Theor Appl Genet (1992) 83:855–863

Transgenic indica rice plants

Jianying Peng, Halina Kononowicz, and Thomas K. Hodges*

Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA

Received June 4, 1991; Accepted July 18, 1991
Communicated by G. S. Khush

Summary. We have established a system to genetically engineer indica rice plants. In order to obtain transgenic plants, genes were introduced into protoplasts isolated from suspension cells of the indica rice var 'IR54' with the aid of polyethylene glycol (PEG). The *neo* gene was on pKAN and the *gusA* gene was on pPUR. The promoter for both genes was CaMV35S. Transformed calli were readily recovered from medium supplemented with G-418. In contrast, kanamycin interfered with plant regeneration from protoplast-callus. Transgenic plants were regenerated from calli resistant to G-418 in several separate experiments and grown to maturity in a growth chamber. Southern blot analysis of DNA isolated from leaves of T_0 plants verified the presence of the transferred *neo* and *gusA* genes in the plant genome. A study of gene expression showed that the CaMV35S-*gusA* gene was active in all of the organs examined. Mendelian inheritance of the introduced *gusA* gene was observed in progeny obtained by backcrossing the T_0 plants to untransformed plants.

Key words: Transformation – PEG – Indica rice – Protoplast culture and regeneration – *neo* – *gusA*

Introduction

Rice (*Oryza sativa* L) is the major nutrition source for about 40% of the world's population, including many of the people living in the developing countries. Japonica and indica rice are the two major subspecies growing in different regions of the world, and they differ in morphological characteristics and grain qualities; each plays an important and irreplaceable role in feeding the world's population. Conventional plant breeding methods have contributed to great improvements to both indica and japonica rice varieties; however, genetic engineering provides an opportunity for introducing foreign genes for pest resistance and better nutritional qualities that are not readily incorporated into new varieties by conventional breeding methods.

The introduction of foreign genes into monocots has so far depended on direct DNA delivery into protoplasts or cells. Among these, polyethylene glycol (PEG)- and electroporation-mediated DNA delivery to protoplasts have shown success in transforming wheat (Lorz et al. 1985), corn (Rhodes et al. 1988; Lyznik et al. 1989), and rice (Toriyama et al. 1988; Zhang et al. 1988; Zhang and Wu 1988; Shimamoto et al. 1989; Datta et al. 1990; Peng et al. 1991). In addition, direct delivery of DNA into cells via particle bombardment was used recently to transform corn cells with subsequent regeneration of transgenic plants (Fromm et al. 1990; Gordon-Kamm et al. 1990).

Combinations of protoplast culture techniques and direct DNA delivery techniques have led several groups of scientists to produce fertile transgenic japonica rice plants (Shimamoto et al. 1989; Hayashimoto et al. 1990). A system to regenerate fertile indica rice plants from protoplasts (Lee et al. 1989) and to co-transfer the selectable *neo* and screenable *gusA* genes into rice protoplasts (Peng et al. 1990) has been reported from this laboratory. In the present article we report the production of transgenic indica rice plants and the transmission of the foreign DNA to offspring.

* To whom correspondence should be addressed

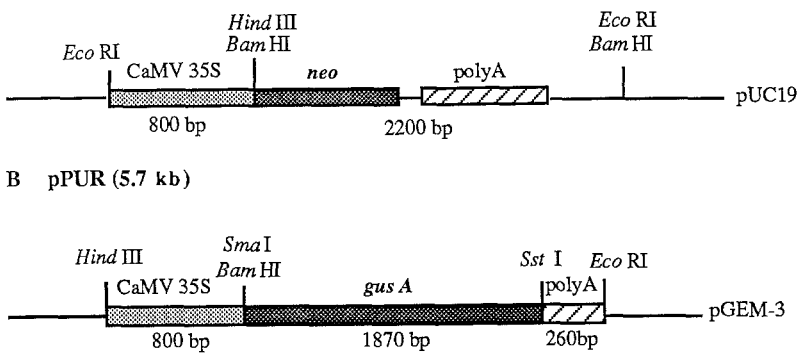

Fig. 1 A, B. Key restriction sites of plasmids pKAN and pPUR

Materials and methods

Cell suspension and protoplast culture

Two cell suspension lines, IR54-5 and IR54-6 were initiated in August 1988 and January 1989, respectively, from 4-week-old calli derived from immature embryos. The cell cultures were initiated and maintained essentially as described by Lee et al. (1989). Briefly, the cell suspensions were cultured in N6 basal medium supplemented with 4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 20 m*M* proline, and 3.0% sucrose, and maintained in the dark on a shaker at 80 rpm. During the first month of culture the medium was replaced twice a week without doing any subculturing. After about 1 month or when the cultures had grown to exceed 4 ml packed cell volume (pcv), subculturing was initiated by transferring 4 ml pcv into 40 ml liquid medium at weekly intervals. During the 2 months following the initiation of subculturing the medium continued to be replaced on the third day after the subculture. After about 3 months the cultures were maintained with just the weekly subculturing. Four days prior to isolating the protoplasts the cell cultures were transferred to AA medium (Müller and Grafe 1978) at 2 ml pcv per 40 ml. The protoplasts were isolated and cultured as described by Lee et al. (1989) with the minor modifications mentioned below.

Transformation and selection

The plasmids used in the transformation experiments were pKAN, which contained the CaMV35S promoter, the *neo* gene, and the T-DNA polyadenylation site (orf 26), and pPUR, which contained the 35S promoter, the *gusA* gene, and the *nos* polyadenylation region (Fig. 1). The plasmid DNA was prepared using a protocol published by Maniatis et al. (1982). For transformation, fresh protoplasts were suspended in CPW medium (Frearson et al. 1973) with 0.4 *M* mannitol. One-milliliter aliquots of protoplasts at a density of 2×10^7/ml were mixed with 50 µg of each plasmid DNA and 1.1 ml 40% PEG in Krens' F solution (Krens et al. 1982) supplemented with 1.0% glucose. The incubation mixture was heat shocked at 45 °C for 5 min, chilled on ice for 20 sec, and then incubated at 30 °C for another 25 min. After incubation, the mixture was slowly diluted with 30 ml Krens'F solution over a 30-min period. Protoplasts were collected after centrifugation at 100 *g* for 15 min and suspended in Kao medium (Kao and Michayluk 1975) modified after Lee et al. (1989) at $2-5 \times 10^6$ protoplasts/ml. Aliquots of 200 µl of protoplasts were plated on Millipore filters (type AA, pore size 0.8 µm) on top of a mixture of modified Kao medium plus 0.8% Sea Plaque agarose (FMC BioProducts, Rockland, Md., USA) containing IR52 cells (Lee et al. 1989) and grown for 3 weeks. Protoplast cultures were then transferred together with the Millipore filters to Petri plates containing selection medium that consisted of MS basal medium (Murashige and Skoog 1962) supplemented with 2.0 mg/l 2,4-D, 0.5 mg/l zeatin, 3.0% sucrose, 0.6% agarose (the agarose used was from BRL, gelling temperature 36°–42°C unless otherwise stated), and 5–20 mg/l antibiotic G-418. After 3 weeks on medium containing G-418, individual calli resistant to G-418 were transferred to new plates containing LS medium (Linsmaier and Skoog 1965) supplemented with 0.5 mg/l 2,4-D, 2% sucrose, and 0.6% agarose to proliferate for an additional 2 weeks. These selected calli were then transferred to regeneration medium composed of N6 basal medium (Chu et al. 1975), 10 mg/l kinetin, 0.1 mg/l naphthaleneacetic acid (NAA), and 3.0% sucrose solidified with 0.6% agarose. Three weeks later, calli with shoots were transferred to MS medium supplemented with 3.0% sucrose, 0.6% agarose, and no plant growth regulators. Regenerated plantlets were allowed to grow in small bottles containing the same medium for 2 more weeks. Growth conditions for the plantlets were as described by Lee et al. (1989).

Growth of transgenic plants and transmission of the foreign gene to T_2 progeny

Plants regenerated from protoplast-derived calli were designated as T_0 plants. T_1 seeds were derived from a cross between T_0 plants and seed-grown plants of 'IR 54', and the T_1 seeds gave rise to T_1 plants. Seeds of self-pollinated T_1 plants were designated as T_2 seeds, and the plants from these seeds were designated as T_2 plants.

Regenerated transgenic plants (T_0) were transplanted to soil in a 6-inch pot and grown to maturity in growth chambers set at 30°/24 °C and 10/14 h for day/night conditions. The standard fertilization and watering schemes used in our laboratory (Koetje et al. 1989; Lee et al. 1989) were followed during the growth period. Because of sterility problems associated with T_0 plants, hand pollination was performed using pollen from seed-grown plants of 'IR54' to produced T_1 seeds. T_1 seeds were harvested, air dried, and heat treated at 52 °C for 3 days to break dormancy, then sterilized as described by Koetje et al. (1989). These T_1 seeds were germinated and grown in medium with ½ strength MS salts plus 3.0% sucrose and 0.6% agarose for 5 days before being planted in soil and grown in growth chambers under the conditions described above.

At the time of transplanting the T_2 plants, the endosperm residue and a root were collected from each plant for histochemical staining to determine GUS activity.

Southern blot analysis and GUS assay

DNA was extracted from leaf blades sampled 1–2 months after transplanting according to Mettler (1987) and then subjected to restriction enzyme digestion, electrophoresis, and Southern blot analysis according to the protocol of Maniatis et al. (1982). The DNA probe used for hybridization to the *neo* gene was the *Bam*HI fragment of pKAN, and to the *gusA* gene was the *Bam*HI-*Sst*I fragment of pPUR (Fig. 1). Fluorometric assays for β-glucuronidase (GUS) activity were performed on extracts from plant tissue following Jefferson's (1987) procedure, and GUS activity was expressed as nmol MU/mg protein per hour. For GUS assays of leaf tissue, the second leaf from the top of a plant was used. Protein content was measured by Lowry's method (1951).

Histochemical analysis of GUS expression

Histochemical staining was performed according to the method described by Jefferson (1987). Small pieces (0.3–0.5 cm) of root, leaf, and stem of transgenic plants were cut by hand, fixed in 0.3% formaldehyde in 10 m*M* MES, pH 5.6, and 0.3 *M* mannitol for 45 min at room temperature. These pieces were washed 4 times in 50 m*M* phosphate buffer, pH 7.0, and incubated in staining solution containing 1 m*M* of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Research Organics, Cleveland, Ohio, USA) in 50 m*M* phosphate buffer, pH 7.0 at 37 °C overnight. After staining, the tissue was cleared of chlorophyll by incubation for 10 min in a solution of 5% formaldehyde, 5% acetic acid, and 20% ethanol followed by incubation in 70% absolute ethanol. Tissue was embedded in Tissue Prep 2 medium (melting point 56.5 °C) and cut into 10-μm sections. The sections were placed in xylene and mounted on microscope slides for photography.

Results

Selection and regeneration of plants from G418-resistant calli

Protoplasts treated with PEG and plasmid DNA grew well when placed on Millipore filters positioned on top of a mixture of modified Kao medium and 'IR52' feeder cells for the first 3 weeks. After being subjected to G-418 selection for the following 3 weeks, most calli stopped growing, while a few putative transformants continued to grow (Fig. 2a). In control plates, the calli derived from protoplasts treated with PEG only (i.e., no DNA) did not continue to grow (Fig. 2b) when subjected to G-418 selection. Because G-418 concentrations ranging from 5 to 20 μg/ml were all effective in selecting the transformants (Table 1), we used 10–20 μg/ml in subsequent experiments. The number of calli resistant to G-418 ranged from 5 to 14.8 per 10^6 protoplasts in different experiments with the overall average being 8.1×10^6 (Table 1).

After being transferred to regeneration medium, G-418-resistant calli formed morphological structures resembling coleoptiles or shoots within 3 weeks. These structures developed into plantlets (Fig. 2c) when transferred to MS medium lacking plant growth regulators. After 5 weeks in the MS medium these plantlets were readily transplanted into soil and grown in environmental chambers. Seventy-four G-418-resistant calli from six separate experiments gave rise to at least 1 plantlet; some calli produced more than 30 plantlets (Table 2). In experiment e and f of Table 2, approximately ten plants were produced by each G-418-resistant callus. Many plants have been grown to maturity (Fig. 2d). In three separate experiments the measurement of morphological characters showed that the transgenic plants had much lower average height and a higher number of tillers per plant than seed-grown plants (Table 3). All the transgenic T_0 plants failed to set seeds upon self-pollination; however, upon cross-pollination, using pollen from seed-grown 'IR54' plants, T_1 seeds were obtained.

Table 1. Protoplast transformation frequency based on the number of resistant calli recovered on medium with G-418

Experiment number	G-418 (μg/ml)	No. of plates[a]	No. of resistant calli recovered		Transformation frequency (10^6)[b]
			Total	Per plate ±SE	
1	5	8	27	3.4±2.2	8.5
1	10	8	46	5.8±5.4	14.5
1	15	8	16	2.0±2.3	5.0
1	20	8	17	2.1±1.8	5.3
2	10	19	45	2.4±0.3	6.0
3	10	44	138	3.1±2.9	7.7
4	10	11	59	5.3±4.2	14.8

[a] Each plate contained 400,000 protoplasts
[b] Transformation frequency = number of transformed calli per 10^6 protoplasts treated

Table 2. Regeneration of transgenic plantlets from G-418-resistant calli of 'IR54'

Experiment number	Number of G-418-resistant calli	
	Selected for regeneration	Regenerated plantlets[a]
a	60	11
b	50	12
c	48	6
d	29	5
e	123	27 (277)
f	25	13 (136)

[a] Each callus produced at least one plant. In experiments e and f, the numbers in parenthesis are the total number of plantlets produced when scored at 8 weeks after the calli were transferred to regeneration medium

Southern blot analysis of T_0 plants

Southern blot analysis of DNA isolated from leaf blades of plants regenerated from G-418-resistant calli confirmed the presence of the introduced *neo* gene (Fig. 3). The untransformed plant did not show any hybridization

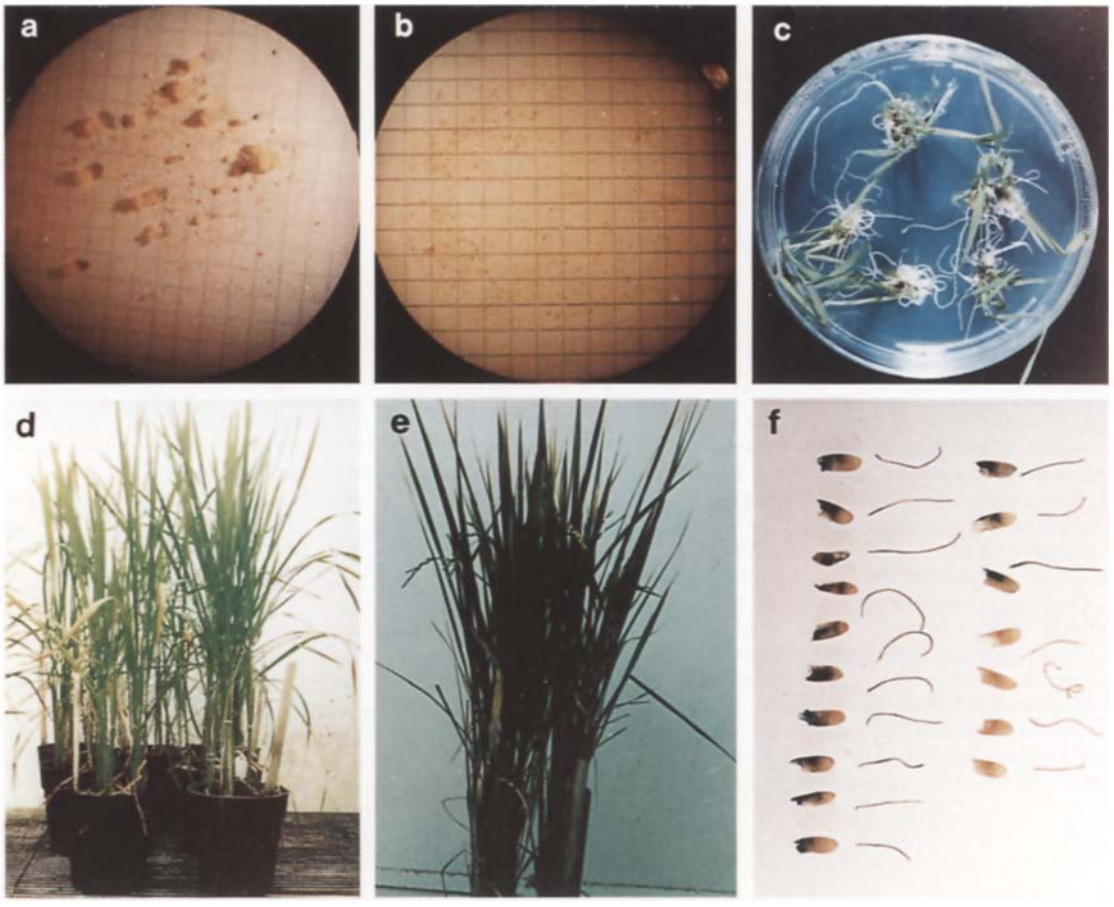

Fig. 2. a–f. Selection of transformed cells and plant regeneration. **a** G-418-resistant calli were recovered from protoplast cultures treated with PEG in the presence of plasmid DNA on medium containing G-418. **b** In control plates (no plasmid DNA was added, but the PEG treatment was applied) calli stopped growing on medium containing G-418. **c** Plantlets regenerated from calli resistant to G-418 6 weeks after being transferred to regeneration medium. **d** Adult plants (T_0) grown in soil in the growth chamber. **e** Mature T_1 plants with seeds, which were later germinated (T_2 generation), and (*f*) their endosperm and roots used for X-Gluc staining to determine GUS activity

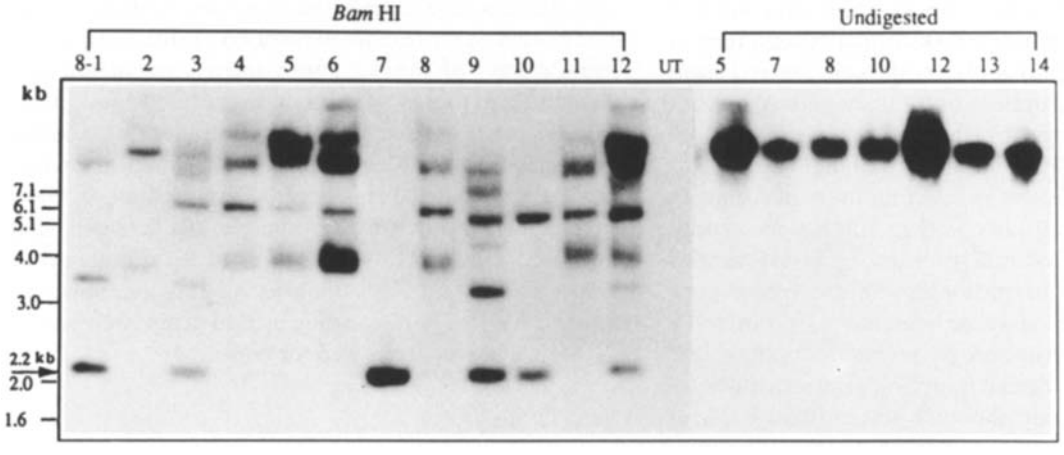

Fig. 3. Southern blot analysis of DNA from T_0 plants. DNA was isolated from leaf blades 1 month after plants were transplanted to soil. DNA samples (10 μg each) on *left side* of the picture were digested with *Bam*HI, while those on the *right side* of the picture were undigested prior to electrophoresis and blotting. The blot was hybridized with the ^{32}P-labelled *Bam*HI fragment of pKAN. Twelve putatively transformed plants (G8-1 to 14) and one untransformed control plant (UT) were analyzed. The *arrow* at 2.2 kb indicates the position of the fragment containing the intact *neo* gene and polyA tail

band. Integration of the foreign gene(s) was illustrated by the formation of a smear in only the high molecular weight region (Fig. 3) when undigested genomic DNA was hybridized with the *neo* probe. Six of the 12 plants illustrated in Fig. 3 showed a hybridization signal in the 2.2-kb region corresponding to the intact *neo* coding sequence. Except for plant G8-7, all of the transformed plants assayed had multiple hybridization bands at molecular weight regions higher than 2.2 kb, which indicated that at least one of the *Bam*HI restriction sites of the plasmid was altered during integration of the gene into the genome. This alteration apparently did not interfere with *neo* function since the calli were selected in the presence of the antibiotic.

In an experiment attempting to produce co-transformed plants with the *gusA* and *neo* genes on two separate plasmids, we obtained two T_0 plants that showed high GUS activity. The *gusA* gene expression was monitored in different organs at different times throughout the growth period (Table 4). Fluorometric assays showed various degrees of background GUS activity in the organs of untransformed plants. In particular, a relatively high background GUS activity was detected in roots. For the transformed plants, specific GUS activity ranged from 500 to 2,700 nmol MU/mg protein per hour, with higher values in roots, stems, and leaf sheaths than in the leaf blades. Samples taken at 45 days after transplanting had the highest GUS activity. Digestion of genomic DNA by different restriction enzymes and Southern blot analysis showed that five to ten copies of the *gusA* and *neo* genes had integrated into the plant genome (Fig. 4). Figure 4 also shows that, for pPUR, a single restriction enzyme digestion (*Hin*dIII or *Sma*I) yielded a major band about the size of the plasmid (about 5.7 kb) as well as two or three other bands. From double restriction enzyme digestion of genomic DNA and probing with the *gusA* gene, a band of the expected size (2.9 kb for *Hin*dIII-*Eco*RI, 2.1 kb for *Bam*HI-*Eco*RI, 1.9 kb for *Sst*I-*Sma*I digestion, respectively), and sometimes additional bands, were observed. For DNA sequences on pKAN plasmid (Fig. 4), it was shown that undigested DNA formed a smear in the high molecular weight region, and double restriction digestion produced a fragment of the expected size (2.2 kb for *Hin*dIII-*Eco*RI and *Hin*dIII-*Bam*HI; 3.0 kb for *Sma*I-*Sst*I) and two or three other fragments.

Table 3. Plant height and number of tillers per plant of transgenic (T_0) 'IR54' rice plants

Experiment number	Sample size	Plant height (cm)		Number of tillers/plant	
		Range	Mean (SD)	Range	Mean (SD)
a	10	52–82	71.2 (9.2)	9–39	15.3 (9.9)
b	12	18–85	62.1 (18.5)	3–30	8.8 (7.8)
c	13	55–94	67.2 (10.4)	3–13	6.8 (2.9)
Control[a]	9	86–103	92.0 (9.5)	4–13	8.3 (4.1)

[a] The control was seed-grown plants that had been grown in the same growth chamber as the transgenic plants

Transmission of the gusA gene and its expression in T_2 plants

T_1 plants grew normally and set seeds (T_2) without artificial pollination (Fig. 2e). T_2 seeds were germinated and Southern blot analysis of DNA isolated from the T_2 leaves provided evidence that the *gusA* gene was indeed transmitted to these T_2 plants (Fig. 5) with integration patterns the same as those of the T_1 parent. Figure 2f presents histochemical staining for GUS activity of remnants of the endosperm of T_2 seeds and roots, after removal of the seedlings for transplanting to soil. Of 45 root samples (from 45 individual plants) tested, 32 showed strong GUS activity, indicating that the *gusA* gene was expressed in the T_2 progeny and probably integrated as a single dominant marker in the plant genome.

Table 4. GUS activity in different tissues of T_0 transgenic 'IR54' rice plants. Specific GUS activity expressed as nmole MU/mg protein per hour

DAT	Plant	Leaf blade	Leaf sheath	Stem	Root	Glume
10	UT	0.88±0.05	0.50±0.13	N.D.	11.9±1.1	–
	G10-8	552±18	1,036±22	N.D.	1,035±35	–
	G10-9	562±19	838±55	N.D.	1,914±187	–
45	UT	0.25±0.10	1.56±0.65	N.D.	12.2±1.1	–
	G10-8	916±16	1,727±129	1,998±725	2,702±190	–
	G10-9	1,510±97	1,747±157	N.S.	N.D.	–
110	UT	2.66±0.27	2.18±0.62	1.18±0.54	9.25±3.0	1.83±0.1
	G10-8	532±60	729±79	712±14	626±121	322±27

DAT, Days after transplanting to soil; UT, untransformed; G10-8 and 9, two transgenic plants; N.D., not determined

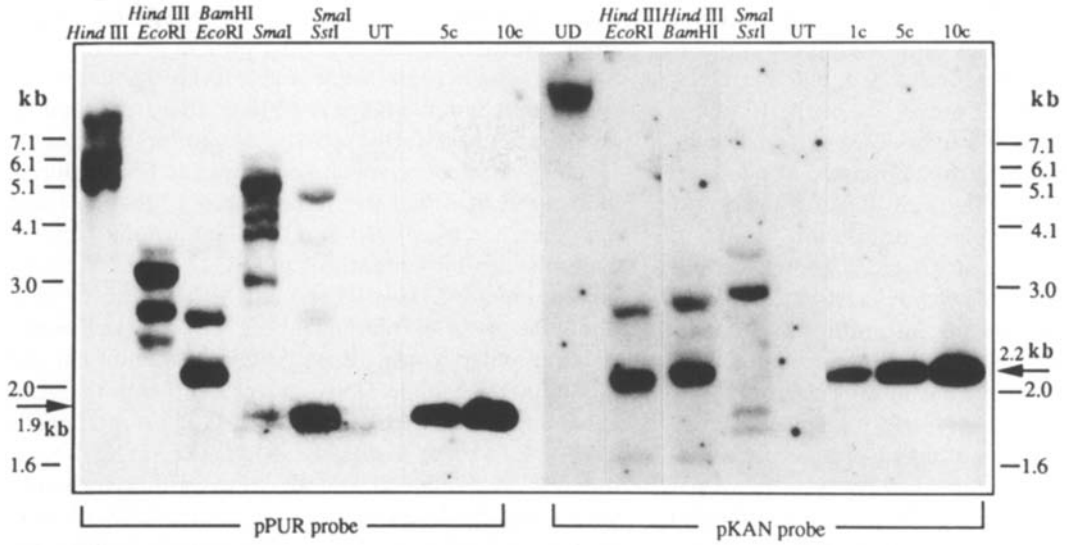

Fig. 4. Southern blot analysis of DNA isolated from leaf blades of 2-month-old T_0 plant G8-10. DNA was digested with various restriction enzymes as shown at the *top of each lane*, and the gel was probed with the ^{32}P labelled *Bam*HI-*Sst*I fragment containing the *gusA* gene of pPUR (marked as pPUR probe) or *Bam*HI fragment containing the *neo* gene of pKAN (marked as pKAN probe). The lanes designated *1c*, *5c*, and *10c* represent the corresponding gene fragments at one, five, or ten copies per rice haploid genome

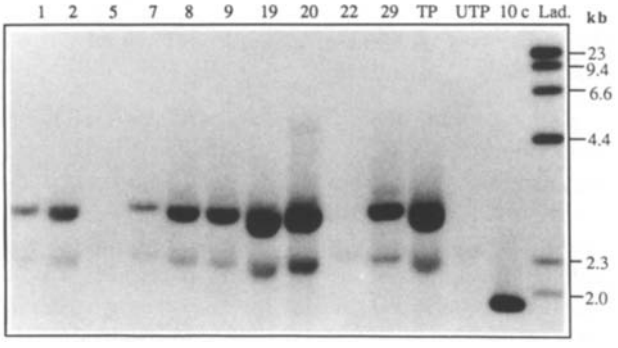

Fig. 5. Southern blot analysis of DNA isolated from leaf blades of 45-day-old T_2 plants for the presence of the *gusA* gene. DNA was isolated from 10 T_2 plants (*1–29*) and the transformed T_1 parent (*TP*) and untransformed parent (*UTP*), digested with *Hin*dIII and *Eco*RI, and subjected to electrophoresis. The probe used was ^{32}P-labelled *Bam*HI-*Sst*I fragment containing the *gusA* gene of pPUR. The lane designated as *10c* was loaded with the *Bam*HI-*Sst*I fragment at ten copies per rice haploid genome. The molecular weight ladder lane (*Lad*) was loaded with λDNA digested with *Hin*dIII and labelled with ^{32}P

Table 5. GUS activity in leaf blades of T_2 plants. Specific GUS activity expressed as nmol MU/mg protein per hour

Plant number	GUS activity	Plant number	GUS activity
UTP	1.3	16	1.9
TP (T_1)	908	18	875
1	1,929	19	1,011
2	1,300	20	702
3	475	21	2.7
5	0.5	22	1.1
6	592	23	762
8	821	24	1,254
9	1,262	26	1,203
12	170	27	9.5
13	1.8	28	1,197
15	0.9	29	1,090

UTP, Untransformed parent; TP, transformed parent; 1 through 29, T_2 plants

Fluorometric assay for GUS activity of the T_2 plants also indicated that the *gusA* gene was highly expressed in 73% of the T_2 plants analyzed (Table 5). The GUS activity of these T_2 plants exhibited a wide range of variability among individual plants.

Histochemical analysis of the gusA gene driven by the CaMV35S promoter

GUS expression in different tissues of T_1 plants are shown in Fig. 6. Figure 6a, e, i, and k illustrate that there was no detectable GUS activity in control (untransformed) leaf, stem, and root cells. Figure 6b, c, and d show that most cells of the leaf exhibited GUS activity, as would be expected since the CaMV 35S was the promoter of *gusA*; however, certain cell types (Fig. 6b), especially phloem cells (Fig. 6d) and guard cells (Fig. 6b insert), had higher levels of blue staining. Staining in other epidermal cells such as bulliform cells was weak in comparison to the guard cells. In the stem (Fig. 6f, g, and h), cells of the vascular tissue as well as cortical and pith cells also exhibited high level of GUS expression. However,

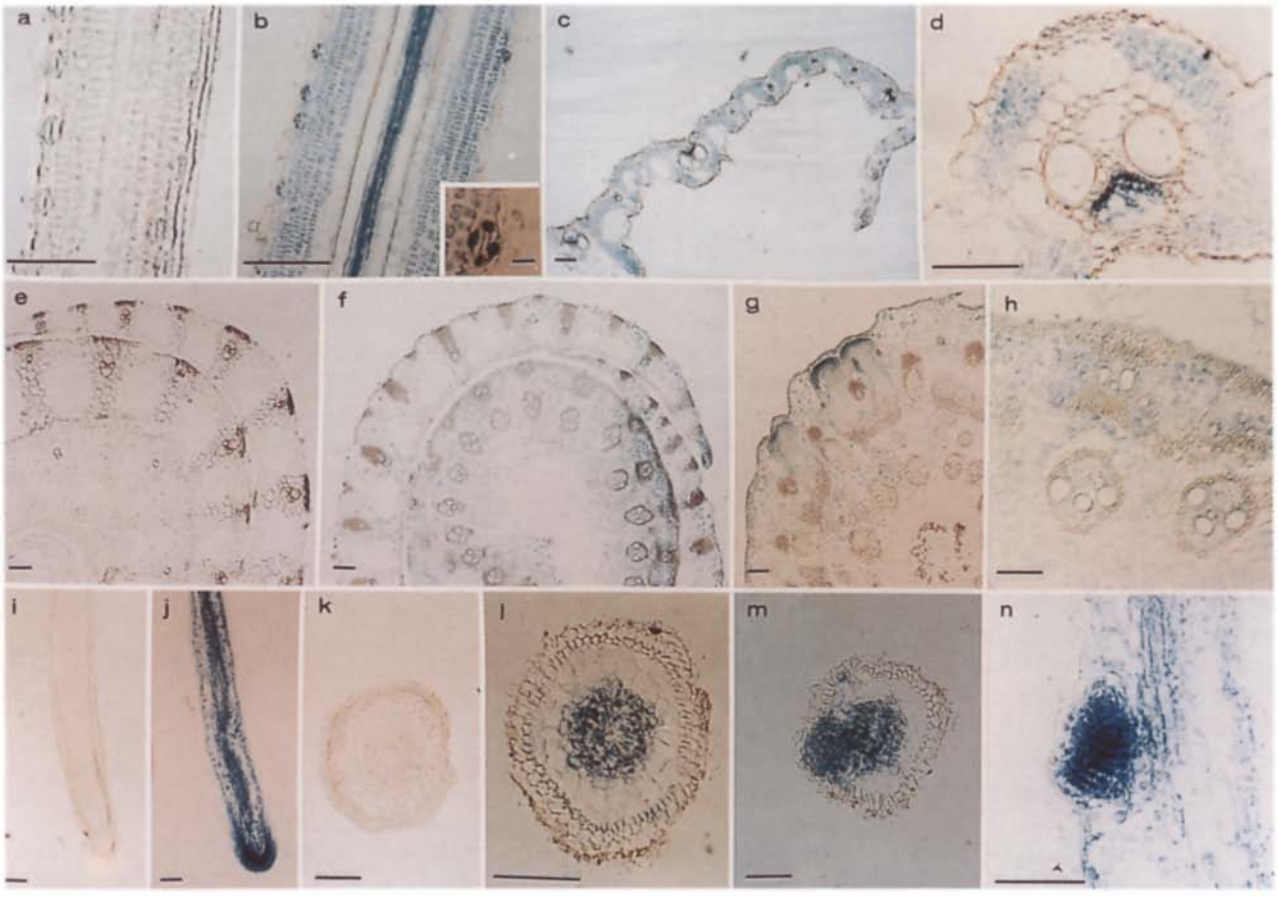

Fig. 6a–n. Histochemical staining of GUS in T_1 plant parts of IR54. *a*, *b*, *c*, *d* are photographs of leaf sections (*insert* of *b* is a photograph of guard cells): *a* and *b* are tangential sections, *c* and *d* are cross sections. Photographs *e*, *f*, *g*, and *h* are cross sections through stem nodes. Photographs *i*, *j*, *k*, *l*, *m*, and *n* are sections of roots: *i*, *j*, and *n* are longitudinal sections, and *k*, *l*, and *m* are cross sections. Photographs shown in *a*, *e*, *i*, and *k* are control sections from untransformed plants. *m* and *n* show high levels of GUS expression in a developing lateral root. The *bar* in each picture represent 100 μm except the *bar* in the *b insert*, which is equivalent to 10 μm

there was a difference in the distribution of staining within the vascular bundles between the stem and the leaf. In the stem, the vascular parenchyma cells throughout the bundle were stained, and the phloem was not specifically stained as intensely in the stem as it was in the leaf. Leaf traces in the stem node (Fig. 6g) also expressed GUS. GUS expression was highest in the apical meristem of the primary roots (Fig. 6j) as well as in developing primordia of lateral roots (Fig. 6m and n). Cells of the vascular cylinder of roots, like those in the leaf and stem, also showed high levels of GUS (Fig. 6l). In general, the blue staining appeared to be more intense in cells with more compact cytoplasm and less so in cells with peripheral cytoplasm, which complicates the interpretation of whether a particular cell type expresses GUS at a higher or lower level than another cell type.

Discussion

The results presented here clearly indicate that DNA uptake into protoplasts mediated by PEG is a reliable and reproducible method for genetically engineering indica rice plants when combined with appropriate protoplast culture protocols and G-418 selection. When the *neo* gene was used as a selectable marker, the use of G-418 as a selective agent was crucial for identifying the transformed cells and regenerating plants from the transformed calli; transformed calli selected with kanamycin did not regenerate into plants (Peng et al. 1990). Similar difficulties with kanamycin selection were reported by Toriyama et al. (1988). Zhang et al. (1988) reported, however, that kanamycin-resistant calli could regenerate into plants, but only on regeneration medium without kanamycin. This together with our observations, illustrates that kanamycin somehow impairs the regeneration

potential of rice calli, and should not be used. Fortunately, this problem does not appear to exist for G-418.

In our system, the transformation efficiency from protoplasts to callus was about 8×10^{-6}. In addition, transgenic indica rice plants were produced at a relatively high regeneration frequency – about 20% of the transformed calli regenerated at least one plant (Table 2), and on average each transformed calli produced about ten plants. This results in one plant per 62,500 protoplasts cultured, a plant transformation efficiency of 1.6×10^{-5}.

We observed various integration patterns of the introduced *neo* gene (Fig. 3). The presence of multiple bands at molecular weights higher than expected is an indication of multiple insertion events and rearrangements of the integrated gene, both of which have been commonly observed in transformed plant materials (Rhodes et al. 1988; Lyznik et al. 1989; Gordon-Kamm et al. 1990).

Fluorometric analysis indicated that the *gusA* gene driven by the CaMV 35S promoter was actively expressed in all plant parts of T_0 transgenic rice plants tested. In different organs, namely, roots, leaf sheath, stem, and glume, we observed various degrees of GUS expression. In general, roots had the highest GUS activity at all developmental stages, an observation different from that reported in japonica rice by Terada and Shimamoto (1990) and Battraw and Hall (1990). The histochemical assays for GUS in the T_1 generation also showed more intensive GUS activity in roots (Fig. 6). Vascular tissues, especially the phloem cells of leaves, exhibited very intensive blue staining in the T_1 plants. Certain specialized cells such as guard cells also showed high GUS activity.

Transmission of the introduced gene was accomplished by crossing the T_0 plants with normal seed-grown 'IR54' plants. It appeared that the mode of transmission of the foreign gene followed a single dominant gene inheritance pattern, as has been reported in tobacco (Potrykus et al. 1985) corn (Fromm et al. 1990), and japonica rice (Shimamoto et al. 1989). Artificial cross-pollination and embryo rescue has also been used for producing transgenic progeny of maize following particle-bombardment delivery of DNA into host cells (Fromm et al. 1990; Gordon-Kamm et al. 1990). We do not understand why the sterility problem exists in T_0 plants; however, the long time involved in developing suspension cultures, the subsequent protoplast culture, and the selection of transformed calli probably contribute to the inhibition of normal pollen development in the T_0 plants. Conversely, since the T_1 plants shed pollen and set seeds normally, the pollen sterility associated with T_0 plants must not be due to the introduced foreign gene(s) *per se*.

In conclusion, we have established an efficient system for co-transforming indica rice protoplasts of an indica variety, 'IR54', with genes on two separate plasmids and producing transgenic indica rice plants. This system resulted in transgenic T_0 plants from which the *gusA* gene was transmitted to T_1 progeny and the foreign gene was inherited in an expected Mendelian manner. Since it is now possible to genetically transform both japonica and indica rice plants, other agronomically-important genes will soon be transferred to rice. The transformation system reported here should provide a good system for studying gene expression and gene regulation in both indica and japonica rice plants. We have recently shown that two japonica varieties, 'Radon' and 'Baldo', can be regenerated and transformed using the protocol developed for the indica variety (Wen et al. 1991). Experiments are now underway to utilize these procedures to study the expression of various genes driven by various promoters in both japonica and indica varieties of rice.

Acknowledgements. This research was supported by a grant from The Rockefeller Foundation.

References

Battraw MJ, Hall TC (1990) Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol 15:527–538

Chu CC, Wang CC, Sun CS, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen sources. Sci Sin 18:659–668

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile indica-rice recovered from protoplasts. Bio/Technology 8:736–740

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of Petunia leaf protoplasts. Dev Biol 33:130–137

Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833–839

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Hayashimoto A, Li Z, Murai N (1990) A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol 93:857–863

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

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126:105–110

Koetje DS, Grimes HD, Wang Y-C, Hodges TK (1989) Regeneration of rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J Plant Physiol 135:184–190

Krens FA, Molendijk L, Wullems GJ, Schilperoort RA (1982) *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. Nature 296:2–74

Lee L, Schroll RE, Grimes HD, Hodges TK (1989) Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. Planta 178:325–333

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue culture. Physiol Plant 18:100–127

Lörz H, Baker B, Schell J (1985) Gene transfer to cereal cells mediated by protoplast transformation. Mol Gen Genet 199:178–192

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:256–275

Lyznik LA, Kamo KK, Grimes HD, Ryan R, Chang KL, Hodges TK (1989) Stable transformation of maize: the impact of feeder cells on protoplast growth and transformation efficiency. Plant Cell Rep 8:292–295

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue-cultured plant cells. Plant Mol Biol Rep 5:346–349

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tobacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Peng J, Lyznik LA, Lee L, Hodges TK (1990) Co-transformation of indica rice protoplasts with *gusA* and *neo* genes. Plant Cell Rep 9:168–172

Peng J, Lyznik LA, Hodges TK (1991) Co-transformation of indica rice *via* PEG-mediated DNA uptake. In: Second Int Rice Genet Symp. IRRI (in press)

Potrykus I, Paszkowski J, Saul MW, Petruska J, Sillito RD (1985) Molecular and general genetics of a hybrid foreign gene introduced into tobacco by direct gene transfer. Mol Gen Genet 199:169–171

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D, Detmer JJ (1988) Genetically transformed maize plants from protoplasts. Science 240:204–207

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276

Terada R, Shimamoto K (1990) Expression of CaMV 35S-GUS gene in transgenic rice plants. Mol Gen Genet 220:389–392

Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technology 6:1072–1074

Wen F, Peng J-Y, Cister RM, Hodges TK (1991) A procedure for regenerating japonica and indica varieties of rice from protoplasts. Plant Molec Biol Rept 9:308–321

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep 7:379–384

Zhang W, Wu R (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor Appl Genet 76:835–840