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A Routine System for Generation of Fertile Transgenic Rice Plants Using Biolistic Method

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Abstract

A routine system based on particle bombardment of embryogenic callus for recovery of fertile transgenic rice (*Oryza sativa* L.) plants was developed. Embryogenic callus was established within 2-3 months from calli derived from mature seeds of Korean rice cultivar, Nagdongbyeo. The callus was bombarded with the plasmid pRQ6 containing the β-glucuronidase gene (*gus*A) and hygromycin phosphotransferase gene (*hph*, conferring resistance to hygromycin B), both driven by CaMV 35S promoter. Placement of cells on an osmoticum-containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 hrs prior to and 16 hrs after bombardment resulted in a statistically significant increase with 3.2-fold in transient expression frequency *gus*A. In five independent experiments, the average frequency of transformation showing GUS activities was 8.86%. A large number of morphologically normal, fertile transgenic rice plants were obtained. Integration of foreign gene into the genome of R_0 transgenic plants was confirmed by Southern blot analysis. GUS and HPT were detected in R_1 progeny and Mendelian segregation of these genes was observed in R_1 progeny.

Key words: Embryogenic callus, fertile transgenic rice, osmotic treatment, particle bombardment

Introduction

The development of efficient methods for introduction of foreign genes into rice is important for both basic studies of the regulation of gene expression and crop improvement. The development of a routine efficient transformation system has, therefore, been the subject of intensive research by scientists committed to genetic improvement of rice (Potrykus et al. 1995). Previously, we reported the recovery of transgenic rice plants using either direct gene transfer of exogenous DNA into protoplasts by PEG (Lee et al. 1995; Lee et al. 1999; Lee et al. 2001) or *Agrobacterium*-mediated gene transfer into mature seed-derived embryogenic callus (Lee et al. 1998; Lee et al. 1999; Lee et al. 2001). However, protoplast system was not only time-consuming but also resulted in the production of reduced fertile or complete infertile primary transformants probably as a consequence of tissue culture-induced variation (Lee et al. 1999). Recently, *Agrobacterium*-mediated transformation also may result in two potential problems, which need to be addressed prior to the release of any genetically engineered plant into the environment. One is that molecular analyses of genomic DNA from engineered plants indicated the presence of vector sequences outside the transferred DNA (T-DNA) borders (Ramanathan and Veluthambi 1995). The second is that *Agrobacterium* was found to persist on the surface and within tissues of soil-grown transformed plants up to 12 months following transformation (Matzik et al. 1996).

One alternative to protoplast and *Agrobacterium* systems for gene transfer studies has been the development of a 'biolistic method' for delivery of DNA or RNA into intact cells or tissues (Sanford et al. 1987). This technology provided the necessary breakthrough for efficient transformation of rice in a genotype-independent fashion, and currently more than 40 varieties have been transformed (Christou 1996). The primary factor that has made the biolistic technology the most widely used

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gene transfer method for engineering recalcitrant species is the capacity to target intact explants for transformation experiments.

Osmotic treatment using osmoticum or a high concentration of agarose in shoot regeneration medium for reducing water content in rice cells was one of the factor discovered to increase the frequency of plant regeneration from suspension-derived cells (Jain et al. 1996) and seed-derived cells (Lee et al. 1999).

In this report, a routine system for generation of transgenic plants from seed-derived embryogenic callus of japonica type Korean rice variety, Nagdongbyeo was described using the biolistic method with osmotic treatment.

Materials and Methods

Plant materials and callus induction

Callus was initiated from mature seeds of japonica cultivar of rice, Nagdongbyeo. Dehusked mature seeds were surface-sterilized in 70% (v/v) ethanol for 1 min and subsequently in 1.0% (w/v) sodium hypochlorite for 50 min. They were then rinsed three times with sterilized water and cultured on NB medium semi-solidified with 2.5 g/L phytagel (Sigma, St Louis, MO, USA) containing macroelements of N6 medium (Chu et al. 1975), microelements and vitamins of B5 medium (Gamborg et al. 1968), 500 mg/L glutamine, 500 mg/L proline, 300 mg/L enzymatic casein hydrolysate, 2 mg/L 2,4-D and 30 g/L sucrose for callus induction. The cultures were incubated in the dark at 26°C. After 3 weeks of culture, embryogenic calli derived from seed scutella were separated and subcultured on fresh NB medium.

DNA/microprojectile preparation and bombardment conditions

Microcalli (100-120) in diameter with 1-2 mm were placed in the center of 9 cm diameter Petri dishes containing NBO medium semi-solidified with 2.5 g/L phytagel, supplemented with 0.2 M mannitol, 0.2 M sorbitol, 2 mg/L 2,4-D and 30 g/L sucrose. After incubation for 4 hrs, the microcalli were immediately subjected to microprojectile bombardment with the Biolistic Particle Delivery PDS-1000/He system (Bio-Rad).

The plasmid pRQ6 contained both *gus*A and *hph* gene, each individually driven by the CaMV 35S promoter and terminated by the *nos* 3′ end. Genes were arranged in a head-to-head orientation (Figure 1) (Sivamani et al. 1996). The manufacturer's instructions were followed for coating the mixture of 1.0 μM (Bio-Rad) and 1.8-2.3 μM (Johnson Matthew) gold microcarriers with the plasmid DNA. Six milligrams (3 mg of 1.0 μM diameter and 3 mg of 1.8-2.3 μM diameter) of gold particles

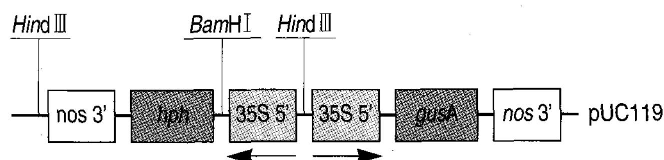

Figure 1. Structure of the plasmid pRQ6: The two genes, *hph* and *gus*A, are placed in a different orientation and both genes are driven with CaMV 35S promters and inserted into pUC119 cloning vector.

were sterilized in 100 μL absolute ethanol for 2 min with vortexing. After washing with sterile water twice, particles were resuspended in 100 μL sterile water. The mixture was prepared as follows: 100 μL 1 of particle suspension were mixed with 5 μL × (1 μg/μL) of plasmid DNA, 50 μL $CaCl_2$ (2.5 M) and 20 μL spermidine (0.1 M). The particle/DNA suspension was vortexed and left for 10 min at room temperature. The DNA-coated particles were pelleted by centrifugation at 10,000 rpm for 10 sec. The supernatant was completely removed and discarded. The pellet was then resuspended in 60 μL absolute ethanol. Particle/DNA mixture (8 μL) was placed in the center of microcarrier. The target cells were placed at a distance of 8 cm from the screen and directly covered with a baffle (500 μM stainless steel mesh). Explants on each plate were bombarded twice. After bombardment, the cultures were maintained on the same medium in the dark at 25-26°C.

Selection and plant regeneration from bombarded cells

Sixteen to twenty hrs after bombardment, the cell aggregates were transferred to NH40 medium semi-solidified with 2.5 g/L phytagel containing 40 mg/L hygromycin B (hyg B; Calbiochem). Cell aggregates were cultured in the dark at 25-26°C for 2 weeks, then were transferred onto the same NH40 medium for selection. Two weeks later, the hygromycin resistant (hyg^r) callus was transferred to PRH50 medium semi-solidified with 2.5 g/L phytagel, supplemented with 5 mg/L ABA, 1 mg/L NAA, 2 mg/L BAP and 50 mg/L hyg B for pre-regeneration treatment. Hygromycin-resistant callus measuring 2-3 mm in diameter was transferred to RNH50 medium solidified with 4 g/L agarose (Sigma type I) containing 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hyg B. Cultures were maintained at 25-26°C under a photoperiod of 16 hrs. After plantlets were regenerated, they were transferred to Magenta boxes containing half-strength MS medium solidified with 4 g/L agarose, supplemented with 0.1 mg/L NAA and 30 g/L sucrose (Murashige and Skoog 1962). When the plants reached 8-10 cm in height, they were transferred to pots and grown to maturity in the greenhouse. The progenies of R_0 plants were planted for morphological and molecular analysis.

Histochemical GUS assays

Histochemical analysis of GUS activity was performed essentially as described by Jefferson et al. (1987). Transient GUS activity was assayed 48 hrs after bombardment by incubating callus for 10-12 hrs at 37°C in reaction buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 100 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100. Small fragments of leaf and root from primary transformants or R_1 seedling were incubated in the same buffer. The chlorophyll was removed by incubation of leaf pieces in 70% ethanol before GUS detection.

Southern blot analysis

Genomic DNA was extracted from leaf and stem tissues of transformed rice plants based on Dellaporta et al. (1983) with modification. Ten μg of genomic DNA from each sample was digested with either *Hin*dIII or *Bam*HI (Figure 1), fractionated on a 0.8% agarose gel, denatured, and transferred onto a nylon membrane. The membrane was pre-hybridized for 1 hr at 65°C in a solution containing 0.25 M sodium phosphate (pH 7.4), 1 mM EDTA, 1% BSA and 7% SDS, and hybridized in the same solution with ^{32}P-labeled *hph* probe. Then the membrane was washed twice with 2×SSC solution at room temperature and once with 0.1×SSC solution containing 0.1% SDS at 60°C for 15 min each time.

Results and Discussion

Effect of osmotic treatment

Osmotic treatment of embryogenic rice callus enhanced transient expression of the *gus*A gene. The osmoticum used was consisted of a mixture of equimolar mannitol and sorbitol. Bombarded tissues were assayed for GUS activity 48 hrs after bombardment and the number of blue spots was counted. The sample treated with osmotic stress revealed 3.2-fold higher frequency of GUS expression in callus compared to the sample without osmotic treatment (Table 1). The average number of blue spots per plate treated osmotic stress was 893 for callus. However, variation among different shots was high, e.g. from 745 to 1,098 per plate for embryogenic calli, which probably reflecting the variation in manipulation of the particle gun. Table 1 shows the results similar to those of Vain et al. (1993) reported for maize. They proposed that osmotic treatment enhanced transient expression and stable transformation of maize by facilitating plasmolysis of the target cells. Plasmolyzed cells may be less likely to extrude their protoplasm following penetration of the cell by particles (Armaleo et al. 1990).

The plasmolyzed state should be maintained for a few hours before and after bombardment to achieve maximum effective-

Table 1. Osmotic effect on *gus*A expression in embryogenic rice callus after particle bombardment

Experiment	Treatment	No. of blue spots for 0.7 g of cells[b]
1	with osmotic treatment[a]	893±177
2	without osmotic treatment	284±36

[a]Osmotic treatment means storage on an NBO medium containing 0.4 M osmoticum
[b]Each value is the mean of 6 replications

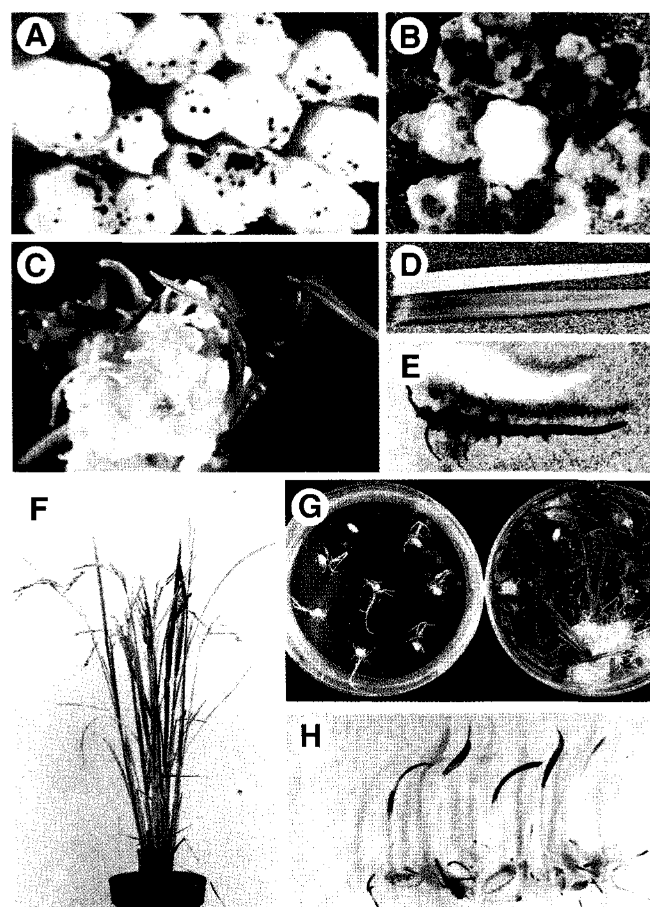

Figure 2. Generation of transgenic rice by particle bombardment-mediated transformation. (A) GUS activity 48 hrs after bombardment of embryogenic callus. (B) Expression of GUS in hygromycin-resistant callus and non-transgenic callus (center). (C) Hyg^r plant regenerated from regeneration medium containing 40 mg/L hyg B. (D) Expression of GUS in segments of the leaf of transformant (lower) and untransformant (upper). (E) Expression of GUS in root segments of transformant (lower) and the untransformant (upper). (F) Mature and fertile transgenic rice plants. (G) Germination of seeds (R_1) of transgenic rice plants (right) and untransformant (left) on the medium containing 30 mg/L hyg B. (H) The staining pattern of a 5-day-old germinating seedlings. Most of the plant body showed strong GUS activity.

ness (Vain et al. 1993). Figure 2A shows transient expression of the *gus*A gene in callus that underwent osmotic treated 24 hrs after bombardment.

Production of hygromycin-resistant callus

Hygromyin B (hyg B) was used as the selection agent. The effect of hyg B on the growth of embryogenic rice callus was examined prior to conducting experiments. Cell growth was severely retarded on solid medium containing either 40 mg/L hyg B or 50 mg/L hyg B. However, if callus was plated on the solid growth medium without hyg B for a few days, the selection on hyg B-containing medium became more difficult. This is presumably because the vigorous growth of the cell clusters caused the cells to be resistant to high concentration of hyg B. Therefore, bombarded cell cultures were kept on high osmotic pressure medium for 16-20 hrs, and then transferred directly to selection medium.

Sixteen to twenty hours after bombardment with the plasmid pRQ6, the cultures were transferred directly to selection medium containing 40 mg/L hyg B. Most microcalli gradually turned brown. But, fresh white cell clusters were identified on the surface of the bombarded tissues after 2 to 3 weeks of culture. Not all the growing clusters were transgenic at this stage, so the fresh cell cluster were carefully removed from dying explant tissue and transferred to fresh selection medium containing 50 mg/L hyg B for further selection. The proliferating regions showed evidence of the uniform expression of GUS (Figure 2B). It appeared that the surrounding non-transformed cells were gradually degenerated under this selection pressure. In order to increase plant regeneration, the hygromycin-resistant callus was transferred to preregeneration medium (PRH50) containing 50 mg/L hyg B after 2 weeks of selection on 50 mg/L hyg B for further growth.

Regeneration of hygromycin-resistant, GUS-expressing plants

When the size of the hyg^r callus reached approximately 2 to 3 mm in diameter, they were transferred to regeneration medium containing 50 mg/L hyg B. In preliminary experiments, it was observed that if hyg B was not added to the regeneration medium, some of the regenerated plants were not transgenic, although the callus was previously grown on medium with hyg B for more than 1 month. After plantlets were produced on the regeneration medium containing hyg B (Figure 2C), they were transferred to rooting medium. GUS assays of segments of leaf or root from the regenerated plants resulted in blue staining of entire samples in most cases (Figure 2D and E), but chimera expressions (periclinal chimeras and dot-like chimeras) were occasionally observed.

Table 2. Efficiency of rice transformation by particle bombardment

Experiment	No. of callus bombarded	No. of regenerated plants	No. of GUS$^+$ plants/total plants analyzed	Transformation frequency (%)
1	1200	170	34/48 (120)	10.0
2	580	80	28/50 (45)	7.8
3	1300	128	16/21 (98)	7.5
4	1000	145	35/40 (127)	12.7
5	1300	113	29/40 (82)	6.3

The results of the bombardment experiments are summarized in Table 2. Totally 636 hygromycin-resistant plants were regenerated from 5,380 of bombarded microcalli in five independent experiments. Among them, 472 hygromycin-resistant plants had detectable histochemical GUS activity with mean 8.9% of transformation frequency from 5 independent experiments, a frequency comparable to other reports in regenerable rice cultures (Li et al. 1993). The hygromycin-resistant and GUS positive plants were transferred to 1/2MSH50 medium to develop into plantlets. All the plants were successfully established in the greenhouse and grown into mature plants. Selfing of R_0 transformants resulted in normal seed set (Figure 2F) and produced more than 100 seeds from three to seven tillers on each plant.

Integration of *hph* gene in transgenic rice plants

To confirm the *hph* gene in the genomic structure of transgenic plants, total genomic DNA was isolated from the leaves of five randomly chosen hygromycin-resistant plants and one untransformed plant. Rice genomic DNA was digested with *Hin*dIII (lanes marked with H) (Figure 3) and probed with a 2.0 kb *Hin*dIII fragment containing the whole *hph* expression cassette (a promoter, *hph* gene and a *nos* terminator). No hybridization signal was detected in DNA from the untransformed control plant (Figure 3; NC). All five hygromycin-resistant plants showed a 2.0 kb hybridization signal, corresponding to the size of the intact *hph* coding sequence (Figure 3, lanes marked with H). The results indicated that the detected hygromycin-resistant phenotype of plants was correlated to the intact *hph* gene in the genome of transgenic plants. In addition to the 2.0 kb fragment, two additional radioactive bands in identical size were also detected in both P3 (H) and P4 (H) plants which means that both P3 and P4 plants might be regenerated from same transformed callus.

DNA of transgenic plants was also analyzed after digestion with *Bam*HI (lanes marked with B). *Bam*HI cleaves the intact

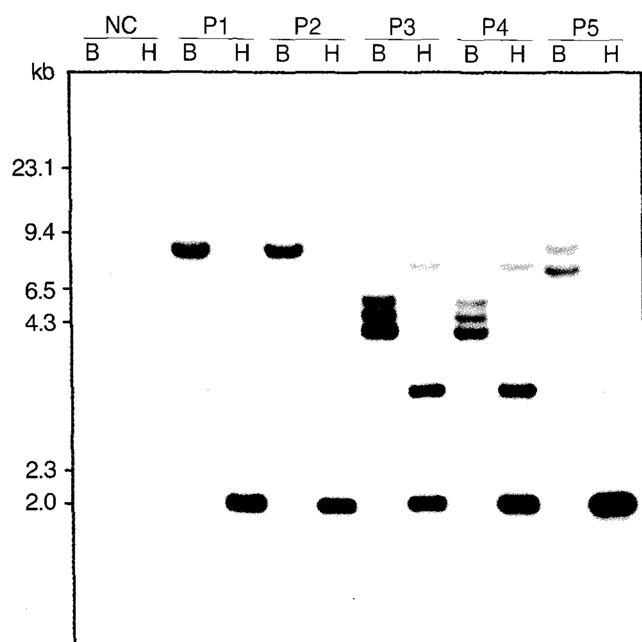

Figure 3. Southern analysis of 5 R_0 rice. Total DNA was isolated from leaves and ten microgram of DNA were digested to completion with *Hin*dIII (lanes marked with H) or *Bam*HI (lanes marked with B). Digested DNA was fractionated by electrophoresis in 0.8% agarose gel and transferred to nylon membrane and hybridized to a ^{32}P-labeled 2.0 kb *Hin*dIII fragment of hygromycin phosphotransferase (*hph*) coding sequence. NC, DNA from untransformed control plant; Pl to P5, DNA from putative transgenic plants P1, -2 -3, -4 and -5, respectively.

plasmid pRQ6 at a single site and generates an 8.25 kb linear molecule (Figure 3, lanes marked with B). The presence of the 8.25 kb fragments hybridized to the *hph* probe should reflect the integration of plasmid concatemers into the rice genome (Figure 3. P5, lane marked with B). The size of the *hph*-hybridizing fragment should indicate the distance from the *Bam*HI site of the integrated plasmid to the nearest *Bam*HI site in the adjacent rice genome. After hybridization with the 2.0 kb *hph* probe, DNA from the untransformed control plant did not produce any detectable signal (Figure 3, NC).

Inheritance of *hph* and *gus*A genes

Expressions of the hyg^r trait and *gus*A gene among the offspring of the transgenic plants were examined by germinating R_1 seeds on the medium containing 30 mg/L hyg B and by GUS assays, respectively. After incubation with hygromycin for 10 days, none of the pregerminated untransformed control seeds grew further (Figure 2G), but a majority of transgenic seeds grew to normal plantlets. Results showed that *hph* and *gus*A genes segregated together in the offspring tested. Most of the lines exhibited 3:1 segregation ratios among the offspring, indicating Mendelian inheritance from either a single genetic locus or closely linked loci of functional *hph* and *gus*A genes (Table 3). The results suggest that several copies of the plasmid DNA were inserted into the small region of the rice genome where recombination occurs infrequently. Inheritance of the *gus*A gene by GUS histochemical assay was also demonstrated. The *gus*A transgene was expressed in most parts (leaf and root) of R_1 seedlings (Figure 2H).

Table 3. Segregation of the *hph* and *gus*A genes expression in transgenic rice

Transgenic rice line	No. of seeds planted	R_1 germin. seedl.[a]	No. of hyg^r seedlings (%)	GUS positive seedl.	x^2	P
ND-1	59	58	43 (73.1)	43	0.023	0.900-0.750
ND-2	74	73	58 (79.9)	58	0.772	0.500-0.250
ND-3	43	43	43 (100)	43	13.458	0.005 <
ND-4	78	77	56 (72.7)	56	0.214	0.750-0.500
ND-5	28	26	20 (76.9)	20	0.046	0.900-0.750
ND-6	24	24	18 (75.0)	18	0.000	0.995-0.950
Control	55	0	-	-	-	-

[a]Transgenic rice seeds (R_1) were planted on 1/2MS salt medium for 5 days, then transferred to hyg B (30 mg/L) containing medium. x^2 tests indicate a good agreement with segregation ratios of 3:1 except the line ND-3

This study conclusively demonstrated that genetic transformation of rice was accomplished by particle bombardment of embryogenic callus. A simple and reproducible system for genetic transformation of a Korean rice variety, Nagdongbyeo, was developed. Since the transformation system is rapid and obviates the need for establishing long-term callus, there is a strong indication that cell suspension and protoplast regeneration procedure could introduce agronomically useful genes directly to other Korean rice cultivars.

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