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Efficient transformation of rice protoplasts mediated by a synthetic polycationic amino polymer

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Abstract A very simple and efficient transformation system for rice was established using a synthetic polycationic amino polymer (polycation). Improvements in the culture conditions, especially filtration of the suspension cells before and after protoplast culture, greatly contributed to a large yield of high-quality protoplasts and an increased ability of the cells to regenerate. Transformation parameters, such as the ratio of DNA and polycation concentrations, preincubation of the DNA and polycation prior to DNA transfer, and precentrifugation and resuspension of protoplasts before DNA transfer, were analyzed. Fertile transgenic plants containing the *bar* gene were selected and shown to demonstrate resistance against high concentrations of bialaphos. Southern blot analysis showed four to nine bands representing the *bar* gene in polycation-mediated transgenic rice plants compared with two to three bands in electroporation-mediated transgenic rice plants. The regeneration efficiency of the polycation-mediated method was compared to that of the electroporation-mediated method; while the polycation-mediated method tended to show a relatively lower regeneration rate, regenerants showed a normal phenotype.

Key words Bialaphos resistance · Polycation · Poly-L-ornithine · Rice transformation

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Introduction

Various methods of gene transfer for rice have been developed, such as polyethylene glycol (PEG) (Zhang and Wu 1988; Hayashimoto et al. 1990; Datta et al. 1992; Peng et al. 1992), electroporation (Toriyama et al. 1988, Zhang et al. 1989; Shimamoto et al. 1989; Tada et al. 1990), and particle bombardment (Christou et al. 1991). Moreover, Hiei et al. (1994) recently developed *Agrobacterium*-mediated transformation in rice. Among these methods, the bombardment and electroporation methods require the use of special equipment for transformation experiments. The polyethylene glycol method is comparatively easy; however, polyethylene glycol itself is sticky and induces undesired cell fusion. On the other hand, the disadvantages of *Agrobacterium* mediated-delivery includes frequent contamination of *Agrobacterium* in transgenic calli and rather complicated vector construction.

We investigated a new alternative to these methods. Recently, a synthetic polycationic amino polymer (polycation) was used in gene delivery to human cells *in vitro* and *in vivo* (Goldman et al. 1997). Polycation is water-soluble, easily miscible in any aqueous vehicle, and not sticky, unlike polyethylene glycol.

Poly-L-ornithine, a kind of polycation, has been used in the infection of plant protoplasts by plant virus (Takebe 1977). Takebe and Otsuki (1969) first found that polycation had a remarkable effect on the infection of tobacco mesophyll protoplasts by tobacco mosaic virus (TMV). This method has also been successfully used for introducing viral RNA (Aoki and Takebe 1969) and artificial macromolecules (Suzuki et al. 1977) into plant protoplasts. Although polycation was shown to be effective for DNA uptake by protoplasts (Suzuki and Takebe 1976, 1978; Fernandez et al. 1978), transformation of higher plants could not have been demonstrated in those days because vectors for plant transformation were not yet available. In this study, we focused on this method again to transform rice protoplasts.

As the polycation method of gene transfer is cost-effective and does not require any special equipment, the method can be widely used even in a laboratory with less sophisticated equipment. Moreover, the polycation method provides the possibility of introducing larger sized DNA molecules into plants than can be done with the *Agrobacterium*-mediated-delivery. Protoplasts have been shown to take-up large molecules such as latex particles (Suzuki et al. 1977).

This method utilizes the chemical property of polycation that binds both DNAs and protoplasts effectively; that is, the contact between the DNA and the surface of protoplasts, both negatively charged, could be facilitated by the positively charged polycation. The original method using polycation to introduce viruses was not sufficient to give a high yield of transgenic plants. To establish a high frequency of transformation, we first developed conditions for a high frequency of regeneration of rice protoplasts, and then we studied a series of parameters for introducing DNA into these rice protoplasts. Finally, we established very simple and highly efficient conditions of transformation using poly-L-ornithine. Thereafter, we produced herbicide-resistant transgenic rice plants with this method and compared it with the electroporation method in terms of fertility and frequency of occurrence of some variations.

Materials and methods

Plant material

The culturing procedure is summarized in Fig. 1 with the media described in Table 1. We induced the callus from embryos of rice (*Oryza sativa* L. *japonica* cvs 'Nipponbare' and 'Mutsuhomare') seeds on callus induction medium (CI medium) based on N6 medium (Chu et al. 1975). After 1 month, a suspension cell culture was made from the embryogenic calli. The suspension cells were maintained by subculturing weekly with the suspension liquid culture medium (L medium). While the suspension culture was maintained, "Filtration manipulation" was carried out several times by means of the selection of calli through callus filtration with an approximately 1-mm-diameter stainless mesh. Only young active calli of a uniform size were filtrated, collected by centrifugation, and cultured. For protoplast isolation, calli were precultured in the preculture medium (P medium) based on MS medium (Murashige and Skoog 1962), for 3–5 days and then incubated in a 0.5 *M* mannitol solution containing 2% Cellulase Onozuka RS (Yakult Co, Japan), 0.05% Pectolyase Y-23 (Seisin Pharmaceutical, Japan), 0.01% $CaCl_2$, and 0.1 m*M* MES (pH 5.6) at 30°C with shaking at 30 rpm in darkness for 1 h followed by standing for 2 h. The regeneration frequency for each line was calculated by counting the number of colonies regenerated to plants per 100 colonies tested.

Plasmids

pBI 221 containing the *uid*A gene linked to the CaMV 35S promoter (Clontech) was used in the β-glucuronidase (GUS) assay. pBI 221 containing the *hph* gene instead of the *uid*A gene was used as a selectable marker of hygromycin. The pINT-bar plasmid vector, which was used for the production of transgenic rice plants resistant to the herbicides bialaphos (Herbiace™, Meiji Seika, Japan) and Basta (AgrEvo, Germany), consisted of the *bar* gene from *Streptomyces hygroscopicus* (Murakami et al. 1986) connected to the intron of a catalase gene from the castor bean (Ohta et al. 1990) under the control of the CaMV 35S promoter.

Transient assay of GUS

Standard assay conditions were as follows: plasmid DNA (pBI 221, 2 mg/l) was incubated in the *K*-phosphate-buffer (5 m*M*, pH 5.6) containing poly-L-ornithine (4–8 mg/l; average MW 169,000 Sigma) and 0.7 *M* mannitol for 10 min to facilitate the binding of DNA and poly-L-ornithine. Protoplasts (3×10^6/ml) suspended in 0.7 *M* mannitol were gently mixed with an equal volume of the DNA solution (containing 2–8 mg DNA/l in 0.7 *M* mannitol) and then incubated standing for 10 min. Protoplasts were then placed into conditioned medium (CM medium) based on R2 medium (Ohira et al. 1973). Samples of the protoplast suspension were incubated in Eppendorf tubes for 24 h at 25°C in the dark. Cells were collected by centrifugation at 3,000 rpm and disrupted by brief vortexing in lysis buffer containing 0.1% Sarkosyl, 0.1% Triton X-100, 2 m*M* DTT, 10 m*M* EDTA, and 50 m*M* Na-phosphate buffer (pH 7.0). The GUS activity of each extract was measured fluorometrically using 4-methyl-umbelliferone (4MU) (Jefferson et al. 1987).

Gene transfer by electroporation

Isolated protoplasts were resuspended in 0.5 *M* mannitol containing purified 5 mg/l plasmid DNA, 10 mg/l carrier DNA (sonicated calf thymus DNA), and 0.1 m*M* $MgSO_4$. Electroporation was performed at 1,000 V/cm in a continuous flow electromanipulator (CET 200; JASCO, Tokyo, Japan). Electroporated protoplasts were kept at room temperature for 1 h, collected by centrifugation, and then cultured at 25°C in the dark.

Culture conditions for transformed protoplasts

To evaluate the efficiency of stable transformation, we used the hygromycin-resistant gene (*hph*) as a selectable marker. Protoplasts with an introduced *hph* gene were embedded in agarose beads (Kyozuka et al. 1987) with CM medium in the dark. Agarose beads containing protoplasts were cultured in CM medium without hygromycin. After 2 weeks, the agarose beads were transferred to secondary protoplast culture medium (SP medium) with 60 mg/l hygromycin B. After 1 month in culture, the agarose beads including cell clusters were transferred to the CI medium containing 60 mg/l hygromycin B and cultured for 2 weeks in the light. Calli were then suspended with the liquid regeneration preculture medium (LP medium) and cultured for 2 weeks. After that, "Filtration manipulation" was carried out on the suspension culture. After 3 days in culture, the suspension calli were transferred to the solid regeneration preculture medium (RP medium) and cultured for 3 weeks. Calli were regenerated by transferring them to the regeneration medium (R medium).

Production of transgenic rice plants resistant to bialaphos

pINT-bar was introduced into rice protoplasts (cv 'Mutsuhomare') either by the poly-L-ornithine-mediated method or by the electroporation-mediated method as described above. The culturing of transformed calli was carried out as described, except that 5 mg/l bialaphos or Basta was used instead of hygromycin B.

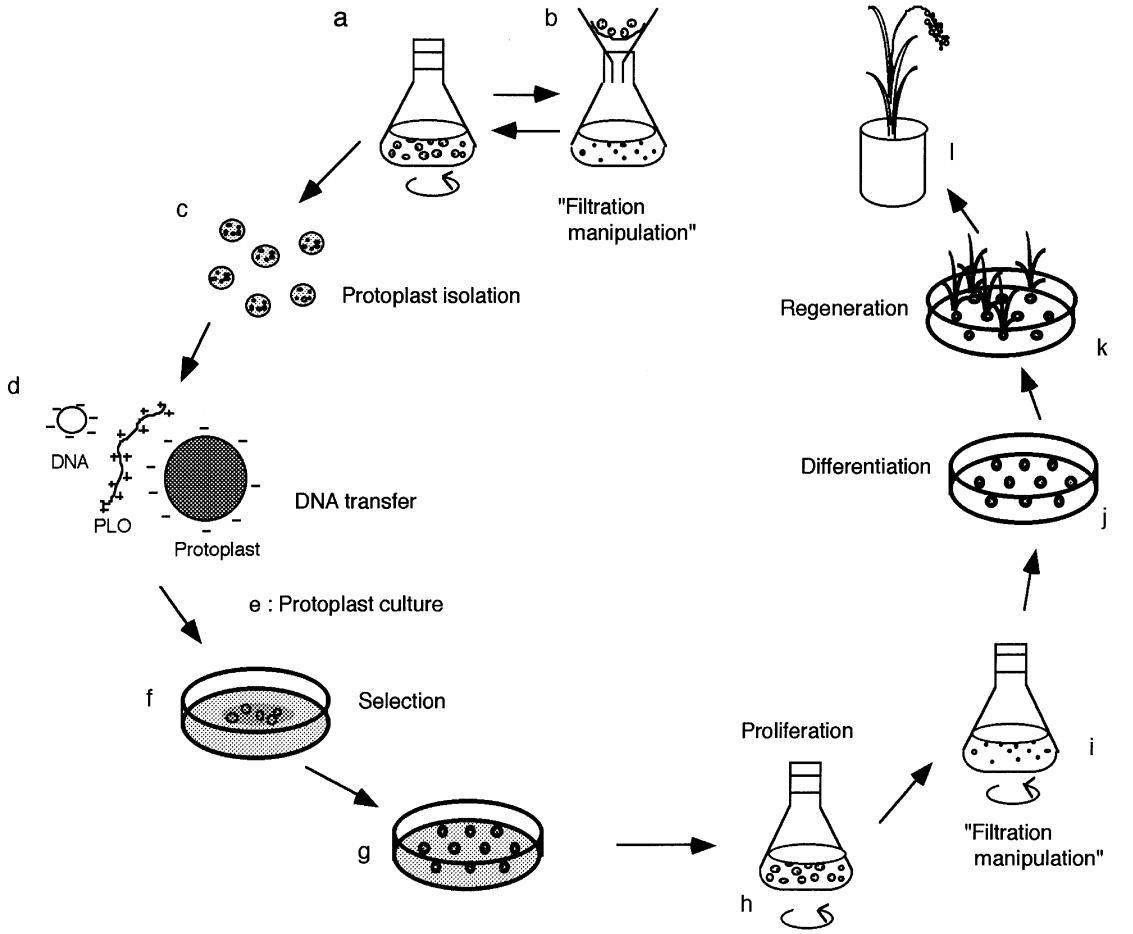

Fig. 1a–l The scheme of the procedure for the production of bialaphos-resistant transgenic rice plants. **a** Induced embryogenic calli were maintained as a suspension culture in L medium (Table 1) for 3–5 months. **b** "Filtration manipulation" was carried out during suspension culture, and calli were cultured in P medium for 3–5 days before the protoplast isolation procedure. **c** Protoplasts were isolated from suspension calli in a 0.5 *M* mannitol solution with 2% Cellulase Onozuka RS, 0.05% Pectolyase Y-23, 0.01% $CaCl_2$, and 0.1 m*M* MES (pH 5.6). **d** pINT-bar plasmids (8 mg/l) were introduced into 1.5×10^6 protoplasts in a 0.7 *M* mannitol solution containing poly-L-ornithine (8 mg/l) and 5 m*M* *K*-phosphate (pH 5.6). **e** Protoplasts were embedded in agarose beads and cultured in CM medium for 2 weeks. **f** Agarose beads including cell clusters were transferred to solid SP medium with 5 mg/l bialaphos for 1 month. **g** Transformed calli were selected on CI medium supplemented with 5 mg/l bialaphos for 2 weeks. **h** Transformed calli proliferated in LP medium without bialaphos for 2 weeks. **i** "Filtration manipulation" was carried out on the calli, then calli were cultured in LP medium for 3 days. **j** Calli were transferred to RP medium and cultured for 2 weeks for differentiation. **k** Shoots were transferred onto R medium, and transgenic rice plants were produced after 1–2 months. **l** Regenerated transgenic plants were cultivated

Southern blot analysis of transgenic plants

Detection of the integrated *bar* gene in transformants was performed by Southern blot analysis (Southern 1975). Genomic DNAs from transformed calli and leaves of transgenic rice were extracted by the CTAB method (Murray and Thompson 1980). Each DNA was digested with *Hin*d III. Southern blot analysis was carried out using Amersham kits with the *bar* gene as hybridization probe.

Verification of resistance in transgenic rice plants

Resistance ability was tested by transferring transgenic rice and control rice (non-transgenic cv 'Mutsuhomare') onto the root elongation medium containing 5 mg/l bialaphos (Herbiace™) and observing them for chlorosis for 2 weeks. We also tested the resistance by spraying the plants with a 1,000-fold Herbiace™ solution corresponding to 0.18% bialaphos. In order to determine the degree of resistance, we tested the transgenic rice plants with the higher concentrations of bialaphos solutions.

Results and discussion

Efficiency of poly-L-ornithine-mediated gene transfer assayed by transient expression with the 35S-GUS gene

We tested the effect of DNA and poly-L-ornithine concentrations on the DNA transfer efficiency (Table 2). The 35S-GUS gene (pBI 221) was transferred into the rice protoplasts by the poly-L-ornithine-mediated

Table 1 Culture media

Medium for each stage	Composition
Callus induction medium (CI)	N6(basic), 2 mg/l 2,4-D, 3% sucrose, 0.5% Gelrite (Wako Pure Chemical Industries, Japan)
Liquid culture medium (L)	N6, 1 mg/l 2,4-D, 3% sucrose
Preculture medium (P)	MS, 1 mg/l 2,4-D, 6% sucrose
Conditioned medium (CM)	R2, 2 mg/l 2,4-D, 0.4 *M* sucrose, extracts from suspension culturing for 4 days with nurse cells (long-term suspension cultures of cv Akenohosi)
Secondary protoplast culture medium (SP)	N6, 0.5 g/l proline, 0.15 g/l casamino acid, 1 mg/l 2,4-D, 0.2 *M* sucrose
Liquid regeneration preculture medium (LP medium)	N6, 1 g/l proline, 0.3 g/l casamino acid, 1 mg/l 2,4-D, 3% sucrose
Solid regeneration preculture medium (RP medium)	N6, 1 g/l aspartic acid, 1 g/l gultamine, 0.2 g/l casamino acid, 0.2 mg/l 2,4-D, 0. 1 mg/l kinetin, 0.5 mg/l ABA, 3% sucrose, 3% sorbitol, 1% agarose (Type I, Sigma)
Regeneration medium (R medium)	N6, 0.2 mg/l IAA, 0.5 mg/l BA, 0.2 g/l casamino acid, 3% sucrose, 1% agarose (Type I)

Table 2 Effects of DNA and poly-L-ornithine (PLO) concentrations and protoplast resuspension on transient GUS expression

Concentration of DNA-PLO (mg/l)	GUS activity (n*M* 4MU[a])	
	Resuspended[b]	Unsuspended
2–2	36.0 ± 6.9	39.9 ± 6.9
2–4	92.8 ± 2.7	48.8 ± 2.7
2–5	102.4 ± 5.1	49.1 ± 12.6
2–8	57.9 ± 5.3	36.6 ± 1.6
2–10	83.0 ± 16.5	37.1 ± 2.5
4–8	239.0 ± 16.6	43.0 ± 4.5
5–8	415.0 ± 38.8	48.7 ± 2.3
8–8	562.2 ± 47.7	145.3 ± 23.4
10–8	518.1 ± 10.7	62.2 ± 6.8
15–8	41.3 ± 2.2	38.8 ± 1.9

[a] GUS activity was shown per 1.5×10^6 protoplasts
[b] Resuspended, Resuspended immediately after centrifugation and kept gently suspended after that. Unsuspended, Left to stand for 30 min after transfer

method, and the transfer efficiency was evaluated on the basis of GUS activity using the transient assay.

When various concentrations of poly-L-ornithine (2–10 mg/l) were tested against a fixed concentration of DNA (2 mg/l), the highest GUS activity (102.4 ± 5.1 n*M* 4MU/1.5×10^6 protoplasts) was observed with 5 mg/l poly-L-ornithine. Likewise, when various concentrations of DNA (4–15 mg/l) were tested against a fixed concentration of poly-L-ornithine (8 mg/l), the highest GUS activity (562.2 ± 47.7 n*M* 4MU/1.5×10^6 protoplasts) was observed with 8 mg/l DNA. Another experiment with fixed concentrations of DNA (2, 5, and 10 mg/l) and various concentrations of poly-L-ornithine (2–20 mg/l) showed the same tendency, suggesting that the ratio of DNA to poly-L-ornithine concentrations was more significant in deciding transgene expression efficiency than the absolute individual concentrations. If the DNA concentration was too much higher than that of poly-L-ornithine, the negative charge of the DNA might not be fully neutralized. Conversely, when too high a concentration of poly-L-ornithine was added, the remaining unneutralized poly-L-ornithine seemed to injure the protoplasts, resulting in decreased GUS activity. The addition of carrier DNA was not necessary for transformation by poly-L-ornithine.

From the protoplast culture experiments, we found that protoplasts aggregated within 1 h of being incubated in the DNA and poly-L-ornithine solution. The aggregation resulted in a reduction in GUS activity, since that could not be separated later. Consequently, we resuspended the protoplasts in CM medium immediately after DNA uptake. After a 24-h incubation, these protoplasts gave significantly higher GUS activity than did the protoplasts which were not resuspended (Table 2). We also found that the frequency of transformation by poly-L-ornithine was higher at room temperature than at a lower temperature. This seemed to be due to the phagocytotic activity of the cells as latex uptake was also dependent on temperature (Suzuki et al. 1977).

Stable transformation by the poly-L-ornithine-mediated method

Stably transformed cells with *hph* were selected on RP medium containing 60 mg/l hygromycin B. Although we had established conditions for highly efficient DNA transfer based on the assay of transient GUS expression, hygromycin-resistant transgenic calli could not be consistently obtained. We found that the following modifications in culture conditions increased the efficiency of stable transformation; namely, DNA and poly-L-ornithine were removed from the protoplast suspension before the protoplasts were embedded in the agarose medium. Also, we found that improving the culturing method after transformation greatly

Table 3 Number of hygromycin-resistant colonies under different culture conditions

Concentration of PLO-DNA (mg/l)	Culture conditions[a]	Number of colonies per 1.5×10^6 protoplasts
4–2	Former method	0
4–2	Improved method	14.3
8–8	Former method	0
8–8	Improved method	85.3

[a] Former method, Medium was not replaced. Improved method, Fresh medium was replaced within 2 days of culture. Both methods involved culture in the presence of 60 mg/l hygromycin B for 1 month

increased the frequency of transformed cells. Initially, we did not change the medium for a length of time following transformation and subsequently got only a few transformed colonies. In contrast, we could obtain a significant number of transformed colonies by changing the culture medium within 2 days after the transformation experiments (Table 3). Thus, the liquid medium surrounding the agarose beads was changed every 24 h after DNA uptake. Washing cells on the day following the transformation experiments was also very effective in improving cell survival. This seemed to result from removing the remaining poly-L-ornithine, which was possibly harmful to the cells. The enhancement in cell activity when these modifications were adopted was also observed microscopically by counting the number of divided cells (data not shown).

The "filtration manipulation" (as described in Materials and methods) was very effective in enhancing the yield of protoplasts from embryogenic calli, and the relatively uniform protoplasts so isolated tended to divide simultaneously. The "filtration manipulation" was also effective in selecting cells that could maintain a high regenerative ability. With this procedure, we could obtain regenerants even from lines having a very low regenerative ability.

Production of bialaphos-resistant rice plants by poly-L-ornithine- and electroporation-mediated methods

To produce transgenic rice plants having the *bar* gene, we selected 19 independent resistant colonies randomly from among the colonies transformed with pINT-bar by the poly-L-ornithine- and electroporation-mediated methods and proliferated them with suspension culture in L medium. From each suspension culture, we prepared 100 colonies and cultured them on plates with regeneration medium (R medium). We observed a slightly higher regeneration efficiency with the electroporation-mediated method than with the poly-L-ornithine method (Table 4). The lower regeneration efficiency of poly-L-ornithine-derived calli may represent deleterious effects of poly-L-ornithine on the normal regenerative activity of calli.

Table 4 Regeneration efficiency of transgenic plants produced by electroporation- and poly-L-ornithine-mediated methods

Electroporation		Poly-L-ornithine	
Transgenic line	Percentage of plant regeneration[a]	Transgenic line	Percentage of plant regeneration
EP1	0	PLO2-2-1	2
EP2	0	PLO2-2-2	0(A4)[b]
EP3	0	PLO2-2-3	0
EP4	8	PLO2-2-4	29
EP5	0	PLO2-2-5	0(A40)
EP6	0	PLO2-2-6	0(A26)
EP7	3	PLO4-2-1	4
EP8	0	PLO4-2-2	0
EP9	47	PLO4-2-3	0
EP10	37	PLO4-2-4	64
EP11	70	PLO8-8-1	0
EP12	55	PLO8-8-2	2
EP13	55	PLO8-8-3	4
EP14	40	PLO8-8-4	4
EP15	18	PLO8-8-5	19
EP16	41	PLO8-8-6	0
EP17	53	PLO8-8-7	0
EP18	51	PLO8-8-8	61
EP19	4	PLO8-8-9	42

[a] Percentage of plant regeneration, Calli producing regenerants per transgenic calli described as percentage
[b] A, Albino plants. Number in parenthesis is the number of albino plants

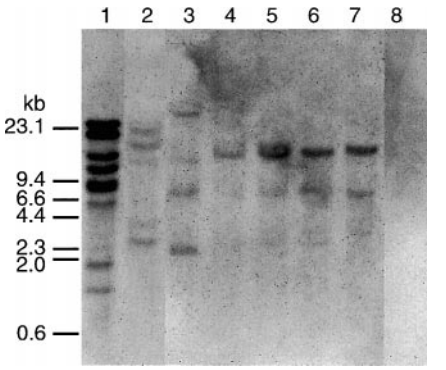

Fig. 2 Southern blot analysis of transgenic rice plants by the poly-L-ornithine- and electroporation-mediated methods. Total genomic DNA isolated from bialaphos-resistant plants was digested with *Hin*d III and hybridized with a fragment of the pINT-bar plasmid. *Lanes 1–3* Transgenic plants produced by the poly-L-ornithine-mediated method (*lane 1* PLO 4-2-4, *lane 2* PLO 8-8-5, *lane 3* PLO 8-8-8). *Lanes 4–7* Transgenic plants produced by the electroporation-mediated method (*lane 4* EP 10, *lane 5* EP 12, *lane 6* EP 13, *lane 7* EP 17). *Lane 8* Non-transgenic plant

The integration of the introduced *bar* gene was demonstrated by Southern blot analysis among three poly-L-ornithine regenerant inbred lines and four electroporation inbred lines (Fig. 2). The PLO 4-2-4, 8-8-5,

Fig. 3 Bialaphos resistance of young transgenic plants on root elongation medium. *Left*, transgenic rice plants; *right*, non-transgenic rice plants. Both were cultured on medium supplemented with 5 mg/l bialaphos. Transgenic rice plants were still growing after 2 weeks, while the non-transgenic plants had died

Fig. 4 Bialaphos resistance of transgenic plants sprayed with Herbiace™ (*from right*; *A* 500-, *B* 200- *C*, 100-fold). *Front*, non-transgenic plants; *rear* transgenic plants. Bialaphos-resistant transgenic plants remained green, while the controls died

Table 5 Resistance of transgenic rice plants to bialaphos

Concentration of bialaphos[a] (mg/l)	Transgenic plants[b]		Non-transgenic plant
	PLO4-2-4	EP10	
0	4/4	4/4	4/4
2	4/4	4/4	1/4
5	4/4	4/4	0/4
10	4/4	4/4	0/4
15	4/4	4/4	0/4
20	4/4	4/4	0/4

[a] Herbiace™ was used as the source of bialaphos
[b] Surviving plants per total number of plants

and 8-8-8 line showed eight, six, and five bands, respectively, whereas the EP 10, 12, 13 and 17 lines showed one to three bands. We also examined the calli of these lines and obtained patterns that were identical to those of the regenerated plants. Poly-L-ornithine induced DNA aggregation by reducing the repulsion of negatively charged DNA and then introducing the aggregates into protoplasts. Such introduced DNA aggregates might cause the occurrence of many bands in the poly-L-ornithine-mediated method.

Resistance of transgenic plants against bialaphos

Young transgenic rice plants survived on the root elongation medium containing 5 mg/l bialaphos, while non-transformants developed chlorosis within 2 weeks on the same medium (Fig. 3). Various concentrations of bialaphos (0–20 mg/l) were tested on two transgenic regenerant lines, PLO 4-2-4 and EP 10, and both of those lines were found to be resistant to at least 20 mg/l of bialaphos (Table 5).

Next, solutions of Herbiace™ (1,000, 500, 200, 100, and 50 times dilutions) were sprayed on transgenic and non-transgenic plants. All of the transformants survived in the presence of 100-times diluted Herbiace™ (10-fold over the standard working concentration), while non-transformed plants were killed with 500-times diluted Herbiace™ (Fig. 4). No differences in the degree of resistance were observed among the transgenic lines tested. These results suggest that the poly-L-ornithine-mediated method is useful for the production of practically useful transgenic plants.

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