A Polyethylene Glycol-Mediated Protoplast Transformation System for Production of Fertile Transgenic Rice Plants

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ABSTRACT

We have established an efficient procedure for protoplast transformation and regeneration of fertile transgenic plants of rice (Oryza sativa L.) cultivars Nipponbare and Taipei 309. Protoplasts were mixed with a plant-expressible hygromycin resistance gene and treated with 25% (w/v) polyethylene glycol. Stringent selection of transformed colonies was applied to 14-day-old regenerated protoplasts in the presence of 95 micromolar of hygromycin B for 12 days. After selection, 450 and 200 resistant colonies were recovered per million treated Taipei 309 and Nipponbare protoplasts, respectively. Southern hybridization analysis of hygromycin-resistant cell lines and regenerated plants indicated that 1 to 10 copies of transferred DNA were integrated at 1 to 4 loci of the rice genome. Southern DNA analysis suggests that the introduced plasmid DNA may form concatemers by intermolecular recombination prior to integration. Four Taipei 309 and 39 Nipponbare transgenic rice plants were regenerated and grown to maturity in the greenhouse. Two Taipei 309 and 35 Nipponbare plants set viable seeds. Agronomic traits of Taipei 309 transgenic plants and inheritance of the hygromycin resistance trait by progeny of the selfed transgenic plants were analyzed.

Application of gene transfer procedures for functional analysis of eukaryotic genes has greatly facilitated our understanding of the mechanisms of gene regulation. Plant regulatory elements involved in transcriptional regulation have been analyzed using Agrobacterium tumefaciens-based transformation systems (9, 11, 23).

We are interested in studying the regulation of gene expression in rice, a monocot plant. Our objective is to develop a homologous gene transfer system in rice to circumvent the difficulties encountered in heterologous dicot gene expression systems (15). A number of laboratories have already reported transformation of rice protoplasts (22, 24) and subsequent regeneration of transgenic plants (21, 25, 26). Production of fertile mature plants and their progeny was also recently demonstrated (19).

We have established an efficient and reproducible procedure for rice protoplast propagation and fertile plant regeneration (10). In this procedure, we have optimized parameters in protoplast transformation. Here, we report an efficient procedure for production of a large number of transgenic rice plants to facilitate quantitative analysis of gene expression.

MATERIALS AND METHODS

Chemicals and Enzymes

Cellulase RS and Macerozyme R-10 were obtained from Yakult Honsha Co. (Tokyo, Japan) and SeaPlaque agarose was acquired from FMC Co. An oligolabeling kit, mixed sequence hexadeoxynucleotides pd(N)₉, and Sephadex G-50 (DNA grade) were obtained from Pharmacia. Nytran membranes were acquired from Schleicher & Schuell. Thymidine 5'-triphosphate (TPP) [methyl-³H] and deoxyctydine 5'-triphosphate (dCTP) [³²P] were purchased from ICN Biochemicals.

Plant Expression Vectors

Plant expression vectors were constructed to express a bacterial gene under the control of plant transcription initiation and termination signals. The nopaline synthase (nos) and cauliflower mosaic virus (CaMV) 35S promoters were used as constitutive promoters in expression vectors pTRA104 and 105, respectively. The transcription termination and polyadenylation sites were derived from Agrobacterium tumefaciens.

The transcription termination and polyadenylation sites were isolated as a 292 bp Sau3a fragment from a tumor morphology large (tml) gene in T-DNA of A. tumefaciens pTi15955. This fragment was located 110 bp downstream from the stop codon of the tml gene from nucleotide sequence positions 10,775 to 11,167 of the T-DNA (2). Sau3a ends of the isolated fragment were filled in with Klenow fragment and ligated with a SacI linker. A 300 bp SacI fragment was cloned into the SacI site of pUC12, forming pTRA103.

The nos promoter was isolated from plasmid pUC9KN (1), kindly provided by Dr. M. Bevan, Plant Breeding Institute, Cambridge, U.K. This plasmid was digested with PstI and the resulting 500 bp fragment was cleaved with Sau3a to release a 270 bp fragment that represents the nucleotide sequence from positions -266 to about +35 from the transcription initiation site of the nos gene. The Sau3a fragment was cloned into the BamHI site of pTRA103, and the resultant expression vector was called pTRA104.

The CaMV 35S promoter was isolated from a plasmid containing the 19S and 35S promoters of the CaMV-S ge-
nucleotide sequence from positions 7141 to 7666 (7). The plasmid was kindly provided by Drs. H. Hirochika and J. Ikeda, National Institute of Agrobiological Resources, Tsukuba, Japan. A BgIII site was present at the 3'-end of the fragment at nucleotide position 7666, 283 bp downstream from the transcription initiation site. A BgIII-digested plasmid was treated with exonucleases III and VII to generate a series of deletions from the BgIII site toward the cap site of the 35S transcript. The deleted plasmids were further digested with PstI, and fragments 350 to 400 bp in length were isolated after PAGE. The isolated fragments were cloned into the PstI and HindIII sites of the vector M13mp19. The DNA sequence of the isolated fragments was determined on single strand DNA by the dideoxy method. One clone carried a 337 bp sequence representing nucleotide positions from −285 to +50 from the transcription initiation site of the CaMV 35S promoter. This sequence was recovered from M13mp19 as a 350 bp HindIII/BamHI fragment and cloned into pTRA103, forming the expression vector pTRA105.

Hygromycin Resistance Gene

A hygromycin resistance gene was used as a selectable marker for rice protoplast transformation. The coding sequence of the *Escherichia coli* hygromycin B phosphotransferase (*hph*) from the plasmid pLG90 was kindly provided by Dr. Linda Gritz, Applied Biotechnology, Cambridge, MA (8). The entire *hph* coding sequence had been cleaved from the vector as a 1.1 kbp BamHI fragment. The initiation and termination codons of the *hph* gene resided 14 bp downstream from the 5'-end, and 100 bp upstream from the 3'-end of the fragment, respectively. The *hph* sequence was inserted into the BamHI site of the expression vector pTRA104, so that the *hph* sequence was downstream from the nos gene promoter, resulting in pTRA131. The *hph* coding region was also placed downstream from the 35S promoter of the expression vector pTRA105, resulting in pTRA132.

Plasmid DNA Isolation

Plasmid DNA for protoplast transformation was isolated by the modified method of Hansen and Olsen (4). Plasmid DNA was further purified by CsCl density gradient centrifugation.

Suspension and Protoplast Culture

Rice (*Oryza sativa* L.) cultivars *Nipponbare* and *Taipei 309* were used for this study. The procedure for callus induction, suspension callus culture, protoplast isolation, and protoplast culture has been described in detail in the previous report (10).

Protoplast Transformation after PEG Treatment

The procedure for PEG-mediated transformation of rice protoplasts was essentially as described by Negrutiu et al. (13). After the final KMC wash (5), isolated protoplasts were resuspended at a density of 4 × 10⁶ protoplasts per mL in a MaMg solution containing 0.4 M mannitol, 15 mM MgCl₂, 1% (w/v) Mes (pH 5.6). Ethanol-precipitated plasmid DNA was dissolved at a density of 10 μg per 20 μL of 10 mM Tris-HCl (pH 7.8), and 1 mM EDTA (TE). Ten μg of the plasmid DNA were applied to one million of protoplasts in 0.25 mL of MaMg solution. Protoplast/DNA mixtures were incubated for 10 min at room temperature. Forty % (w/v) PEG (mol wt 8,000) in a given amount of MaMg solution was gently applied to the protoplast/DNA mixture to achieve the desired PEG concentration. After 15 min of incubation at room temperature, protoplasts were suspended in 40 mL of KMC solution and centrifuged at 130g for 8 min. Two million PEG-treated protoplasts were resuspended in 1 mL of General protoplast medium. The protoplast suspension was then mixed with an equal volume of prewarmed protoplast medium containing 2.5% (w/v) Seaplaque agarose. The agarose mixture was poured into a Petri dish (60 × 15 mm) (18). The solidified agarose sheet was cut into 10 mm square blocks and was cultured in 5 mL of liquid protoplast medium with nurse cells as described elsewhere (10).

Oligolabeling

Oligolabeling was performed according to the protocol provided in the Pharmacia oligolabeling kit. Probe DNA fragments were isolated by electrophoresis or glass-powder elution. Plasmid DNA (50–200 ng) was denatured by boiling or alkaline treatment, and mixed with 1 μg of random primer pd(N)₆ in a solution containing 0.5 mM each of deoxynucleotide triphosphates. The polymerization reaction was initiated by addition of 2 units of Klenow fragment and 50 μCi of TTP [methyl-³H] or dCTP [α-³²P], and continued for 3 h at room temperature. At the end of the reaction, 43 to 71% of total radioactivity was incorporated into DNA. The specific activity of the DNA probes was prepared ranged from 1 to 7 × 10⁶ cpm per μg of ³H-labeled DNA, and from 0.4 to 1.5 × 10⁶ cpm per μg of ³²P-labeled DNA.

³H-Labeled Plasmid DNA Transfer to Protoplasts

³H-labeled DNA was used to monitor DNA transfer to protoplasts after PEG treatment or electroporation. Ten μg of ³H-labeled plasmid DNA were applied to one million protoplasts in 0.5 mL of MaMg solution. After 10 min of incubation at room temperature, protoplasts were treated with PEG in a series of concentrations. Protoplasts were washed three times in 14 mL of KMC solution and then lysed in 1 mL of distilled water. Radioactivity of protoplast lysates was counted for 10 min in a Beckman LS1801 scintillation counter. Background counts for each PEG concentration were obtained from solutions treated exactly in the same manner as the protoplast samples, except without protoplasts. Transfer of radioactivity to protoplasts was estimated by subtracting the background counts from counts in the protoplast lysates.

Selection for Hygromycin Resistance

Hygromycin selection for resistant colonies was performed on 14-d-old regenerated protoplasts. Protoplasts were em-
bedded in agarose blocks (10 × 10 × 0.7 mm) after isolation and all subsequent treatments were done as a bead-type culture (18). Protoplasts were grown in nurse culture for 10 d and in liquid protoplast culture for an additional 4 d. Regenerated protoplasts were then selected for 12 d in General protoplast medium containing 95 µM hygromycin B. Agarose blocks were transferred to General medium containing 0.25% (w/v) Sigma type I agarose and 3% (w/v) sucrose and were maintained for 2 to 4 weeks or until the colony size reached 2 mm in diameter. The number of hygromycin-resistant colonies was counted in at least 10 agarose blocks after 2 weeks of soft agarose culture. The apparent transformation efficiency of protoplasts was defined as a percentage of the number of hygromycin-resistant colonies per total number of protoplasts originally plated in agarose blocks.

**Transgenic Plant Regeneration**

Transgenic plants were regenerated from hygromycin-resistant protoplast colonies in Murashige and Skoog basal medium (12) containing 555 µM inositol, 500 mg/L casein hydrolysate, 2.9 µM indole-3-acetic acid, and 3.6 µM 6-benzylaminopurine (3). The cultures were maintained from 40 to 50 d at 27 °C under constant light (56 μmol m⁻² s⁻¹). Subcultures were repeated every 20 to 25 d.

**Progeny Analysis of Hygromycin Resistance**

Inheritance of the hygromycin-resistance trait was analyzed in seeds of selfed transgenic plants. Seed were dehulled using a mortar and pestle. Dehulled seeds were sterilized in 70% (v/v) aqueous ethanol for 1.5 min then 2.6% (v/v) sodium hypochlorite-water for 35 min with vigorous agitation, followed by three rinses with sterile distilled water. Sterilized seeds were plated in MS medium containing 190 µM of hygromycin B and cultured for 15 d at 27 °C under light.

**Rice DNA Isolation**

Rice DNA was isolated from suspension cells or leaves of transgenic plants by Rogers and Bendich’s procedure with minor modifications (16). The DNA solution was adjusted with CsCl of the density to 1.55 g/mL and was incubated at 20 °C for 1 h. After removal of cell debris by low-speed centrifugation, the total DNA solution was centrifuged at 187,000g for 40 h at 20 °C. A single DNA band thus formed was extracted with 20 × SSC-saturated isopropanol seven times and was dialyzed three times against 2 L of TE buffer at 4 °C. The yield of DNA ranged between 50 and 100 µg per 10 g tissue.

**Southern Blot Hybridization**

Southern blot hybridization was performed essentially as described by Southern (20) and by Schleicher & Schuell’s protocol. Five µg of rice DNA was digested with BanHI or HindIII. Digested DNA samples were fractionated by 0.8% (w/v) agarose gel electrophoresis. The fractionated DNA was transferred to a Nytran membrane and hybridized to a 32P-labeled 1.1 kb BanHI fragment corresponding to the coding sequence of the hph gene (2–7 × 10⁷ cpm). Two control rice DNA samples were reconstructed by adding unlabeled probe DNA to concentrations corresponding to 2 and 10 copies of transformed DNA per diploid rice genome (0.60 pg). The intensities of the bands in x-ray films were measured by a Biorad Video Densitometer model 620. The copy number of transformed DNA was estimated by comparing the band intensities between transformed DNA and reconstructed samples.

**RESULTS AND DISCUSSION**

**Selection for Hygromycin Resistance**

We initially tested responses of rice suspension cells to three potential selection agents (data not presented) (6). The lowest concentrations for complete inhibition of the suspension cell growth were 22 nm for methotrexate, 19 µM for hygromycin, and 1.7 mM for kanamycin. These three chemicals had similar inhibitory effects on the growth of 14-d-old regenerated protoplasts (Fig. 1). The minimum concentrations required for complete inhibition of protoplast growth were 22 nm for methotrexate, 70 µM for hygromycin, and 1 mM for kanamycin. We chose hygromycin as a selection agent for rice protoplasts, because methotrexate was not effective for stringent selection of tobacco, and because endogenous neomycin phosphotransferase activity was detected in rice.

The optimal concentration of hygromycin for stringent selection was determined to be 95 µM based on our selection results. Almost all protoplast-derived colonies were hygromycin-resistant when retested after being selected in the presence of 95 µM hygromycin B. Furthermore, all hygromycin-resistant colonies that have been selected by 95 µM of hygro-

![Figure 1. Effect of hygromycin B on the growth of 14-d-old regenerated protoplasts. Isolated protoplasts were first grown for 10 d in General protoplast medium (10) with nurse cells, and then in liquid protoplast culture for additional four days. Fourteen-d-old regenerated protoplasts were tested for 2 weeks in General protoplast medium containing various concentrations of hygromycin. The surviving colonies were counted in 10 agarose blocks under an inverted microscope for each concentration.](image-url)
mycin contained transferred DNA by Southern hybridization analysis as described below. When selection was applied earlier than 14 d, the number of surviving colonies was significantly reduced. When selection was applied later than 14 d, the selection efficiency was greatly diminished.

Radioactive DNA Transfer to Protoplasts

Physical transfer of DNA to protoplasts after PEG treatment was monitored using labeled DNA to assess the effectiveness of the transformation procedures. Protoplasts were incubated with 3H-labeled DNA and treated with PEG at different concentrations. After extensive washing, radioactivity in protoplast lysates was determined. Radioactivity in protoplasts increased with increasing PEG concentrations from 0 to 30% (w/v) (Fig. 2). Up to 23% of the applied radioactivity was transferred to protoplasts after treatments with the maximum PEG concentration used. More rigorous washing of treated protoplasts or centrifugation of protoplast lysates to remove membrane fractions did not significantly lower the radioactivity in protoplast lysates. Thus, the observed protoplast-associated radioactivity appears to represent the physical incorporation of labeled DNA molecules into the cytoplasm, rather than a tight binding to the plasma membrane of protoplasts. The amount of radioactivity transferred to protoplasts after electroporation was over 100-fold lower than that after the PEG treatment (data not presented).

Protoplast Transformation after PEG Treatment

The PEG concentration was found to be the most important parameter in PEG-mediated transformation of rice protoplasts. Increasing the PEG concentration from 0 to 30% (w/v) reduced the protoplast plating efficiency from 11 to 3% in

Figure 2. Transfer of labeled plasmid DNA to protoplasts after PEG treatment. One million protoplasts were mixed with 10 μg of 3H-labeled plasmid DNA (732,530 cpm) and then treated with PEG in various concentrations. Protoplasts were washed three times in 14 mL of distilled water. Background counts for each PEG concentration were obtained from solutions treated exactly in the same manner as the protoplast samples, except without protoplasts. The radioactivity counts were the average of two independent experiments.

Nipponbare protoplasts (Fig. 3A). Taipei 309 protoplasts responded similarly, but with slightly higher plating efficiencies of 14% without the PEG treatment and 5% with the 30% (w/v) PEG treatment (Fig. 3B). By contrast, the number of hygromycin-resistant colonies increased with increasing concentrations of PEG in both cultivars. A maximum 486 and 242 resistant colonies were recovered at the 30% (w/v) PEG concentration per million PEG-treated Taipei 309 and Nipponbare protoplasts, respectively. The apparent absolute transformation frequency of rice protoplasts reached 2 to 5 × 10⁻⁴ in PEG-mediated transformation. In fact, PEG-mediated transformation yielded over 10-fold more hygromycin-resistant Nipponbare colonies than the electroporation-based method that has been recently reported (19). Although the highest transformation frequency occurred at a PEG concentration of 30% (w/v), at this concentration it was difficult to prevent PEG from precipitation or crystallization. Thus, we chose a final PEG concentration of 25% (w/v) for subsequent transformation experiments.

Promoter and DNA Concentration Effects

The hygromycin resistance gene under the control of CaMV 35S promoter in pTRA132 was three times more effective in

Figure 3. Inverse correlation between the plating efficiency and apparent transformation efficiency of Nipponbare (A) and Taipei 309 (B) protoplasts in various concentrations of PEG. The protoplast plating efficiency was calculated as the number of regenerant colonies per million protoplasts. Apparent transformation efficiency was indicated as the number of resistant colonies per million protoplasts. The plating efficiency and apparent transformation efficiency of Nipponbare protoplasts were determined as the average of three and two independent experiments, respectively. Data for Taipei 309 were obtained from a single experiment.
producing resistant colonies than the one under the nos promoter in pTRA131 (Fig. 4). After PEG-mediated transformation with pTRA132, the numbers of resistant colonies were 197 and 176 per million Nipponbare protoplasts in the presence of 95 and 190 μM hygromycin, respectively. The corresponding colony numbers were 76 and 59 after transformation with pTRA131. These results were consistent with the findings that the CaMV 35S promoter was expressed more effectively than the nos promoter in tobacco and maize cells (17).

The apparent transformation frequency of Taipei 309 protoplasts increased with higher applied concentrations of plasmid DNA. There were 98, 226, 243, and 492 hygromycin-resistant colonies per million PEG-treated protoplasts after transformation with 5, 10, 15, and 20 μg of pTRA132, respectively. We have not tested more than 20 μg DNA per 20 μL TE buffer because DNA was difficult to dissolve in a small volume. We used 10 μg DNA per million protoplasts in all other experiments reported here.

Southern Hybridization Analysis

Transformation of hygromycin-resistant colonies was confirmed by Southern hybridization analysis of Taipei 309 genomic DNA. Total DNA was isolated from five independent suspension cell lines and leaves of three transgenic plants derived from these cell lines. Taipei 309 DNA was digested with HindIII, then the gel-fractionated DNA was transferred to nylon filters and probed with a 1.1 kbp BamHI fragment representing the hph coding sequence (Figs. 4B and 5A). The plasmid pTRA132 contains a unique HindIII site (Fig. 4A). Should individual copies of plasmid DNA be inserted at multiple loci of the rice genome, the number of variable border fragments detected will be the lower limit for the number of integrated plasmid copies. The size of border fragments will depend on the integration site in the plasmid molecule and the location of neighboring HindIII site in the rice genome (Fig. 5A). Should tandem copies of plasmid DNA be inserted at a single locus, a border and 4.4 kbp internal fragments will be generated. The intensity ratio of 4.4 kbp internal to border band will indicate the copy number of integrated plasmid DNA.

BamHI digestions should generate a 1.1 kbp band, indicating the presence of the intact hph coding sequence. Should the hph coding sequences be disrupted in the integration or subsequent rearrangement event, fragments with variable sizes would be detected (Fig. 5B).

The results from HindIII digestion indicated that one (T132-4c) (Fig. 5A, lane 5), two (T132-1 and 2) (lanes 2 and 3) and five copies (T132-4p) (lane 6) of the internal 4.4 kbp band were detected. This suggests that the introduced plasmid DNA might have formed concatemers by intermolecular recombination prior to integration into the rice genome. Less than one copy of the 3 kb band was detected in T-132-3 (Fig. 5A, lane 4). Based on the all digestion results, the copy number of the integrated plasmid was estimated to be less than one (T132-3) (Fig. 5, lane 4), two to three (T132-4c) (lane 5), four (T132-1 and -2) (lanes 2 and 3), and 10 copies (T132-4p) (lane 6). A single two-copy concatemer appeared to be present in the rice genome in T132-4 (lane 5). Two-copy concatemers may have inserted into two locations of the genome in T132-1 and 2 (lanes 2 and 3). Several concatemer molecules may be integrated at four loci of the rice genome in T132-4p (lane 6).

Similar evidence for plasmid concatenation was previously reported in transgenic tobacco plants after protoplast transformation by electroporation (14). In tobacco, linear plasmid molecule appeared to form both head-to-head and head-to-tail concatemers. A tandem repeat of T-DNA was also detected after transformation of tobacco with Agrobacterium tumefaciens nopaline-type Ti plasmid (9, 23). Thus, the integration of transferred DNA after concatemer formation appears to be a general feature of plant cell transformation.

DNA from hygromycin-resistant calli and corresponding transgenic plants were analyzed in three independent cell

![Figure 4. A, Physical structure of the hygromycin resistance gene in the plasmid pTRA132; B, probe DNA used in southern analysis was a 1.1 BamHI fragment corresponding to the coding sequence of the hygromycin resistance gene. Sizes of expected DNA fragments in BamHI and HindIII digestions of genomic DNA.](https://www.plantphysiol.org)
The results from *HindIII* and *BamHI* digestions indicated that the hybridization patterns of most samples were essentially identical (data not presented). This indicated that transferred DNA was stably maintained in the rice genome during plant regeneration. However, when T132-4 DNA was isolated from cell lines propagated for 11 months without selective pressure, the copy number was greatly reduced from 10 to 2 copies (Fig. 5, A and B, lanes 5 and 6). In addition, the hybridization band pattern of leaf DNA (lane 6) was distinct from that of suspension cell DNA (lane 5). The evidence suggests that some transferred DNA was lost and rearranged during the prolonged cell suspension culture in the presence of 2,4-D.

**Fertile Transgenic Rice Plants**

Four *Taipei 309* transgenic plants were regenerated from three transformed cell lines and grown to maturity in the greenhouse. Two phenotypically normal plants were regenerated from one transformed line, T132-4. Both plants set flowering panicles and produced abundant viable seeds (Fig. 6). Agronomic traits of these two plants were normal: plant height, 69 and 84 cm; tiller numbers per plant, 12 and 9;
average panicle length, 19 and 16 cm; average spikelet numbers per panicle, 89 and 63; and seed fertility, 62 and 94%. Inheritance of the hygromycin-resistance trait by progeny was demonstrated by genetic analysis of seeds of two selfed T132-4 plants. The segregation ratios of resistant to sensitive traits in progeny were 48 to 25 and 38 to 17, respectively. Thus, the progeny analysis indicated that the hygromycin-resistance trait is located at one locus or one closely linked loci of the T132-4 genome.

Two other Taipei 309 plants regenerated from transformed cell lines T132-1 and T132-2 displayed some abnormal phenotypic characteristics. They were sterile despite persistent attempts to obtain viable seeds in the greenhouse. The plant T132-1 was taller than average, and had wide, thick leaves, increased kernel size and longer awn. These changes are typical of polyploid or aneuploid plants (3). The architecture of T132-2 plant was abnormal in that the tiller angles were much wider than the average. These abnormal phenotypes have been frequently observed among somaclonal rice variants (3).

Thirty-nine *Nipponbare* transgenic plants were grown to maturity and set flowering panicles in the greenhouse. Fourteen plants were transformed with pTRA131 and 25 plants with pTRA132. After selfing, 35 plants produced viable seeds. Analysis of seed fertility and other agronomic traits of these transgenic plants and inheritance of the hygromycin-resistance gene will be reported in a subsequent publication.

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LITERATURE CITED