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Mini transposon vector mediated foreign gene expression in *Mesorhizobium huakuii* subsp. *rengei*

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Among the transposable elements, mini-Tn5 transposon vector has proven to be of greater utility for insertion mutagenesis of variety of Gram negative bacteria. The mini-Tn5 vector containing promoter less *egfp* gene and gentamycin resistant gene was used for the present study. The transposon vector was introduced to *M. huakuii* from E. coli S17 by conjugation. The conjugants were screened for stable expression of *egfp* both in free-living and in nodules of *Astragalus sinicus*. The result showed that the conjugant #3 showed stable expression of green fluorescent both in free-living and bacteroid stage. The visualization of *sym* plasmid of wild strain and conjugants showed that conjugant #3 had a fragmentation of large sized plasmid into two but without affecting the nodulating ability. These results clearly indicated that mini-Tn5 vectors (Transposon vectors) the best alternate tools for plasmid vectors for integration of foreign genes in chromosomal DNA or symbiotic plasmid and expression, both in free-living and bacteroid stage of *Rhizobium*.

Keywords : Conjugation, *Rhizobium*, *sym* plasmid, Transposon vectors

Astragalus sinicus (Chinese milk vetch or renge-soh in Japanese) is a winter growing green manure legume. Many strains of this species have been widely used in the rice fields for fertilizing the soil in the southern part of China, Korea and Japan. This plant harbours *Mesorhizobium huakuii*[1] or *M. huakuii* subsp. rengei[2] that results in formation of nitrogen fixing root nodules. Introduction of foreign genes in *M. huakuii* is interesting to improve the nitrogen fixation, plant biomass production, bioremediation of polluted soil, etc. However, the introduction of foreign genes to *Rhizobium* needs a special attention because of different life cycles, i.e., free-living in soil and bacteroid stage in nodules. For the expression of introduced genes in rhizobia under bacteroid stage, at least the following points are indispensable—(i) high stability of introduced genes, (ii) usage of an active promoter in all stages especially bacteroid stage. Many vectors mediated gene transfer have been reported in *Rhizobium*[3]. In case of plasmid vectors, we have to consider the stability of both plasmid transferred and *sym* plasmid. The transposable elements can also be used in gene manipulation of *Rhizobium*[4]. In the present experiment, it has been tried to use mini-Tn5 vector to integrate foreign gene *(egfp)* in *M. huakuii* subsp. *rengei* and its expression in free-living and bacteroid stage.

Bacterial strains and plasmids—*Mesorhizobium huakuii* subsp. *rengei* strain B3 (referred as B3 hereafter) was isolated from nodules of *A. sinicus* cv. Japan grown in rice fields of Higashi-Hiroshima, Japan[2]. The host cells used for plasmid amplification were *Escherichia coli* JM109 (*recA endA1 gyrA96 thi hsdR17 supE44* Δ (*lac proABrelA*1) and *E. coli* S17-1 *λpir* (*Tp*^{r} *Sm*^{r} *recA thi pro hsdR*$^-$ *M*$^+$ *RP4-2-Tc:Mu:Km Tn7*) . The vector miniTn5 (pUT) conferring promoterless *egfp* gene and *aacC1* (gentamycin resistant) provided by Dr K Saeki, Professor (Biotechnology), Osaka University, Osaka was used (Fig. 1).

Media and culturing condition—*Rhizobium* strains were maintained and grown in Tryptone Yeast Extract medium[5] at 30°C. *E. coli* strains were grown in LB medium. *A. sinicus* was grown on nitrogen free modified NFR medium[2]. *A. sinicus* seeds (Yutou-Obansei Renge, Takayama Seeds Co., Kyoto) were surface sterilized by immersion in 0.5% sodium hypochlorite for 5 min. followed by washings with sterile water and used.

Gene manipulation—The plasmid purification, transformation and agarose gel electrophoresis were done as the standard protocols described by Sambrook and Russel[6].

Conjugation—The pUT vector containing *E. coli* S17- 1 *λpir* and B3 were grown in appropriate medium to get 0.5 OD. The cells were washed twice with $MgSO_4$ (10 m*M*) and finally resuspended in $MgSO_4$ (10 m*M*). Equal volume of *E. coli* and B3 were mixed together in Eppendoff tube (1.5 ml) and

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filtered through millipore membrane (0.4 μ) by vacuum pump. The membrane containing cells was placed in TY medium and incubated at 30°C for overnight. The membrane containing cells were then resuspended in $MgSO_4$ (10m*M*) by vortex and spread on TY medium containing 10 μg/ml of gentamycin (Gm) and 10 μg/ml of nalidixic acid (Nal). The plates were incubated at 30°C for 3-4 days for appearance of conjugants (Fig. 2).

Analysis of conjugants—Conjugants and strain B3 were grown in TY plates for 3 days at 30°C and observed under UV light for the presence of green fluorescence due to *egfp* expression. The conjugants and strain B3 were inoculated to *A. sinicus* plant following the standard procedure of plant infection test[7] and the nodules were transversely cut and observed under fluorescence microscope for emission of green fluorescence due to *egfp* gene expression. Visualization of *sym* plasmid of wild and four conjugants was carried out by in-gel lysis method[8].

Eight conjugants were obtained after four days of incubation at 30°C. All the conjugants were re-streaked in GM + Nal plates for testing the stability of the conjugants. Only four showed the stable expression of GM^r and Nal^r, whereas other 4 lost their insert. The plant infection test with *Astragalus sinicus* revealed that these conjugants had normal nodulating and nitrogen fixing ability and the inserted gene did not affect the nodulation and nitrogen fixing genes. Among the four, conjugant #3 had the *egfp* gene expression (as emission of green fluorescent light under UV microscope). Similarly, when the colonies were observed under UV illuminator, the same conjugant #3 showed the *egfp* expression (Fig. 3). Since, the *egfp* gene of pUT vector was promoterless, the integration of *egfp-accC1* cassette in *Rhizobium* promoter led to the expression both in free-living and bacteroid stage. Analysis of symbiotic plasmids of conjugants showed that the splicing of symbiotic plasmid of conjugant #3, meant that the pUT vector modified the *sym* plasmid also, but without affecting the nodulating ability (Fig. 4).

The transposon element would have variety of utilities such as random mutagenesis, single stable insertion, promoter probe, cloning, conjugal element for mobilization, marker for localization on physical

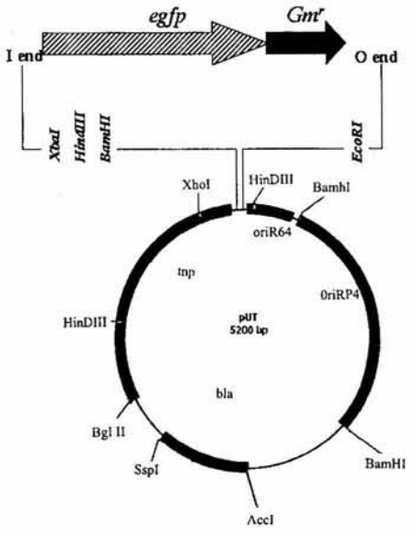

Fig.1—Physical map of delivery vector of promoter-probe *egfp* mini-transposon vector (pUT). Mini-transposon portions were shown over the suicide vector, pUT portion. Genes in transposon were presented as thick arrows. The promoter-less *egfp* (lined arrow) and *aacC1* (Gm^r) (block arrow) were located in mini-transposon region.

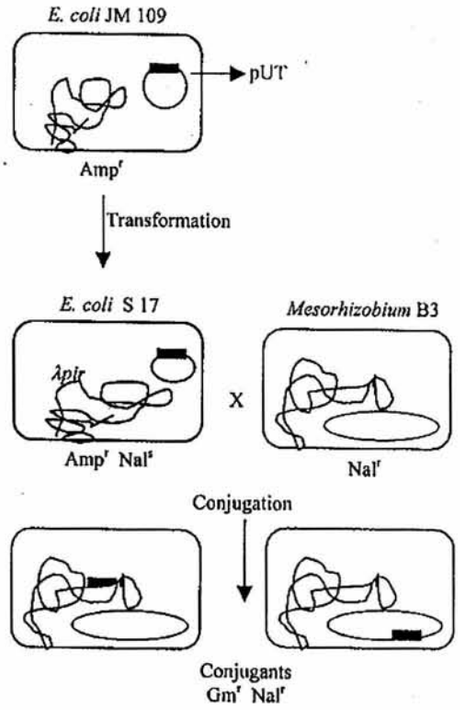

Fig.2—Conjugation procedure of *egfp* mini transposon vector. The thick (black) line indicates the minitransposon portion of pUT vector with *egfp* and *accC1*. The large circle in B3 shows the *sym* plasmid of *Mesorhizobium*. The delivery vector pUT can replicate only in the presence of π protein supplied by *λpir* in *E. coli* S17. Since *Rhizobium* shows Nal^r, the conjugants were selected by growing in Gm^r Nal^r containing TY plates. The integration of *egfp-accC1* cassette will take place either in chromosomal DNA or *sym* plasmid of *Mesorhizobium*

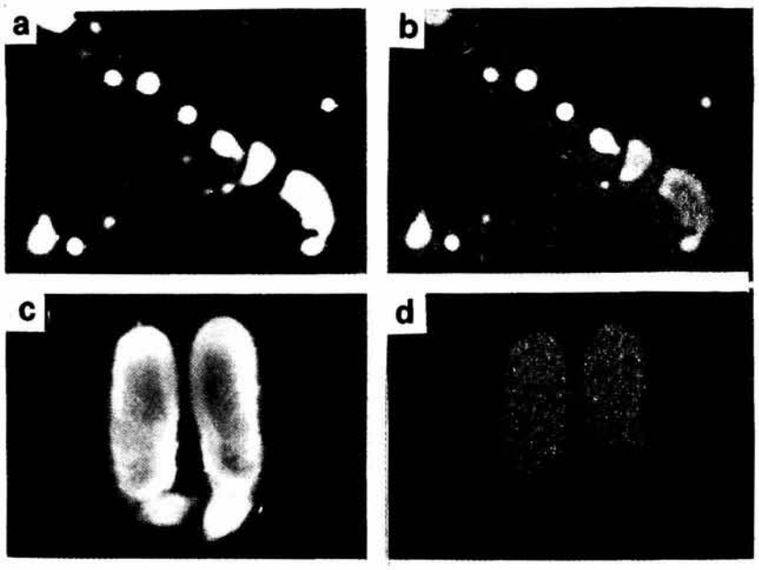

Fig.3—The expression of *egfp* gene in conjugant #3 in free-living and bacteroid stage. The colonies of conjugant #3 under light (a) and under fluorescent light (b). The transverse section of nodule developed by conjugant #3 under light (c) and under fluorescent light (d).

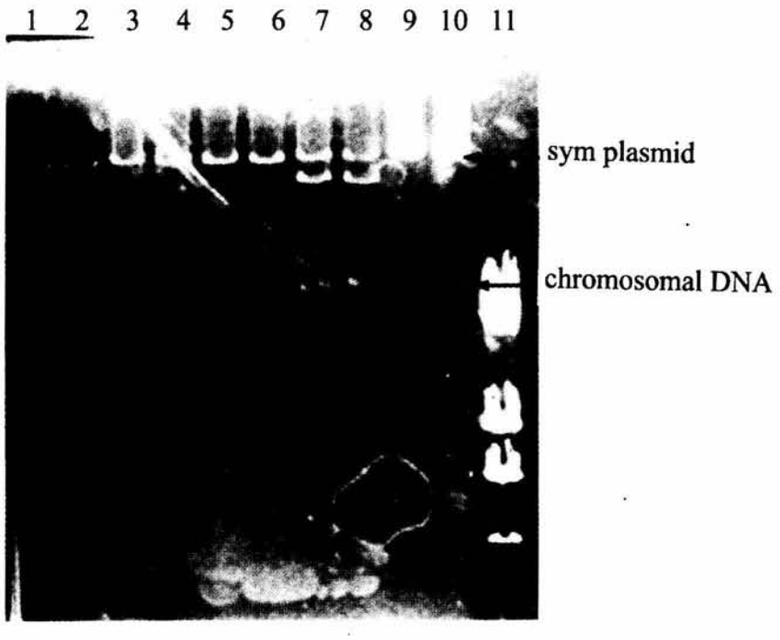

Fig.4—*sym* plasmid visualization of wild and conjugants of B3. Lane 1 & 2 – B3 wild; Lane 3 & 4 – conjugant #1; Lane 5 & 6 – conjugant #2; Lane 7 & 8 – conjugant #3; Lane 9 & 10 – conjugant #4; Lane 11 - λ DNA digested with HinD III (marker).

map for bacterial genome and facilitation of sequencing of chromosomal site of insertion. The stable expression of transposon vector in many Gram negative bacteria has proven to be of greater utility not only for the insertion mutagenesis but also for foreign gene expression too[9]. The derivatives of these transposon with selection marker as vectors for the cloning of genes and their stable chromosomal integration in selected host bacteria of Gram negative has been reported earlier[10]. However, the foreign gene insertion in rhizobia needs special attention for expression in nodules of host legume too. The transposon mediated gene transfer in *R. meliloti* with low frequency has also been reported[4]. In the present study, frequency of conjugation for pUT transposon vector was 10.3×10^{-5} per donor cell, which was relatively fair and the conjugants had very stable expression both in free-living and bacteroid stage and present results agree with the idea of use of transposon for foreign gene expression in *Rhizobium*[11].

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