Bradyrhizobium elkanii ICA 8001 *GUS* A: A NEW STRAIN TO EVALUATE NODULATION GENE EXPRESSION

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ABSTRACT. The reporter *gus* A gene from *Escherichia coli*, encoding ß-glucuronidase enzyme, is used in this work to genetically transform *Bradyrhizobium elkanii* ICA 8001 strain by a triparental mating conjugation. The conjugates obtained were tested by a *nod* expression test and confirmed by nodulation plant experiments. From 10 conjugates obtained, only two colonies expressed GUS activity and only one of them corresponded with *Bradyrhizobium*. A new strain was obtained: *B. elkanii* ICA 8001 transformed with *gus* A in its chromosome. The Nod A-*gus* expression analysis is compared with the nodulation factor synthesis for three different culture media. This result constitutes an important tool to study nodulation genes in the most used strain in Cuban inoculants for soybean.

Key words: *gus*, *Bradyrhizobium elkanii*, nodulation, genetic markers, genetic transformation

RESUMEN. El gen marcador *gus* A de *Escherichia coli*, gen que codifica para la enzima ß-glucuronidasa, es utilizado en este trabajo para transformar genéticamente la cepa *Bradyrhizobium elkanii* ICA 8001, mediante una conjugación triparental. Los conjugados obtenidos fueron evaluados mediante la prueba de expresión *nod* y confirmados a través de un experimento de nodulación en plantas. De 10 conjugantes obtenidos, solo dos colonias expresaron actividad GUS y de ellas, solo una correspondió a *Bradyrhizobium*. Se obtuvo una nueva cepa: *B. elkanii* ICA 8001 transformada con el gen *gus* A en su cromosoma. Se compara el análisis de expresión Nod A-*gus* con la síntesis de factores de nodulación en tres medios de cultivo diferentes. Este resultado constituye una herramienta importante en el estudio de los genes de nodulación en la cepa más utilizada en inoculantes cubanos para soya.

Palabras clave: *gus*, *Bradyrhizobium elkanii*, nodulación, marcadores genéticos, transformación genética

INTRODUCTION

The *Escherichia coli gus* A gene, encoding ß-glucuronidase, was first used as a reporter gene in plants (1). It is currently being used in a wide variety of biological systems, including plant-interacting bacteria.

GUS activity is not present in higher plants nor in bacteria of agricultural importance such as *Meso*-, *Sino*-, *Azo*-, *Brady*, *Rhizobium*, *Agrobacterium*, *Azospirillum* and *Pseudomonas* (2).

Originally, ß-glucuronidase was biochemically characterized in the bacterium *Escherichia coli* and later the *gus* A was isolated from *E. coli* strain K_{12} (3). In *E. coli*, *gus* A is part of an operon, together with two other genes, *gus* B and *gus* C, encoding a glucuronide-specific permease and a membrane-associated protein of unknown function, respectively. The *E. coli gus* A gene is 1809 bp long and the ß-glucuronidase has a predicted monomer molecular weight of 68, 300 in agreement with the experimentally determined molecular weight of 73,000. The enzyme is probably active as a tetramer *in vivo*.

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The ß-glucuronidase of *E. coli* specifically hydrolyzes ß-linked D-gucuronides to D-glucuronic acid and aglycone.

The use of fusions between a gene of interest and a reporter gene with an easily detectable phenotype, such as *gus* A, offers several advantages for the study of gene expression. While the data generated with GUS fusions to 5'control sequences of higher plant genes give only partial information on the transcriptional control of the gene of interest (4), this problem is not significant in prokaryotes. ß-glucuronidase has become a widely used reporter system to measure expression of diverse genes by means of gene fusions.

In many studies, GUS has been used to mark the soil bacterium of interest or to analyze the expression of a specific gene in plant (5). G*us* A fusions was employed for combined gene expression and rhizobacterial localization studies (6).

Many other GUS constructs are suitable for studies of plant-bacteria interactions (7, 8).

The main benefit when using GUS reporter system in microbial rhizosphere ecology is the ability to localize GUS marked bacteria on plants using histochemical GUS substrates.

Taking into account the advantages of this useful marker and specifically in the nodulation gene study, the aim of this paper was to transform *B. elkanii* ICA 8001 strain inserting the *gus* A gene in its chromosome and to evaluate the effectiveness as marker in the nodulation gene induction.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are described in Table I. *E. coli* was grown in Luria-Bertani (LB) medium at 37ºC (9). *Bradyrhizobium* was cultured at 30ºC in liquid TY medium (supplied with 7 mM $CaCl_2$) (10) for triparental mating or in YEM (11), Traditional (12) and Bradyfact (13) liquid media for the analysis of Nod factors. Appropriate antibiotics: kanamicin (Km) (30µg.mL^{-1}) and nalidixic acid (Nal) (30µg.mL^{-1}) were added.

Table I. Bacterial strains and plasmids

Strains or plasmids	Relevant properties	Source/Reference
	Strains	
E. coli DH5α	Nal^s F(-), endA1, hsdR17[r(-)(k),m(+)(k)], supE44, thi1, lambda(-), recA1, gyrA96, relA1, del(arg F - lac zya) U169, phi80dlacZdelM15	Gibco BRL
E. coli HB101	Nal^s F- hsdS20 (rB- mB-) supE44 ara14 galK2 lacY1 proA2 rpsL20 (StrR) xyl-5 mtl-1 recA13 mcrB thi-1 leuB6 (In vitrogen)	Gibco BRL
Bradyrhizobium elkanii ICA 8001	Wild-type strain isolated from soybean	Institute of Animal Science (ICA)
	Plasmids	
pGUS 32Km	Nm^r Rhizobium sp. BR816 nodABC-gusA Multiple copies of nodD in Rhizobium tropici CIAT899 and BR816 Km^r reporter plasmid	Van Rhijn *et al.* (1993) (14)
pRK 2013	Nm^r ColE1 helper plasmid with RK2 transfer genes (tra) for triparental mating	Firguski and Helinski (1979) (15)

Conjugation test. Triparental mating was carried out using the donor strain DH5α/pGUS and the helper strain HB101/pRK2013 from *E. coli* and the *B. elkanii* wild-type strain as an acceptor (16). *B. elkanii* conjugation mixtures were plated out on peptone-yeast extract medium with Km and Nal (30µg.mL^{-1}) to select for the *Bradyrhizobium* colonies harbouring pGUS32Km.

β *glucuronidase assay*. G*us* expression was monitored in wild-type strain, in 10 transconjugates obtained and in the medium used as control (17).

Quantitative analysis of GUS A activity was carried out with *p*-nitrophenyl-ß-D-glucuronide (pNPG) as the substrate (previously described in 18) in microtiter plates and GUS A activity was examined in VERSA max microplate reader (molecular devices). Results were statistically analyzed by a randomized complete design using Duncan's Multiple Range Test to discriminate differences between media. Values followed by the same letter are not significantly different.

Plant experiments. *Glycine max* cv. "William 82" seedlings were planted in a medium containing KCl (140 mg), K_2HPO_4 (50 mg), KH_2PO_4 (100 mg), $MgSO_4.7H_2O$ (490 mg), $CuSO_4.5H_2O$ (0.037 mg), $ZnSO_4.7H_2O$ (0.11 mg), $MnSO_4.2H_2O$ (0.1 mg), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.005 mg), H_3BO_3 (0.35 mg), $FeSO_4.7H_2O$ (1.25 mg), citric acid (1.25 mg) and $CaSO_4.2H_2O$ (350 mg) per litre. Plants were inoculated with 200 µL of the wild-type strain and two conjugates from the expression test (number 2 and 5) grown in YEM medium modified with inducer compounds (19). Medium without cells was used as control. Plants were grown in the growth room with a 12-h photoperiod (day/night temperature 26ºC/22ºC; relative humidity 70 %) (20). Four weeks after inoculation, nitrogen fixation capacity of the inoculated plant was determined by means of the acetylene reduction assay using a gas chromatographer (5890 A; Hewlett-Packard, equipped with a "PLOT fused silica" column). Other parameters as number of nodules, fresh and dry weights of nodules per plant were determined. Results were statistically analyzed by a randomized complete design using Duncan's Multiple Range Test to discriminate differences between media. Values followed by the same letter are not significantly different.

Determination of Nod factor profile. Nodulation factors were radioactively labelled and they were isolated by following a slightly modified protocol (21). 100 µL from *Bradyrhizobium* cultures, growth for two nights, were inoculated in 900 µL of each fresh culture medium: YEM, Traditional and Bradyfact, and the concentration was adjusted to 5x10^8 CFU per medium milliliter. They were pre-incubated to 30°C with agitation, during 1h. Each sample was supplemented with genistein 10 µM as inducer and incubated during two hours at the same temperature and agitation. After induction, the isotopic label was carried out adding 125 µL of ^{14}C [2-^{14}C] acetic acid as sodium salt. Cells were labelled for 36h. Nodulation factors were isolated twice with 500 µL n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were applied on reverse-phase TLC plates (RP-18 F_{245}, Merck). H_2O/acetonitrile (1:1, vol/vol) was used as the mobile phase. Radioactivity was visualized by autoradiography using Hyperfilm- β max (Amershan Life Sciences) after four days of exposure.

Data analysis. The statistical analysis was carried out with the Statistic Package for Social Science (SPSS) version 7.5 for Windows.

RESULTS AND DISCUSSION

Triparental mating produced 10 different isolates with characteristics of *Bradyrhizobium*. These isolates grew in PSY medium with Km and Nal. The colonies isolated appeared seven days after inoculation in plates, time when

Bradyrhizobium grows. Colonies from *E. coli* appear 24 hours after inoculation. Then, the 10 conjugates obtained were analyzed in the expression test to evaluate GUS activity.

The results of expression analysis as ß-glucuronidase activity (expressed in miller units) are shown in Table II.

Only two conjugates expressed GUS activity: number 2 and number 5. Number 2 with a high expression and number 5 with a low expression. The rest of conjugates, the wild-type and the control, did not show any expression *gus*.

This result suggests that the plasmid carrying *gus* A was inserted in the chromosomes of cells from colonies 2 and 5, the other isolates could be *Bradyrhizobium* colonies, but without insertion.

Plant experiments constituted another test to determine if the *gus* conjugates obtained were really *Bradyrhizobium* or *E. coli* plasmid remains. In the second case, the expression GUS would be positive, but the nodulation test would be negative.

Plant experiment results are described in Table III, which evidenced no differences in all nodulating parameters evaluated between conjugate number 2 and wild-type strain; it means this isolate obtained corresponds to *Bradyrhizobium elkanii* ICA 8001.

It is also interesting the similarity in the values obtained for both of them. That indicates the genetic transformation does not influence on the wild-type strain nodulation and fixation behaviour.

Conjugate number 5, however, does not produce nodules on soybean. That means this isolate does not correspond to *Bradyrhizobium*. It is possible the *gus* expression found for it is due to some remains of *gus* vector present on it.

In Table IV the values of ß-glucuronidase activity using the new strain are compared with the profile of Nod factors produced in three different culture media composition to *B. elkanii* ICA 8001.

Table IV. Effect of different medium compositions on ß-glucuronidase activity (A) and Nod factors synthesis (B)

Culture Media	YEM (A)	YEM (B)	Traditional (A)	Traditional (B)	Bradyfact (A)	Bradyfact (B)
Miller units	-6.384		1.669		17.676	
	-5.407		1,360		22.209	
	-6.138		1.891		18.433	
	-5.990		1,428		17.831	
	-5.949		1.241		48.96	
	-6.006		1.507		33.619	
	-5.381		1.554		32.685	
	-5.778		1.482		33.222	
	0		1.587		19.037	
	0		1.446		37.121	
AV						
	0		1.517		28.079	

Table II. Expression GUS (miller units) in different conjugates obtained from triparental mating

	Strain or conjugate											
	Wild-type	1	2	3	4	5	6	7	8	9	10	Control
GUS	1.534	1.064	34.14	1.032	0.526	4.808	0.295	0.472	1.019	-0.09	-1.09	-0.49
activity	0.056	0.276	36.03	-0.920	-0.470	3.387	-0.250	-0.41	-0.320	-0.970	-1.75	-0.36
	-3.390	1.930	34.68	-0.630	-0.210	3.295	-0.370	-1.04	0.414	-0.740	-1.56	-0.83
	-1.620	-0.200	40.42	0.401	0.024	3.085	-0.200	-1.06	-0.740	-0.740	-0.29	-0.42
	0.546	0.875	55.75	0.589	0.500	4.789	-0.100	0.088	1.415	0.170	-0.48	-0.04
	-0.680	1.371	42.29	0.558	0.323	5.260	0.152	-0.30	-0.000	-0.070	-0.07	-0.59
	-1.680	0.982	46.33	0.099	-0.380	3.882	0.163	0.079	0.374	0.001	-0.35	-0.42
	0	0.767	36.32	0	0	3.644	0	0	0.092	0	0	0
AV***	-0.65 c	0.88 c	40.74 a	0.13 c	0.03 c	4.01 b	-0.03 c	-0.27 c	0.28 c	-0.30 c	-0.69 c	-0.39 c
SE	0.54	0.22	2.60	0.22	0.13	0.29	0.08	0.19	0.24	0.15	0.23	0.09

Values followed by the same letter are not significantly different
***P<0.005

Table III. Effect of wild-type and two conjugates on soybean nodulation and nitrogen fixation

Treatments	Number of nodules per plant	Fresh weight of nodules per plant (g)	Dry weight of nodules per plant (g)	ARA (µmol/ethylene/plant/h)
Wild-type	35.66 a	0.25 a	0.04 a	6.33 a
Conjugate 2	33.33 a	0.27 a	0.02 a	6.10 a
Conjugate 5	0 b	0 b	0 b	0 b
Control	0 b	0 b	0 b	0 b
SE x	5.23***	0.04***	0.007***	1.04***

Values are the mean of five independent repeats, values followed by the same letter are not significantly different. SEx *** means P<0.005

Results show a correspondence between the *nod* genes expression by ß-glucuronidase activity and by Nod factor production determined in TLC and autorradiography. Medium YEM did not expresse GUS activity and TLC showed no spots of Nod factors produced. Traditional medium induced a low enzymatic activity and two or three spots of Nod factors, while Bradyfact medium induced a major ß-glucuronidase activity and correspondingly a high Nodulation factor production with at least five different structures of this biomolecule.

This result validates the use of this transformed strain in the new technique to evaluate *nod* expression genes and once more time demonstrates the superiority of Bradyfact as an inducer medium for *Bradyrhizobium* (13).

There is clear evidence a new strain *B. elkanii* ICA 8001 transformed with *gus* A in its chromosome was obtained. This fact constitutes an important way to study compounds that induce nodulation genes in the most used strain in Cuban inoculants.

On the other hand, recent studies have demonstrated the influence of culture medium composition on nodulation factor production for *Bradyrhizobium* (non-published results, 22). In this sense, to determine *nod* gene induction, the most used technique is the autorradiography with radiolabelling. This TLC analysis constitutes an expensive and risky technique, which can be now substituted for the induction test using the new strain. Time is another advantage using *gus* strain: while TLC analysis of Nod factors needs at least one week to visualize the spots corresponding to Nod factors, the results of *nod* induction test need only six hours to evaluate the enzymatic activity in the studied strain.

So far, this work is the first aim to transform the most used *Bradyrhizobium* strain in our country, now available to molecular studies related with nodulating genes and maybe to other studies as ecology, competitiveness, etc.

The studies concerning to improve soybean inoculants have now a new tool: *B. elkanii* ICA 8001 *gus* A.

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