Molecular Breeding 7: 25–33, 2001.

Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment

Shunhong Dai[1,2], Ping Zheng[1], Philippe Marmey[1], Shiping Zhang[1], Wenzhong Tian[2], Shouyi Chen[2], Roger N. Beachy[1] & Claude Fauquet[1,*]

[1]*ILTAB (ORSTOM/TSRI), Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.;* [2]*Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China; *Author for correspondence (fax: 314-5164582; e-mail: CMF@danforthcenter.org)*

Received 7 May 1999; accepted in revised form 7 August 2000

Key words: Agrobacterium, Biolistic, Rice, Transgene analysis

Abstract

We compared rice transgenic plants obtained by *Agrobacterium*-mediated and particle bombardment transformation by carrying out molecular analyses of the T_0, T_1 and T_2 transgenic plants. *Oryza sativa japonica* rice (c.v. Taipei 309) was transformed with a construct (pWNHG) that carried genes coding for neomycin phosphotransferase (*nptII*), hygromycin phosphotransferase (Hyg^r), and β-glucuronidase (GUS). Thirteen and fourteen transgenic lines produced via either method were selected and subjected to molecular analysis. Based on our data, we could draw the following conclusions. Average gene copy numbers of the three transgenes were 1.8 and 2.7 for transgenic plants obtained by *Agrobacterium* and by particle bombardment, respectively. The percentage of transgenic plants containing intact copies of foreign genes, especially non-selection genes, was higher for *Agrobacterium*-mediated transformation. GUS gene expression level in transgenic plants obtained from *Agrobacterium*-mediated transformation was more stable overall the transgenic plant lines obtained by particle bombardment. Most of the transgenic plants obtained from the two transformation systems gave a Mendelian segregation pattern of foreign genes in T_1 and T_2 generations. Co-segregation was observed for lines obtained from particle bombardment, however, that was not always the case for T_1 lines obtained from *Agrobacterium*-mediated transformation. Fertility of transgenic plants obtained from *Agrobacterium*-mediated transformation was better. In summary, the *Agrobacterium*-mediated transformation is a good system to obtain transgenic plants with lower copy number, intact foreign gene and stable gene expression, while particle bombardment is a high efficiency system to produce large number of transgenic plants with a wide range of gene expression.

Introduction

Agrobacterium-mediated transformation and transformation using direct delivery of DNA are the two methods widely used for genetic transformation. *Agrobacterium*-mediated transformation has been established for many years in dicotyledous plants, and recently has been applied to monocotyledous plants (Hiei et al. 1994; Smith and Hood 1995; Ishida et al. 1996; Hiei et al. 1997; Tingay et al. 1997). Meanwhile, direct DNA delivery transformation methods were developed for monocotyledous plants and have become one of the most effective plant transformation systems applied to major cereals (Christou et al. 1991; Spencer et al. 1992; Li et al. 1993; Vasil 1994; Chen et al. 1998a). Many transformation methods have been applied to rice (Christou et al. 1991; Ayres and Park 1994; Hiei et al. 1994; Smith and Hood 1995; Zhang et al. 1996; Zheng et al. 1996). In recent years, protocols for particle bombardment have been well developed, modified and routinely used (Christou et al. 1991; Li et al. 1993; Zhang et al. 1996; Zheng et al. 1996; Chen et al. 1998a) and *Agrobacterium*-mediated transformation has been adapted to rice (Chan et al.

1993; Hiei et al. 1994; Park et al. 1996; Rashid et al. 1996).

Patterns of integration, inheritance and expression of transgenes in plants upon *Agrobacterium*-mediated and direct DNA delivery-mediated transformation have been reported by many laboratories (Goto and Toki 1993; Flavell 1994; Hiei et al. 1994; Elmayan and Vaucheret 1996; Chen et al. 1998a; De Neve et al. 1997; Kononov et al. 1997; Dean et al. 1998; Kohli et al. 1998). The direct DNA delivery systems tend to result in integration of multiple copies of transgenes at single loci and rearrangement of transgenes (Finnegan and McElroy 1994; Flavell 1994; Pawlowski and Somers 1996; Kohli et al. 1998), while most of *Agrobacterium*-mediated transgenic plants had a lower copy number of transgenes and a more predictable pattern of integration (Smith and Hood 1995). However, Kononov et al. (1997) reported that approximately 75% of the transgenic tobacco plants from *Agrobacterium*-mediated transformation contained sequence from the binary vector 'backbone' sequences in plant genome. Furthermore, there was not a clear correlation between transgene expression and transgene copy number (Dean et al. 1988; Hobbs et al. 1993). While single copies of transgenes may tend to be more stably expressed than multiple gene copies or scrambled inserts, there were additional factors that influenced transgene expression (Iglesias et al. 1997). Transgenic plants that exhibit classical Mendelian inheritance ratios were reported from both types of transformation methods (Pawlowski and Somers 1996; Hiei et al. 1997). There were also cases of non-Mendelian patterns of inheritance in transgenic plants from both transformation methods (Spencer et al. 1992; Goto et al. 1993; Peng et al. 1995).

In this paper, we propose to do a comparative analysis of transgenic rice plants obtained from both *Agrobacterium*-mediated transformation and particle bombardment. For this, we used for both techniques the same genotype, the same batch of calli, the same plasmid and the same experimentation.

Materials and methods

Bacterium strain and plasmid

Agrobacterium tumefaciens strain LBA4404 was used (Hoekema et al. 1983). Plasmid pWNHG (from Dr P.M. Waterhouse, CSIRO, Australia) was a binary vector that contains a NPT gene *(nptII)*, a hygromycin-resistance gene (*hpt*) and a GUS gene (*uidA*) (Figure 1); it was introduced into LBA4404 by electroporation.

Agrobacterium-mediated transformation

Agrobacterium strain LBA4404 (pWNHG) was grown on YM medium containing 50 mg/l hygromycin B and 50 mg/l kanamycin at 24 °C for 3 days. *Agrobacterium* cells were collected and re-suspended in NB liquid medium (Li et al. 11993) with 100 μM acetosyringone, with an optical density (OD) of 0.5–1.0 at 600 nm. Rice calli induced from Taipei 309 mature seeds were placed in bacterial suspension for 10 min. After briefly draining the calli on sterilized paper filter, the calli were transferred to NB solid medium containing 100 μM acetosyringone and co-cultured at 28 °C in the dark for 2 days. The co-cultured calli were washed three times with sterile water containing cefotaxime at 250 mg/l and transferred to selection medium. The selection medium was the same as for particle bombardment except that it contained cefotaxime at 250 mg/l. Preregeneration and regeneration were carried out as for particle bombardment (Li et al. 1993; Zheng et al. 1996; Chen et al. 1998a).

Particle bombardment transformation

Particle bombardment transformation was performed on the same batch of calli that were used for *Agrobacterium*-mediated transformation. Transgenic plants were produced as described (Li et al. 1993) with modification (Zheng et al. 1996; Chen et al. 1998a).

GUS assays

Both histochemical staining and fluorometric measurement of *GUS* activity in leaves of transgenic plants were carried out as described (Jefferson 1987).

Southern blot analysis

Genomic DNA extraction was performed as described (Dellaporta et al. 1983). For each sample, 10 μg of genomic DNA were digested by appropriate enzymes. Southern blots were carried out as described (Zhang et al. 1996). As probe for the *uidA* gene, a 0.6 kb *Bam*HI/*Eco*RV fragment within the GUS-coding region was cut from plasmid pAct1-D (McElroy et al. 1990). As probe for the *hpt* gene, a 0.8 kb *Eco*RI fragment within the *hpt* coding region was cut from plLTAB310 *(hpt* gene driven by 35S promoter) and

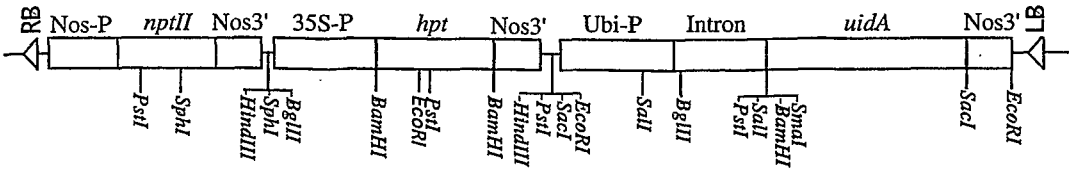

Figure 1. Map of the T-DNA of plasmid pWNHG. RB, T-DNA right border; LB, T-DNA left border; *nptII*, coding region of the neomycin phosphotransferase gene; *hpt*, coding region of the hygromycin phosphotransferase gene; *uidA*, coding region of the β-glucuronidase gene; Nos-P, promoter of the nopalin synthase gene; Nos 3′, 3′ region of the nopalin synthase gene; 35S-P, promoter of the 35S RNA of cauliflower mosaic virus; Ubi-P, promoter of the ubiquitin gene; intron, first intron of the ubiquitin gene.

used as probe. For the *nptII* gene probe, a 0.6 kb PCR product amplified from the *nptII* coding region by using primer 1 5′-GAACAAGATGGATTGCACGC-3′ and primer 2 5′-GCTCTTCAGCAATATCACGG-3′ as forward and reverse primers, respectively.

Analysis of hygromycin resistance in plants progeny

Seeds from T_0 plants were sterilized and germinated on MS medium without 2,4-D for two weeks. Leaves of seedlings were collected for GUS histochemical assay and further analysis. The remaining part of the plant was transferred to 1/2 MS medium containing 50 mg/l hygromycin B for selection. Resistant plants were counted two weeks after transfer. The survey was repeated one week later.

Results

Rice transformation

To minimize the influence of uncontrollable factors during development of transgenic plants, a single plasmid (pWNHG) and the same batch of calli were used for both *Agrobacterium* and particle bombardment-mediated transformation. All the tissue culture procedures were the same, except that the selection medium for *Agrobacterium*-mediated transformation contained cefotaxime to repress growth of *Agrobacterium*. Hygromycin B-resistant transgenic lines developed from both methods of transformation were confirmed by PCR analysis of the *hpt* gene. The efficiency of transformation was estimated by establishing the ratio between the number of hygromycin resistant transgenic plants obtained versus the number of calli used for transformation. A 7% efficiency was obtained with *Agrobacterium*-mediated transformation, and a 22% efficiency with particle bombardment. Transgenic plants from *Agrobacterium*-mediated transformation and particle bombardment were designated as A_n and G_n, respectively. A total of 15 transgenic plants from each transformation method were subsequently transferred to the green house for further analysis. However, only 13 plants from *Agrobacterium*-mediated transformation and 14 plants from particle bombardment survived and were analyzed.

Gene copy number

The copy number of the three foreign genes within T-DNA region of pWNHG was analyzed by southern blot assays. To detect the copy number of *hpt*, genomic DNAs of transgenic lines were digested with *SacI* and probed with a 0.8 kb *Eco*RI fragment from *hpt* coding region. To detect the *uidA* gene, genomic DNAs were digested with *Pst*I and probed with a 0.6 kb *Bam*HI/*Eco*RV fragment from *uidA* coding region. To detect the *nptII* gene, genomic DNAs were digested with *Hin*dIII and probed with a 0.6 kb PCR product within the *nptII* coding region.

The average copy number of the *hpt* gene was 1.7 and 2.5 among transgenic lines obtained by *Agrobacterium*-mediated transformation and by particle bombardment, respectively (Table 1). The average copy number of genes per line was similar for most of the plants obtained by *Agrobacterium*-mediated transformation; in contrast the number of genes copies was more variable for lines obtained by particle bombardment (Table 1, Figure 2A and B).

Integrity of foreign genes and GUS expression levels

The integrity of *hpt* and *uidA* was analyzed by Southern blot analysis. The intact *uidA* cassette was released by digesting plant DNA with *Eco*RI (Figure 1) and *the hpt* gene was released by *Hin*dIII (Figure 1).

All of the transgenic lines except one, G18, contained at least one intact copy of the *hpt* gene regardless of the transformation method used (Figure 3A and B). Similarly, all *Agrobacterium*-mediated transgenic plants had an intact copy of the *uidA* except

Table 1. Estimated numbers of *uidA*, *hpt*, and *nptII* genes in transgenic rice lines A. transgenic lines from *Agrobacterium*-mediated transformation; G, transgenic lines from particle bombardment-mediated transformation.

Line	Copy number			Line	Copy number		
	uidA	*hpt*	*nptII*		*uidA*	*hpt*	*nptII*
A2	3	1	1	G5	4	8	4
A3	2	2	2	G7	5	2	2
A4	2	2	3	G10	3	3	3
A5	1	1	1	G14	2	1	1
A6	2	2	2	G15	3	3	2
A8	1	1	1	G16	2	2	3
A9	2	2	2	G17	5	3	3
A14	1	1	1	G18	2	1	0
A15	2	2	1	G19	3	2	3
A18	5	5	5	G21	2	1	1
A19	1	1	1	G22	3	2	2
A38	1	1	NT	G24	3	3	3
A39	2	1	1	G28	6	2	2
				G29	3	2	NT
Average	1.9±1.1	1.7±1.1	1.8±1.2	Average	3.3±1.3	2.5±1.7	2.2±1.1

NT, not tested.

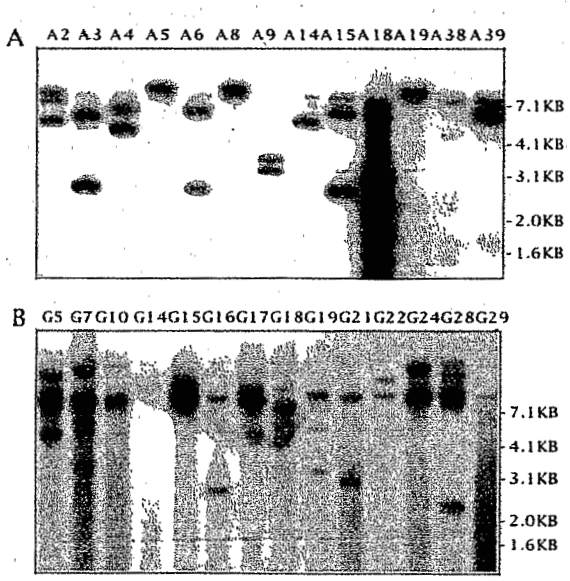

Figure 2. Southern blot analysis showing copy number of *uidA* in transgenic lines. For each sample, 10 μg of genomic DNA were digested with *Pst* I and probed with a 0.6 kb *Eco*RV/*Bam*HI fragment of pAct1-D. A. A2-A39, samples of transgenic lines from *Agrobacterium*-mediated transformation. B. G5-G28, samples from particle bombardment.

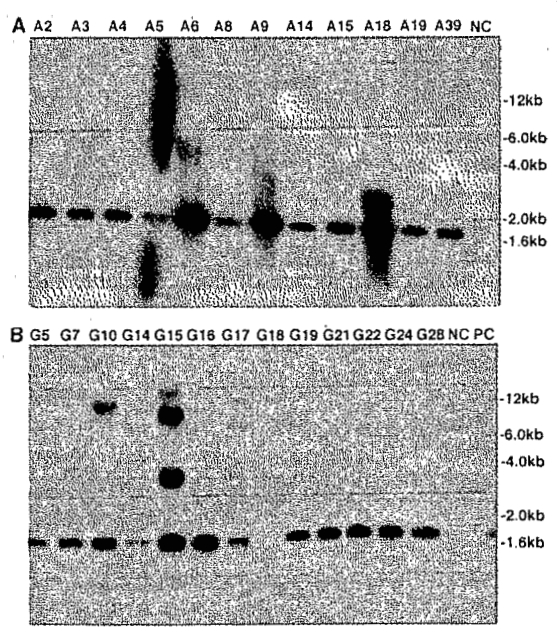

Figure 3. Southern blot analysis showing integrity of *hpt* gene in transgenic lines. For each sample, 10 μg of genomic DNA were digested with *Hin*dIII and probed with a 0.8 kb *Eco*RI fragment of p35H. A. NC, negative control (TP309); A2-A39, samples of transgenic lines from *Agrobacterium*-mediated transformation. B. NC, negative control (TP309); pc, positive control, *hpt* gene released from plasmid pWNGH; G5-G28, samples from particle bombardment.

line A38 (Figure 4A). In contrast, the number was reduced to about 50% for the transgenic lines obtained via particle bombardment (Figure 4B). The average

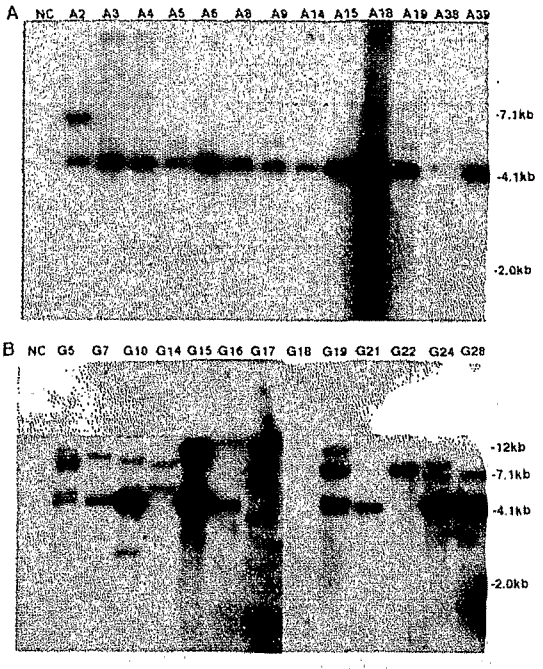

Figure 4. Southern blot analysis showing integrity of *uidA* gene in transgenic lines. For each sample, 10μg of genomic DNA were digested with *Eco*RI and probed with a 0.6 kb *Eco*RV/*Bam*HI fragment of pAct1-D. A. NC. negative control (TP309): A2-A39. samples of transgenic lines from *Agrobacterium*-mediated transformation. B. NC. negative control (TP309): G5-G28, samples of transgenic lines from particle bombardment.

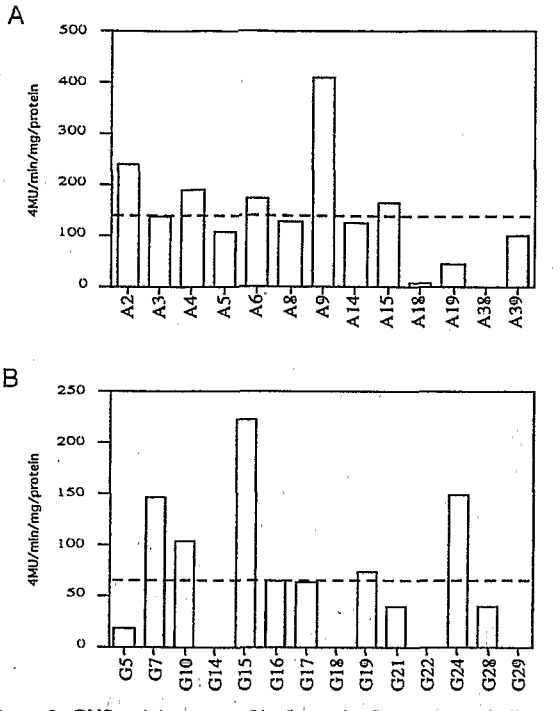

Figure 5. GUS activity assay of leaf samples from transgenic lines. A. A2-A39. samples from *Agrobacterium*-mediated transformation lines. B. G5-G28. samples from particle bombardment.

level of GUS activity for the lines developed via *Agrobacterium*-mediated transgenic lines was slightly higher than the average level of particle bombarded transgenic lines (Figure 5). In addition, the level of GUS activity was more stable between transgenic lines obtained from *Agrobacterium*-mediated transformation than between transgenic lines developed from particle bombardment. Plant lines that lack an intact copy of *uidA* likewise did not show GUS activity (e.g., A38, G14, G18, G22 and G29).

Segregation patterns

Segregation patterns of GUS activity and resistance to hygromycin in T_1 and T_2 generations were analyzed. All of the 12 fertilized lines developed by *Agrobacterium*-mediated transformation showed a Mendelian segregation pattern of gene expression (Table 2). Among them, A_2 exhibited two active loci of *uidA*, A_9 and A_{18} had two active loci of *uidA* and *hpt*, and the other lines had one active locus for each gene. The T_1 generation of A_8 gave an aberrant segregation pattern in the GUS histo-chemical assay. Because of seed contamination by fungi, not all of the T_1 plants of line A_8 were tested for resistance to hygromycin B (hyg^r). Uncontaminated T_1 plants gave a normal hyg^r segregation pattern; and hyg^r and GUS activity co-segregated in all these plants. To establish a correlation between GUS expression and presence of the *uidA* in this line, 34 T_1 plants were analyzed by Southern blot analysis and GUS staining. All plants that contained the *uidA* were GUS-positive and all the Southern-negative plants were GUS-negative. GUS segregation patterns were analyzed in the T_2 generation of eight A_8 GUS-positive T_1 lines. Each of the lines gave a Mendelian segregation pattern (Table 3). Three were homozygous lines (A_{8-1}, Ag_{8-6} and Ag_{8-8}) and the remaining lines were heterozygous. The aberrant segregation of GUS gene in T_1 plants of line A_8 was apparently due to the population seeds tested.

The patterns of segregation of GUS and hyg^r of 9 fertilized lines generated by particle bombardment were also investigated (Table 2). Most lines, except G_{10} and G_{14}, showed a Mendelian pattern of segregation. Line G_{10} exhibited a 1:1 segregation pattern of GUS and hyg^r and the genes co-segregated in individual T_1 progeny plants of G_{10}. To investigate the correlation between *uidA* integration and GUS expres-

Table 2. Segregation of hygromycin B resistance and GUS activity in T_1 progeny plants of transgenic rice lines.

T_0 plants		Segregation in T_1 generation			
Lines	Hyg^r/GUS	Hyg^r/Hyg^s	GUS^+/GUS^-	$ratio_{(GUS)}$	χ^2_{GUS}
A2	+/+	NT*	54/2	15:1	0.686
A3	+/+	64/12	64/12	3:1	3.439
A5	+/+	67/15	67/15	3:1	1.968
A6	+/+	56/25	56/25	3:1	1.486
A8	+/+	18/6*	37/22	?	
A9	+/+	29/3	29/3	15:1	0.533
A14	+/+	62/20	62/20	3:1	0.016
A15	+/+	63/29	63/29	3:1	2.087
A18	+/+	61/3	61/3	15:1	0.267
A19	+/+	50/18*	58/20	3:1	0.017
A38	+/–	53/19	0/72	–	–
A39	+/+	75/20	75/20	3:1	0.790
G10	+/+	24/37	24/37	1:1	2.770
G14	+/–	0/42	0/42	–	–
G15	+/+	42/15	42/15	3:1	0.053
G16	+/+	NT*	13/3	3:1	0.333
G17	+/+	34/8	34/8	3:1	0.794
G18	+/–	39/17	0/56	–	–
G 19	+/+	47/17	47/17	3:1	0.083
G21	+/+	61/17	61/17	3:1	0.427
G22	+/–	65/22	0/77	–	–

NT, not tested; *partial plants contaminated during hygromycin resistence selection.

Table 3. Segregation of GUS activity in T_2 progeny plants of A8 an G10.

T_1 Plants	Hyg^r/GUS	GUS activity in T_2 generation		
		GUS^+/GUS^-	ratio	χ^2
A8-1	+/+	33/0	1:0	–
A8-2	+/+	32/11	3:1	0.008
A8-3	+1+	24/8	3:1	0.000
A8-4	+1+	41/7	3:1	2.778
A8-5	+/+	38/10	3:1	0.444
A8-6	+1+	56/0	1:0	–
A8-7	+/+	31/17	3:1	2.778
A8-8	+1+	47/0	1:0	–
G10-1	+/+	7/5	1:1	0.343
G10-2	+/+	7/6	1:1	0.077
G10-3	+/+	20/16	1:1	0.444
G10-4	+/+	24/17	1:1	1.195
G10-5	+/+	13/17	1:1	0.533
G10-6	+/+	6/10	1:1	1.000
G10-7	+/+	12/15	1:1	0.333
G10-8	+/+	9/10	1:1	0.053

sion in T_1 progeny of G_{10}, 51 individual plants were analyzed by GUS histochemical assays and southern blot analysis using the *uidA* probe. A11 plants that contained *uidA* were GUS-positive, and all the Southern blot-negative plants were GUS-negative. To confirm the segregation pattern of G_{10} in the T_2 generation, eight *uidA* Southern blot-positive T_1 lines were analyzed by GUS histochemical assay. All lines showed a 1:1 segregation pattern of GUS in the T_2 generation (Table 3). T_1 progeny of G_{14} were all sensitive to 50 mg/l hygromycin B selection; however, the T_0 plant of G_{14} was resistant to hygromycin selection and contained the *hpt* gene (Figure 3B).

In most of the transgenic lines derived via both transformation methods, transgenes follow the Mendelian rules of inheritance. In case of *Agrobacterium*-mediated transgenic lines, there was greater likelihood that genes were integrated in several loci, while genes were mostly co-integrated in lines generated via particle bombardment. We only observed an abnormal segregation pattern for the line G_{10}.

Table 4. Fertility of T_0 rice transgenic lines.

Line	Number of panicles	Total seeds	Plump seeds	Setting percentage
A2	4	182	74	40.7
A3	7	226	183	81.0
A4	3	162	0	0.0
A5	6	217	176	81.1
A6	3	152	106	70.0
A8	6	276	217	78.6
A9	4	134	41	30.6
A14	5	219	132	60.3
A15	4	193	114	59.1
A18	2	115	73	63.5
A19	4	123	87	70.7
A38	2	126	95	75.4
A39	6	203	178	87.7
Average	4.3±1.6	179.1±40.8	113.5±62.2	61.4±24.6
G5	3	97	0	0.0
G7	3	63	0	0.0
G10	3	138	91	65.9
G14	5	170	51	30.0
G15	6	140	64	45.7
G16	5	168	22	13.1
G17	2	135	56	41.5
G18	2	111	95	85.6
G19	4	150	109	72.7
G21	5	180	141	78.3
G22	6	190	144	75.8
G24	4	93	0	0.0
G28	1	31	0	0.0
G29	2	73	0	0.0
Average	3.6±1.6	124.2±47.7	55.2±53.7	36.3±34.2

Fertility of transgenic lines

The transgenic lines were planted in the same green house and observed for agronomic characteristics. There were no obvious differences of agronomic characters between transgenic lines, except that transgenic lines derived from particle bombardment showed a higher percentage of sterile plants (5 out of 14) than transgenic lines from *Agrobacterium*-mediated transformation (1 out of 13). In addition, the degree of fertility (i.e., number of seeds per plant) was also lower in Gn versus An lines (Table 4).

Discussion

In this paper, we compared two transformation systems for rice using the same experimental procedures. We considered differences in transformation efficiency, transgene copy numbers, gene expression and fertility of transgenic lines.

In our experiment, the efficiency of *Agrobacterium*-mediated transformation (7%) was lower than the one with particle bombardment (22%). Our efficiency of *Agrobacterium*-mediated transformation was not as high as reported by Hiei et al. (1994). In comparison with particle bombardment, efficiency is still a limitation of *Agrobacterium*-mediated transformation in many laboratories. Furthermore, *Agrobacterium*-mediated transformation is still a genotype-dependent method. Although transgenic *japonica* and *indica* rice plants have been obtained by *Agrobacterium*-mediated transformation (Hiei et al. 1994, Park et al. 1996, Rashid et al. 1996), the number of varieties transformed by *Agrobacterium* is lower than the number of varieties transformed with particle bombardment (Zhang et al. 1996, Zheng et al. 1996; Christou 1997).

The influence of transgene copy number on level of gene expression is known to be complex. Although it was anticipated that increasing transgene copy number would increase expression level, it is now known that multiple gene copies frequently lead to co-suppression and gene silencing (Vaucheret et al. 1998). Transgene copy number can be positively or negatively associated with transgene expression (Hobbs et al. 1993). In some cases, a single copy of the transgene can be silenced (Elmayan and Vaucheret 1996). However, it is general considered that selecting plants with low gene copy number decreases the possibility of transgene co-suppression. In our experiments, *Agrobacterium*-mediated transgenic plants have relatively low gene copy numbers and only two transgenic lines, A_9 and A_{18} showed very high or low level of GUS activity, respectively. A_9 had two intact copies of the *uidA* and A_{18} had 5 copies, at least one of which was an intact copy. Transgenic lines from particle bombardment had multiple copies of the *uidA* sequences and had a low level of GUS activity. It is suggested that the low level of gene expression may due to gene silencing mechanisms.

All of the co-transformed genes co-segregated in transgenic lines obtained from particle bombardment. This is in agreement with previous authors (Pawlowski and Somers 1996, Kohli et al. 1998) who showed that particle bombardment favored integration of mul-

tiple copies of transgenes at a single locus. The co-integration does not regard if the co-transformed transgenes were from the same vector or from different vectors. Kohli et al. (1998) proposed a two-phase model to explore the transgene integration mechanism of DNA direct delivery transformation. This model, together with exonuclease/ligase model (McElroy et al. 1990), can well explain the phenomenon that, in most cases, transgenes in transgenic plants developed by direct DNA delivery transformation system have multicopies and locate at a single locus. The transgenes cointegration and co-segregation phenomenon in transgenic plants developed from direct DNA delivery transformation systems makes the job easy to genetically select transgene pure lines from progenies. It is also useful for map-based breeding program (Kohli et al., 1998). However, it is also hard to eliminate selection markers through genetic approach unless we have special design to remove selection genes from transgenic plants.

Agrobacterium-mediated transformation gives a greater frequency of multi-locus insertion of transgenes. Using a 'super-binary' vector and *Agrobacterium*-mediated transformation, Komari et al. (1996) reported a 47% frequency of transferring two T-DNAs of the vector, and the T-DNAs segregated independently in more than half of the co-transformants. De Neve et al. (1997) co-cultured *Arabidopsis* with two *Agrobacterium* strains each carrying a different T-DNA, and found that the two T-DNAs segregated independently in 15 out of 27 co-transformed plants.

The integrity of the foreign DNA is different for the two types of transformations. T-DNA is integrated in most of the cases as a unit onto rice chromosome. Even though 'backbone' DNA of Ti plasmid vector may also integrate onto plant chromosome (Kononov *et al.* 1997), and, in another hand, partial of T-DNA may miss when T-DNAs were integrated onto chromosome (Kononov et al. 1997), transgenic plants from *Agrobacterium*-mediated transformation still have better chance to get intact copy of transgenes than transgenic lines from bombardment. From our data, only A2, A9 and A18 show partial T-DNA integration. By contrast, the integrity of transgenes in transgenic plants from particle bombardment showed a high variation for the non-selectable genes. The re-arrangement and multimerization of transgenes resulted in direct and inverted repeats of co-integration, as well as integration of partial transgenes (Pawlowshi and Somers 1996; Kohli et al. 1998). This suggested that transgene DNA was integrated onto plant chromosome in a random way and Kohli's two-phase integration model provided a good explanation of transgene integration (Kohli et al., 1998). Re-arrangement of transgene will affect the function of transgene (Vaucheret et al. 1998).

Fertility of transgenic plants is very important for either using transgenic plants as genetic material for a breeding program or using transgenic plants for basic research. Even though, in some cases, the fertility of transgenic lines with a poor fertility in the T_0 generation could be restored to a certain degree in subsequent generations (Dai and Tian, unpublished data), it is critical to get seeds from transgenic plants. To this point, the fertility was usually better for *Agrobacterium*-mediated transgenic plants than it was for transgenic plants from particle bombardment.

Particle bombardment can be a very efficient method of transformation, and it is often genotype-independent method. Furthermore, multiple plasmids can be used to co-transform multigenes (Hadi et al. 1996, Chen et al. 1998b), and large fragments of DNA, including those in yeast artificial chromosome (Van Eck et al. 1995) can be introduced. Also, co-transformation, cosegregation may provide a simplified way to apply transgenic materials to breeding programs (Kohli et al., 1998). However, points like the number of gene fragments that are inserted, the low fertility of transgenic plants, and the rearrangement of gene sequences still need to be improved with this method.

While *Agrobacterium*-mediated transformation is an established system in dicotyledonous plants, the behavior of foreign genes is relatively well documented; *Agrobacterium* co-transformation method may provide an easy way to produce selection marker free transgenic plants. The low efficiency of transformation and adaptability to monocotyledonous plants are major disadvantages.

Both transformation methods have their own advantages and disadvantages. The goal of the project (study of the expression of a gene (or a promoter); numbers of required, numbers of target genes to be used) will guide the scientist in choosing between the two systems.

Acknowledgements

We thank Ms Lili Chen. S.D. and P.Z. were supported by Rockefeller Foundation. P.M. and C.F. were supported by ORSTOM (French Scientific Research Institute for Development through Cooperation).

References

Ayres N. and Park W. 1994. Genetic transformation of rice. Crit. Rev. Plants Sci. 13: 219–239.

Chan M., Chang H., Ho S., Tong W. and Yu S. 1993. *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric oc-amylase promoter/β-glucuronidase gene. Plant Mol. Biol. 22: 491–506.

Chen L., Zhang S., Beachy R.N. and Fauquet C.M. 1998a. A protocol for consistent, large scale production of fertile transgenic rice plants. Plant Cell Rep. 18: 25–31.

Chen L., Marmey P., Taylor N., Brizard J-P., Espinoza C., D'Cruz P., Huet H., Zhang S., de Kochko A., Beachy R.N. and Fauquet C.M. 1998b. Expression and inheritance of multiple transgenes in rice plants. Nature Biotechnol. 16: 1060–1064.

Christou P, Ford TL and Kofron M. 1991. Production of transgenic rice [*Oryza sativa* L.] plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/technology 9: 957–962

Christou P. 1997. Rice transformation: bombardment. Plant Mol. Biol. 35: 197–203.

De Neve M., De Buck S., Jacobs A., Van Montagu M. and Depicker A. 1997. T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J. 11: 15–29.

Dean C., Jones J., Favreau M., Dunsmuir P. and hedbrook J. 1988. Influence of flanking sequences on variability in expression levels of an introduced gene in trangenic tobacco plants. Nucl. Acids Res. 16: 9267–9283.

Dellaporta S.L., Wood J. and Hicks J.B. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19–21.

Elmayan T. and Vaucheret H. 1996. Expression of single copies of a strongly expressed 35s transgene can be silenced post-transcriptionally. Plant J. 9: 787–797.

Flavell R.B. 1994. Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91: 3490–3496.

Finnegan J. and McElroy D. 1994. Transgene inactivation: plants fight back. Bio/technology 12: 883–888.

Goto F., Toki S. and Uchimiya H. 1993. Inheritance of a co-transferred foreign gene in progenies of transgenic rice plants. Transgenic Res. 2: 300–305.

Hadi M.Z., McMullen M.D. and Finer J.J. 1996. Transformation of 12 different plasmids into soybean via particle bombardment. Plant Cell Rep. 15: 500–505.

Hiei Y., Ohta S., Komari T. and Kumashiro T. 1994. Efficient transformation of rice [*Oryza sativa* L.] mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271–282.

Hiei Y., Komari T. and Kubo T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. 35: 205–218.

Hobbs S.L.A., Warkentin T.D. and DeLong C.M.O. 1993. Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21: 17–26.

Hoekema A., Hirsch P.R., Hooykaas P.J.J. and Schilperoort R.A. 1983. A binary plant vector strategy based on separation of *vir* and T-region of *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180.

Iglesias V.A., Moscone E.A., Papp I., Neuhuber F., Michalowski S., Phelan T., Spiker S., Matzke M. and Matzke A. 1997. Molecular and cytogenetic analysis of stably and unstably expressed transgene loci in tobacco. Plant Cell 9: 1251–1264.

Ishida Y., Saito H., Ohta S., Hiei Y., Komari T., Kumashiro T. 1996. High efficiency transformation of maize *(Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14: 745–750.

Jefferson R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387–405.

Kohli A., Leech M., Vain P., Laurie D.A. and Christou P. 1998. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc. Natl. Acad. Sci. USA 95: 7203–7208.

Komari T., Hiei Y., Saito Y., Murai N. and Kumashiro T. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. 10: 165–174.

Kononov M., Bassuner B. and Gelvin S. 1997. Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J. 11: 945–957.

Li L., Qu R., de Kochko A., Fauquet C.M. and Beachy R.N. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250–255.

McElroy D., Zhang W., Wu R. 1990. Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163–171.

Park S.H., Pinson S.R.M. and Smith R. 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol. 32: 1135–1148.

Pawlowski W.P. and Somers D.A. 1996. Transgene inheritance in plants genetically engineered by microprojectile bombardment. Mol. Biotechnol. 6: 17–30.

Peng J., Wen F., Lister R.L. and Hodges T.K. 1995. Inheritance of *gusA* and *neo* genes in transgenic rice. Plant Mol. Biol. 27: 91–104.

Rashid H., Yokoi S., Toriyama K. and Hinata K. 1996.Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. Plant Cell Rep. 15: 727–730.

Smith R.H. and Hood E.H. 1995. *Agrobacterium tumefaciens* transformation of monocotyledons. Crop Sci. 35: 301–309.

Spencer T.M., O'Brien J.V., Start W.G., Adams T.R., Gordon-Kamn W.J. and Lemaux P.G. 1992. Segregation of transgenes in maize. Plant Mol. Biol. 18: 201–210.

Tingay S., McElroy D., Kalla R., Fieg S., Wang M., Thornton S. and Brettell R. 1997. *Agrobacterium tumefaciens*-mediated barley transformation. Plant J. 11: 1369–1376.

van Eck J.M., Blowers A.D. and Earle E.D. 1995. Stable transformation of tomato cell cultures after bombardment with plasmid and YAC DNA. Plant Cell Rep. 14: 299–304.

Vasil I.K. 1994. Molecular improvement of cereals. Plant Mol. Biol. 25: 925–937.

Vaucheret H., Béclin C., Elmayan T., Feuerbach F., Godon C., Morel J.B., Mourrain P., Palauqui J.C. and Vernhettes S. 1998. Transgene-induced gene silencing in plants. Plant J. 16: 651–659.

Zhang S., Chen L., Qu R., Marmey P., Beachy R.N. and Fauquet C.M. 1996. Regeneration of fertile transgenic indica (group1) rice plants following microprojectile transformation of embryogenic suspension culture cells. plant Cell Rep. 15: 465–469.

Zheng H., Dai S., He S., Tian W. and Li L. 1996. Genetic transformation of *indica* rice using the biolistic method. Chin. J. Genet. 23: 201–208.

Molecular Breeding

New Strategies in Plant Improvement

ISPMB **International Society for Plant Molecular Biology**

7, No. 1, January 2001

ISSN 1380-3743
CODEN MOBRFL

Academic Publishers