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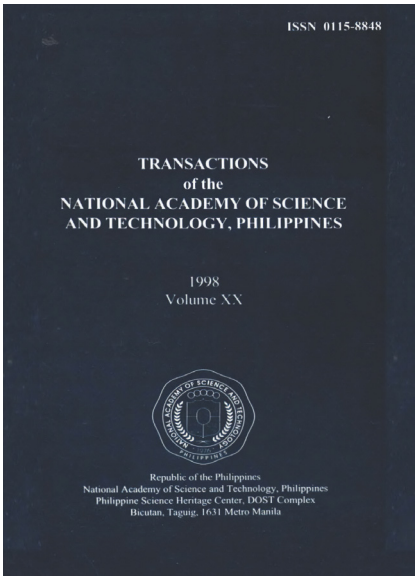

Gene Delivery System in Rice

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GENE DELIVERY SYSTEMS IN RICE

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ABSTRACT

The increasing population rate (2.5% per year growth rate) and shrinking area devoted to rice production (-1.8% per year decline) in the Philippines pose a big challenge to plant breeders to expedite genetic improvement in rice. Genetic engineering is a powerful and novel means, complementing the traditional methods in improving plant characters by introducing foreign genetic material or by enhancing the expression of endogenous genes. Improved varieties are developed in a shorter time, repeatable results are obtained, and there is an immense source of genetic variability. Monocots were not transformable using *Agrobacterium tumefaciens*, a natural transformation vector for dicots. Hence, other methods were developed such as polyethylene glycomediated DNA and electroporation-mediated transformation of protoplasts, and the particle bombardment of tissues. The recent utilization of the *Agrobacterium* system in transforming rice paved the way to a more efficient, less costly, and more reliable method. In rice the development of these transformation systems was reflective of the scientific knowledge of the era. The development of each gene delivery technology conducted in rice, the advantages and disadvantages, and the important contributions in rice improvement based on experimental results and published data will be discussed.

Key words: Genetic engineering, transformation, rice, protoplasts, polyethylene-glycol, electroporation, particle bombardment, *Agrobacterium tumefaciens*, monocots, crop improvement.

INTRODUCTION

Rice production and consumption is a very important issue in Asia since 92% of the world's rice is produced and consumed there and a doubling of the Asian population is predicted to occur in about 50 years. Yields of future varieties must increase consistently in order to attain self-sufficiency in rice production. Solving this problem will entail development of rice varieties which have higher yields, excellent grain quality, resistance to biotic and abiotic stresses; and the inputs e.g., land, water, and fertilizer should be used more effectively.

To expedite this genetic improvement in rice, genetic engineering can be used as a powerful and novel means to complement the traditional methods of plant improvement. Genetic engineering is a method of modifying plants for the improvement of characters by increasing genetic variability by the introduction of foreign genetic material and by enhancing the expression of endogenous genes within a shorter time than conventional breeding. Through genetic engineering, 45 countries have released their first transgenic plants (James 1997), which contain resistances to biotic and abiotic stresses and improved product quality.

Genetic transformation of many dicotyledonous plant species has been routine through the use of the natural vector *Agrobacterium tumefaciens*. Monocotyledonous species, including the economically important cereals, are generally considered non-transformable through *A. tumefaciens* (Binns and Thomashow 1988). With these limitations, other methods of transforming cereals were developed including the polyethylene glycol-mediated DNA transformation (Uchimiya et al. 1986; Peng et al. 1990), and electroporation-mediated transformation (Toriyama et al. 1988; Rao et al. 1995) of protoplasts, and the particle-bombardment method (Christou et al. 1991; Li et al. 1993) of tissue masses. Many attempts have been made to transform rice using *A. tumefaciens* (Raineri et al. 1990; Gould et al. 1991; Chan et al. 1992; 1993; Li et al. 1992; Hiei et al. 1994), but until only recently, the results were limited or equivocal. Independent studies conducted using the different transformation methods revealed significant differences in terms of transgene copy number, sterility of transgenic plants, transgene methylation, and stability of the transgene.

MATERIALS AND METHODS

Protoplast Isolation, Transformation, Cell Selection, and Culture

Protoplasts were isolated from Radon cell suspension culture following the procedures described by Lee et al. 1989 with some minor modifications by Peng et al. (1990). Protoplasts were floated in 6.5 M sucrose to separate viable from dead protoplasts and debris. Transformation methods were as described by Peng et al. (1990). Freshly isolated protoplasts were suspended in 1 mL CPW at a density of 2 x 10^7 cells/mL. The plasmid pCPPGUS (Zmc13 promoter/*gus*A) and pPUR (CaMV35S/*gus*A/*nos*) were co-transformed independently with p35SBarB (CaMV35/*gus*A/*bar*B) at 50 µg DNA per mL protoplast suspension. The treated protoplasts were suspended in liquid Kao medium (Kao and Michayluk 1975; Lee et al. 1989) and plated at a density of 5 x 106 protoplasts per mL. About 200 to 250 µL of treated protoplast suspension was plated on a Millipore filter (type AA, 0.8 mm) placed on top of either IR52 or Taipei 309 feeder cells embedded in 0.8% Sea Plaque (FMC Bioproducts, Rockland ME 04841) agarose in Kao medium. After 10 to 14 days of growth, the Millipore filters containing the calli were transferred to Kao medium without feeder cells for another 7 to 10 days of growth. The Millipore filters containing the calli were then transferred to selection me-

dium LSP containing 8 mg/L phosphinothricin (PPT, the selectable agent for bar gene resistance) for a 3-week selection period (Rathore et al. 1993). PPT-resistant calli were transferred to LS0.6 medium (Peng et al. 1992) for a 3-week proliferation period. PPT-resistant, embryogenic calli were subsequently transferred to an MS regeneration medium (Murashige and Skoog 1962) containing 2.5 mg/L kinetin and 30 g/L sucrose. Regenerated shoots were transferred to an MS medium without hormones for root development. Regenerated T_0 plants were grown in the growth chamber as described by Lee et al. (1989).

Test for Resistance to the Herbicide Basta

Two-month-old putatively transformed and untransformed regenerated plants were tested for Basta (herbicide with phosphinothricin as the active ingredient) resistance. An 8- to 10-cm portion of a young, newly emerged leaf was briefly dipped in a solution containing 0.25% Basta (500 mg/L PPT), and scored after one week for herbicide damage.

Agrobacterium tumefaciens Strains

A. tumefaciens strains At656 (pTiEHA101) and At657 (without a Ti-plasmid) (Li et al. 1992; Ritchie et al. 1993; Gelvin and Liu 1994) contained the binary vector pCNL56. The binary vector contained the chimeric promoter of mannopine synthetase and a truncated CaMV35S promoter (mass-35S)/B glucuronidase with an intron (*gus*A-int)/octopine synthetase terminator (ocs) and the neomycin phosphotransferase gene (*npt*II) gene in the T-DNA which confers resistance to G418 (geneticin) and kanamycin.

A. tumefaciens strain LBA4404 (pTOK233) was obtained from Japan Tobacco Inc. The binary vector pTOK233 contained the virB, *vir*C, and *vir*G genes derived from the supervirulent Ti-plasmid pTiBo542 (Jin et al. 1987), and the *hpt*, *npt*II, and the *gus*A-int within the T-DNA region.

Preparation of *A. tumefaciens* Culture, Plant Materials, and Co-cultivation Methods

A. tumefaciens suspensions were prepared from a 4-day culture in AB medium (Chilton et al. 1974) containing sucrose or glucose for At656 or LBA 4404, respectively (Cangelosi et al. 1989). The bacteria were preinduced overnight with 100 to 200 μM acetosyringone (AS) in PIM2 medium (Aldemita and Hodges, 1996). *A. tumefaciens* were grown to give an absorbance of 600 nm of 1.6 to 1.9, and AS was adjusted to give a final concentration of 200 μM to the bacterial suspension immediately before co-cultivation.

IR54, TCS10, IR72, Nortai, and Radon immature embryos with sizes ranging from <1.5 to >2.5 nm were isolated from sterile young panicles and plated on NgGAs (Hiei et al. 1994) with 10 μL *A. tumefaciens* suspension placed on each immature embryo.

Callus Induction, Selection, and Plant Regeneration

A. tumefaciens-inoculated Nortai and TCS10 immature embryos were transferred to LS0.5 (Hiei et al. 1949) 2 days after co-cultivation with At656 (pNCL56). Radon, TCS10, and IR72 infected immature embryos were plated onto N6CS medium 3 days after co-cultivation with LBA4404 (pTOK233). The antibiotics carbenicillin (250 mg/L) and cefotaxime (100 mg/L) were added to the callus induction medium to kill the *A. tumefaciens* (Li et al. 1992).

Immature embryos inoculated with LBA4404 were allowed to grow for three weeks without selection and 1 to 2 mm pieces of calli were transferred to LS0.5 containing 30 mg/L hygromycin for a 3-week selection period, followed by a 3-week proliferation in a medium without hygromycin. Hygromycin resistant embryogenic calli were transferred to MS (Murashige and Skoog 1962) regeneration medium containing 30 g/L sucrose, 2.5 mg/L kinetin, and 0.1 mg/L NAA (MS2.5K), and incubated in the dark for one week, before exposure to light. Regenerated plants were then transferred to soil in the growth chambers with 70% relative humidity, 30°C/27°C day/night temperatures, and a 12 h daylength.

GUS Assays

Histochemical and fluorimetric enzyme assays were conducted to detect and quantify stable GUS activity on explants 10 weeks after transformation. For histochemical staining, cells and tissues were incubated in X-gluc solution (Jefferson 1987) at 37°C overnight (14-16 h) and scored for evidence of GUS activity; the presence of the blue colored substrate. GUS activity of leaves from transformed plants were determined fluorimetrically following procedures by Jefferson et al. (1987). GUS activity (fluorescence of the 4-methylumbelliferon) was detected using a UV lamp.

NPTII Assays

Dot blot NPTII analysis was conducted according to Peng et al. (1993) using crude protein extracted from calli derived from immature embryos of Nortai infected with At656. A 100 μL aliquot of the protein extracts prepared as described in the GUS activity determination assays, were loaded onto each of the wells of a 96-well microtiter plate (Corning Catalog number 25861, Corning, New York 14831) with a multichannel pipette. Fifty μL of the assay mixture was added to each well and incubated at 37°C for 2 h. A 50 μL aliquot of the reaction mixture from each well of the microtiter plate was blotted onto the P81 paper, air-dried, washed, and taped on a dry piece of 3MM paper. It was then wrapped in a Saran wrap prior to autoradiography for 24 h at -70°C with the said of an intensifying screen (Dupont, Cronex-Plus).

Genomic DNA Isolation, Southern Blot, and Progeny Analyses

Genomic DNA was isolated from the putatively transformed callus and plants following the method of McCouch et al. (1988). The procedures for restriction enzyme digestion, electrophoresis, and the Southern blot analyses were according to Sambrook et al. (1987). Twenty μg genomic DNA was used for Southern blot analysis. T1 seeds of transformed Radon and TCS10 plants were planted to determined the segregation ratios for GUS activity, and to eliminate the possibility of *A. tumefaciens* contaminants (or inhabitants) being responsible for the results.

RESULTS AND DISCUSSION

Choice of an Efficient Selectable Marker

In genetic transformation experiments, the choice of an efficient, effective, and reliable selectable agent is a critical step. More importantly, the selectable agent should also allow the regeneration of transformed plants.

There are many selectable markers commonly used and available in the market today; neomycin phosphotransferase gene (*nptII*) for kanamycin or geneticin resistance, *bar* for phosphinothricin resistance, hygromycin phosphotransferase gene (*hpt*) for hygromycin resistance, among others. The *nptII* gene was successfully used in dicot transformation systems which was carried over in the development of the monocot transformation systems. In the initial studies on *A. tumefaciens*-mediated transformation, *A. tumefaciens* was used to infect immature embryos of Nortai and TCS10. A high callus transformation efficiency was obtained (Table 1), and transformation was confirmed through Southern blot and NPTII dot blot assays (Figures 1 and 2, respectively). However, successive attempts to regenerate plants from the transformed calli failed.

In another study, using the polyethylene glycol-mediated transformation of protoplast, co-transformation of protoplasts with a pCPPGUS and pKhan (cauliflower mosaic virus promoter (CaMV35S)/*nptII*) of IR54 and Radon protoplasts yielded transformed but unregenerable calli (data not shown). These results confirmed the earlier observations (Raineri et al. 1990; Chan et al. 1993; Ritchie et al. 1993) that for some unknown reasons the *nptII* gene is not a suitable selectable marker in monocot transformation.

The selectable marker *bar* gene was successfully used in direct-DNAuptake by other workers (Christou et al. 1991; Datta et al. 1992; Rathore et al. 1993). The *bar* gene-containing plasmid, p35SBarB was used as the selectable marker in our study on the evaluation of the corn pollen-promoter activity in transgenic rice. Selection for transformed cells in medium containing phosphinothricin (PPT) greatly facilitated the selection of calli transferred to proliferation medium, which is important in minimizing the number of escapes. The growth of PPT-resistant and putatively transformed calli in selection medium was very distinct from the calli in control plates (Figure 3). The concentration of PPT used in this experiment was

Table 1. Efficiency of obtaining stably transformed calli from immature embryos of different sizes of Nortai and TCS10 based upon GUS activity following inoculation of the embryos with *Agrobacterium* strain At656 (pCNL56).

Variety	Embryo size (mm)	No. of immature embryos (IE)		Number of calli plated in			
		Treated	Plated in 30 mg/L G418*	50 mg/L G418*	Prolif. medium	Reg'n medium	Eff. ** (%)
Nortai[a]	<1.5	120	93	617	317	250	39/120 (32.5)
	1.5-2.5	144	108	574	205	196	52/144 (36.1)
	>2.5	50	24	122	67	48	2/50 (4.0)
TCS10[b]	>1.5	34	17	96	6	6	2/34 (5.9)
	1.5-2.5	8	7	31	0	0	0

[a]Total of three experiments
[b]Total of two experiments
I.E. = immature embryos
*Embryos plated in 30 mg/L G418 for 3 week selection. Calli formed were cut into 1 to 2 mm pieces and plated in 50 mg/L G418
GUS activity was determined ten weeks after treatment with *A. tumefaciens*.

$$**\%\ \text{Eff.} = \frac{\text{no. of embryos that produced calli that were GUS}^{+}}{\text{number of embryos plated}} \times 100$$

higher (8 mg/L PPT) than the one used by Rathore et al. (1993) (2.5 to 5 mg/L PPT). However, the efficiency of obtaining Basta^{R} plants obtained was comparable; 94.4% (Rathore et al. 1993) and 86.2% (Table 2).

Hygromycin selection has been used routinely to identify transformed protoplast (reviewed by Ayres and Park 1994); it allows a good discrimination between transformed and nontransformed cells and does not seem to induce albinoisms or fertility problems. The hygromycin gene was used in this study as a selectable marker in developing an *A. tumefaciens*-mediated transformation of rice. Efficiencies of obtaining transformed calli, based upon hygromycin-resistance and GUS activity, were 40% for Radon, 6.3% for TCS10, and 7.9% for UR72 (Table 3). Using the same criterion (i.e., HYG^{+}-GUS^{+}) for scoring plant transformation, average transformation efficiencies were 27% for Radon, about 4% for IR72 and about 1% for TCS10. Although the latter efficiencies may seem low, a range for

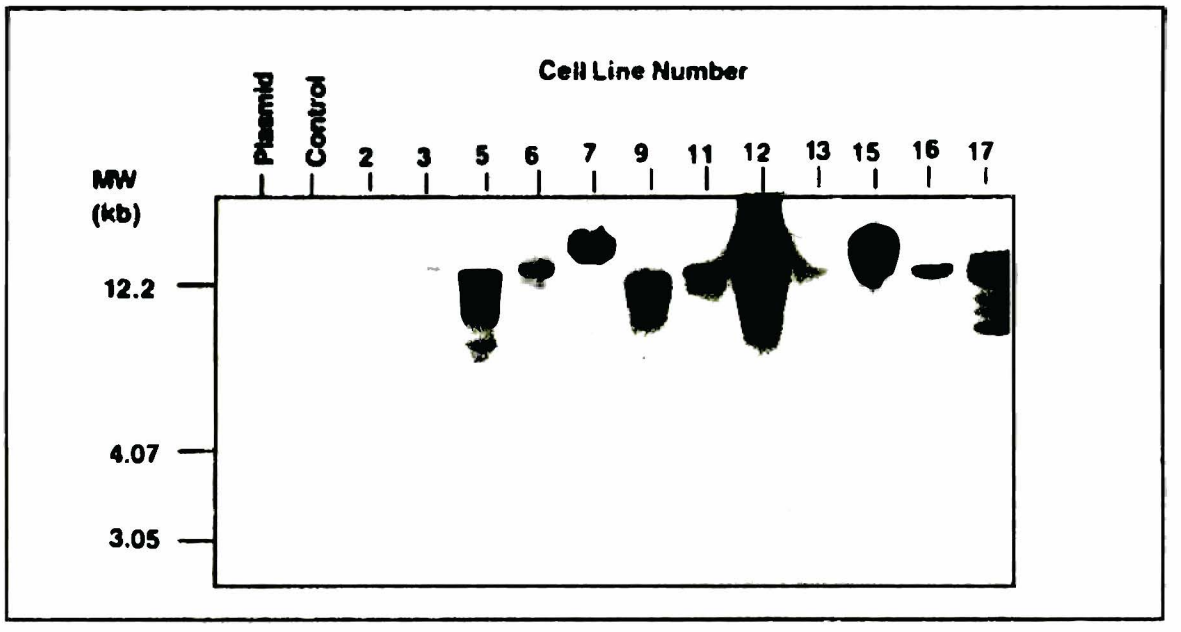

Figure 1. Southern blot of genomic DNA of cells derived from immature embryos of Nortai inoculated with At656 (pCNL56). The genomic DNA was restricted once with *Sal*I which cuts the T-DNA once outside the *gus*A gene and probed with the 1.8 ib *gus*A fragment. Lane 1 = 15.9 kb pCNL56 digested with *Sal*I, Control = DNA obtained from uninoculated embryo, Cell line number 2 = DNA obtained from untransformed cell line of an inoculated embryo, Cell line numbers 3 to 17 = DNA obtained from transformed cell lines from inoculted embryos.

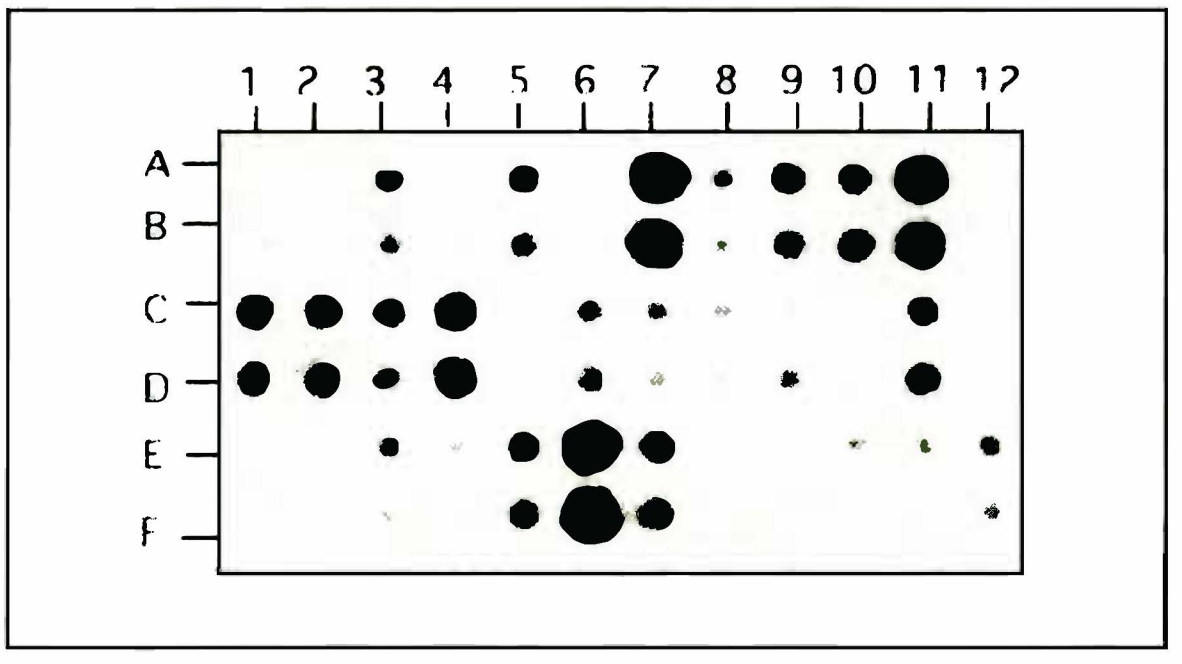

Figure 2. An NPTII dot blot assay of crude protein extracted from cell lines derived from Nortai immature embryos inoculated with At656 (pCNL56). Each treatment was done in duplicate; A1 = no protein, A2 = cells from uninoculated embryos, A3 = (+) control (corn cells expressing NPTII), A4 to C12 = Cell line numbers of putative transformants. Black spots mean presence of the gene.

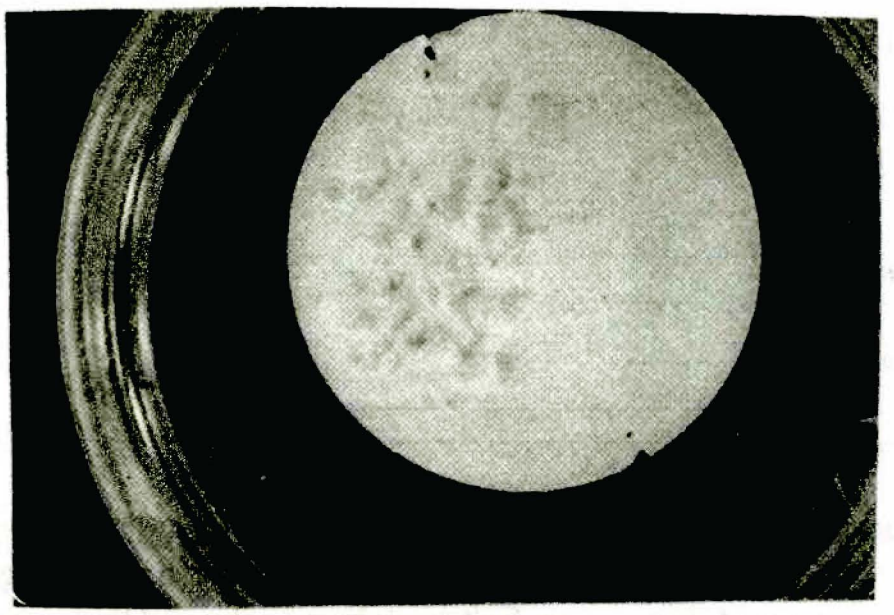

Figure 3. Selection of transformed cells and plants on PPT: (a) callus growth from DNA-treated protoplasts in 8 mg/L PPT (b) callus without PPT.

Table 2. Number of Basta^R, *gus*A$^+$, and plants expressing GUS in mature pollen.

Number of plants analyzed				
Total number regenerated	Independent transformants	Basta^R	*gus*A$^+$ by Southern blot	Expressing GUS in pollen
48	29	25	18	13

Table 3. *A tumefaciens*-mediated transformation efficiency in immature embryos of three rice varieties infected with LBA 4404 (pTOK233).

Variety	Expt. No.	No. of immature embryos		No. I.E. with G$^+$ calli/I.E. plated (%)	**No. I.E. with G$^+$ plants/ I.E. plated (%)**	No. of plants per I.E.	Total G$^+$ plants/ total plants (%)
		plated	with calli				
Radon	1	62	42	20/62 (32.2)	**18/62 (29.0)**	1-16	86/101 (85.1)
	2	70	62	43/70 (61.4)	**24/70 (34.3)**	1-18	125/165 (75.8)
	3	75	53	24/75 (32.0)	**22/75 (29.3)**	1-26	100/149 (67.1)
	4	35	34	14/35 (40.0)	**7/35 (20.0)**	1-9	16/56 (28.6)
	5	28	14	7/28 (25.0)	**2/28 (7.1)**	1-30	19/37 (51.3)
Total		270	205	108/270 (40.0)	**73/270 (27.0)**	1-30	346/508 (68.1)
TCS10	1	203	119	13/203 (6.4)	**1/203 (0.5)**	1	1/2
	2	84	63	5/84 (6.0)	**1/84 (1.2)**	1	1/2
Total		287	182	18.287 (6.3)	**2/(287) (0.7)**	I	2/4

Table 3. (continued)

Variety	Expt. No.	No. of immature embryos		No. I.E. with G^+ calli/I.E. plated (%)	**No. I.E. with G^+ plants/ I.E. plated (%)**	No. of plants per I.E.	Total G^+ plants/ total plants (%)
		plated	with calli				
IR72	1	60	48	4/60 (6.7)	**5/60 (8.3)**	2-8	6/19 (31.6)
	2	105	53	9/105 (8.6)	**2/105 (1.9)**	3-4	3/7 (42.38)
Total		165	101	13/165 (7.9)	**7/165 (4.2)**	2-8	9/26 (34.6)

*I.E. = immature embryos: G^+ = GUS positive

A. tumefaciens cells were preinduced overnight in 100 μM AS. Immediately prior to adding *A. tumefaciens* to the embryos, AS were adjusted to 200 μM. The co-cultivation medium also contained 100 μM AS.

1-5 embryos producing at least one independent transformer per 100 embryos cultured for *indica* varieties is quite satisfactory.

Gene Integration Affecting Transgene Expression

Southern blot analysis of genomic DNA of transgenic plants obtained from polyethyleneglycol co-transformation of protoplasts with pCPPGUS and p35SBarB cut with *Eco*RI (Figure 4) showed a wide range in the number of gene integration events as indicated by the hybridization bonds with the *gusA* gene probe. The number of introduced genes ranged from 1 to more than 10 copies and the banding patterns were indicative of rearrangements of the integrated gene, which is observed commonly in transformed plant materials through direct-DNA uptake (Rhodes et al. 1988; Lyznik et al. 1989; Peng et al. 1992; Rathore et al. 1993).

Some of the transgenic plants which were found to contain the *gus*A gene in Southern blot analyses expressed GUS (determined histochemically) in the pollen grains, i.e., transgenic plant numbers 2, 15, 25, 27, and 34 (Figure 5). On the other hand, plant numbers 11, 12, 13, 14-2, and 17 did now show GUS activity in the pollen (data not shown) although they were *gus*A^+ by Southern blot (Figure 4). This probably indicates transgene methylation and inactivation might have occurred in these transgenic plants (Finnegan and McElroy, 1994).

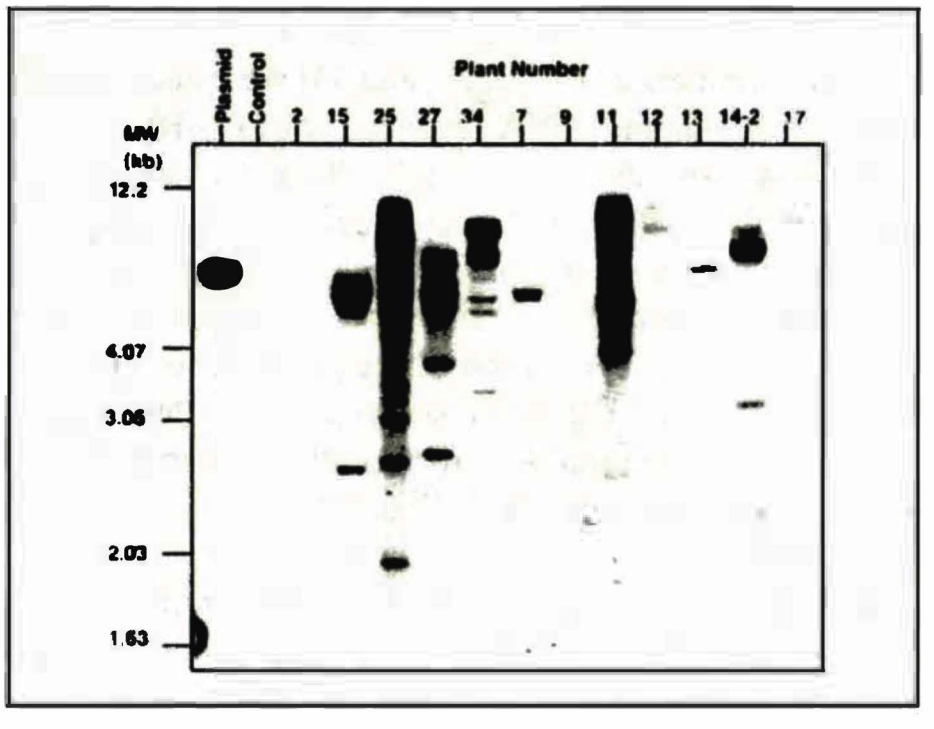

Figure 4. Southern blot of genomic DNA of transgenic plants (2-17) after a restriction digest with *Eco*RI and probed with a 1.8 kb *gus*A fragment. Lane 1, expected size (5.6 kb) of plasmid pCPPGUS; Lane 2, untransformed control.

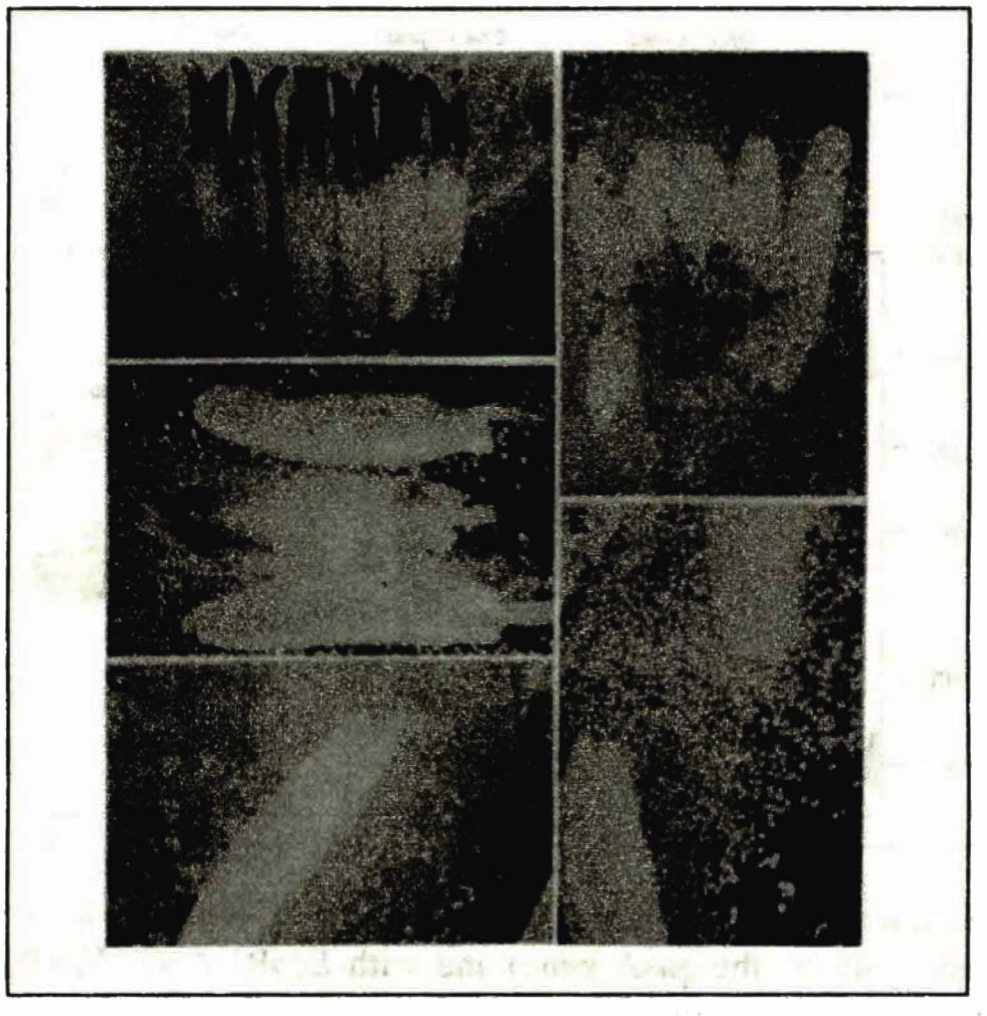

Figure 5. Histochemical GUS staining of mature pollen of transgenic plant number 2(A), 15(B), 25(C), 27(D), and 34(E).

The genomic DNA from transgenic plants that showed GUS activity in the mature pollen (plant numbers 2, 15, 25, 27, and 34) were analyzed further. A Southern blot of undigested genomic DNA, digested with *Eco*RI (cuts once in the plasmid outside the gusA), and digested with *Eco*RI and *Hind*III (excises the 2.5 kb fragment containing the -260 *Zmg* 13 promoter/*gus*A/*nos* fragment) is shown in Figure 6. The 2.5 kb fragment (arrow) was present in all the lanes of genomic DNA digested with *Eco*RI and *Hind*III. GUS activities in the pollen appeared to be correlated with the complexity of integration of the *gus*A gene. Plant number 2, which had only one integration site (Figure 6), showed the greatest number of pollen expression GUS (visual examination) Figure 5A). Plant number 25, of which only one pollen with GUS activity was identified (Figure 5), had multiple integration events (Figure 4). The rest of the T_o plants showed intermediate GUS activity.

On the other hand, Southern blot analyses of hygromycin-resistant and GUS positive plants obtained from *A. tumefaciens*-mediated transformation, revealed that the *gus*A gene integration patterns were simple (Figure 7A). In Figure 7A, genomic DNA restricted with *Xba*I and probed with the *gus*A coding sequence showed the presence of two or three bands for each plant, and most of the bands were larger than 8.3 kb, indicating integration into the genome. Integration of *gus*A into the rice genome was shown to be as T-DNA by Hiei et al. (1994) and is

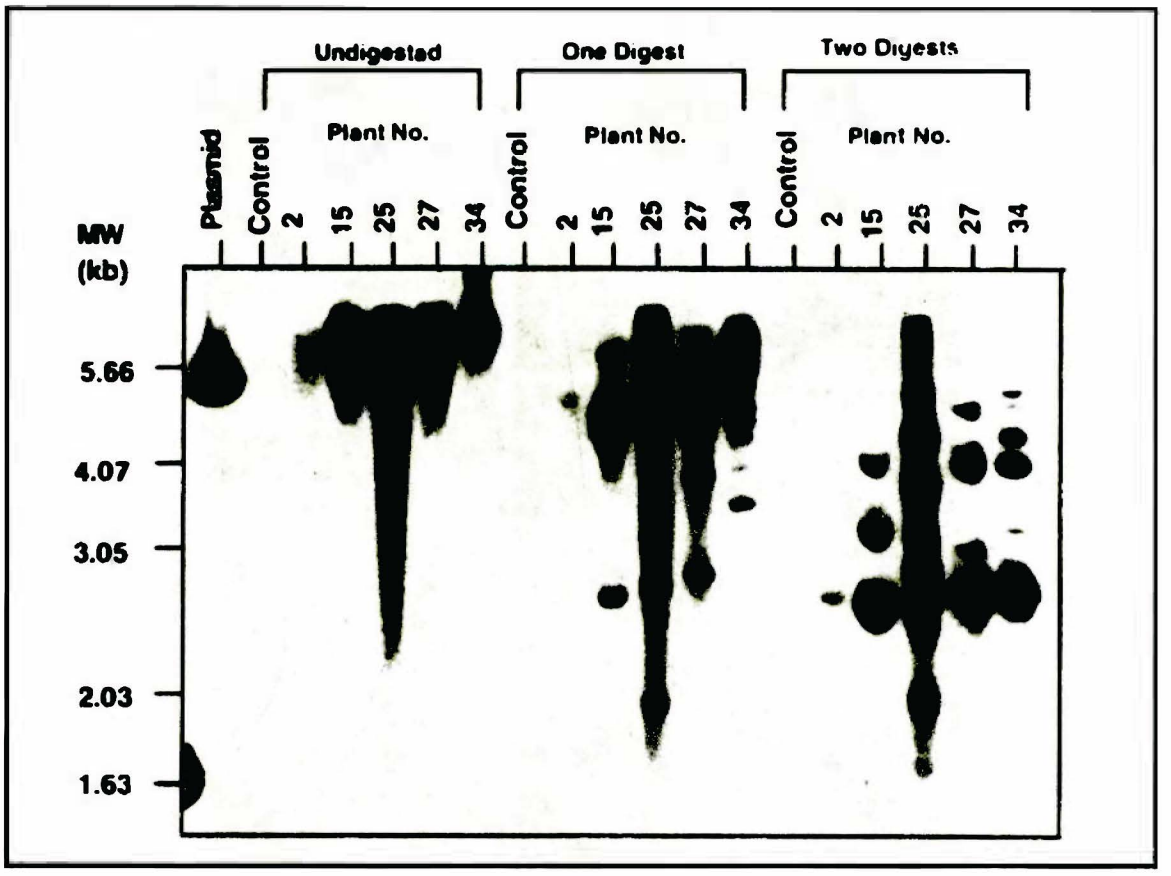

Figure 6. Southern blot of genomic DNA undigested, digested with *Eco*RI (cuts once outside the *gus*A gene) and with *Eco*RI AND *Hind*III (releasing the 2.5 kb fragment -260 Zmg 13/*gus*A/*nos*) of plants expressing GUS in mature pollen. Lane 1, expected size (5.6 kb) of plasmid pCPPGUS; Lane 2, untransformed control.

presumably random, as in dicots, but in comparison to direct DNA uptake methods (Peng et al. 1992; Rathore et al. 1993; Rao et al. 1995; Alvarez 1997), the number of copies of genes integrated, and the frequencies of gene rearrangements appear to be much fewer (Figure 7B).

Sterility of Transgenics

Recovery of useful transgenic plants with a workable fertility and agronomically good morphology is a measure of successful transgenic study. It is thus important to study the conditions that will promote the recovery of such transgenics.

It has been observed in this study and in other earlier transformation work that the use of direct-DNA uptake methods such as biolistics and polyethyleneglycol or electroporation-mediated transformation of protoplasts produce transgenic plants with a high degree of sterility (Peng et al. 1992, Alvarez 1997). In the present study, the transgenic plants recovered from polyethylene glycol mediated transformation of protoplasts produced very few fertile seeds even if most of the plants were morphologically normal (data not shown). However, by using the natural vector *A. tumefaciens,* transgenic plants produced have very little sterility problems (data not shown).

The decreased fertility may be due to a combination of transformation method used, the general sensitivity of pollen to the transgenes, and the possible cell mutations that occurred during the establishment of the suspension culture. The use of cell suspensions as materials for transformation through biolistics or as source of protoplasts in polyethylene-glycol or electroporation-mediated transformation may result in a high frequency of sterility. An increase in sterility in T_0 plants regenerated from PEG-mediated transformation is proportional to the age of the suspension culture used (Battraw and Hall 1992; Peng et al. 1992).

In *A. tumefaciens*-mediated transformation, regenerable primary calli, immature embryos, and young inflorescences can be used as explants overriding the need to develop cell suspensions.

Mendelian Inheritance in the Transgenic Progenies

Phenotypic and molecular analysis of the progenies of the transgenic plants is a basis of the stability of the transgene. Reports indicate that the transformation method used and the copy number may affect the stability of the transgene in the progenies. Previous studies conducted by Peng et al. (1990) and Rathore et al. (1993) indicated that the occurrence of non-Mendelian inheritance in the progenies of kanamycin-resistant and Basta-resistant transgenic plants, respectively, obtained from polyethylene-glycol-mediated transformation of protoplasts was due to the multiple copies of the transgene in the primary regenerant. In addition there were also a lot of methylation events observed. Similar observations were obtained from progenies of transgenic plants produced through the particle bombardment method (Alvarez 1997).

On the other hand, progenies of transgenic plants produced through *A. tumefaciens*-mediated transformation exhibited a Mendelian inheritance. Progenies of Radon 1 which contains a single copy of the *gus*A gene (Figure 7A) gave the expected ratio of 3 GUS^+: 1 GUS^- (Table 4), the segregation ratio for a single locus integration of the transgene in the rice genome.

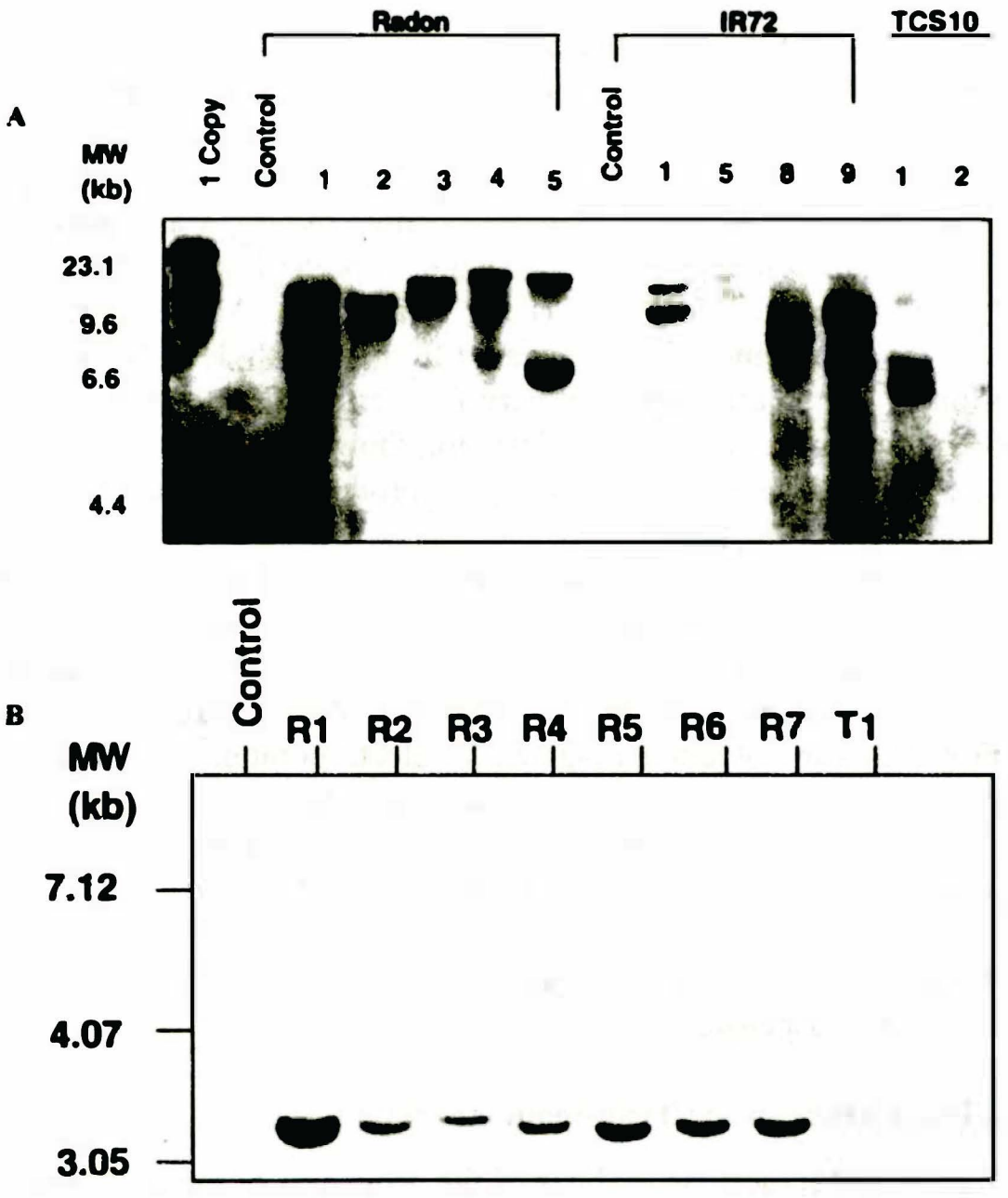

Figure 7. Southern blot of genomic DNA of plants transformed with *Agrobacterium tumefaciens* strain LBA4404 (pTOK233). (A) Genomic DNA of transgenic Radon, IR72, and TCS10 plants restricted with XbaI (cuts at one site within the T-DNA but outside the *gus*A gene and in the binary vector). The lane labeled one copy contains the plasmid cut with *Xba*I which yields a fragment of 12.3 kb. (B) Genomic DNA of transgenic Radon and TCS10 plants restricted with *Hind*III (cuts at two sites releasing the 3.14 kb fragment 35S-*gus*A-int-*nos*). Both A and B were probed with a 1.8 kb *gus*A fragment.

Table 4. Progeny analysis of transgenic T1 seedlings derived from Radom 3 and TCS10 parent (T_0) transformed with *Agrobacterium tumefaciens* strain LBA4404 (pTOK233).

Parents and (Panicle No.)*	Progeny of plants tested for GUS activity		
	GUS^+ seedlings	GUS^- seedlings	Total
Radon 3- (10)	23	11	34
Radon 3- (1)	25	2	27
Radon 3- (2)	32	14	46
Total	80	27	107
Expected**	80.25	26.75	
TCS10 1-(1)	8	2	10
TCS10 1-(3)	21	4	25
TCS10 1-(4)	14	4	18
TCS10 1-(2)	16	2	18
Total	59	12	71
Expected**	53	18	

*Numbers in parentheses are the panicle numbers from T_0 parent plants.

**Expected is the number based on a predicted 3:1 segregation ratio for a single gene. For Radon segregants, the Chi-quare = 0.0032 giving a probability of 0.95-0.99, and for TCS10 segregants, the Chi-square = 2.6792 giving a probability of 0.10-0.20.

CONCLUSION

Genetic engineering to improve the resistance of rices of important diseases have been conducted in other laboratories. To date, China is the only country where field release of transgenic rice containing insect resistance gene has been approved. Besides the restriction problems imposed on transgenic products in other countries, the stability of the transgene is also a problem. In most cases, the transformed rices that are in the pipeline for field release were obtained through the protoplast system or the particle bombardment method.

The preceding discussion indicated the problems associated with the use of the direct-DNA uptake methods; multiple copy number of the transgene, sterility in the regenerants, non-Mendelian inheritance of the transgenes in the progenies, and the relatively non-cost effective transformation efficiency. With these problems, the development of the *A. tumefaciens*-mediated transformation procedure for rice is a necessary step in the quest for a more effective transformation system.

Some well-funded laboratories are designed to use the transformation system that will suit their needs, expertise, notwithstanding the cost. Laboratories in developing countries need a transformation system that is cost effective and that

produces stable transgenics. It is therefore recommended that the natural vector *A. tumefaciens* be used in introducing economically important genes into *indica* and *japonica* rices.

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