Use of the *gus*A Gene to Study N_2-fixing Bacteria

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ABSTRACT

The transposon mTn5SS*gus*A20 from *E. colis* S17-1 lamda-pir as a donor strain was inserted into the genome of *Bradyrhizobium japonicum* strains THA-7, USDA 110 and *Bradyrhizobium* sp. strains TAL 182, TAL 1376, TAL 182 as the recipients. Plate matings were carried out as described by Wilson (1995). Transconjugants were checked by inoculating into the host plants grown in Leonard's Jars with N-free solution (Munn's) and 28-day old nodules were soaked in GUS-extraction buffer plus the GUS substrate (X-Gluc 100 μg/ml). The results indicated that mating efficiencies were in between 10^7-10^8. All nodules inoculated with transconjugants turned blue within 2-48 hours while nodules from parental strains remained unstained.

Key words: GUS gene, transposon, transconjugant

INTRODUCTION

To overcome the problem of identifying the closely related strains of rhizobia, the use of the GUS gene to mark in the rhizobial genome is interesting. This method is simpler, sensitive, and has no effect on growth of plants, animals or ecological system. The technique of using GUS marker gene was described by Wilson (1995). The GUS transposon could be inserted into a bacterial genome by conjugation. Insertion of the transposon occurs when donor and recipient bacteria grow together on medium where both can grow, then selection of the transconjugants is obtained by using minimal media. Steps and details of the procedure were written in "GUS as a marker to track microbes" by Wilson in Recent advances in nitrogen fixation research : role of *gus* reporter gene by Wilson and Jefferson (1995). Smit and Elsas (1990) studied on bacterial mating and reported that the log mumber of donor related with the log number of recipient and transconjugant cells. Ui Gam Kang et al. (Unpublished data) conducted the experiment on "Nodulation competitiveness and nitrogenase activity of *gusA* marked *Rhizobium meliloti* in alfalfa". He concluded that the *gusA* marked nodules could be easily determined due to the blue-coloured precipitate in the nodules. The purpose of this experiment were: I) determination of transfering efficiency of *gusA* gene to N_2-fixing bacteria, ii) assessment of transconjugant strains on nodulation and colour development.

MATERIALS AND METHOD

Bacterial strains

Bradyrhizobium japonicum strains; USDA 110 and THA-7, *Bradyrhizobium* sp., TAL 182 and TAL 1376 were used as the recipient strains. *E. coli* S17-1 lamda pir/pCAM 120 carrying Tn5 *SSgusA20* was used as the donor strain.

Conjugation (Bacterial Mating)

Rhizobial strains were cultured on yeast mannitol borth for 3 days at room temperature while *E. coli* was grown for 1 day. Bacterial cells were certrifuged and washed. Plate matings were done on yeast mannitol agar. Transconjugants were selected for blue colonies on Brown and Dilworth +X-Gluc+spectinomycin (25 μg/ml) as described by Wilson (1995). Mating efficiencies of rhizobia were calculated by number of transconjugants divided by number of recipients.

Quantitative GUS Assays

Four transconjugant strains were cultured in

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yeast manitol broth (YMB) in flasks and shaked at 120 rpm for 4 days. The GUS activity of these 4 strains were determined as written by Wilson (1995).

Inoculation, Nodulation and Colour Development

To check the transconjugant strains for further use, the rhizobial strains for soybean, USDA 110: *gusA20*, for cowpea TAL 182 : *gusA20*, and the parental strains were cultured in YMB for 4 days.

Seeds of soybean and cowpea were sterilized with ethanol 70% and clorox 4% for 1 minute and 3 minutes respectively, washed with autoclave-distilled water for 9 times and germinated. Each two seedlings were transplanted in each Leonard Jar containing N-free solution in the greenhouse. One mililiter of each parental strains and transconjugant strains were inoculated on seeds. Plants were maintained in the greenhouse from April 29 until May 27. Then plants were harvested, roots with nodules were immersed in GUS-extraction buffer+X-Glue (100 μg/ml) with aeration (Wilson, 1995). Colour development of nodules were determined.

RESULTS AND DISCUSSION

Number of recipients ranged from log 8.34 to log 8.80 (>10^8 cfu). The transconjugants as shown by blue colonies on the selective agar plates (BD+X-Gluc+spectinomycin) were counted. Mating efficiencies calculated by number of transconjugants divided by number of recipients were shown in Table 1. Data from Table 1 showed that mating efficiencies of four rhizobial strains were between 10^{-7}-10^{-8}. This means that the conjugation will occur at the high number of rhizobia (recipient strains). Smit (1980) found that the log number of recipients related with the log number of transconjugants. They concluded that matings on plates did not occur at low donor and recipient cell densities (<10^4 cells per plate). This experiment supported Smit's finding. The results also confirmed Ui Gum Kang (Unpublished data) which found that the *gusA20* could be inserted into *R. melitoti*. The conjugation is not difficult and affordable, one microbiologist can work on it. The *gusA20* could be inserted to strains of rhizobia and also the other gram-negative bacteria.

The GUS activities of 4 transconjugants were shown in Table 2. Two strains; USDA 110: *gusA*20

Table 1. Mating efficiencies of N_2-fixing bacteria.

Donor strains	Recipient strains	Number of recipients (log10/ml)	Mating efficiencies*
E.coli (*gusA20*)	USDA 110	8.38	$2.9x10^{-7}$ - $3.5x10^{-7}$
	THA-7	8.80	$1.95x10^{-7}$ - $1.62x10^{-8}$
	TAL 182	8.77	$1.96x10^{-7}$ - $6.7x10^{-7}$
	TAL 1376	8.34	$2.0x10^{-7}$ - $9.0x10^{-8}$

* average of 3 replicates

$$\text{mating efficiency} = \frac{\text{no.of transconjugants}}{\text{no. of recipients}}$$

Table 2. The GUS activity of 4 transconjugant strains.

Transconjgant strains	GUS activity* n mol/minute
USDA110 : gusA20	20.065
THA7 : gusA20	2.155
TAL182 : gusA20	58.081
TAL1376 : gusA20	2.155

* average of 2 replicates

and TAL 182: *gusA*20 expressed higher GUS activities than the other two which had low activities and did not show yellow color of paranitrophenol (pNP). Feldhaus et al. (1991) measured GUS activity of *B. thetaiotaomicron* and found that if different plasmids, expression were different for this experiment only one minitransposon was used. Also measurement of the GUS activity confirmed that if the transconjugants showed blue colonies, the GUS activities should be detected.

When inoculating the transconjugant rhizobia to the host plants, nodules formed on the roots, the GUS marked strain of rhizobia were identified. Roots with nodules were immersed in the GUS-substrate+X-Gluc (100 μg/ml) and aerated with an aquarium pump (Wilson et al., 1995). All nodules from, soybean and cowpea with the GUS-marked strains changed to blue colour within 2-48 hours while nodules from parental strains remained unstained. Nodules from cowpea showed blue colour within 2 hours while nodules from soybean stained blue colour within 48 hours after incubation at room temperature (Fig. 1). The region of blue colour concentrated at the outer region of nodules as shown in Fig. 2. This experiment also supported Wilson et al. (1995) which found that the expression of markedstrain NGR 234:gusA11 was limited at the outer region of nodule of pigeonpea, which different from strain NGR234: gusA31; the expression was maximal at the central

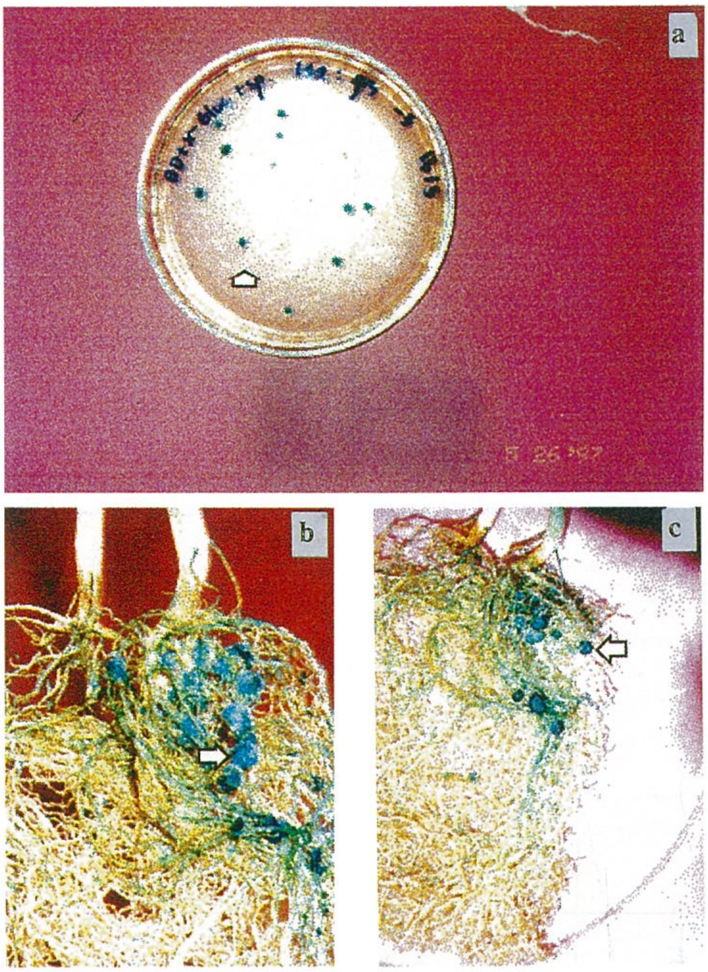

Fig. 1. a : Blue colonies of transconjugants on BD+X-Gluc+spectinomycin plates.
b, c : Blue nodules occupied by the GUS marked strain.

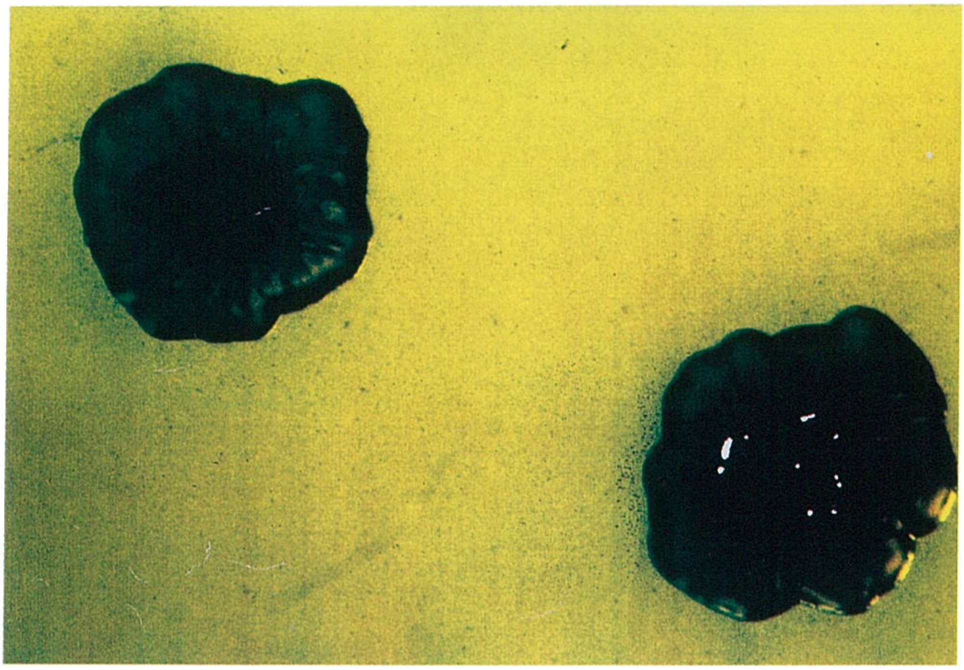

Fig. 2. Hand section showed soybean nodules infected with USDA 110:*gusA20*, the expression maximized at the outer layer of nodules.

regions of the nodule.

blue nodules incubated in the Gus-extraction buffer.

CONCLUSION

The minitransposon mTn5SS*gusA*20 can be introduced into the rhizobial cells by conjugation, when donor and recipient cells at high densities grow together on the agar medium. Strain marked with the *gusA* can be identified by the development of the blue colonies on the selective medium and also

ACKNOWLEDGMENT

This report was sponsored by Department of Agriculture, Thailand. The author wish to thank K.J. Wilson and R. Jefferson (CAMBIA) for good advisory and comments also Dr. S. Akao (NIAR) for providing the donor strain.

REFERENCES

Anonymous. 1995. Recent Advances in Nitrogen Fixation Research : Role of gus Reporter Gene. 112 pages.

Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski. 1980. Broad host range DNA cloning system for gram-negative bacteria : construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci 77, 7347-7357.

Feldhaus, M.J. Vivan Hwa, Q. Cheng and A.A. Salyers. 1991. Use of an *Escherichia coli B-glucuronidase* gene as a reporter gene for investigation of bacteroides promoters. Jour. Bacteriol. 4540-4543.

Smit, E. and J.D. van Elass. 1990. Determination of plasmid transfer frequency in soil : consequences of bacterial mating on selective agar media Current Microbiology. vol. 21, 151-157.

Wilson, Kate J. 1994. Molecular techniques for the study of rhizobial ecology in the field. Soil Biol. Biochem. vol. 27,501-514.

Wilson, Kate J, A. Sessitsch, J.C. Corbo, Ken E. Giller, A.D.L. Akkermans and R.A. Jefferson. 1995. *B-Glucuronidase* (GUS) transposons for ecological and genetic studies of rhizobia and other gram-negative bacteria. Microbiology vol. 141, 1691-1705.