

Application of the GUS marker gene technique to high-throughput screening of rhizobial competition

Kate J. Wilson, Adriana Parra, and Lina Botero

Abstract: The GUS marker gene system has been developed for the study of bacterial ecology, particularly rhizobial competition. For high-throughput field screening of rhizobial competition, the technique must be robust and reliable under diverse conditions, with diverse cultivars and strains. Here we demonstrate its applicability to the evaluation of competition on five different *Phaseolus vulgaris* cultivars with 10 different *Rhizobium* strains. We describe refinements of the GUS assay, which make it more affordable and applicable to field-based studies, and use the assay to examine the effect of sample size on the accuracy of nodule occupancy measurements.

Key words: GUS gene, *Rhizobium*, rhizobial competition, microbial ecology.

Résumé : Le système du gène marqueur GUS a été développé pour étudier l'écologie bactérienne et en particulier la compétition pour les rhizobia. Pour présenter une forte capacité de criblage sur le terrain, la méthode doit demeurer puissante et fiable dans diverses conditions avec différents cultivars et différentes souches. Notre recherche démontre la faisabilité de cette méthode dans l'évaluation de la compétition entre cinq cultivars différents de *Phaseolus vulgaris* et 10 souches différentes de *Rhizobium*. Nous suggérons certaines améliorations de la méthode qui la rendent plus facile d'accès et plus applicable aux études sur le terrain, et nous utilisons cet essai pour mesurer l'effet de la taille de l'échantillon sur la précision des mesures d'occupation des nodules.

Mots clés : gène GUS, *Rhizobium*, compétition pour les rhizobia, écologie microbienne.

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Introduction

The use of introduced marker genes for the study of microbial ecology is becoming increasingly common. We have focused particularly on the use of *gusA*, encoding β -glucuronidase (GUS). The advantages are that there is no background activity in plants, or in most bacteria and fungi that interact with them, making GUS an excellent marker for plant-microbe interactions. There are both quantitative and histochemical assays available, the latter enabling precise spatial localization of marked bacteria or fungi. The assay is cumulative in that detectable product builds up with pro-

longed activity of the enzyme, and hence it is very sensitive. Finally, the assay is straightforward, requiring no special equipment or other conditions. For these reasons, *gusA* has been used to study the ecology of a number of Gram-negative bacteria (Christiansen-Weniger and Vanderleyden 1993; Hurek et al. 1994; Kang et al. 1997; Sessitsch et al. 1997; Streit et al. 1992, 1995; Vande Broek et al. 1992; Wilson et al. 1991, 1994, 1995; Yuhashi et al. 1997) and fungi (Couteaudier et al. 1993; Hammond-Kosack and Jones 1994; Green and Jensen 1995) that associate with plants.

Earlier we described a series of GUS transposons that can be used for delivery of the *gusA* gene into a variety of Gram-negative bacteria, the goal being to facilitate the study of native strains of bacteria that may be particularly well adapted to local environmental conditions. These transposons contain the *gusA* gene driven by a variety of promoters, for use in different environmental conditions. For example, some transposons are optimal for the study of free-living and associative bacteria, while others are designed for the study of active nitrogen-fixing *Rhizobium* and *Bradyrhizobium* strains in legume root nodules (Wilson 1995; Wilson et al. 1995).

The long-term goal is to have the GUS marker system applicable to the study of rhizobial competition in the field, as an alternative to current approaches. These include the use of antibiotic-resistant strains and the ELISA technique, both of which are laborious and frequently suffer from high background levels making identification of the inoculated strain difficult. To date, the GUS system has primarily been applied in laboratory situations with standard strains, which

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K.J. Wilson¹ Cambia, GPO Box 3200, Canberra
ACT 2601, Australia.

A. Parra² and **L. Botero**³ Phytonutrition Bean Program,
Centro Internacional de Agricultura Tropical (CIAT),
A.A. 6713, Cali, Colombia.

¹Author to whom all correspondence should be addressed:
Australian Institute of Marine Science, Cape Ferguson,
Queensland, PMB No. 3, Townsville Mail Centre,
QLD 4810, Australia (e-mail: k.wilson@aims.gov.au).

²Present address: Department of Microbiology, Washington
State University, P.O. Box 644233, Pullman,
WA 99164-4233, U.S.A.

³Present address: Montana State University, Department of
Land Resources and Environmental Sciences, 623 Leon
Johnson Hall, Bozeman, MT 59717, U.S.A.

have been worked with for many years. Moreover, only small numbers of host plants have been used, and incubation could be carried out in ample X-glcA assay buffer. To be of use in field experiments, the system must be capable of handling much larger sample numbers with far greater throughput, and with accuracy and cost efficiency that are equal to or better than those of existing methods for analyzing rhizobial competition. In the present work, the efficacy of different GUS transposons in analyzing competition for nodule occupancy was evaluated on five different *Phaseolus vulgaris* cultivars, with 10 different native strains of bean-nodulating rhizobia from Latin America. Furthermore, since the substrate for GUS X-glcA is expensive, mechanisms of enhancing the efficiency of substrate use were also determined.

Materials and methods

Bacterial strains and media

Rhizobium etli CIAT632, CIAT7001, CIAT151, CIAT2560, KIM55, CIAT652, CIAT895, and CIAT7202 and *R. tropici* CIAT899 and CIAT7069 were obtained from the CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia.) strain collection. *Escherichia coli* S17-1 (Tn5gusAKJW107) (Wilson et al. 1992; Anyango et al. 1998), S17-1 λ -pir (mTn5SSgusA10), S17-1 λ -pir (mTn5SSgusA11), S17-1 λ -pir (mTn5SSgusA20), S17-1 λ -pir (mTn5SSgusA30), S17-1 λ -pir (mTn5SSgusA31), and S17-1 λ -pir (mTn5SSgusA40) (Wilson et al. 1995) were used as donors to transfer the different GUS transposons to *Rhizobium* recipients. *Escherichia coli* strains were routinely maintained on LB medium (Ausubel et al. 1998) at 37°C, and *Rhizobium* strains on yeast extract mannitol (YM) medium (Vincent 1970) at 28°C. Minimal medium used for selection was modified Brown and Dilworth medium (Wilson et al. 1995). Where appropriate, media were supplemented with the following (in $\mu\text{g}\cdot\text{mL}^{-1}$): ampicillin 50, spectinomycin 50, streptomycin 50 (*E. coli*), and rifampicin 30 and spectinomycin 50 (*Rhizobium*).

Introduction of GUS transposons into rhizobial recipients

Plate matings were carried out as previously described (Wilson et al. 1995) between *E. coli* donor strains carrying the transposons and recipient *Rhizobium* strains. Two different means of selection were used. In the first set of matings, spontaneous rifampicin-resistant derivatives of the recipient strains were isolated, and transconjugants were then selected on YM plates containing 30 $\mu\text{g}\cdot\text{mL}^{-1}$ rifampicin to counterselect the donor and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin to select for the transposon. In later matings, transposons were introduced directly into the original (rifampicin-sensitive) rhizobial recipients. Minimal medium (modified Brown and Dilworth medium, Wilson et al. 1995), on which the donor *E. coli* cannot grow, was supplemented with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin and was used to select transconjugants. Putative transconjugants were purified by restreaking on selective medium, and single colonies were subsequently streaked on YM plates containing X-glcA at 25 $\mu\text{g}\cdot\text{mL}^{-1}$ to check for GUS expression by the appearance of blue colonies (resulting from cleavage of X-glcA by GUS). In the case of transconjugants marked with mTn5SSgusA10, in which the *gusA* gene is regulated by the lac repressor (LACI), the LAC inducer IPTG (isopentyl-thio-galactoside) was also included at 1 mM.

Phaseolus vulgaris cv. Bat 76 seeds were surface sterilized in 95% (v/v) alcohol for 1 min, 3.5% (w/v) sodium hypochlorite for 3 min, and rinsed seven times with sterile water. They were germinated for 72 h prior to planting in seedling growth pouches con-

taining 100 mL sterile nitrogen-free Sandman nutrient solution (Sandman 1970). *Rhizobium* strains for inoculation were grown in YM liquid medium for 48 h at 23°C. The concentration of the inoculum was determined using a Petroff-Hausser counting chamber and was adjusted to 10^8 cells $\cdot\text{mL}^{-1}$. Plants were inoculated with 1 mL after 7 days of germination. For each marked *Rhizobium* strain, four replicate pouches of the following three treatments were set up: 1:1 mixture of the wild-type and GUS-marked strains; wild type alone; GUS-marked strain alone. Plants were grown in a temperature- and humidity-controlled growth chamber with 25°C day (12 h) and 19°C night. Plants were harvested 15–20 days after inoculation and were assessed for nodule occupancy by the marked strain. All four replicates of the same treatment were combined in one pouch, the shoots were removed, 150 mL of GUS staining buffer (see below) was added, and the pouch was sealed and incubated at 28°C for 48 h.

Competition studies in the glasshouse

Three separate experiments were carried out in the glasshouse with strains chosen from the growth pouch evaluation. In the first two experiments, GUS-marked rifampicin-resistant derivatives were evaluated, whereas in experiment three, GUS-marked derivatives, which had been generated directly from the original wild-type parents by selection of transconjugants on minimal media, were used. *Phaseolus vulgaris* cultivars from the CIAT collection, which differed in their ability to fix nitrogen (based on other experiments carried out at CIAT), were chosen: A 285 (poor nitrogen fixation), G12168 (moderate nitrogen fixation), DOR 41, Carioca, Riz 32 (good nitrogen fixation). Seeds were surface sterilized, and inoculum was prepared as described above. Inoculated seedlings were grown in sand in pots and watered with Sandman N-free medium. Four replicates of each single strain inoculum as well as wild-type and GUS-marked derivatives inoculated in a 1:1 ratio were set up per harvest date. Pots were arranged in a randomized complete-block design.

At harvest, shoots were removed from roots, and the top of the taproot was labeled with masking tape. The sand surrounding the root was shaken off, and the roots were rinsed in water to remove adhering sand. All sand used for the growth of plants was treated by autoclaving, and all water used for rinsing the roots was treated with sodium hypochlorite to eliminate any GUS-marked bacteria. In the laboratory, nodulated roots were placed together in a 3-L beaker containing 2 L of GUS-staining buffer: 50 mM potassium phosphate pH 7.0; 1 mM EDTA; 0.1% w/v sarkosyl; 0.1% v/v Triton X-100; 50 $\mu\text{g}\cdot\text{mL}^{-1}$ X-glcA; 0.02% w/v sodium azide. After experimentation with different staining conditions (see Results), it was determined that the best treatment was vacuum infiltration of the roots in the buffer for 10 min, followed by overnight staining in GUS buffer at room temperature. The staining solution was kept well aerated using a small aquarium pump. The percentage nodule occupancy was determined by counting the total number of blue-stained nodules compared with the total number of all nodules on each root.

Measurement of specific GUS activity of nodules

Four individual nodules were picked per plant, their fresh weight was determined, and then they were crushed in a 1.5-mL Eppendorf tube containing 100 μL of 50 mM potassium phosphate pH 7.0; 1 mM EDTA; 0.1% w/v sarkosyl; and 0.1% v/v Triton X-100. Fourty microlitres of this nodule suspension was then added to an Eppendorf tube containing 760 μL of the same buffer, with the addition of 1 mM (final concentration) *p*-nitrophenyl- β -D-glucuronide (pNPG). The reactants were mixed and dispensed into five wells of a microtitre dish, 150 μL per well. The progress of the reaction was monitored by following the development of yellow color, and the reaction in successive wells was stopped at different time points by the addition of 50 μL of 0.2 M NaCO₃. The

absorbance was measured at 405 nm on a Bio-tek EL-308 ELISA reader, and GUS activity was calculated as previously described (Wilson et al. 1992, 1995). In some cases where the GUS activity of individual nodules was very high, a tenfold dilution of the nodule suspension was used in the assay. This analysis was carried out on the CIAT899 treatments in competition experiment three, 35 days after inoculation.

Effect of sample size on estimation of nodule occupancy

Plants induced by *R. tropici* CIAT7069, marked with mTn5SSgusA10, mTn5SSgusA20, mTn5SSgusA31, or mTn5SSgusA40, and inoculated either as single strains or in competition with the wild-type unmarked parental strain, were harvested 31 days after inoculation. Thirty-two nodules were picked from each root system, 16 from the upper root and 16 from the lower root. Individual nodules were placed in separate wells of a 96-well microtitre plate containing GUS staining buffer with 1 mM pNPG in place of X-glcA as the substrate. The root with the remaining attached nodules was placed in GUS staining buffer with X-glcA as the substrate. After overnight incubation, nodules in microtitre plates were scored for the presence or absence of GUS-marked strains indicated by the presence or absence of yellow color. Nodules remaining on the roots were scored by the percentage of blue nodules formed in the X-glcA staining buffer. The percentage nodule occupancy was then calculated using data from the first 16, 24, and 32 nodules respectively in the microtitre plates (each subsample contained equal numbers of nodules from the upper and lower parts of the root) and from the total number of nodules (those assayed in the microtitre plates plus those on the remainder of the root).

Statistical analysis

Statistical analysis was carried out using the SAS package. Where treatments were balanced, analysis of variance (ANOVA) was applied, whereas the general linear model (GLM) was used when treatments were unbalanced. Percentage data that fell outside the range 30–70% were subjected to an arcsine transformation prior to analysis.

Results and discussion

Marking of diverse strains

An initial goal of this research was to ensure that the GUS transposons could be used to mark a wide variety of indigenous strains of bean-nodulating rhizobia. The full suite of GUS transposons was used, including all the mini-transposons (except for mTn5SSgusA21) (Wilson et al. 1995) and the earlier prototype GUS transposon Tn5gusAKW107 (Wilson et al. 1992; Anyango et al. 1998). In the initial round of matings, spontaneous rifampicin (rif)-resistant mutants of the recipient rhizobial strains were first isolated to enable rif resistance to be used as a selectable marker for the recipient strains after conjugation of the transposons from the donor *E. coli* strain. Although this was a successful strategy, several of these rifampicin-resistant derivatives turned out to have impaired symbiotic properties prior to the introduction of the GUS transposon (see below). Hence, in later matings recipients were selected based on their ability to grow on minimal media, which did not support the growth of the auxotrophic donor *E. coli* strains. In total, 70 different conjugations were carried out, and no difficulties were encountered in marking any of the *Phaseolus*-nodulating strains tested.

Assessment of suitability of strains for use in competition studies

Prior to larger scale competition studies, GUS-marked strains were inoculated in a 1:1 ratio with the unmarked parental strains (rif or non-rif resistant depending on the selection used for transconjugants) on *Phaseolus* plants grown in growth pouches. Fifteen to twenty days after inoculation, nodules were assessed for occupancy by the marked strain. GUS-marked strains, which produced completely blue nodules when used as a single strain inoculum, and formed approximately 50% of the nodules when co-inoculated with the parental strain, were chosen for a second evaluation in the glasshouse. Between one and three independently marked strains were evaluated in this system to enable the selection of a strain that appeared not to be strongly affected in competitive ability by insertion of a GUS transposon.

Large-scale competition studies

Three consecutive large-scale competition studies were set up in the glasshouse with the following aims: (i) to develop optimal staining conditions for large-scale GUS assays; (ii) to assess the utility of the different GUS transposons in measuring competitive ability for a variety of rhizobial strains; (iii) to determine whether there was any variability associated with host cultivar used; (iv) to measure the specific GUS activity associated with different transposons; and (v) to provide material for an experiment on the effect of sample size on measurements of nodule occupancy.

(i) Development of optimal staining conditions

To minimize the amount of the substrate X-glcA used, staining was carried out in the smallest volume of buffer needed to completely immerse the harvested roots. However, this gave poor staining of nodules even at the first harvest date, when the nodules were young with high GUS activity. As cleavage of X-glcA by GUS is a two-step reaction, requiring oxidative dimerization of the initial cleavage product to produce the blue precipitate, it was possible that oxygen was limiting the completion of the reaction. This hypothesis was supported by the observation that nodules on some roots could be observed to turn blue very quickly when removed from the buffer for examination, i.e., when exposed to air. This problem was most effectively addressed by aeration of the buffer during overnight staining, using a simple aquarium pump. The inclusion of potassium ferricyanide, by contrast, did not enhance staining. Although ferricyanide is an oxidizing agent, it also appears to inhibit GUS activity and should be omitted when maximum staining sensitivity is required (Wilson et al. 1995).

With the use of the aquarium pump to ensure adequate aeration of buffer, nodule occupancy could be assayed on a large number of roots in a small volume of buffer, and hence at an affordable cost. In the third experiment, 96 plants with average nodule numbers of approximately 500 nodules per root were placed in 1.7 L of buffer containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ of X-glcA, i.e., a total of 85 mg of X-glcA was used. The cost of the other buffer components is minor. Given that the procedure avoids the time involved in picking nodules and carrying out ELISA assays, it is likely to compare very favorably in cost.

Table 1. Percentage of nodule occupancy of GUS-marked strains in competition with the respective wild-type *Rhizobium* strain inoculated on common bean (*Phaseolus vulgaris*).

Transposon	Strain	15 days after planting			30 days after planting		
		Wild type + GUS strain	WT Rif ^r + GUS strain	GUS strain alone	Wild type + GUS strain	WT Rif ^r + GUS strain	GUS strain alone
Tn5gusAKJW107	CIAT632	26 ± 12	35 ± 11	98 ± 5	23 ± 26	20 ± 2	82 ± 30
mTn5SSgusA11	CIAT151	39 ± 11	n.t. ^b	100	23 ± 13	n.t.	79 ± 17
	CIAT7001	41 ± 7	81 ± 14	100	24 ± 13	67 ± 17	97 ± 9
mTn5SSgusA30	CIAT151	40 ± 20	n.t.	88 ± 20	23 ± 15	n.t.	79 ± 19
	CIAT632	28 ± 26	20 ± 4	100	20 ± 16	23 ± 11	95 ± 10
	CIAT7001	41 ± 7	68 ± 15	100	18 ± 10	68 ± 11	91 ± 16
	CIAT899 (isolate a)	26 ± 42	n.t.	100	2 ± 4	n.t.	93 ± 16

Note: Data are means ± SEM of 12 replicates (4 replicates per cultivar). General linear model analysis indicated significant effects of date, strain, transposon, competitor (original wild-type or rif-resistant derivative), transposon × competitor, and strain × competitor ($p < 0.01$).

^aWT Rif^r, wild-type rifampicin-resistant strain.

^bn.t., not tested.

While optimizing the staining conditions, it was noted that nodules on roots that had been harvested up to 1 week earlier retained the capacity to turn blue if exposed to the correct conditions, i.e., exposure to oxygen and, if necessary, immersion in fresh staining buffer. This illustrated the great stability of the GUS enzyme and indicates that there will be no difficulty in using the system in field experiments, even if there is a delay of 24 h or more between harvest in the field and assay of the roots in the laboratory. In all these experiments, sodium azide was added to block growth of contaminating microorganisms and prevented induction of endogenous GUS activity in any other microorganisms present. Sodium azide was found to be more effective than chloramphenicol, which was included earlier in GUS-staining buffer to block induction of GUS in contaminating bacteria (Wilson et al. 1995). Sodium azide also prevents the roots from rotting.

(ii) *Competition studies: effects of strain, cultivar, transposon, and harvest date*

Summary data from the large-scale competition experiments are presented in Tables 1–3. In each experiment, mixed inoculum treatments were set up with marked and unmarked strains inoculated in equal numbers, and nodule occupancy, as judged by X-glcA staining of whole roots, was determined at harvest. Control treatments were the marked and unmarked strains tested as single-strain inocula.

It can be seen that nodule occupancy by the GUS-marked strain was not always detected as being 100% even where single-strain inocula were used. This could be due either to cross-contamination by unmarked strains, or to a failure of expression of the GUS enzyme. Most likely it was due to low level cross-contamination, as blue nodules were occasionally detected on plants inoculated with the wild-type strains, indicating contamination by GUS-marked derivatives.

The three experiments are not strictly comparable, as strains were tested in the greenhouse as they became available, and hence there are different combinations of cultivars, strains, and transposons in each experiment. However, some general conclusions can be drawn. In all three experiments there were significant effects of transposon and strain. The effect of cultivar appears to be minimal; no significant ef-

Table 2. Percentage of nodule occupancy of GUS-marked strains in competition with the respective wild-type *Rhizobium* strain inoculated on common bean (*Phaseolus vulgaris*).

Transposon	Strain	Wild type + GUS strain	GUS strain alone
Tn5gusAKJW107	CIAT151	22 ± 9	75 ± 28
mTn5SSgusA11	CIAT652	49 ± 9	100
	CIAT151	25 ± 10	94 ± 11
mTn5SSgusA10	CIAT7001	30 ± 9	99 ± 3
	CIAT899	5 ± 4	90 ± 30
	KIM5S	18 ± 4	99 ± 3
	CIAT2560	5 ± 4	100
mTn5SSgusA20	CIAT632	47 ± 11	99 ± 4
	CIAT7001	27 ± 8	100
	CIAT899 (isolate a)	65 ± 8	100
	CIAT2560	6 ± 4	100
mTn5SSgusA30	CIAT652	10 ± 30	100
	CIAT895	46 ± 7	99 ± 3
	CIAT899 (isolate b)	29 ± 11	100
	KIM5S	16 ± 5	100

Note: Data are means ± SEM of 12 replications (4 replications per cultivar). General linear model analysis indicated significant effects of strain, transposon, transposon × strain ($p < 0.01$). Plants were harvested 22 days after planting.

fects were detected, except in experiment three, and then only at the $p < 0.05$ level. In both experiments (one and three) with more than one harvest date, there was a significant effect of date. In most cases, the latter represented a decrease in percent nodule occupancy by the marked strain by the second date, although in a few cases the nodule occupancy actually increased. These changes are probably due to unavoidable low levels of cross-contamination between pots in the glasshouse, as the single-strain inocula also show decreases in percent nodule occupancy detected. As this decrease does not appear to be related to the specific transposon, it is probably not due to GUS expression from any of the transposons declining in the duration of these experiments.

The observation that there were significant effects of strain and transposon is somewhat surprising, given that the

Table 3. Percentage of nodule occupancy of GUS-marked strains in competition with the respective wild-type *Rhizobium* strain inoculated on common bean (*Phaseolus vulgaris*).

Transposon	Strain	20 days after planting		35 days after planting	
		Wild type + GUS strain	GUS strain alone	Wild type + GUS strain	GUS strain alone
mTn5SSgusA10 ^a	CIAT7069	43 ± 8	100	43 ± 18	92 ± 12
	CIAT899	62 ± 6	100	51 ± 7	87 ± 27
	CIAT7202	56 ± 5	98 ± 1	49 ± 4	99 ± 3
	CIAT895	57 ± 11	100	49 ± 16	91 ± 11
mTn5SSgusA20 ^a	CIAT7069	76 ± 10	100	67 ± 13	98 ± 7
	CIAT899 (isolate <i>b</i>)	58 ± 6	100	59 ± 9	93 ± 8
	CIAT7202	56 ± 6	100	56 ± 4	100
	CIAT895	57 ± 8	99 ± 5	58 ± 8	87 ± 22
mTn5SSgusA30	CIAT899 (isolate <i>c</i>)	69 ± 6	100	62 ± 6	93 ± 10
	CIAT7202	59 ± 10	98 ± 5	54 ± 6	100
mTn5SSgusA31 ^a	CIAT7069	26 ± 6	98 ± 7	40 ± 17	89 ± 25
	CIAT899	60 ± 6	100	50 ± 5	95 ± 7
	CIAT7202	54 ± 3	95 ± 19	50 ± 3	93 ± 10
	CIAT895	49 ± 9	98 ± 6	55 ± 18	79 ± 21
mTn5SSgusA40	CIAT7069	57 ± 6	100	55 ± 4	90 ± 14
	CIAT899	67 ± 6	100	63 ± 5	85 ± 15
	CIAT7202	55 ± 5	89 ± 30	39 ± 13	77 ± 15

Note: Data are means ± SEM of 12 replicates (4 replicates per cultivar). General linear model analysis indicated significant effects of date, strain, transposon, transposon × strain, date × transposon × strain ($p < 0.01$); and of cultivar and date × transposon ($p < 0.05$).

^aAnalysis of variance on the treatments that used transposons mTn5SSgusA10, mTn5SSgusA20, and mTn5SSgusA31 indicated significant effects of transposon, transposon × strain, date × transposon × strain ($p < 0.01$); and of date and strain ($p < 0.05$).

strains tested were preselected for their ability to form approximately 50% of the nodules in competition with the parental strain in growth pouches. It could be due to factors such as slight variation in numbers of cells added at inoculation. The exact number of GUS-marked and wild-type cells in the inoculum can be assessed by plating dilutions of the inoculum onto solid media containing X-glcA, and the sensitivity of competitiveness measurements to slight variations in the inoculum ratio is illustrated by Sessitsch et al. (1997). Alternatively, some of the marked strains may be slightly altered in competitive ability because of the transposon insertion disrupting genes affecting competitiveness, even though this was not detected in the initial screening. The results do suggest that screening in growth pouches can only be used for a preliminary assessment of whether marked strains show altered competitiveness phenotypes and that further screening may be necessary. This is not surprising as it is a common observation that nodule occupancy results change when transferring competition experiments from the very artificial environment of growth pouches to other more natural environments, e.g., Anyango et al. (1998).

One significant difference worth noting is that different results were obtained in competition experiment one, when transconjugants obtained from rif-resistant derivatives of wild-type strains competed against the original wild-type parent, or the rif-resistant derivative. In most cases, the marked strain showed a much stronger ability to compete against the rif-resistant derivative than against the original parent. The implication is that selection of spontaneous rif-resistant strains may also affect competitive behaviour, an observation that has been made before (Pankhurst 1977;

Bromfield et al. 1995). The use of induced antibiotic-resistant rhizobia for selection of transconjugants should therefore be avoided if at all possible.

(iii) Specific GUS activity in nodules induced by strain CIAT899 marked with different GUS transposons

The series of GUS transposons using different promoters to drive GUS expression was constructed with the aim of optimizing detectable GUS activity in different circumstances to satisfy the needs of different experimental goals, e.g., the study of root-surface colonization versus symbiotic nitrogen fixation within a nodule (Wilson 1995; Wilson et al. 1995). For example, two transposons, mTn5SSgusA30 and mTn5SSgusA31, have *nifH* promoters from *Rhizobium* and *Bradyrhizobium*, respectively, driving the *gusA* gene to maximize GUS expression in active, nitrogen-fixing nodules. To test whether there are indeed differences in GUS expression due to the different promoter activities, quantitative GUS measurements were made on individual nodules induced by strain CIAT899 marked with the different transposons. The nodules were assayed 33 days after inoculation, using nodules induced on all three cultivars included in competition experiment three (Table 4).

The results showed quantitative differences with significant effects of both transposon and cultivar, and a significant transposon by cultivar interaction (all $p < 0.01$). The reason for the difference between cultivars is unknown, but it is noteworthy that cultivar G12168 gave a lower quantitative GUS activity with the transposons that have *gusA* driven by either the constitutive or the symbiotic promoters. However, this did not translate to any reduction in the detection of

Table 4. Quantitative GUS activity in nodules induced by GUS-marked derivatives of CIAT899.

Transposon	Regulation of <i>gusA</i> on transposon	Cultivar	GUS activity per nodule ^a	GUS activity per mg fresh weight nodule tissue ^b
mTn5SS <i>gusA</i> 10	Inducible	Riz 32	1.42 ± 0.16	0.12 ± 0.01
		G12168	1.23 ± 0.13	0.13 ± 0.01
		A285	1.49 ± 0.09	0.12 ± 0.01
mTn5SS <i>gusA</i> 20	Constitutive	Riz 32	419.72 ± 92.78	31.90 ± 4.48
		G12168	44.17 ± 5.32	6.27 ± 4.08
		A285	344.66 ± 101.79	27.63 ± 7.59
mTn5SS <i>gusA</i> 30	Symbiotic	Riz 32	351.67 ± 52.23	33.59 ± 6.96
		G12168	116.01 ± 3.49	17.74 ± 2.63
		A285	125.11 ± 13.94	18.93 ± 1.15
mTn5SS <i>gusA</i> 31	Symbiotic	Riz 32	106.01 ± 11.15	10.55 ± 0.27
		G12168	54.09 ± 17.76	11.13 ± 2.45
		A285	141.43 ± 26.54	14.55 ± 3.08
mTn5SS <i>gusA</i> 40	Promoter probe	Riz 32	41.86 ± 5.73	4.40 ± 0.85
		G12168	47.89 ± 12.34	8.92 ± 2.99
		A285	49.42 ± 14.51	6.15 ± 2.17

Note: Four nodules were analyzed separately per plant, and an average GUS value per nodule was calculated for that plant. The data shown are the average of four replicate plants ± SEM. Data are from plants harvested 35 days after planting. Analysis of variance indicated significant effects of transposon, cultivar, and transposon × cultivar ($p < 0.01$).

^anmols pNPG cleaved·min⁻¹·nodule⁻¹.

^bnmols pNPG cleaved·min⁻¹·mg nodule fresh weight⁻¹.

GUS-positive nodules in competition experiments with this cultivar (data not shown).

The results confirmed that the different promoters do lead to different levels of GUS expression. For example, mTn5SS*gusA*10, which requires induction by IPTG for full expression, showed the lowest levels of expression, whereas mTn5SS*gusA*20, which gives strong constitutive expression, gave the highest levels. Both symbiotic promoters give strong expression, and the promoter probe transposon mTn5SS*gusA*40 gives an intermediate level of expression. The latter will vary depending on the endogenous promoter that drives GUS expression from mTn5SS*gusA*40. These assays were performed at the second harvest (35 days after planting) in competition experiment three. The results thus contrast with those of Streit et al. (1995) and Anyango et al. (1998) who found a substantial decline in GUS expression in *Phaseolus* nodules induced by strains marked with Tn5*gus*AKJW107, which has the same promoter-*gusA* construct as mTn5SS*gusA*20, by this stage. This was presumed to be due to restriction of GUS expression to undifferentiated bacteria, and a consequent decline in expression as the bacteria differentiate into bacteroids (see also Wilson et al. 1995). It is not clear in the present case whether a higher proportion of undifferentiated bacteria remain, or whether there is some other difference brought about by the two different transposons. One possibility is that of read-through from some external promoter in mTn5SS*gusA*20, where the promoter-*gusA* construct lies close to one end of the transposon.

(iv) Does sample size affect the accuracy of nodule occupancy measurements?

In all methods used to date for nodule occupancy studies, it has only been possible to assay subsamples of nodules for logistical reasons. These subsamples are generally 20–30

nodules per plant, when a plant may contain several hundred nodules on its root system. The errors associated with such small subsamples are potentially large (see Wilson (1995) for a discussion of the standard error of a binomial distribution). We therefore carried out an experiment to measure sampling errors empirically, to determine whether the use of small sample sizes did in fact bias the data. The percentage nodule occupancy for all three cultivars co-inoculated with *R. tropici* CIAT7069 and with different marked derivatives of this strain was calculated based on sample sizes of 16, 24, and 32 nodules, and on determining nodule occupancy in every nodule on the root using the X-glcA-staining assay. Controls were also included that were inoculated only with the GUS-marked strain.

A summary of the results is given in Tables 5 and 6. The data in Table 5 were subjected to a split-plot analysis of variance, with sample size being the subplot factor. This indicated significant effects of transposon and sample size ($p < 0.01$). The transposon effect is consistent with that observed when analyzing all the data from competition experiment three (Table 3). The effect of sample size is primarily due to variation in the values obtained when every nodule on the root is analyzed, rather than a subsample, as split-plot analysis of the data obtained from the three subsamples ($n = 16, 24, \text{ or } 32$) only shows a small effect of sample size ($p < 0.05$). It can be seen from Table 6 that even when single-strain inocula were used, the GUS assay did not always identify 100% of the nodules at this harvest date. It is noteworthy that in all cases where the values in Table 6 fall below 100% detection, they are lowest when all nodules on the root were assayed. This is most likely due to some nodules being either too small or too senescent to express detectable GUS activity. Thus the higher values observed when subsamples were analyzed almost certainly reflect a bias associated with sampling, namely that researchers inevitably

Table 5. Effect of sample size on estimation of percentage of nodule occupancy by GUS-marked derivatives of strain CIAT7069 co-inoculated with wild-type CIAT7069.

Transposon	Cultivar	n = 16	Sample size		
			n = 24	n = 32	n = all
mTn5SSgusA10	Riz 32	50 ± 4	48 ± 4	49 ± 4	37 ± 9
	G12168	44 ± 4	45 ± 4	44 ± 4	47 ± 12
	A285	63 ± 5	57 ± 6	52 ± 6	46 ± 7
mTn5SSgusA20	Riz 32	83 ± 8	83 ± 7	81 ± 8	72 ± 4
	G12168	75 ± 8	71 ± 11	71 ± 11	62 ± 9
	A285	77 ± 10	79 ± 9	82 ± 7	67 ± 7
mTn5SSgusA31	Riz 32	46 ± 14	36 ± 10	35 ± 7	40 ± 4
	G12168	30 ± 6	32 ± 9	33 ± 8	40 ± 8
	A285	41 ± 20	39 ± 20	39 ± 20	40 ± 14
mTn5SSgusA40	Riz 32	77 ± 2	73 ± 6	74 ± 6	56 ± 2
	G12168	66 ± 4	63 ± 3	56 ± 2	56 ± 2
	A285	61 ± 7	61 ± 4	60 ± 3	53 ± 3
Grand mean		59 ± 2.59	57 ± 2.56	56 ± 2.43	51 ± 2.22

Note: Data are the average of four replicates ± SEM. Plants were harvested 32 days after planting. Split-plot analysis indicates a significant effect of transposon and sample size ($p < 0.01$).

Table 6. Effect of sample size on estimation of percentage of nodule occupancy by GUS-marked derivatives of strain CIAT7069 inoculated alone.

Transposon	Cultivar	n = 16	Sample Size		
			n = 24	n = 32	n = all
mTn5SSgusA10	Riz 32	88 ± 13	86 ± 14	86 ± 14	66 ± 12
	G12168	97 ± 3	97 ± 3	98 ± 2	92 ± 4
	A285	100 ± 0	98 ± 2	98 ± 2	94 ± 6
mTn5SSgusA20	Riz 32	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	G12168	100 ± 0	99 ± 1	99 ± 1	93 ± 6
	A285	100 ± 0	100 ± 0	100 ± 0	100 ± 0
mTn5SSgusA31	Riz 32	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	G12168	91 ± 9	93 ± 7	94 ± 6	68 ± 18
	A285	100 ± 0	100 ± 0	100 ± 0	99 ± 1
mTn5SSgusA40	Riz 32	100 ± 0	100 ± 0	100 ± 0	88 ± 10
	G12168	100 ± 0	100 ± 0	100 ± 0	89 ± 6
	A285	100 ± 0	100 ± 0	100 ± 0	93 ± 7

Note: Data are the average of four replicates ± SEM. Plants were harvested 33 days after planting.

choose well-formed nodules that they can readily pick from the roots to analyze. This operator bias in sampling may not be undesirable, because all methods will only work on nodules that are sufficiently developed for analysis, but are not yet senescent: ELISAs, RAPDs, isolation of bacteria for antibiotic resistance, and marker gene expression are all liable to fail if the nodules are either underdeveloped or senescent.

Thus it appears that estimation of percent nodule occupancy is not greatly improved by increasing the sample size, providing there is adequate replication of samples (four in this case). The standard error was reduced with increasing sample size, but the difference was small (Table 5). Hence, another approach to calculating percent nodule occupancy using the GUS system might be to pick a sample of nodules and assay that subset for GUS expression either using XglcA as a substrate, or using pNPG as a substrate in microtitre plates. Alternatively, it might be possible just to cut a section of a root for staining in GUS buffer. Both approaches would reduce both substrate use and time required for nod-

ule counting, and still provide a reasonable estimation of nodule occupancy.

In summary, our work demonstrates that the GUS system works well in the glasshouse with a variety of native *Phaseolus*-nodulating *Rhizobium* strains and *Phaseolus* cultivars. The only problems encountered were those already documented elsewhere, namely that some strains bearing GUS-transposon insertions will be affected in competitive ability (e.g., Sessitsch et al. 1997) and that GUS activity declines in senescent nodules making them undetectable by this assay (Streit et al. 1995; Wilson et al. 1995). We confirm the expectation that the different transposon constructs give rise to different quantitative as well as qualitative patterns of GUS expression. Finally, we have demonstrated that the GUS assay can be refined to make it more affordable, and more applicable to large-scale field experimentation by increasing the efficiency of substrate use and by assaying subsamples of nodules to reduce both the amount of substrate and the time required for counting nodules.

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