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Comparative Analysis of Rice Transformation Using *Agrobacterium tumefaciens* and *Rhyzobium leguminosarum*

Syamsidah Rahmawati[1,4*], Osmat Azzam Jefferson[2], Didy Sopandie[3], Suharsono[4] and Inez Hortense Slamet-Loedin[1]

[1] Research Center for Biotechnology, Indonesian Institute of Sciences, Bogor, Indonesia
[2] CAMBIA G301, 2 George Street, Brisbane, QLD 4000, Australia
[3] Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia
[4] Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia

Abstract

This study was aimed to study the effectiveness of *Rhizobium* transformation system compared to the most widely used *Agrobacterium* mediated transformation system on three rice cultivars, Ciherang (Indica), Nipponbare (Japonica), and Rojolele (Javanica). Six day old calli induced from immature embryos were inoculated with *Rhizobium leguminosarum* bv trifolii ANU845 and *Agrobacterium tumefaciens* LBA288 that harbored with vector pCAMBIA 5106. This plasmid contained a minimum set of transfer machinery genes and had a *gusplus* and an *hpt*II gene driven by 35S CaMV promoter in the T-DNA. The results showed that the transformation frequencies (number of PCR positive plants per number of calli inoculated) ranging from 0 to 12.05 % depend on the genotype and transfer agent used. The highest transformation frequency (12.05%) was obtained in Ciherang transformed with *R. leguminosarum*. Most of the transgenic rice obtained by *Rhizobium* transformation were normal in morphology and fertile similar to those obtained by *Agrobacterium* transformation. Integration, expression and inheritance of transgenes were demonstrated by molecular and genetic analysis in T_0 and T_1 generations
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Key words : *Rhizobium leguminosarum*, immature embryos, *Agrobacterium tumefaciens*

Introduction

Currently there are two approaches for introducing new genes into plant cells: the biological approach using *Agrobacerium* and the physical approach such as gene gun and electroporation. *Agrobacterium*-mediated transformation is the most widely used technique today because of the high possibility to obtain plants with single copy of transgene. Both approaches, however, have been included in inventions, with patent claims to gene parts and as well as delivery tools (Chilton 2005). To circumvent patents on plant transformation technologies, the use of bacteria other than *Agrobacterium* strains is highly expected. The successful use of non-*Agrobacterium* strains (*Rhizobium spp* NGR234, *Shinorhizobium meliloti*, and *Mesorhizobium loti*) as a transformation agent for plants was for the first time published by Broothaerts *et al*. (2005), although the transformation frequency was low. Since then, there has been no other reports on the use of non-*Agrobacterium* spesies for plant transformation available.

Broothaerts *et al*. (2005) reported that when suitable engineered non-*Agrobacteria* could like *Agrobacterium* transfer genes into

***Corresponding author** :
Syamsidah Rahmawati
Research Center for Biotechnology, Indonesian Institute of Sciences, Jl. Raya Bogor KM 46, Cibinong 16911, Indonesia
Phone: +62-21-8754587, Fax: +62-21-8754588,
E-mail: syamsidah_rahmawati@yahoo.com

the plant genomes. Rather than using the pathogen *Agrobacterium*, they used plant associated bacteria, *Rhizobia*. *Rhizobia* are soil bacteria that fix nitrogen from its inert molecular form (N_2) and convert it into nitrogenous compounds useful for the plant. These symbiotic bacteria were modified to make them competent for the gene transfer.

The gene transfer process itself is utilizing the virulence genes of a giant extrachromosomal circular plasmids called Ti (Tumor-inducing) plasmids. Virulence genes deliver a specific part of the plasmid called T-DNA (Transferred DNA) to the plant cell nucleus. Broothaerts *et al.* (2005) further modified the Ti plasmid to allow it to be more easily mobilized into a diverse group of *Rhizobial* genera, including *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*, and tested the gene transfer on different plant species. These modified non-*Agrobacterium* species could genetically transformed several plant species, although the transformation efficiency was low. Rice transformed with *Sinorhizobium meliloti*, showed b-glucuronidase activity in 0.6% of infected calli (compared to 50 to 80% for *Agrobacterium*). Appropriate choices factors influence transfer of T-DNA and its integration to plant genomes, such as; explant, vectors-plasmid, bacteria strain, addition *vir*-genes inducing synthetic phenolic compounds, culture media composition, suppression and elimination of bacterial infection after co-cultivation, and desiccation of explant (Roy *et al.* 2000; Opabode 2006), may could enhance the transformation frequency. The low frequencies of *Agrobacterium*-mediated transformation of many recalcitrant plant species have been substantially increased through such manipulation over the past 20 years (Gelvin 2005).

In the study reported here, we choosed *Rhizobium leguminosarum* bv trifolii ANU845 harbored with cointegrate vector pCAMBIA5106. This plasmid carrying a minimum set of *vir* genes and the T-DNA. This bacteria was tested on three cultivars (Ciherang sub-species Indica, Nipponbare sub-species Japonica, and Rojolele sub-species Javanica) representing a wide range of rice germplasm. The *Rhizobium* transformation system was compared to the *Agrobacterium tumefaciens* and the previously reported *S. meliloti*. In this report we demonstrate that *R. leguminosarum* could like *A. tumefaciens* transfer genes into a wide range of rice genome and there is a considerable enhancement of the frequency of transformation. Integration, expression, and the inheritance pattern of transgenes are demonstrated and discussed. The efficient transformation of this system indicates that it will be a good alternative to obtained transgenic rice plants in the future.

Materials and Methods

Rice varieties and plant tissues

Seeds of Ciherang, Rojolele, and Nipponbare were grown under natural condition in a green house. Six day old calli induced from immature embryos (10 – 12 days after anthesis) were used for transformation. Immature seeds were dehusked and sterilized in 70% ethanol for one min and in 2.5% sodium chloride (NaClO) solution containing a drop of Tween 20 for 15 min. The seeds were rinsed several times in sterile water to remove traces of NaClO solution. Embryos were collected by squeezing the seeds using a sterile pinset in a laminar flow cabinet, blotted dry on a sterile filter paper and placed on a callus induction medium before incubated in a dark condition. A modified NB medium (Table 1) supplemented with, 2 mg/l 2,4-D, 1 mg/l NAA, 1mg/lBAP, was used as callus induction medium for Ciherang (Hiei *et al.* 2006).

While modified NB medium and LS medium supplemented with 1 mg/l 2,4-D were, respectively, used for Rojolele and Nipponbare (Hiei & Komari 1994; Slamet Loedin *et al.* 1996)

Bacterium strains and plasmids

Agrobacterium tumefaciens LBA288 (pCAMBIA0105), LBA288 (pCAMBIA5106), *Rhizobium leguminosarum* bv trifolii ANU845

Table 1. Medium composition for callus induction

Purpose	Cultivar	Medium	Composition
Callus induction	Rojolele	LS	MS major salts, MS minor salts, LS vitamins, 30 gl^{-1} sucrose, 2.5 mgl^{-1} 2,4-D, 2.5 gl^{-1} phytagel, pH 5.8
	Nipponbare	NB1	N6 major salts, B5 minor salts and vitamins, 0.3 gl^{-1} casamino acids, 0.5 gl^{-1} L-prolin, 0.5 gl^{-1} L-glutamin, 30 gl^{-1} sucrose, 2.5 mgl^{-1} 2,4-D, 2.5 gl^{-1} phytagel, pH 5.8
	Ciherang	NB2	N6 major salts, B5 minor salts and vitamins, 0.5 gl^{-1} casamino acids, 0.5 gl^{-1} L-prolin, 20 gl^{-1} sucrose, 10 gl^{-1}D-glucose, 2 mgl^{-1} 2,4-D, 1 mgl^{-1}NAA, 1mgl^{-1}BAP, 5.5 gl^{-1} agarose type I, pH 5.8
Infection	all	R2	R2 major salts, R2 minor salts and LS vitamins, 10 gl^{-1} D-glucose, 2.5 mgl-1 2,4-D, 0.5 mM acetosyringone, pH 5.2
Co-cultivation	Rojolele	LS	MS major salts, MS minor salts, LS vitamins, 30 gl^{-1} sucrose, 2.5 mgl^{-1} 2,4-D, 0.1 mM acetosyringone, 2.5 gl^{-1} phytagel, pH 5.2
	Nipponbare	R2-As	R2 major salts, R2 minor salts and LS vitamins, 10 gl^{-1} D-glucose, 2.5 mgl-1 2,4-D, 0.1 mM acetosyringone, 2.5 gl^{-1} phytagel, pH 5.2
	Ciherang	NB-As	N6 major salts, B5 minor salts and vitamins, 0.5 gl^{-1} casamino acids, 0.5 gl^{-1} prolin, 20 gl^{-1} sucrose, 10 gl^{-1} D-glucose, 2 mgl^{-1} 2,4-D, 1 mgl^{-1}NAA, 1mgl^{-1}BAP, 0.1 mM acetosyringone, 5.5 gl^{-1} agarose type I, pH 5.2
Selection	Rojolele	LS	MS major salts, MS minor salts, LS vitamins, 30 gl^{-1} sucrose, 2.5 mgl^{-1} 2,4-D, 100 mgl^{-1} hygromycin,100 mgl^{-1} cefotaxime and 150 mgl^{-1} timentin 2.5 gl^{-1} phytagel, pH 5.8
	Nipponbare	R2	R2 major salts, R2 minor salts and LS vitamins, 30 gl^{-1} sucrose, 2.5 mgl-1 2,4-D, 50 mgl^{-1} hygromycin,100 mgl^{-1} cefotaxime and 150 mgl^{-1} timentin 2.5 gl^{-1} phytagel,, pH 6.0
	Ciherang	NBM	N6 major salts, B5 minor salts and vitamins, 0.5 gl^{-1} casamino acids, 0.5 gl^{-1} prolin, 0.3 gl^{-1} L-glutamin, 20 gl^{-1}D- maltose, 36 gl^{-1} D-mannitol, 2 mgl^{-1} 2,4-D, 1 mgl^{-1}NAA, 0.2 mgl^{-1}BAP, 50 mgl^{-1} hygromycin, 100 mgl^{-1} cefotaxime and 150 mgl^{-1} timentin, 5 gl^{-1} Gelrite, pH 5.8
Pre-regeneration	Ciherang	NBPR	N6 major salts, B5 minor salts and vitamins, 0.5 gl^{-1} casamino acids, 0.5 gl^{-1} L-prolin, 0.3 gl^{-1} L-glutamin, 30 gl^{-1}D- maltose, 2 mgl^{-1} 2,4-D, 1 mgl^{-1}NAA, 1 mgl^{-1}BAP, 7 gl^{-1} Gelrite, pH 5.8
Regeneration	Rojolele, Nipponbare	LS	MS major salts, MS minor salts, LS vitamins, 40 gl^{-1} sucrose, 0.5 mgl^{-1} IAA, 0.3 mgl^{-1} BAP, 3,75 gl^{-1} phytaagel, pH 5.8
		RNM	N6 major salts, B5 minor salts and vitamins, 0.3 gl^{-1} casamino acids, 0.3 gl^{-1} L-prolin, 0.3 gl^{-1} L-glutamin, 30 gl^{-1}D- maltose, 1 mgl^{-1}NAA, 3 mgl^{-1}BAP, 4 gl^{-1} agarose type I, pH 5.8
Planlet development	Ciherang	½MS	Half strength of MS major salts, MS minor salts, B5 vitamins, 10 gl^{-1} sucrose, 2 mgl^{-1} NAA, 2.5 gl^{-1} phytagel, pH 5.8
	Rojolele, Nipponbsre	½MS	Half strength of MS major salts, MS minor salts, B5 vitamins, 10 gl^{-1} sucrose, 0.05 mgl^{-1} NAA, 2.5 gl^{-1} phytagel, pH 5.8

(pCAMBIA5106), and *Sinorhizobium meliloti* (pWBTi1, pCAMBIA1105.1) were developed at CAMBIA Australia. *Agrobacterium tumefaciens* strain LBA288 is an avirulent strain which has no Ti plasmid. Plasmid pCAMBIA0105 is a binary plasmid which has a *gusplus* and an *hpt*II gene in the T-DNA region. *Agrobacterium tumefaciens* LBA288 carrying vector pCAMBIA0105 was used as a negative control for DNA transfer. Plasmid pCAMBIA 5106, on the other hand is a cointegrate plasmid, which has both a minimum set of *vir* genes and the T-DNA. The T-DNA contains a *gusplus*, which had an intron in the coding region, and an *hpt*II gene (Figure 1). *Sinorhizobium meliloti* containing both pWBTi1 and pCAMBIA1105.1 (Broothaerts *et al*. 2005) was used as a control.

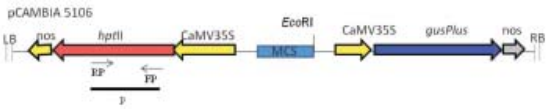

Figure 1. T-DNA of vector pCAMBIA 5106. A couple of primer, forward (FP) and reverse (RP) primer, were used to amplify coding region of *hpt*II gene. Product of PCR (P) was used as probe in Southen blot

Transformation and regeneration

Transformation and regeneration of Ciherang calli were carried out as described by Hiei & Komari (2006). Whereas transformation and regeneration of Rojolele and Nipponbare were conducted as described by Slamet-Loedin *et al.* (1996) and Hiei *et al.* (1994) except for the use of infection medium, infection technique and the antibiotics for removal of bacteria. *Agrobacterium* or *Rhizobium* were grown for 3 days on LB medium or YM, respectively, supplemented with specific antibiotics at 28^{o}C. The Bacteria are collected and suspended in R2 infection medium (Table 1) that contained 0.5 mM acetosyringone untill it reached an OD_{600} of 0.275. As many as 5µl of the bacterial suspension was dropped on top of each calli. Transformed calli were then selected on an approriate medium containing selective agent and antibiotics (Table 1). Regenerated planlets were culture on half-strenghth MS medium to induce root formation and eventually transferred to soil in pots and grown to maturity in a greenhouse.

GUS Assay

Histochemical staining of GUS activity in calli 6-days after co-cultivation and leaves of transgenic plants were carried out as described previously (Hiei *et al*., 1994).

HPT Leave Assay

A green and healthy leave was selected from each plant tested. The leaves were

marked with a water-proof marker. Five microliters of hygromycin solution (0.2 mg/l hygromycin, 0.5% gelatin, 0.001% triton-X) was dropped on a mark line and repeated twice. Water was used as a control. Observations were made on the fourth day. Leaves with necrotic symptoms showed no expression of hygromycin phosphotransferase. Leaves that remain green, on the other hand, indicated expression of hygromycin phosphotransferase.

Polymerase chain reaction

DNA was extracted from leaves as described by van Heusden *et al.* (2000). A couple of primers 5'-GCATCTCCCGCCGTGCAC-3' and 5'-GATGCCTCCGCTCGAAGTAGCG-3' as forward and reverse, respectively, were used to amplify the coding region of *hpt*II gene. Amplification of *hpt*II fragment was performed in a total of 12.5 µlreaction mixtures containing 1 µl of DNA sample, 2.5 µM of each primer, and 6.5 µl of GoTaq®Green Master Mix (Promega). The PCR reactions were carried out as following conditions: initial denaturation at 95°C for 3 min for the first cycle, which was followed by 35 consecutive cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension for 1 min at 72°C. At the end of the PCR process, another 10 min at 72°C was extended to complete the polymerization of DNA. PCR products were separated in 1% agarose gels.

Southern blot analysis

Plant Genomic DNA for Southern analysis was extracted from the freshly collected leaves of PCR-positive transgenic plants as described by Lodhi *et al*. (1994). Each DNA sample was digested with *Eco*RI. Southern blot was carried out as described previously (Sambrook *et al*. 1989). As probe for the *hpt*II gene, a 492 bp PCR product amplified from the *hpt*II coding region was used. Probe was labelled using the Alkphos direct labeling and detection system from GE Healthcare (Amersham Bioscience) following the manufacturer instructions.

Morphology and fertility of transgenic lines

Plant height, number of tiller, productive tiller, total seeds and filled grains were observed on selected lines developed using *Agrobacterium* and *Rhizobium* transformation system at the end of the experiment.

Results

Regeneration and transformation frequencies

Regeneration and transformation frequencies of three rice cultivars transformed with *Agrobacterium tumefaciens* LBA288 and *Rhizobium leguminosarum* bv trifolii ANU845 were evaluated. Histochemical GUS assay was carried out to asses the transient expression of *gusplus* gene in the calli 6 days after cocultivation. As expected, no GUS activity was found on calli infected with *Agrobacterium tumefaciens* strain LBA288 harbored with vector *pCAMBIA0105* and on uninfected calli.

Blue spot indicated GUS activity, conforming GUS expression, was observed in callus tissues co-cultivated with *A. tumefaciens* and *R. leguminosarum* carrying plasmid pCAMBIA 5106 (Figure 2). It was observed that rice calli incubated with *Rhizobium leguminosarum* bv trifolii ANU845 gave higher frequency of GUS expression compare to that of *Agrobacterium tumefaciens* LBA288 in these three rice cultivar (Figure 3).

In this experiment, 0.91% GUS activity was detected on Rojolele calli cocultivated

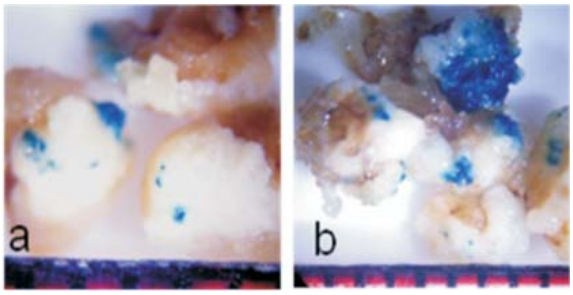

Figure 2. Calli expressing GUS after incubated with **a**. *Agrobacterium tumefaciens* LBA288 (pCAMBIA5106) and **b.** *Rhizobium leguminosarum* ANU845 (pCAMBIA5106). The interval between bars 10^{-3} m.

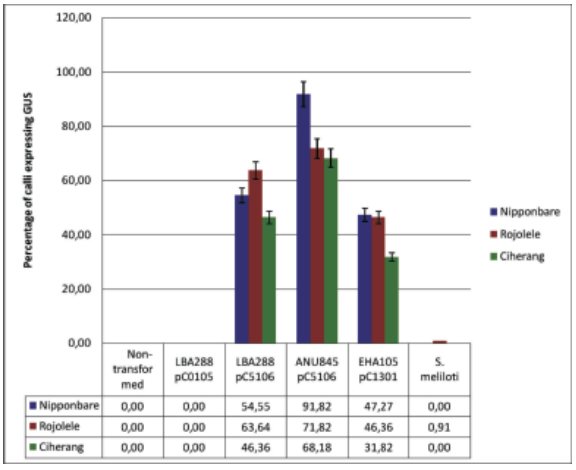

Figure 3. The average of immature derived calli of Ciherang, Nipponbare, and Rojolele showing GUS activity six days after cocultivation with different strains of bacteria (*A. tumefaciens* strain LBA 288, *R. leguminosarum* strain ANU845, and *S. meliloti*). As many as 10 calli were tested per treatment and repeated 11 times

with *S. meliloti* which is similar to previous study (Broothertsa *et al.* 2005). The highest frequency of GUS expression in *Rhizobium*-transformed calli was obtained in rice plant model Nipponbare (91.82%), followed by Rojolele (71.82%), and Ciherang (68.18%).

There were large differences in the frequencies of hygromycin-resistant calli among the three cultivars (Table 2).

The frequencies were higher in Rojolele compare to Ciherang and Nipponbare. Our previous experiments showed that Rojolele calli were more resistant to hygromycin compare to other rice varieties (data not shown). To select hygomycin resistant calli, the transformed calli were cultured on selection medium that contained higher concentration of hygromycin (100 mg/l). However, in all of these three cultivars, the frequencies of hygromycin resistant-calli developed by *Rhizobium* transformation were higher compare to that of by *Agrobacterium* transformation.

Regeneration frequencies between the three cultivars were large differences (Table 2). Regeneration frequencies were high in Ciherang but low in Nipponbare and Rojolele. This result indicated that

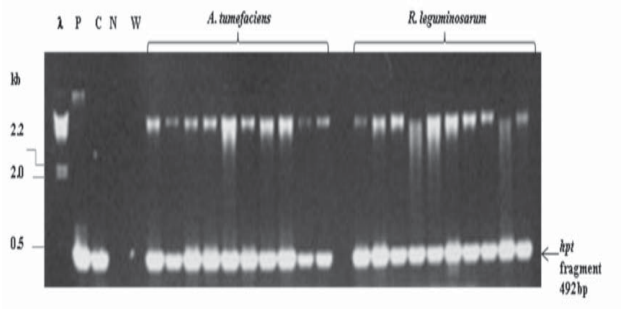

Figure 4. Amplification of fragment of *hpt*II gene from selected transgenic rice transformed with *A. tumefaciens* or *R. leguminosarum* carrying plasmid pCAMBIA 5106. DNA was isolated from rice leaves. **l**. DNA marker l *Hin*dIII, **P**. Plasmid pCAMBIA5106, **C**. Rice plants positive *hpt*II, **N**. Untransformed plants, **W**. Water

Tabel 2. Transformation frequencies of three rice cultivars transformed with *Rhizobium leguminosarum* and *Agrobacterium tumefaciens*.

Cultivar	Bacterial strain/ plasmid*	Number of calli inoculated	Number of experiment	Hygromycin resistan calli	Regenerated plants	PCR positive independent plants**	Transformation frequency (%)***	independent plants expressing GUS	independent plants resistant to hygromycine
Ciherang	*Rl*	440	11	96	57	53	12.05	38	43
	At	440	11	43	16	15	3.41	13	13
	Sm	440	11	6	2	1	0.23	0	1
Nipponbare	*Rl*	440	11	127	37	35	7.95	23	30
	At	440	11	50	6	5	1.14	3	5
	Sm	440	11	19	0	0	0	0	0
Rojolele	*Rl*	440	11	154	9	9	2.05	6	6
	At	440	11	103	5	5	1.14	3	4
	Sm	440	11	27	0	0	0	0	0

**Rl*: *Rhizobium leguminosarum* bv trifolii ANU845 (pCAMBIA5106), *At*: *Agrobacterium tumefaciens* LBA288 (pCAMBIA5106), *Sm*: *Shinorrhizobium meliloti* (pWBTi1, pCAMBIA0105.1R)
** Independent plants: plants coming from different calli
*** Number of PCR-positive plants/number of calli inoculated

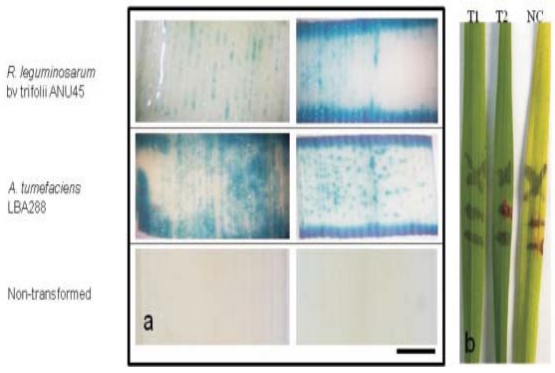

Figure 5. Transgenic plant leaves showing GUS activity at various level **(a)**, and HPT activity (**b**). **T1** green leaves indicating expression of hygromycin phosphotransferase, **T2** necrotic leaves indicating no expression of hygromycin phosphotransferase, and **NC** negative control of untransformed plant. Bar representing 10^{-3} m.

the transformation system is efficient for Ciherang. Regeneration frequencies of calli incubated with *Rhizobium* were observed higher than that of *Agrobacterium* in all of these three rice varieties. All the transgenic plants were analyzed for the presence of the *hpt*II gene by PCR (Figure 4) and assayed for the expression of GUS and HPT. Regenerated plants showed both GUS and HPT activity at various level in their leaves (Figure 5).

A number of plants that were GUS-negative in the histochemical assay and or negative in HPT activity were found containing *hpt*II gene in the PCR, indicating that the *gusplus* and or the *hpt*II gene may have been silencing. This phenomenon has been reported previously in rice (Lin & Zhang 2005) and other plants (Li *et al*. 2002). Only small number (less than 5%) of plants was found to be non transformed or "escapes".

From Table 2 it can be seen that the transformation frequencies (number of PCR positive plants per embryos inoculated) among these three cultivars were also large differences, ranging from 1.14% to 12.05% depend on the plant genotype and the bacterial agent used. The transformation frequencies of calli co-cultivated with *Rhizobium* were observed higher than that of *Agrobacterium* in all these three rice varieties. The transformation frequencies of Ciherang and Nipponbare incubated with *Rhizobium* were relatively high, 12.05% and 7.95% respectively. In contrast, the transformation frequencies of Ciherang and Nipponbare co-cultivated with *Agrobacterium* were 3.41% and 1.14%, respectively. Transformation frequency of Rojolele was, however, low both incubated with *Agrobacterium* (1.14%) or *Rhizobium* (2.05%). These transformation efficiencies are much higher than that (0.6%) previously reported by Broothaerts *et al* (2005). In this experiment, two transformed rice calli (from a total of 440 calli) that cocultivated with *S meliloti* containing pTiWB1 and pCAMBIA 1105.1R were regenerated and only one of them was PCR positive for *hpt*II gene, which consistent with the previous study reported by Broothaerts *et al*. (2005).

Gene copy number

The copy number of *hpt*II gene was analyzed by southern blot analysis (Figure 6). As many as 20 PCR-positive plants developed by *Agrobacterium* or *Rhizobium* transformation were selected. The average copy number of the *hpt*II gene per plant obtained by *Rhizobium*-mediated transformation was 1.20,

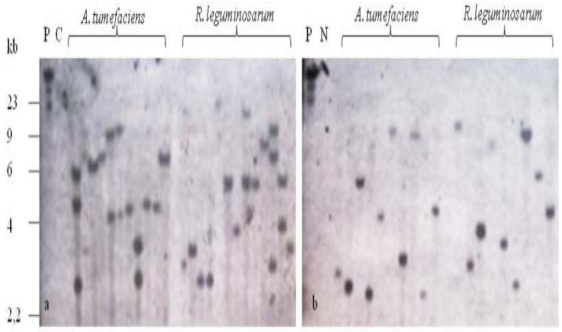

Figure 6. Examples of Southern hybridization of transgenic Ciherang (**a**) and Nipponbare (**b**) rice plants transformed with *R. leguminosarum* bv trifolii ANU845 (pCAMBIA5106) and *A. tumefaciens* LBA288(pCAMBIA5106). DNA sample, which were PCR positive, digested with *Eco*RI . PCR product amplified from the *hpt*II coding region was used as a probe. **P** Uncut Plasmid pCAMBIA 5106, **C** Untransformed Ciherang, and **N** Untransformed Nipponbare

which was similar to plants developed by *Agrobacterium* transformation (1.35) (Table 3). The frequency of transgenic plants containing one copy number of *hpt*II was 70% and 85% for plants cocultivated with *Agrobacterium* and *Rhizobium*, respectively.

Segregation pattern

Segregation pattern of *hpt*II gene in T_1 generation was analyzed. T_1 progenies of four lines originated from plants transformed with *Rhizobium* transformation that showed GUS expression and PCR positive for *hpt*II gene were selected. As many as 30 progenies from each line were PCR tested for the presence of the *hpt*II gene. Results showed that the *hpt*II gene inherited following the Mendelian-segregation pattern at a segregation ratio of 3:1 for line Rc162, Rc208, and Rc214, while 15:1 for line Rc203 (Table 4). This indicates that the transgene integrated in the rice genome as a single and two independent locus, respectively (Clemente *et al*. 2000).

Table 3. Estimated numbers of *hpt*II gene in selected transgenic rice lines developed by *Rhizobium* (R) and *Agrobacterium* (A) transformation.

Line A	Copy number of *hpt*II gene	Line B	Copy number of *hpt*II gene
An37	1	Rn41	1
An172	1	Rn6	1
An28	1	Rn24	1
An31	1	Rn7	1
An82	1	Rn43	1
An35	1	Rn52	1
An23	1	Rn17	1
An29	1	Rn231	1
An30	1	Rn28	1
An31	1	Rc94	1
Ac126	3	Rc214	1
Ac148	2	Rc171	1
Ac120	1	Rc156	1
Ac130	1	Rc208	1
Ac136	2	Rc205	1
Ac179	2	Rc159	2
Ac150	1	Rc181	1
Ac119	2	Rc203	3
Ac127	1	Rc106	2
Ar2	2	Rr32	1
average	1.35		1.20

Table 4. Segregation of *hpt*II gene in the T_1 progenies of transgenic lines.

Transformants	Number of T1 progenies PCR + hpt	Total number of plants analyzed	Segregation ratio	X^2	*P*
Rc162	24	30	3:1	0.4	0.05*
Rc208	20	30	3:1	1.10	
Rc203	30	30	15:1	2	
Rc214	23	30	3:1	0.04	

*very significat at 5% level. X^2 table (*df*=1, α0.05) = 3.841

Figure 7. Morphological performance of **a.** Nipponbare, **b.** Ciherang, and **c.** Rojolele transformed with *A. tumefaciens* LBA288 (pCAMBIA5106) (***At***) and *R. leguminosarum* (pCAMBIA5106) (***Rl***). **d.** Ciherang plants flowering normally after transformed with *R. leguminosarum* ANU845 (pCAMBIA5106).

Table 5. Growth and fertility of T_1 transgenic lines

Lines	Plant Height (cm)	Tiller	Productive tiller	Total seeds	Filled grains
Ciherang					
Rc171	67	14	11	1078	674
Rc210	64	10	10	735	519
Rc267	72	11	7	443	171
Rc211	61	15	15	1001	631
Rc160	67	12	8	523	288
Rc84	63	10	7	710	264
Rc189	68	15	15	1063	490
Rc182	66	11	10	710	401
Rc186	70	16	11	882	333
Rc89	62	14	10	769	354
Average	**66,00**	**12,80**	**10,40**	**791,40**	**412,50**
Ac128	56	12	9	573	184
Ac122	76	12	9	716	395
Ac118	70	7	6	552	334
Ac152	62	13	11	828	385
Ac177	68	11	7	695	297
Ac137	62	16	12	992	513
Ac180	74	16	13	996	354
Ac192	72	13	9	930	486
Ac149	75	12	8	599	253
Ac125	66	8	7	617	331
Average	**68,10**	**12,00**	**9,10**	**749,80**	**353,20**
Nipponbare					
Rn47	57	17	14	715	452
Rn45	54	21	14	410	40
Rn232	58.5	18	18	857	697
Rn4	50	14	11	510	317
Rn18	57	15	13	625	459
Average	**54,50**	**17,00**	**14,00**	**623,40**	**393,00**
An29	55	16	15	673	431
An38	50	16	15	726	477
An39	49	18	18	687	457
An36	45	22	16	446	285
An54	52	16	14	567	362
Average	**50,20**	**17,60**	**15,60**	**619,80**	**402,40**

Morphology and fertility of transgenic lines.

The morphology and fertility of transgenic lines were observed. There were no obvious difference of morphological performance between transgenic lines derived from *Rhizobium* transformation and *Agrobacterium* transformation. All transgenic lines observed were normal and fertil (Figure 7). Data on growth and fertility of transgenic plants is presented on Table 5.

Discussion

In this experiment two rice transformation systems, *Agrobacterium* and *Rhizobium* transformation, were compared. This was intended to evaluate whether *Rhizobium* capable of transferring genes into rice genome as efficient as *Agrobacterium*. We considered the regeneration and transformation frequecy, transgene copy number, inheritance pattern, and morphological performance and fertility. To minimize the influence of uncontrollable factors during development of transgenic plants, a single plasmid (pCAMBIA5106), and the same batch of calli were used for *Agrobacterium* and *Rhizobium*-mediated transformation. Both *A. tumefaciens* strain LBA288 and *R. leguminosarum* bv trifolii ANU845 are avirulent. Each rice genotype, however, was cultured on an appropriate medium since there is no universal medium adaptable to all rice genotype has been developed (Ge *et al*. 2006). Our preliminary studied also showed that each rice genotype grew better in different culture medium (data not shown).

Compared with earlier transformation system using *S meliloti* (Broothaerts *et al*. 2005), the transformation frequency using *Rhizobium* described here has increased remarkably, transformation efficiency ranging from 2.05 to 12.05% depend on the plant genotype used. The highest transformation frequency (12.05%) was found in rice Ciherang transformed with *R. leguminosarum* bv trifolii strain ANU845 (pCAMBIA5106). This is very interesting since Ciherang is a widely accepted rice variety by Indonesian farmer's today for it's good quality of rice. At this moment, Ciherang is in the second position after IR64 based on planting area, and the trend has increased every year since it was released in 2000. The development of tissue culture and transformation methods will help accelerating the improvement of agronomic trait of Ciherang in the future. The development of tissue culture techniques of Ciherang has been initiated (Purnamaningsih, 2006). However, no reports on successfully transformation methods was available .

The results of this study showed that *Rhizobium leguminosarum* that harbored with pCAMBIA5106 was capable of transferring genes into a wide range of the rice genome. The transformed plants were analyzed through GUS activity, HPT assay, PCR, and Southern blot. Based on those molecular analysis of T_0 and T_1 generation plants, it is proved that the transgenes have been integrated into the plant genomes (Figure 4 and 6), expressed in the plant cells (Figure 3 and 5), inherited on to the next generation following the Mendelian inheritance pattern (Table 4) and the transgenic plants grew normally and fertile (Table 5 and Figure 7d). These indicate that the transformation proceed normally as those *Agrobacterium*–mediated transformation.

From these results, it can be concluded that *Rhizobium leguminosarum* bv trifolii ANU845 was succesfully transferred the *gusplus* and *hpt*II genes into three rice varieties, including indica rice which was known to be recalcitrant to tissue culture and transformation. *Rhizobium leguminosarum* bv trifolii ANU845 can be used as an alternative agent for genetic transformation of plants in the future. Like *Agrobacterium*, *Rhizobium* is a genotype dependent transformation agent thus it is important to examine other *Rhizobium* species or biovar that maybe more suitable for certain genotype.

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