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Efficient gene introduction into rice by electroporation and analysis of transgenic plants: use of electroporation buffer lacking chloride ions

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Summary. We have developed a method for reproducibly obtaining transgenic rice at a high frequency (10^{-4}): electroporation with a buffer in which chloride ions are replaced with organic acids. Co-transformation frequencies of the β-glucuronidase (GUS) and hygromycin phosphotransferase (HPT) genes located on two separate plasmids were higher than 50%. Transgenic rice plants contained multiple copies of introduced genes integrated into their genomes in a complex manner. GUS enzyme activity was not proportional to gene copy number. Introduced HPT genes were detected and expressed in the progeny of transformants.

Key words: Electroporation – Rice – Co-transformation – β-Glucuronidase – Hygromycin phosphotransferase

Introduction

Transgenic plants have been obtained by electroporation not only in dicot plants such as tobacco (Riggs and Bates 1986) and *Brassica napus* (Guerche et al. 1987), but also in the gramineae including *Oryza sativa* (Toriyama et al. 1988; Zhang et al. 1988; Shimamoto et al. 1989), *Zea mays* (Rhodes et al. 1988) and *Dactylis glomerata* L. (Horn et al. 1988), which are refractory to transformation by *Agrobacterium*. However, the high voltage electric pulse used in this method causes the destruction of protoplasts and leads to low plating efficiency. As a result the transformation frequencies reported in rice and the other gramineae have been generally low 10^{-6} to 10^{-5} transformants/electroporated cell (Toriyama et al. 1988; Hauptmann et al. 1988; Dekeyser et al. 1989; Shimamoto et al. 1989).

Methods described in the literature generally utilize high concentrations of chlorides (e.g., KCl, NaCl, $CaCl_2$ and $MgCl_2$) in their electroporation buffers (Fromm et al. 1985, 1986; Ou-Lee et al. 1986; Dekeyser et al. 1989; Rhodes et al. 1988, Shimamoto et al. 1989) despite the fact that the electrolysis of chlorides produces Cl_2, a toxic gas. We hypothesized that the elimination of Cl_2 gas formation might increase cell survival rates and subsequent transformation frequencies. In this article we report higher survival rates and reproducibly high transformation frequencies were obtained by electroporation in a buffer in which organic acids replaced the chloride ions. In addition, we analyze the transgenic rice plants obtained by our method.

Materials and methods

Isolation and culture of protoplasts

Protoplasts were isolated from suspension cells of rice (*Oryza sativa* cv 'Norin-8' or 'Sasanishiki') and purified according to the method of Akagi et al. (1989). Electroporated protoplasts were cultured following Fujimura et al. (1985).

Plasmids

pBI221 (Fig. 1a, Clontech) containing the β-glucuronidase (GUS) gene linked to the CaMV35S promoter and pUC19-HPT [Fig. 1b, provided by Dr. A. Kato (Nat. Inst. Agrobiol. Res.)] containing the hygromycin phosphotransferase (HPT) gene linked to the CaMV35S promoter were used in their super-coiled form.

Electroporation

Purified protoplasts were suspended at a population density of 2×10^6/ml in the electroporation buffer described below, and 0–20 μg/ml of plasmid DNA was then added. This suspension was incubated at 0 °C for 20 min and then transferred into an

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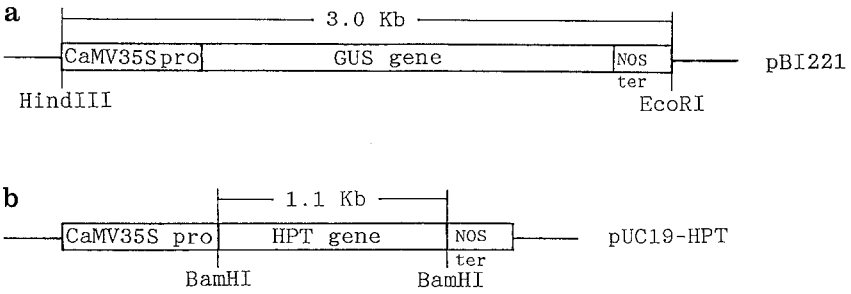

Fig. 1 a, b. Diagramm of pBI221 (**a**) and pUC19-HPT (**b**). Only the restriction sites used in this work are indicated.

ice-cold electroporation chamber which had two parallel gold-plated stainless steel electrodes spaced 4 mm apart attached to the bottom of a glass petri dish. An exponential electric pulse was applied to the chamber from the capacitor (880 μF). On the basis of preliminary transient expression experiments with the GUS gene (data not shown), an initial voltage of 475 V/cm with the time decay constant $T_{1/2}$ (defined here as the duration time for decaying voltage to half of the initial voltage) of 30.0 msec was chosen for all experiments described here. Electroporated protoplasts were incubated on ice for 20 min and collected by centrifugation at 900 rpm for 3 min. These protoplasts were resuspended at a density of 10^6/ml in protoplast culture medium.

GUS assay

Transient expression. After incubation for 48 h protoplasts were collected by centrifugation and homogenized in the buffer. GUS activity were assayed in extracts following the method of Jefferson et al. (1987). After a 1-h incubation at 37 °C with the substrate, 1 m*M* 4-methyl umbelliferyl glucuronide (4-MUG), the amount of product, 4-methyl umbelliferon (4-MU), was determined by fluorometric assay.

Stable expression. The expression of GUS gene in callus or plant tissues was detected by a staining method (Jefferson et al. 1987) utilizing 1 m*M* 5-bromo-4-chloro-3-indlyl-β-D-glucuronide (X-Gluc). GUS activities were determined in leaf extracts by the same fluorometric assay as that described for transient expression. Protein content was determined using the Bio-Rad protein assay.

Selection of hygromycin resistant colonies

At day 14 of protoplast culture hygromycin was added to the medium at a concentration of 50 μg/ml, and after another 2 weeks the colonies were transferred to fresh medium containing hygromycin (50 μg/ml). After further culture colonies attaining 2 mm in diameter were transferred onto plant regeneration medium (Fujimura et al. 1985) lacking hygromycin.

Southern blot analysis

Plant DNA was extracted from leaves according to the method of Rogers and Bendich (1985) and then purified by CsCl gradient centrifugation. The purified DNA was digested with restriction enzymes, electrophoresed on 0.9% agarose gels and transferred onto nylon membranes. Hybridization were carried out using Nonradioactive, DIG-ELISA DNA Labeling and Detection kit (Boehringer Mannheim).

Test of hygromycin resistance in progenies

Selfed seeds from each hygromycin resistant plant were germinated in water containing hygromycin (20 μg/ml). After 10 days surviving seedlings were counted and planted in soil.

Table 1. Effect of buffer composition on transient expression of the GUS gene

Experiment	Buffer[a]	GUS activity[b] (moles 4-MU/h per 7.5×10^5 protoplasts)		
		0	20 (μg pBI221/ml)	
		(a)	(b)	(b−a)
1	KCL	221	174	−47
	ASP	142	184	+42
	AA	141	200	+59
2	KCL	254	248	−6
	ASP	202	238	+36
	AA	206	306	+100
3	KCL	193	206	+13
	ASP	175	224	+49
	AA	181	230	+49

[a] Compositions of which are described in text
[b] Each value is the mean of duplicate samples that were taken 48 h after electroporation

Results

Electroporation buffer composition

We initially introduced the GUS and HPT genes into rice protoplasts by electroporation in a buffer containing 70 m*M* KCl, 5 m*M* $CaCl_2$, 5 m*M* 2-[*N*-morpholio] ethanesulfonic acid (MES) and 0.4 *M* mannitol (pH 5.8) (referred to as the KCL buffer). When the GUS gene was introduced in KCL buffer, the protoplasts often showed lower fluorometric values of GUS activity than control protoplasts electroporated without DNA (Table 1). When the HPT gene was introduced in KCL buffer, the colonies formed were selected with hygromycin to determine the transformation frequencies. Under our selection conditions no colonies were formed after 2 months of culture from protoplasts electroporated without DNA. Transformation frequencies with DNA were low (from 10^{-6} to 10^{-5}) and irreproducible (Table 2). In some experiments no transformants were obtained.

The Formation of Cl_2 gas in KCL buffer at the moment of electroporation was evident from its characteristic odor. In order to depress its formation, we devised

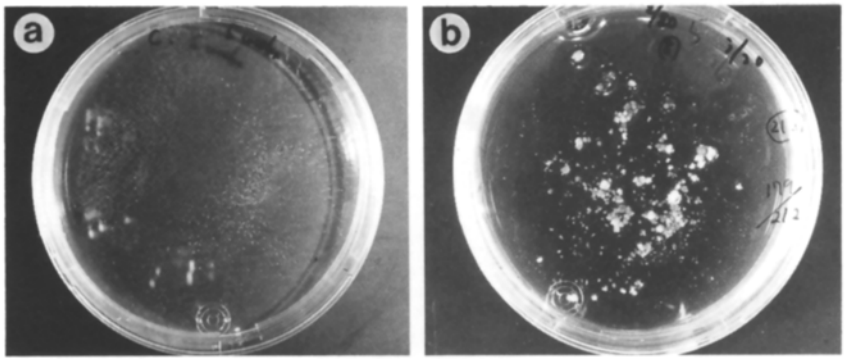

Fig. 2a, b. Selection of hygromycin resistant colonies. Protoplasts were electroporated in the absence (**a**) or presence (**b**) of plasmid DNA (pBI221 and pUC19-HPT) and selected with hygromycin as described in the text. The photographs show plates for single electroporations five weeks later

Table 2. Transformation frequency

Experiment	Buffer	Colony formation efficiency[a, b] (%)	Transformation frequency[a, c]
1	KCL	3.57 ± 0.70	$3.65 \pm 0.34 \times 10^{-6}$
	ASP	9.50 ± 1.14	$1.33 \pm 1.17 \times 10^{-4}$
2	Cont	9.80 ± 1.28	0
	KCL	0.58 ± 0.09	0
	ASP	8.98 ± 1.23	$1.37 \pm 1.34 \times 10^{-4}$

Cont, Non-electroporated protoplasts
[a] Each value is the mean ± standard error of triplicate samples
[b] Percentage of initial protoplasts forming colonies in the absence of hygromycin after 3 weeks of culture
[c] Each value was determined after 5 weeks of culture and is based upon the number of protoplasts initially present in the electroporation buffer

Table 3. Co-transformation of the GUS and HPT genes (5 weeks after electroporation)

Experiment	pBI221 + pUC19 -HPT (μg/ml)	GUS^+/Hyg^R	Co-transformation frequency (%)
1	10+10	16/109	14.7
2	10+10	8/ 12	66.7
3	20+10	7/ 21	33.3
4	20+10	76/175	43.4
5	20+10	179/212	84.8
6	20+10	20/ 36	55.6
7	20+10	113/203	55.7

GUS^+, No. of colonies staining blue with X-Gluc; Hyg^R, no. of hygromycin-resistant colonies tested

new buffers: one contains 70 m*M* aspartic acid monopotassium salt, 5 m*M* calcium gluconate, 5 m*M* MES and 0.4 *M* mannitol (pH 5.8) (referred to as the ASP buffer) and the other contains 35 m*M* aspartic acid monopotassium salt and 35 m*M* glutamic acid monopotassium salt in place of the 70 m*M* potassium aspartate used in ASP buffer (referred to as the AA buffer).

Using these buffers we were able to reproducibly detect the transient expression of the GUS gene (Table 1). By introducing the HPT gene and selecting for hygromycin resistance, we reproducibly got transformants at a high frequency (10^{-4}) when the ASP buffer was used (Table 2, Fig. 2a, b). The Survival of the electroporation process as reflected in the frequency of colony formation was relatively low in the KCL buffer. In contrast, those electroporated in ASP buffer formed colonies as efficiently as non-electroporated protoplasts (Table 2). Microscopic observation indicated that protoplasts electroporated in the KCL buffer suffered obvious damage. In contrast, those protoplasts electroporated in ASP buffer could scarcely be distinguished from non-electroporated protoplasts.

Co-transformation

The GUS and HPT genes were introduced simultaneously into protoplasts in ASP buffer, and hygromycin selection was applied to the resulting colonies. Surviving colonies were assayed for GUS activity using X-Gluc to determine the co-transformation frequency (Table 3). On average more than 50% of hygromycin resistant colonies exhibited GUS activity.

Analysis of regenrated plants

One hundred and eleven plants (23.0%) were independently regenerated from hygromycin-resistant calli (483) transferred to regeneration medium. Leaves from each of these plants were tested for GUS activity by staining with X-Gluc: 43 plants (38.7%) stained positively, as did all of their tissues that were examined –, root, leaf, stem, glume and awn. GUS activities were determined more quantitatively by an assay of the leaf extracts with 4-MUG (Table 4). GUS activities among non-transformant and non-stained plants were low (25 to 134 pmoles 4-MU/min per mg protein), while those of the stained plants were very high, though variable (1,497 to

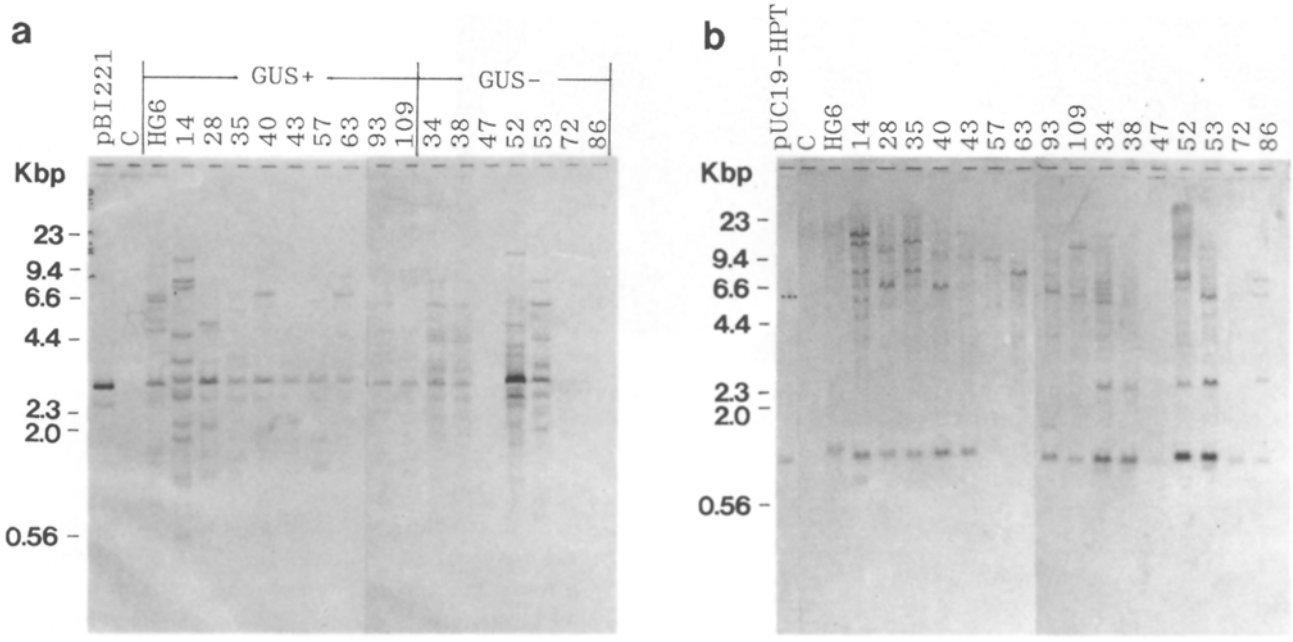

Fig. 3a, b. Southern blot analysis of DNA extracted from plants regenerated from hygromycin-resistant calli. *Numbers* refer to individual regenerated plants. **a** Total DNAs (5 μg) digested with EcoRI and HindIII were probed with the GUS structural gene. X-Gluc Stainable (GUS^+) and non-stainable (GUS^-) plants were used. *Lane pBI221* was a molecular weight standard. The amount of DNA corresponds to approximately 15 copies per diploid rice genome; *lane C* nontransformant. **b** Total DNAs (5 μg) digested with BamHI were probed with the HPT structural gene. *Lane pUC19-HPT* was a molecular weight standard. The amount of DNA corresponds to less than three copies per diploid rice genome; *lane C* nontransformant

10,531 pmoles 4-MU/min per mg protein). The measured GUS activity of HG93 was 420-fold that of HG47, which contained no GUS gene.

Total DNA was isolated from leaves of the regenerated plants, digested with EcoRI and HindIII or BamHI, and Southern blots were performed using the GUS or HPT structural genes sequences as probes (Fig. 3a and b, respectively). Only No. 43 displayed a single band corresponding to the intact CaMV35S promoter, the GUS structural gene and the NOS terminator (3.0 kb). The other plants that stained positively with X-Gluc contained multiple copies of the GUS genes, many of which did not correspond in restriction pattern with the initial genes construct. Of the seven regenerated plants tested whose leaves did not stain positively with X-Gluc, four (HG34, HG38, HG52 and HG53) nevertheless contained copies of the GUS gene. Most notably, HG52 contained many copies of the gene. HG47, HG72 and HG86 certainly contained less than two and probaby no full-size GUS insert. The presence of hybridizing sequences larger than the original construct indicates that these sequences were integrated into the genome. The HPT gene also displayed a complex integration pattern similar in manner to that of the GUS gene. Most plants contained a 1.1 kb hybridizing fragment that corresponded to the initial HPT gene construct. In the case of HG57 and HG63, no intact HPT gene sequences were detected, even

Table 4. GUS activity in leaf extracts from transgenic plants

Plant	GUS activity[b]	Plant	GUS activity[b]	Plant	GUS activity[b]
c[a]	134	HG19	7,005	HG63	9,840
HG3	30	20	5,874	67	4,127
47	25	24	2,559	68	2,281
73	36	25	4,033	75	6,457
100	107	32	5,430	78	1,788
6	7,154	35	4,154	81	7,957
8	1,921	36	4,397	93	10,531
9	4,509	40	3,983	105	3,563
13	5,639	42	3,578	107	4,451
14	1,497	43	6,842	109	6,516
15	5,798	57	5,650	111	7,695

Leaves of first five plants listed (C, HG3, HG47, HG73 and HG100) did not perceptibly stain with X-Gluc; the remaining plants all stained

[a] Non-transformant

[b] pmoles 4-MU/min per mg protein

Table 5. Transmission of hygromycin resistance to the progeny

Seed	No. of seeds tested	No. of resistant seeds	No. of sensitive seeds
S-1 self	20	17	3
S-7 self	40	29	11

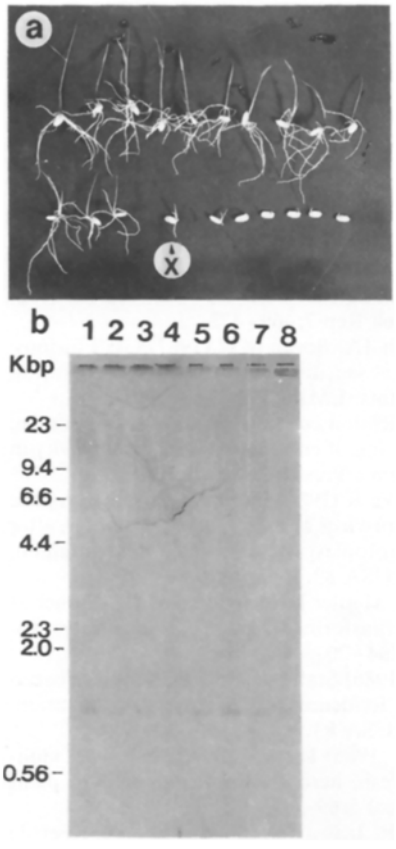

Fig. 4 a, b. Transmission of the HPT gene to progeny. **a** Seedling resistance to hygromycin. Selfed seeds of the hygromycin resistant transformant S-7 were germinated in a solution of 20 μg hygromycin/ml for 10 days. Fourteen resistant and six sensitive seedlings are shown in this fig. The seedling denoted with an X was shown to contain the HPT gene by Southern blot analysis. **b** Southern blot analysis of DNAs from S-7 and its progeny. Total DNAs (5 μg) digested with BamHI were probed with the HPT structural gene. *Lane 1* S-7, *lane 2–8* individual hygromycin resistant progeny of S-7, *lane 7* the seedling denoted X in **Fig. 4 a**

though these plants were regenerated from hygromycin-resistant calli. The sensitivity of detection does not allow us to exclude the possibility that a single unaltered sequence was present. Alternatively, the altered HPT gene sequences in these plants may have conferred resistance.

Although an accurate determination of introduced gene copy number cannot be made from this experiment, clearly GUS activity levels did not correspond simply to the sequence copy numbers maintained in regerated plant genomes. This lack of correspondence is demonstrated most clearly for regenerant HG93 (compare Table 4 and Fig. 3 a).

Transmission of expressed HPT gene among the progeny

Selfed seeds from two hygromycin resistant plants (S-1 and S-7) were germinated in 20 μg/ml of hygromycin solution for 10 days. Under these conditions, radicles of non-resistant germinating seeds elongated very little and then died within 2 weeks (Fig. 4 a). The approximate ratio for the segregation of hygromycin resistance among the selfed seedling of both transformants were thus determined (Table 5). DNAs isolated from leaves of S-7 and its hygromycin resistant progeny were subjected to Southern blot analysis (Fig. 4 b). A 1.1 kb fragment hybridizing to the HPT gene probe was observed in S-7 and all resistant progeny. These results demonstrate the stable transmission and expression of the introduced HPT gene among transformant progeny.

Discussion

Electroporation buffers of differing compositions have been described in various reports, but there has been no detailed examination of the effect of the buffer composition on electroporation efficiency. We have examined the effects of substituting organic acids for chloride ions in electroporation buffers. Using such buffers we have successfully obtained high plating efficiencies and consequently high transformation frequencies. The buffer used contained aspartic acid and/or glutamic and gluconic acid, all of which were assumed to result in a lower cell toxicity than chloride. From our results we infer that (1) the Cl_2 gas formed in the buffer during electrolysis has considerable toxic effects on the cells, and (2) during electroporation buffer solutes may enter the cells. Therefore, the buffer used for electroporation must not contain toxic elements. For these reasons, the organic acids described above are more suitable buffer constituents than chloride.

The inclusion of several organic acids simultaneously might result in a safer buffer for electroporation than simple solutions, as single amino acids at high concentrations in the cell sometimes inhibit amino acid metabolism (Miflin 1977).

The frequency of plant regeneration from callus formed from protoplasts electroporated in ASP buffer was as high as that from untreated protoplasts, and a large number of fertile transgenic plants were obtained. Neither growth inhibition nor the mutagenesis of calli and plants resulted from electroporation in ASP buffer or AA buffer. Preliminary experiments with eggplant and carrot indicate that these buffers will be generally useful for electroporation of other plant species (data not shown).

Co-transformation by electroporation has been reported by others (Wirtz et al. 1987; Shimamoto et al. 1989). In our work, some plants with both rearranged and apparently intact GUS sequences intergrated in their genomes exhibited no GUS activity. This fact suggests that actual co-transformation frequencies are higher than those indicated in Table 3.

GUS gene expression under control of the CaMV35S promoter was detected in all of the tissues of transgenic rice plants that were examined; this is in agreement with the report of Aoyagi et al. (1988). Considerable differences in GUS activity were observed among transformants, perhaps reflecting position effects. In some cases plants with apparently intact and multiple rearranged copies of the gene exhibited no activity above background, and in other cases high expression levels were observed in plants with relatively few gene copies. Disparities between gene copy numbers and expression levels have also been reported by DeBlock et al. (1987) and Streber and Willmitzer (1989). In contrast to the report of Shimamoto et al. (1989), we observed a rather complex pattern of foreign gene integration into the genome. The reason for this difference is not apparent.

An efficient and reproducible transformation system for rice has been established. Practical exploitation of this system must depend upon the isolation and introduction of useful genes and a greater understanding of the sequences and factors responsible for tissue- and/or stage-specific expression.

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