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Transformation System of Rice Suspension-Cultured Microcolonies by Electroporation

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For establishing a transformation system of rice (*Oryza sativa*), after three days of culture embryogenic suspension-cultured cell clusters were enzymatically macerated for 2 hours in electroporation buffer containing 2% cellulase and filtered through 550, 400, 250 and 100 μm stainless mesh. Filtered embryogenic microcolonies of 100-250 μm with pBI121 were electroporated at 400 V/cm for 1.2 ms. Four weeks after the electroporation, stable transformed calli were obtained at a frequency of 72% on the selection medium containing 100 mg/L kanamycin. GUS gene in the genomic DNA among 20 out of 22 putative transformed calli lines were detected by PCR analysis. The expression of GUS gene into the kanamycin-resistance calli was confirmed by spectrophotometric assay and histochemical assay of GUS activity. In a histochemical study of the transgenic rice regenerants, it was shown that the GUS activity directed by the CaMV 35S promoter was localized mainly in leaf vein and root apex.

Keywords: transformation system, embryogenic microcolonies, electroporation, *Oryza sativa*, PCR, GUS expression

Rice (*Oryza sativa* L) is the major nutrition source for about 40% of the world population, including most people living in the developing countries. Rice varieties of Japonica and Indica are the major subspecies growing in different regions of the world. Through conventional breeding methods, they play an important and irreplaceable role to crop improvments. But recently, 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.

In past few years, it has been possble to introduce and express foreign genes in plants, especially through the use of Agrobacterium mediated transformation, PEG, Pollen tube, microinjection, particle bombardment and electroporation method (Rainer *et al.*, 1990; Dekeyser *et al.*, 1990; Luo and Wu 1988; Vasil *et al.*, 1993; Lindsey and Jones 1987a; D'Halluin *et al.*, 1992). In dicotyledonary plants, Agrobacterium-mediated transformation system can be used to generate many transgenic plants, but for monocots, which include the agronomically important cereal crops, the use of this method has been hinderd by the limited host range of the bacterium (Terada and Shimamoto, 1990). For this reason, the methods to deliver foreign genes into the protoplasts of cereal plants by polyethylene glycol-facilitated DNA uptake and particle bombardment have been applied to rice varieties, but these applications are limited by plant regeneration system from protoplasts which is generally genotype-dependent and difficult culture techniques (Klein *et al.*, 1987; McElroy *et al.*, 1990; Peng *et al.*, 1990).

The electroporation method is based on the use of short electrical impulses of high field strength that increase the permeability of protoplast membrane (Zimmermann and Vienken 1982). Plant genetic engineering by electroporation was progressed to provide better quality and economically valuable informations to crop plants (Fromm *et al.*, 1986; Lindsey and Jones 1987b; Zhang *et al.*, 1988). These informations were accumulated rapidly in progress of plant genetic engineering field. The electroporation method are applicable to gene transfer directly into the protoplasts of a wide range of plants. However, the regeneration of plants from protoplasts remains to be a difficult problem. Even

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in rice, it takes approximately eight months and includes the low frequency to regenerate mature plants from protoplasts. To date, several electroporation methods have been reported that transfer genes into cultured cell clusters or meristematic organs of monocots, bypassing difficult protoplast culture techniques (Dekeyser *et al.*, 1990; D'Halluin *et al.*, 1992).

We proposed the electroporated gene transfer system into suspension-cultured microcolonies which was simple and high regeneration frequency because cultured microcoloines were more viable and better grew via embryogenic callus to plant than that of protoplasts. Also, we investigated the electroporation effect of transient GUS gene expression in transformed rice regenerants.

MATERIALS AND METHODS

Suspension Culture

Callus of *Oryza* sativa was induced from leaf base region (Kim *et al.*, 1995). Embryogenic cell suspensions were developed from these calli and culture in LS221 liquid medium containing 2 mg/L 2,4-dichlorophenoxacetic acid, 0.2 mg/L kinetin and 0.1 mg/L gibberellic acid. Cells were subcultured at one-week intervals by adding 30 mL of inoculum to 70 mL of fresh medium in 250-mL Erlenmeyer flasks. All cell suspensions were maintained for more than 4 months on the rotary shaker at 100 rpm in the dark at 27°C.

Electroporation

After subculture, four-day-old cell clusters were filtered through a 870 μm stainless steel mesh. Embryogenic suspension cell clusters were enzymatically macerated for 2 hours in electroporation buffer (0.8 g NaCl, 0.02 g KCl, 0.025 g KH_2PO_4, 0.115 g Na_2HPO_4, 100 g glucose, pH 7.1) containing 2% cellulase and filtered through 550, 400, 250 and 100 m stainless mesh. 0.5-1.0 g of filtered microcolonies were resuspended in 10 ml of electroporation buffer. Plasmid pBI121 was isolated and purified according to Sambrook *et al.* (1989).

For electroporation, resuspended solution was placed on ice bath for 5-10 min and the supernatant was removed. One mL of filtered embryogenic microcolonies (100-250 μm) were mixed with 20 μg of plasmid DNA in 20 μL of TE buffer. 500 μL of microcolonies-plasmid mixture solution was transferred to each well of Nunc multi-well chamber and placed on ice bath for 10 min. The electroporation was carried out by discharging with a field strength of 200-1,000 V/cm for 1.2 ms.

Culture Condition

After the electroporation, electroporated microcolonies were incubated on ice for 15 min and they transferred on AA2 or KPR medium (Muller and Grafe, 1978). Selection of kanamycin resistant cell lines was initiated 2-3 days after electroporation in the Nunc multi-well. Media of electroporated microcolonies were changed to a fresh medium containing antibiotics every week and resistant calli were isolated after about 4 weeks of culture. Before being transferred on a MS regeneration medium with 1.0 mg/L NAA and 2.0 mg/L kinetin (Murashige and Skoog, 1962), isolated kanamycin resistant calli were cultured further on the selection medium. These individual colonies were placed, using fine forceps, onto the surface of MS regeneration medium.

PCR Assay

For polymerase chain reaction (PCR) analysis, genomic DNA was isolated from transformed calli by CTAB (Murray and Thompson, 1980). PCR was carried out using 21-mer oligonucleotides, 5'-GGTGG-GAAAGCGTTACAAG-3' and 5'-GTTTACGCG-TTGCTTCCGCCA-3' representing sequences on the GUS gene, were used as primers (Hamill *et al.*, 1991). Each reaction mixture (100 μL) contained 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.1 mM of each oligonucleotide primer, 2.5 units of Taq polymerase, 50 mM KCl, 10 mM Tris-HCl, pH 8.8 and 100 ng genomic DNA. Each cycle consisted of 1 min at 94°C, 1.5 min at 60°C and 2 min at 72°C. Reactions were terminated following a final extension step of 7 min at 72°C. After 35 repeats of the thermal cycle, amplification products were analysed on 1.2% agarose gels. The gels were stained with ethidium bromide and visualized with UV light.

Spectrophotometric Assay

Total proteins were extracted from tissues for assays in 50 mM sodium phosphate (pH 7.0), 10 mM β-mercarptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% triton X-100 (extraction buffer) by freezing with liquid nitrogen

and grinding with PVP. The extracts were centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants for measuring the concentrations of protein extracts were dertermined by Bradford method (1976). The spectrophotometric reaction was carried out with a reaction volume of 5 ml containing 20 g of protein and 1 mM ρ-nitrophenyl β-D-glucuronide. The reaction was carried out at 37°C and aliquots of one mL were removed at zero time and at subsequent times. The reaction was terminated with the addition of 0.4 ml of 2.5 M 2-amino-2-methylpropanediol. Absorbance was measured at 415 nm against substrate blank.

Histochemical GUS Assay

Histochemical assay was performed according to the method described by Jefferson *et al.* (1987). Calli, leaf and roots, with a scalpel to about 5-10 mm, were then fixed in a solution of 0.3% paraformaldehyde, 10 mM MES (pH 5.6) and 0.3 M mannitol for 2 hrs and finally washed with a solution of 0.5 mM sodium phosphate (pH 7.0). Tissue samples were incubated for 24 hr at 37°C in a solution containing 50 mM sodium phosphate (pH 7.0), and 0.1% 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc). After staining, rinse sections in 70% ethanol for 5 min and then mount on slide glass for microsopy. Sectioning of tissues were done by using the two piece blades.

RESULTS AND DISCUSSION

Callus Induction and Electroporation

Calli were induced from the leaf base region of germinated rice (*Oryza sativa*) on LS medium supplemented with 2.5 mg/L 2,4-D in the dark at 27°C. Rice calli, like many other species, are capable of two types of callus growth: embryogenic and nonembryogenic (Nabors *et al.*, 1983). Embryogenic callus is usually produced on the surface region of the callus and frequently gives rise to somatic embryos. Nonembryogenic callus grows in an unorganized manner and may, occasionally, give rise to shoots and roots by organogenesis. After 4-5 weeks of culture embryogenic calli of pale yellow and globular type were collected using fine forceps and used for the initiation of cell suspension cultures in LS221 liquid medium with 2 mg/L 2,4-D, 0.2 mg/L kinetin and 0.1 mg/L GA3 (Muller and Grafe, 1978). Cell clusters were subcultured every week by adding 30 mL of fresh medium.

Table 1. The frequency (%) of callus formation in 100 mg/L kanamycin

Voltage	No. of plates	No. of resistant calli[a]	Transformation frequency[b]
0	142	0	0
200	312	149	47
400	473	348	72
600	297	198	67
800	305	72	23
1000	324	39	12

[a]The number of kanamycin resistant calli was scored after 3 weeks.
[b]Transformation frequency = Number of calli on medium with kanamycin compared to the number of microcolonies.

We electroporated with pBI121 contaning GUS gene as a reporter and NPTII gene as a selection marker for a stable transformation. Embryogenic suspension cells were enzymatically macerated for 2 hours in electroporation buffer containing 2% cellulase and filtered through 100-250 μm stainless mesh. 0.5-1.0 g of these filtered embryogenic microcolonies of 100-250 μm were resuspended in 10 ml of electroporation buffer. One mL of these filtered microcolonies suspension was mixed with 20 μg of plasmid DNA in 20 μL of TE buffer. 500 μL of microcolonies-plasmid mixture solution were transferred to each well. To improve the efficiency of foreign gene introduction, the effect of different voltage was evaluated for expression of the GUS gene in rice cells (Table 1). A middle voltage between 200-600 V/cm produced approximately the same viability and the maximal electroporation efficiency. The transient expression of GUS gene was increased with the increasing voltage. However, at higher voltage than 800 V/cm, cell viabilities were damaged rapidly (Fromm *et al.*, 1986; Dekeyser *et al.*, 1990). Effective electroporation in suspension-cultured microcolonies was able to be carried out by discharging with a field strength of 400 V/cm for 1.2 ms.

Selection and Analysis of GUS Activity in Transformed Microcolonies

After the electroporation, selection of kanamycin-resistant cell lines from each well was done after 2-3 days of culture. Spectrophotometric GUS activity in Table 2 showed that suspension-microcolonies were transformed by electroporation. Within 3 days of culture, the electroporated microcolonies showed the

low levels of GUS activity but the cultured microcolonies for 3 more than weeks had higher GUS activity at a highest frequency of 72%. These results concurred with the view that GUS protein

Table 2. Time course of GUS activity after electroporation of rice microcolonies

Experiments	ρ-nitrophenol (nM)[a]											
	1	2	5	10	20	days	1	2	5	10	20	days
1	4.7	6.4	10.3	18.3	19.6		2.3	2.7	3.6	6.6	9.4	
2	5.2	6.9	12.4	16.7	17.8		2.1	2.6	3.2	6.1	8.9	
3	7.2	7.6	11.5	11.5	18.5		2.4	2.9	4.1	7.3	9.8	

[a]Activities were represented per mg of tissue fresh weight.

Fig. 1. Histochemical assay of GUS activity in rice calli. A, GUS stained electroporated-microcolonies after 2 days of culture; B, completed GUS gene expression of transformed calli after 5 days of culture; C, untransformed control microcalli; D, GUS staining of transformed microcalli derived from suspension-microcolonies after 6 weeks of culture.

has been persisted for a long time, and accumulated within tissues.

The electroporated microcolonies were assayed visually for GUS expression by counting the number of blue cells among 1, 3, 5, 10 and 20 days after transformation with plasmids. The number of blue cells was examined at the different time intervals between electroporation and histochemical GUS assay after the electroporation (Table 2). The number of blue cells was low within 2 days (Fig. 1A) and for these initial lag period there were few cells displaying visible GUS expression. The number of cells displaying visible GUS activity increased after 5 days of culture (Fig. 1B). But after 3 weeks of culture we found no significant increase in the number of cells displaying GUS activity.

Individual colonies from the kanamycin selection medium were placed, using fine forceps, onto the surface of regeneration medium. When treated with X-gluc after 6 weeks of culture, transformed microcalli grew and were GUS-positive (Fig. 1D). On the average, 22 out of 30 kanamycin resistant microcalli lines showed blue color with X-gluc in each well, whereas eight non-blue color microcalli lines were GUS-negative (Fig. 1C).

Transformation Analysis by PCR

The presence of GUS gene in the genomic DNA from kanamycin resistant calli was detected by PCR amplification of the 1.2 kb fragment, by using primers based on sequences from the coding region of the GUS gene. As in Fig. 2, putative transformant calli lines of RT1-10, were analyzed by PCR. Size marker of GUS gene (1.2 kb) as a positive control was shown in RT1-5, and RT7-9 lines except RT6 and RT10, indicating that GUS gene is retained in subculture. The PCR analysis of all lines derived from each well, revealed that 20 out of 22 GUS-positive and kanamycin resistant calli lines retained the GUS gene. This means that the GUS gene was introduced in about 67% of kanamycin resistant calli lines. However, two lines of the kanamycin resistant calli which didn't express GUS gene, was suggested that partial deletion or position of inserted GUS gene on the genome affected negatively on the GUS gene expression in these kanamycin resistant calli (Peng *et al.*, 1990). These results indicate that most of kanamycin resistant calli lines are transformed.

Plant Regeneration from Kanamycin Resistant Calli and Histochemical GUS Assay

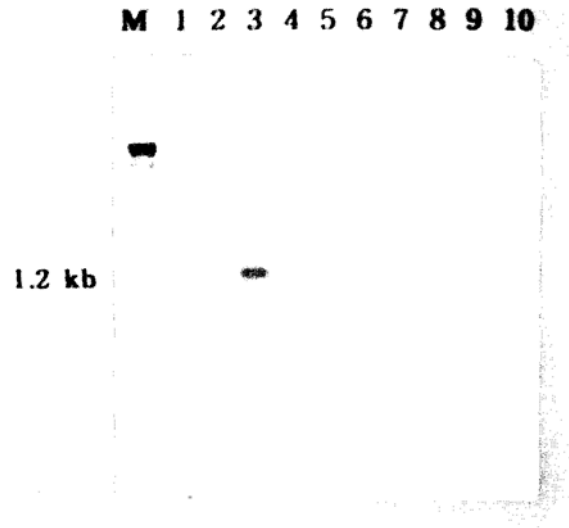

Fig. 2. The detection of GUS gene in the genomic DNA of 10 putative transformant calli lines of rice by PCR. PCR was performed with 30 cycles of amplification: 94°C denature/55°C anneal/72°C extention. For each sample, 10 μL from a total 50 μl reaction was run on 1.2% agarose gel at 100 V for 60 min. M, Size marker; Lane 1-10, putative transformant calli lines RT1-10, respectively. 1.2 kb = Size of GUS gene as a positive control.

Before transferred on MS regeneration medium with 1.0 mg/L NAA and 2.0 mg/L kinetin, microcalli were cultured further in the liquid selection medium which differed with those culture method reported previously by Abdullah *et al.* (1986), and Zhang *et al.* (1988). To select the resistant calli lines in liquid selection medium improved the frequency for obtaining positive transgenic plants. After 4 weeks on a liquid selection medium containing 100 mg/L kanamycin in suspension culture medium, approximately 120 independent kanamycin resistant calli were selected totally from 12 wells of Nunc multi-well chamber. Except the selected calli, all the other calli failed to grow. Being further cultured on the selection medium for 4 weeks, kanamycin resistant calli were grown actively when compared to untransformed calli to be inhibited by kanamycin (Zhang *et al.*, 1988). Some transformed calli derived from each microcolony on selection medium containing 100 mg/L kanamycin, showed organogenic. It is suggested that the calli growing in liquid selection medium with antibiotics can be maintained as embryogenic calli which have a regeneration ability. Most of them showed a high activity in *in situ* GUS assay. Also, these embryogenic calli formed multiple embryo-like structures. Within 4 weeks after the

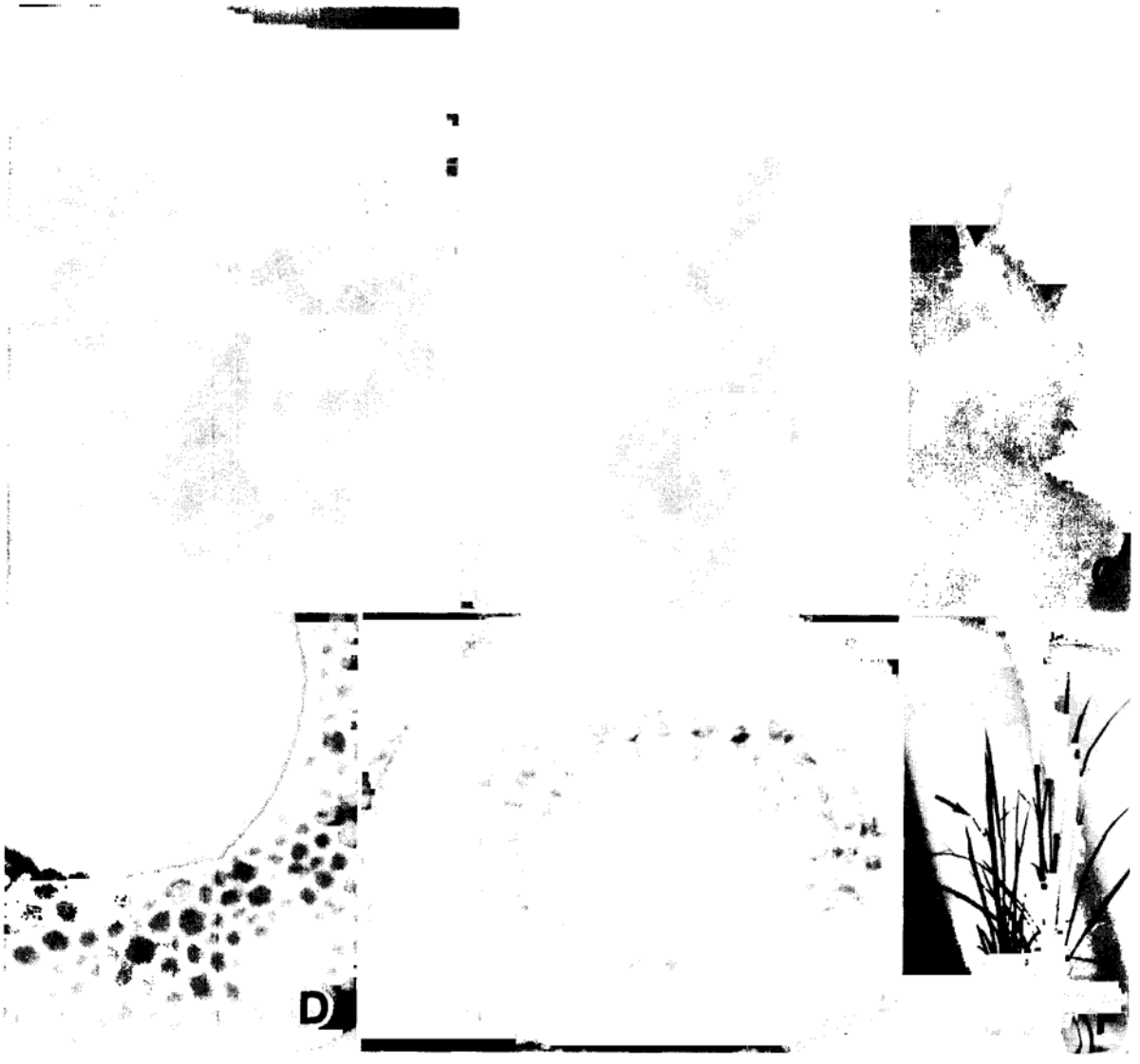

Fig. 3. Rice plantlet regenerated from the transformed microcalli line RT3 and their histochemical assay of GUS activity. A, Transformed callus on the selection medium containing of 100 mg/L kanamycin after 3 weeks of subculture; B, Plantlets regenerated from the transformed callus and their shoot used for X-Gluc staining to determine GUS expression; C, Root hairs; D, Abundant GUS activity of vascular bundle in transverse section of young leaf of transgenic RT3 plant line; E, GUS staining in transverse section of young root apex of RT3 plant; F, transgenic RT3 plant.

calli were transferred onto a regeneration medium, embryo-like structures were first observed on some calli (Fig. 3A). These structures developed into tiny shoots after 3-4 weeks from intial culture onto the sulface of regeneration medium (Fig. 3B). Most of roots developed bilaterally and simiutaneously from the bases of shoots, whose appearance was similar to organogenesis (Fig. 3C). So far, more than 61 plants were acclimatized and then transferred to potting soil. Finally 47 regenerated plants were cultivated in a green house (Fig. 3E). Transgenic regenerants through the electroporation method had similar morphological characteristics to that of seed plant and regeneration frequency from the selected microcalli was about 39%. The protoplast transformation process for producing transgenic rice plants, requires time period of 10 months but these method using suspension-cultured microcolonies only requires 12 weeks of short culture period to obtain transgenic plant.

To determine the expression pattern of CaMV 35S in regenerated transformed plant lines from RT1-

10 transformed callus lines, even though GUS activities in these callus lines previously confirmed, leaf sheaths and roots of the transgenic rice RT3 plant line were sectioned and then stained histochemically by X-gluc. In leaf sheath and root of RT3 plant line, we observed various degree of GUS expression, whereas in non-transformed control plant, GUS activity was not detected. Vascular tissues in transverse section of young leaf sheaths of transgenic RT3 plant line revealed abundant GUS activity. Leaf apex had also high GUS activity, but leaf mesophyll cells showed weak. Especially, Leaf vein exhibited very intensive blue staining (Fig. 3D). Root apex had the highest GUS activity at young developmental stage in the transgenic plants (Fig 3E). Certain specialized cells such as epidermal and phloem cells also showed high GUS activity.

In transgenic plants, it was reported that the expression pattern of CaMV 35S promoter gene depended on the division capacity of cells, was not active in all types. In all tissues that we examined, there was a good correlation between higher GUS activity driven by the CaMV 35S promoter and cell division capacity. Our data suggested that CaMV 35S promoter activity might be located to actively dividing tissues such as meristem, and vascular bundle of shoot and root in transgenic rice.

In conclusion, we have proposed the transformation system using the electroporaton of microcolonies, which is simple and efficiency in rice suspension-cultured cells.

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