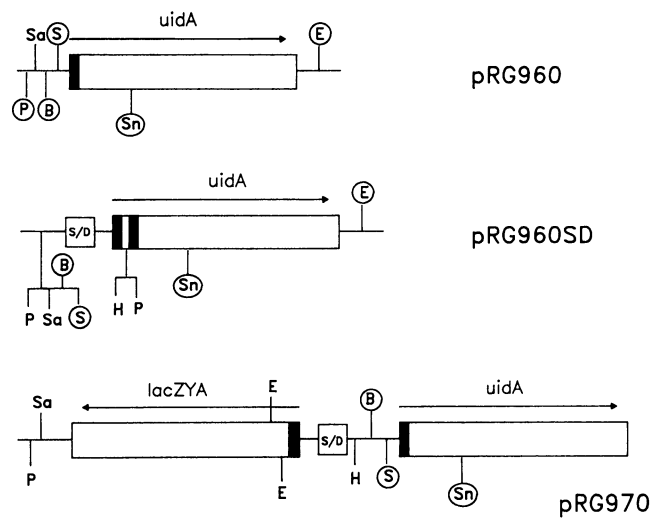

Fig. 2. Schematic representation of reporter gene(s) and cloning sites in pRG960, pRG960SD, and pRG970. The genes are represented as boxes; arrows indicate the direction of transcription. The Shine and Dalgarno (S/D) sequence and translation initiation sites (■) of *lacZ* and *gusA* are indicated. Unique restriction sites are encircled. Sa, *Sal*I; P, *Pst*I; B, *Bam*HI; S, *Sma*I; E, *Eco*RI; H, *Hin*dIII; Sn, *Sna*BI.

stabilize pGV910 replication. After growth for 20 generations without selecting for pGV910, 13.5% lost the pGV910 resistance markers, whereas when pRK290 was present and selected for, only 2% of the cells lost the pGV910 markers.

These results indicate that pRK290 and pGV910 are compatible in the three strains tested. When both plasmids are present together, their replication has an equal or an increased stability as compared to the same host carrying either of the plasmids alone.

pGV910-derived promoter selection vectors. Three promoter probe plasmids in which DNA fragments can be linked to the *E. coli lacZ* gene or the *E. coli gusA* gene have been constructed. The plasmid pRG960 (Figs. 1,2) is derived from pRG930 by inserting the *E. coli gusA* gene, without its own promoter, but with an ATG initiation codon. A 1.87-kb *Bam*HI-*Sac*I fragment containing the *gusA* gene was isolated from pBI101 (Jefferson 1987), ligated into *Bam*HI-*Sac*I digested pUC19 DNA, and consecutively inserted as a 1.87-kb *Pst*I-*Eco*RI fragment into pRG930, yielding pRG960. Hence, the *gusA* ATG start codon is preceded by unique *Pst*I, *Bam*HI, and *Sma*I cloning sites. As this linker sequence upstream from the ATG codon does not contain any stop codons, both transcriptional and translational fusions can be generated.

Plasmid pRG960SD is pRG960 with a Shine and Dalgarno sequence upstream from the *gusA* open reading frame (Figs. 1,2). There are no stop codons present between the unique *Bam*HI and *Sma*I cloning sites and *gusA*. pRG960SD was constructed by exchanging the upstream sequences of *gusA* in pRG960 with a corresponding fragment from the Tn*5-gusA1* transposon carried by pSB165 (Sharma and Signer 1990). An 800-bp *Xho*II fragment from Tn*5-gusA1* that contained the −20 to +800 nucleotides of *gusA* was ligated into the pUC19 *Bam*HI site. Both this intermediate construct and pRG960 contain unique *Sma*I and *Sna*BI sites, the latter being located within the structural *gusA* gene (Fig. 2). This fragment was substituted in pRG960 to obtain pRG960SD.

The plasmid pRG970 (23.6 kb) carries two reporter genes, *lacZ* and *gusA* (see Figs. 1,2). The ATG initiation codon of *lacZ* is immediately preceded by a TAA stop codon; only transcriptional *lacZ* fusions can be obtained. The Shine and Dalgarno sequence GGAGG (Stormo *et al.* 1982) is located from position −10 to −6 relative to the *lacZ* initiation codon (Fig. 2).

Any fragment, cloned into the unique *Bam*HI or *Sma*I sites will be flanked by the 5′ end of *lacZ* on one side and by the 5′ end of *gusA* on the other. By measuring the β-galactosidase and GUS activities, it is possible to detect divergent promoter configurations.

For its construction, a 6.6-kb *Bam*HI-*Pst*I fragment containing *lacZYA* from MudIIpR13 (Ratet *et al.* 1988) was ligated into *Bam*HI-*Pst*I digested pRG930. To introduce an initiation codon in frame with *lacZ*, we exchanged a *Bam*HI-*Sac*I fragment that contained the 5′ region of *lacZ* with a corresponding *Bam*HI-*Sac*I fragment from pTC1 (J. Botterman, personal communication). Finally, we inserted the *Bam*HI-*Pst*I fragment carrying the *lacZYA* genes into *Bam*HI-*Pst*I digested pRG960.

These constructs are best maintained in the *rec*A *E. coli* strain HB101 to avoid recombination between *gusA* on the vector and on the *E. coli* chromosome.

To test pRG960, pRG960SD, and pRG970 as promoter probe vectors, we introduced a *nodA* promoter fragment from *A. caulinodans*. It has previously been demonstrated by using *lac* fusions that expression of the common *nod* operon of *A. caulinodans* is induced in the presence of *S. rostrata* exudate or of the flavanones naringenin (Goethals *et al.* 1989) or liquiritigenin (Messens *et al.* 1991). A 1.27-kb *Bam*HI fragment, extending from position −887 to position +386 relative to the putative *nodA* initiation codon, contains *cis*-acting sequences required for this induction (Goethals *et al.*, in press).

This fragment was isolated from pUCN3 (Goethals *et al.* 1989) and inserted in *Bam*HI-digested pMP220 (Spaink *et al.* 1987), pRG960, pRG960SD, and pRG970. pMP220-31 contains the *nodA* promoter oriented towards *lacZ*; in pRG960-32 and pRG960SD-32, the promoter is oriented towards *gusA*. pRG970-31 and pRG970-32 contain the *nodA* promoter oriented towards *lacZ* or *gusA*, respectively.

From the nucleotide sequences of the parental plasmids, we expect all *gusA* and *lacZ* fusions to be transcriptional. These different constructs were assayed in ORS571 for β-galactosidase and/or GUS activities in the presence or absence of 10 μM naringenin. In comparison to pMP220, the plasmids pRG960, pRG960SD, and pRG970 direct a lower basal expression of their reporter genes, a useful characteristic for a promoter selection system (Table 4). Upon addition of 10 μM naringenin, ORS571(pRG960-32), ORS571(pRG960SD-32), and ORS571(pRG970-32) showed induced GUS activity, and ORS571(pRG970-31) showed an induced β-galactosidase activity (Table 4). These results indicate that the *gusA* and *lacZ* genes are correctly transcribed and translated in our constructs. When a Shine and Dalgarno sequence was present, expression was more efficient.

GUS activity in nodule tissue. *S. rostrata* stems were inoculated with ORS571(pRG960), ORS571(pRG960-32), ORS571(pRG960SD), and ORS571(pRG960SD-32). Ten-day-old nitrogen-fixing nodules were examined for GUS activity (for a detailed description of the morphogenesis of stem nodules on *S. rostrata*, see Duhoux 1984).

No GUS activity could be detected in non-nodule tis-

Table 4. β-Galactosidase and β-glucuronidase activities of induced and noninduced *Azorhizobium caulinodans* ORS571[a]

	β-galactosidase units		β-glucuronidase units	
Plasmid	**Noninduced**	**10 μM NAR**	**Noninduced**	**10 μM NAR**
pMP220	186	203	ND	ND
pMP220-31	102	900	ND	ND
pRG960	ND	ND	15	15
pRG960-32	ND	ND	40	137
pRG960SD	ND	ND	30	29
pRG960SD-32	ND	ND	18	764
pRG970	25	25	8	8
pRG970-31	256	751	36	32
pRG970-32	87	61	22	289

[a] Inductions were done for 14 hr as described by Goethals *et al.* (1989). β-Galactosidase and β-glucuronidase units were determined as described in text. For construction details, see text. NAR, naringenin; ND, not determined.

sue or in nodules induced by ORS571(pRG960) or ORS571(pRG960SD) (data not shown). This is in agreement with the low levels of GUS activities measured in these strains *ex planta* and with the observation that higher plants generally lack GUS activity.

Ten-day-old nodules induced by ORS571(pRG960-32) showed dark-blue spots on the surface after incubation with X-gluc (Fig. 3). A possible explanation is that the nodule is covered with bacteria, and nodule cells exude *nod* gene inducing molecules. When sections of these nodules were examined by dark-field microscopy, GUS activity in the central bacteroid tissue was found to be very low in comparison to the activity in the vicinity of young dividing plant cells (Fig. 3). In bright field, the GUS activity is seen as blue crystals (data not shown).

When 10-day-old nodules induced by ORS571

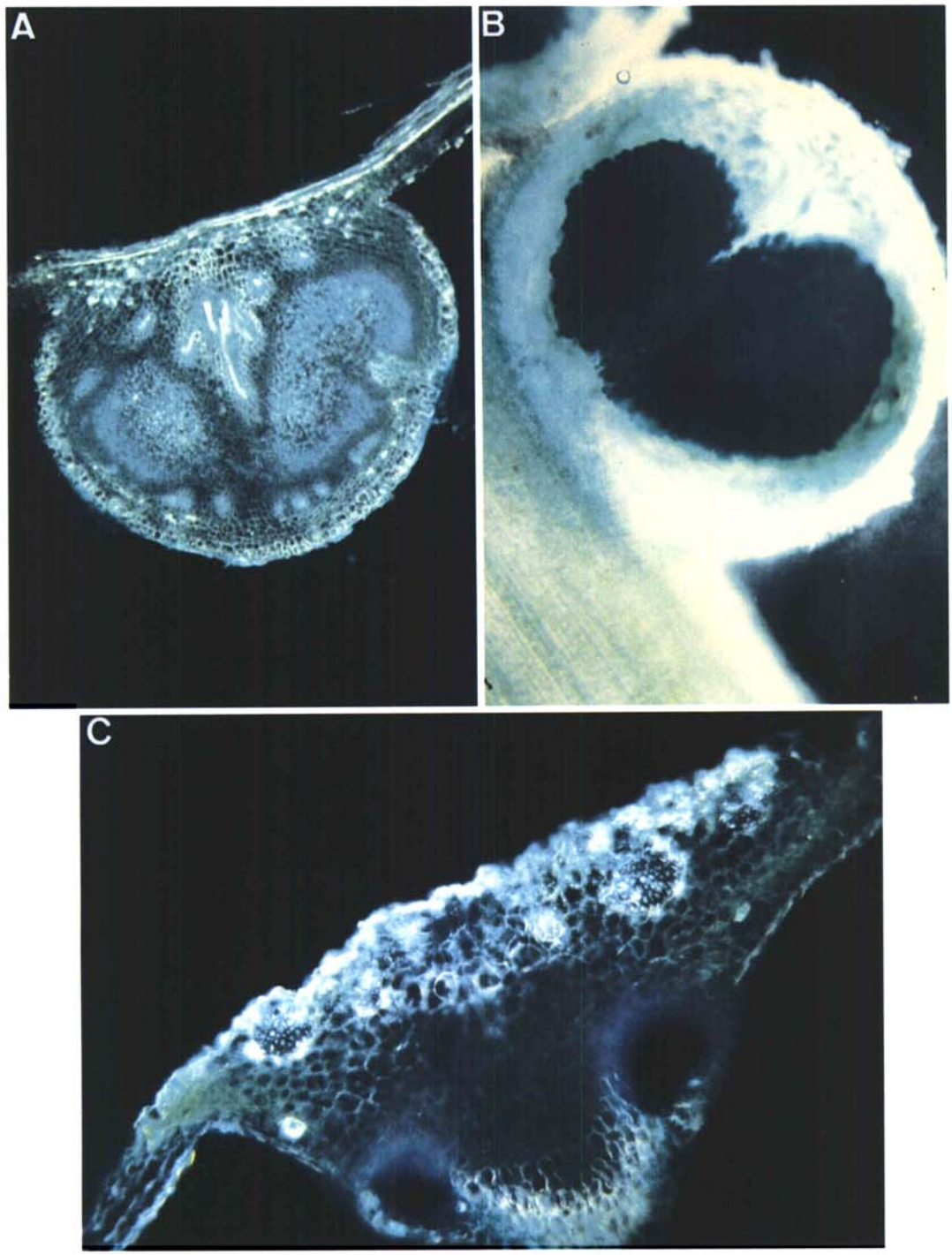

Fig. 3. Micrographs of semithin sections of stem nodules. **A** and **B** are sections of 10-day-old stem nodules of *Sesbania rostrata* induced by ORS571(pRG960-32) and ORS571(pRG960SD-32), respectively. **C** is a section of a 5-day-old nodule induced by ORS571(pRG960SD-32). The β-glucuronidase activity is seen as a blue color.

(pRG960SD-32) were sectioned, the entire bacteroid portion of the nodules stained deep blue (Fig. 3B). This difference between pRG960-32 and pRG960SD-32 probably reflects the expression efficiency of both vectors and might be overcome by decreasing the substrate concentration or lowering the incubation time. In Figure 3C, a section of a 5-day-old stem nodule induced by ORS571(pRG960SD-32) is shown. GUS activity is seen in the nodule sections that are invaded by azorhizobia, namely the cavities produced by the perforation of the root primordium through the stem epidermis and around the central, newly induced nodule primordium.

In conclusion, the results we have presented demonstrate that the vectors described are useful either as general cloning vectors, as part of a binary vector system, or as reporter vectors, and are specifically valuable to study bacterial gene expression in plant tissues.

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