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BRIEF COMMUNICATION

Particle mediated DNA delivery and transient expression of GUS gene in plated cells of rice

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Abstract

Culture conditions for a fine dispersion of plated cells of *Oryza sativa* L. cv. IR 20, have been worked out. These plated cells developed microcalli containing large number of somatic embryos and subsequently plantlets. By using single cells and clusters of 2 - 4 cells, an efficient DNA-delivery by microprojectile bombardment into cells and its transient expression were assessed by employing a plasmid construct containing β-glucuronidase gene.

Additional key words: micro-callus, *Oryza sativa*, particle-bombardment, suspension culture.

In order to incorporate traits of agronomic importance into rice, several studies have been undertaken to establish suitable genetic transformation protocols, *e.g.*, co-cultivation of rice tissues with *Agrobacterium tumefaciens* (Hiei *et al.* 1994), direct DNA-delivery into protoplasts either by polyethyleneglycol (Biswas *et al.* 1994) or high voltage electroporation (Potrykus 1991) and by microprojectile bombardment into meristematic-tissues, such as immature embryos and shoot apices (Christou 1993). However, the success of these genetic transformation protocols depended on *1*) the susceptibility of rice, a monocotyledonous cereal, to

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; GUS - β-glucuronidase; IAA - indole-3-acetic acid; LM medium - Li and Murai medium; MS medium - Murashige and Skoog medium; X-Gluc - 5-bromo-4-chloro-3-indolylglucuronide.

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Agrobacterium mediated transformation and *2*) induction of plantlet regeneration *in vitro*. Employing multicellular tissues as target tissue for DNA-delivery by the microprojectile-bombardment has been of choice since it circumvents the handling of protoplast cultures of rice, which is known for its recalcitrance *in vitro*. However, usage of multicellular tissues, though preferred for their easy regenerability, often led to chimeric expression of foreign gene(s) when used as target tissue for DNA-delivery. Cao *et al.* (1992) employed a suspension-culture derived multicellular clump (< 500 μm) of a japonica rice as the target tissue for DNA-delivery by microprojectile bombardment. They concluded that efficient DNA-delivery into a regenerable system of cell clumps, in contrast to immature zygotic embryos, is best suited for genetic transformation studies in order to avoid chimeric expression of introduced gene in the regenerated plantlets. This is because of stringency by which transformed cells could be selected and also to allow for high-frequency growth of transformed cells under selection conditions (Jain *et al.* 1996). Especially, in a situation where multicellular tissues, such as immature embryos or shoot tips, consisting of both transformed and normal cells, were subjected to selection pressure, growth of transformed cells depended on the relative proportion of transformed cells to the sensitive cells. When sensitive cells were proportionally high they tended to inhibit the further growth of transformed cells by cross-inhibition thus decreasing the transformation efficiency. Here we report the *1*) development of culture conditions for a fine dispersion of plated cells of rice, which consisted of single cells and clusters of 2 - 4 cells, *2*) usage of the finely dispersed cells for DNA-delivery by microprojectile bombardment and *3*) effect of the parameters of particle delivery process on the efficiency of DNA-delivery and transient expression of β-glucuronidase (GUS) gene in the plated cells.

Suspension cultures of indica rice (*Oryza sativa* L.) cultivar IR 20 were established from mature-seed embryo derived callus which was initiated from dehusked seeds under dark and maintained on MS medium (Murashige and Skoog 1962, Manoharan and Gnanam 1992) supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D; 2.5 mg dm^{-3}) + kinetin (0.5 mg dm^{-3}) + choline chloride (5 mM) + casein hydrolysate (300 mg dm^{-3}) + agar (0.8 %) at pH 5.8. Essentially, 2 - 3 month old callus (about 1 g) was transferred to specified liquid media (25 cm^3 in 150 cm^3 Erlenmeyer flask and incubated at 26 ± 1 °C at 120 rpm under continuous white light (irradiance of 420 μmol m^{-2} s^{-1}). Suspension cultures were subcultured at 5-d intervals. For cell-plating experiments, a fine cell-suspension was prepared from the supernatant of 1-month-old cultures, on 4th day after subculture, by centrifugation at 500 *g* for 5 min. Subsequently, the cells were resuspended at a density of 3.2×10^6 cells cm^{-3}. The cell suspension (1 cm^3) was uniformly layered on a semisolid medium in Petri dishes (diameter 90 mm). The inoculum was homogeneous and consisted of single cells as well as clusters of 2 to 4 cells (Manoharan and Gnanam 1992). In all the experiments, the nutrient medium for cell suspension and cell plating were the same. Nutrient media formulations such as N_6 (Chu *et al.* 1975), R_2 (Ohira *et al.* 1973) and LM (Li and Murai 1990) were employed. Choline chloride (5 mM), proline (5 mM) and 2,4-D (1 mg dm^{-3}) were supplemented to all suspension- and cell

plating-media. Glutamine (860 mg dm^{-3}) and casein hydrolysate (300 mg dm^{-3}) were included as specified in Table 1. Plated cells were incubated at 26 ± 1 °C under continuous white light. Plating efficiency was scored after 15 d of plating. After 25 d, when the microcalli were formed, subculturing of plated cell-cultures was done and frequency of somatic embryos in microcalli was determined. Frequency of somatic embryos were determined microscopically in a total of randomly picked 25 microcalli per culture dish in each of the experiments. For inducing plantlet regeneration, calli were transferred to MS medium + choline chloride (5 mM) + casein hydrolysate (300 mg dm^{-3}) + indole-3-acetic acid (IAA; 1 mg dm^{-3}) + kinetin (4 mg dm^{-3}) + agar (0.8 %) at pH 5.8.

An intron containing plasmid construct (*pIG 221*) with GUS-gene under the control of cauliflower mosaic virus *35S* promoter was employed (Ohta *et al.* 1990). Plasmid DNA was extracted and purified by a large-scale isolation procedure followed by PEG-precipitation (Sambrook *et al.* 1989). DNA was coated to 1.0 μm gold particles in an assay solution containing gold particles (3 mg), plasmid DNA (5 μg), spermidine (2.7 mM) and $CaCl_2$ (1.7 M) in a final volume of 0.075 cm^3. The DNA coated particles were finally resuspended in 0.060 cm^3 of ethanol and distributed to 6 macrocarrier films.

About 3.2×10^6 cells were layered on semisolid medium in 0.5 cm^3 of nutrient medium at the centre of the Petri dish. Microprojectile bombardment was carried out using *Bio-Rad Biolistic PDS 1000/He* system (*Bio-Rad Laboratories*, Hercules, USA). Helium pressure for particle delivery was varied by using corresponding rupture discs. The flight distance of macrocarrier, *i.e.* film coated with gold particles (6 mm) and chamber vaccum (660 mm Hg) were kept constant. Flight distance of gold particles at 6, 9 and 12 cm (corresponding to sample position 2, 3 and 4; Table 2) were employed. Cells were subjected to single bombardment and subsequently incubated. After 72 h of DNA delivery, the cells were histochemically assayed for GUS-gene expression (Jefferson 1987) by using 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) as the substrate. Percentage of GUS-gene expressing cells was determined microscopically by counting GUS-positive cells among a total of randomly picked 425 cells counted in each experiment. Results are the mean of 3 independent experiments which had six replicates each.

All organic chemicals were obtained from *Sigma Chemical Company*, St. Louis, USA. X-Gluc was obtained from *BioSynth AG*, Staad, Switzerland. Inorganic chemicals were locally available analytical reagents.

Experiments were undertaken to optimise the plating conditions for rice cells in order to make use of this system for particle mediated DNA-delivery. Several nutrient media formulations and gelling matrices were employed for this purpose. Among the nutrient media tested, Li and Murai (LM) medium along with a set of growth supplements was found to be optimal which lead to the formation of microcalli at a plating efficiency of about 173 microcalli per culture dish for an inoculum of 3.2×10^6 cells (Table 1). It was also found that somatic embryogenesis was induced only in the cultures grown on LM medium which had about 46 somatic embryos per microcallus. Among the gelling matrices tried (agar 0.8 %, agarose

1.2 % and phytagel 0.4 %), phytagel was found ideal due to high plating efficiency and also for getting the required gel rigidity for the particle delivery process (data are not presented). It was found that agar/agarose based semi-solid media, at tested concentrations, got disturbed under the conditions of the particle delivery. The embryogenic microcalli on transfer to a regeneration medium developed plantlets. Experiments were subsequently performed to find out the effect of operational conditions of microprojectile bombardment on the plating efficiency of cells. For this, the plated cells were subjected to the particle delivery under varying pressures with only plain particles. It was observed that, in a total of 12 experiments which were performed employing plated cell cultures grown on LM medium, the response of plated cells in the formation of microcalli and subsequent regeneration was the same as compared to the control (which was not subjected to the particle-delivery). The plating efficiency in those experiments ranged between 153 - 167 microcalli per culture dish.

In order to assess delivery of foreign DNA into cells, transient expression of GUS-gene was monitored histochemically in the subsequent experiments. It was observed

Table 1. Plating efficiency (number of microcalli or number of somatic embryos per culture dish) in cell cultures of rice on various nutrient media.

Medium	Additives	Plating efficiency exp. 1	exp. 2	exp. 3	Somatic embryos
N_6	casein hydrolysate	138 ± 13	130 ± 10	145 ± 11	0
N_6	glutamine	68 ± 6	65 ± 5	69 ± 6	0
R_2	casein hydrolysate	0	0	0	0
LM	casein hydrolysate+ glutamine	170 + 10	173 + 15	171 + 12	46 + 3

Table 2. Effect of the parameters of particle delivery (pressure and sample position) on the delivery efficiency (% of GUS-gene expressing cells) in three independent experiments with 6 replicates.

Pressure [kg cm^{-2}]	Sample position	Delivery efficiency exp. 1	exp. 2	exp. 3
46	2	0	0	0
46	3	0	0	0
46	4	0	0	0
63	2	11 ± 2	10 ± 1	9 ± 1
63	3	16 ± 2	14 ± 1	14 ± 1
63	4	11 ± 1	12 ± 2	11 ± 1
77	2	27 ± 3	27 ± 4	26 ± 2
77	3	30 ± 3	28 ± 2	26 ± 3
77	4	28 ± 2	27 ± 2	28 ± 3
91	2	16 ± 1	15 ± 1	14 ± 1
91	3	14 + 2	13 + 1	14 + 1
91	4	15 ± 1	14 ± 2	16 ± 1

that transient expression of GUS-gene in cells was positive at a range of particle-delivery pressures, *i.e.* 63, 77 and 71 kg cm^{-2} (Table 2) with expression levels varying between 11 and 28 %. However, pressure at 46 kg cm^{-2} did not lead to GUS-gene expression in the cells. The delivery efficiency of GUS-gene into the rice cells (Table 2) was comparable to that reported for immature embryos of wheat, on the basis of number of chromogenic - GUS gene expressing cells and spots on the immature embryos, respectively (Vasil *et al.* 1993). Thus, the present study, on the employment of fine dispersion of plated-cells, illustrated an useful system of target cells for DNA-delivery by microprojectile bombardment which could be employed in genetic transformation studies.

References

Biswas, G.C.G., Iglesias, V.A., Datta, S.K., Potrykus, I.: Transgenic indica rice (*Oryza sativa* L.) plants obtained by directed gene transfer to protoplasts. - J. Biotechnol. **32**: 1-10, 1994.

Cao, J., Duan, X., McElory, D., Wu, R.: Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. - Plant Cell Rep. **11**: 586-591, 1992.

Christou, P.: Particle gun mediated transformation. - Current Opinion Biotechnol. **4**: 135-141, 1993.

Chu, C.C., Wang, C.C., Sun, C.S., Hus, C., Yin, K.C., Chu, C.Y., Bi, F.Y.: Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. - Scientia sin. **18**: 659-668, 1975.

Hiei, Y., Ohta, S., Komari, T., Kumashiro, T.: Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of T-DNA. - Plant J. **6**: 271-282, 1994.

Jain, R.K., Jain, S., Wang, B., Wu, R.: Optimisation of biolistic method for transient gene expression and production of agronomically useful transgenic basmati rice plants. - Plant Cell Rep. **15**: 963-968, 1996.

Jefferson, R.A.: Assaying chimeric genes in plants: the GUS-gene fusion system. - Plant mol. Biol. Rep. **5**: 387-405, 1987.

Li, Z., Murai, N.: Efficient plant regeneration from rice protoplasts in general medium. - Plant Cell Rep. **9**: 216-220, 1990.

Manoharan, K., Gnanam, A.: Growth stimulation by conditioned medium and spermidine in low density suspension cultures of rice. - Plant Cell Physiol. **33**: 1243-1246, 1992.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Ohira, K., Ojima, K., Fujiwara, A.: Studies on the nutrition of rice cell culture. 1. A simple defined medium for rapid growth in suspension culture. - Plant Cell Physiol. **14**: 1113-1121, 1973.

Ohta, S., Mita, S., Hattori, T., Nakamura, K.: Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. - Plant Cell Physiol. **31**: 805-813, 1990.

Potrykus, I.: Gene transfer to plants: Assessment of published approaches and results. - Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 205-225, 1991.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular Cloning: A Laboratory Manual. 2nd Edition. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.

Vasil, V., Srivastava, V., Castillo, A.M., Fromm, M.E., Vasil, I.K.: Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. - Bio/Technology **11**: 1553-1558, 1993.