Parameters influencing the regeneration capacity of calluses derived from mature indica and japonica rice seeds after microprojectile bombardment

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Summary

The microprojectile bombardment procedure has allowed the stable transformation of indica and japonica rice varieties, although at different frequencies of transformation depending mainly on their regeneration capacity and on the specific parameters of the transformation protocol. A study of the process of regeneration to whole plants from primary calli derived from mature indica and japonica rice seeds, via embryogenesis, has shown that somatic embryos are produced by division and differentiation of the external cell layers of callus tissues. Adjusting the bombardment conditions to optimize gene delivery to those regenerable cells, we have evaluated the influence of parameters such as the target distance, particle penetration and the effect of osmotic treatment on the regeneration capacity of bombarded cells.

Abbreviations: BAP – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxy acetic acid, GUS – β -glucuronidase, MS – Murashige and Skoog medium; NAA – naphthalene acetic acid

Introduction

Rice is one of the most important crop plants in the world. Traditionally it has been considered as a monocotyledonous model plant in terms of *in vitro* culture response and genetic manipulation. The first transgenic rice plants were obtained using direct DNA transfer methods such as electroporation or PEG-mediated gene delivery into protoplasts, although these procedures were strongly dependent on the genotype and the regeneration method. After microprojectile bombardment provided the necessary breakthrough for efficient transformation of indica and japonica varieties (Christou, 1996, 1997), more than forty rice varieties have been transformed using genes of agronomic importance (Potrykus et al., 1995; Oard et al., 1996; Wünn et al., 1996).

Microprojectile bombardment uses the acceleration of small DNA-coated particles to deliver foreign DNA into target cells able to regenerate whole plants, with the subsequent integration of the introduced DNA (Sanford et al., 1993). The physical nature of the biolistic transformation system allows easy manipulation of the parameters involved in the procedure in order to optimize the bombardment method for each type tissue, species and variety. Numerous reports have appeared analysing the bombardment factors influencing gene delivery into cells, especially of monocots, to improve transformation frequencies (Vain et al., 1993; Christou & Ford, 1995; Kemper et al., 1996; Jain et al., 1996b). However, the effect of these parameters on the regeneration capacity of the bombarded cells has not been reported in detail, although many of the factors may act by influencing regeneration capacity.

Table 1. Frequency of callus formation and plant regeneration from primary calluses derived from mature seed of different varieties of *Oryza sativa* L.

Rice variety	Total n° of seeds	N° of calluses formed (%)	N° of regenerable calluses (%)	Total n° of plants regenerated	Average n° of plantlets regenerated per regenerable callus
Perla	190	150 (78.9) ^a	129 (86.0)	555 ^b	4.3 ^b
IAA-Cu-14	200	150 (75.0) ^a	98 (65.3)	206 ^b	2.1 ^b
Senia	130	100 (76.9) ^a	38 (38.0)	67 ^b	1.7 ^b
Leda	215	160 (74.4) ^a	97 (60.6)	214 ^b	2.2 ^b
Thainato	280	200 (71.4) ^a	50 (25.0)	100 ^b	2.0 ^b
Thaibonnet	300	200 (66.6) ^a	55 (27.5)	55 ^b	1.0 ^b

^a The number of calluses formed counted after 8 days of culture in the callus induction medium.

^b The number of plantlets regenerated from calluses was counted after transfer to soil.

We report here the influence of various microprojectile bombardment parameters on the regeneration capacity of primary rice calluses derived from mature seeds, from which whole plants can be regenerated via somatic embryogenesis.

Materials and methods

Culture and regeneration of rice plants

Mature seeds of 6 different *Oryza sativa* L. varieties were used throughout this study; japonica vars. Leda, Senia, Thainato and Thaibonnet (IVIA, Valencia, Spain) and, indica vars. Perla and IIA-Cu-14 (IIA, La Havana, Cuba). After surface sterilization in 40% Domestos (Lever, Spain) for 30 min, followed by several rinses in sterile water, dehusked mature seeds were induced to form callus on N6 medium (Chu et al., 1975), containing 1 g/l casein hydrolysate, 30 g/l sucrose, 2 mg/l 2,4-D and 0.45% agarose or 0.3% Phytigel as gelling agents for japonica and indica, respectively. Incubation was performed in darkness at 26°C. Primary calluses obtained after 1–4 weeks of culture, were transferred to MS regeneration medium supplemented with 3 mg/l kinetin, 0.5 mg/l BAP, 0.5 mg/l NAA, 30 g/l sucrose and 0.45% Phytigel. The incubation was carried for the first 48 h in darkness and then under 16 h light/8 h dark at 26°C for 4–6 weeks, subculturing every 2 weeks. Regenerated plantlets were separated and cultured on MS medium with 30 g/l sucrose, 0.3% Phytigel without hormones and after 2 weeks, plantlets were transferred to soil.

The culture response was followed by histological observations through light microscopy. The explants were fixed in ethanol:acetic acid (3:1) for 12 h and after a complete dehydration in ethanol dilution series, samples were embedded in Histo-technik 8100 resin (Kulzer). Sections of 10 µm were stained with 0.1% safranin. The regeneration capacity was expressed as percent of regenerated plantlets per area of callus (10 mm²) derived from mature seed.

Bombardment of primary rice calluses and GUS detection

Primary rice calluses were bombarded once with a DuPont PDS-1000/He Biolistic Delivery System, using the GUS-encoding plasmid p35S1-GUS, a pBSK based vector containing the *uidA* reporter gene (Jefferson, 1987) controlled by the CaMV35S promoter fused to the first intron of the *Adh1* gene (Fromm et al., 1990) and the polyadenylation site of the nopaline synthase gene. Gold particles of 1.0 µm diameter were coated with the DNA plasmid as described by Taylor & Vasil (1991). In each bombardment 15 calli of 2–3 mm in length were placed onto MS regeneration medium in 6-cm diameter dishes and bombarded with 0.154 µg gold and 0.350 µg DNA, after a vacuum of 700 mm Hg was attained.

The effect of different concentrations of osmoprotectants (0.3, 0.4, 0.6, 0.8, 1.0 and 1.2 M mannitol and/or sorbitol) and target distances (6, 9 and 12 cm from stopping plate to callus tissue) with a helium pressure adjusted at 7.5 were evaluated 48 h after bombardment, through transient GUS expression assays.

Table 2. Effect of osmotic treatment for 4h before and 16 h after bombardment, on transient GUS expression and regeneration capacity in primary calluses of rice variety Perla

Osmotic treatment (M)	^a N° blue spots per 10 mm ² of calluses	^b % of regenerable calluses	^c N° of regenerated plants per callus
* Control	0.0	80.1	9.4 ± 0.8
No osmoticum	10.4 ± 2.2	51.0	4.8 ± 0.3
(Mannitol)			
0.3	22.1 ± 7.4	57.4	–
0.4	26.4 ± 9.1	56.6	5.4 ± 0.4
0.6	36.8 ± 9.3	–	–
0.8	50.9 ± 8.0	55.2	7.7 ± 0.6
1.0	62.5 ± 8.0	59.7	12.6 ± 1.1
1.2	63.9 ± 10.6	41.3	14.2 ± 1.2
(Sorbitol)			
0.3	19.5 ± 5.3	–	–
0.4	18.7 ± 4.7	49.9	6.4 ± 0.5
0.6	35.5 ± 9.5	–	–
0.8	49.1 ± 8.1	42.1	12.7 ± 0.9
1.0	44.1 ± 7.5	19.6	14.3 ± 1.0
1.2	55.4 ± 12.1	20.2	16.5 ± 1.3
(Mannitol+Sorbitol)			
0.4	24.5 ± 3.9	46.6	6.0 ± 0.4
0.6	43.2 ± 9.5	–	–
0.8	54.1 ± 9.9	45.0	8.7 ± 0.6
1.0	49.9 ± 6.4	44.5	6.1 ± 0.4
1.2	66.8 ± 8.2	37.2	8.1 ± 0.6

* Control non-bombarded.

– Not evaluated.

^a Values are expressed as the number of blue spots per 10 mm² of callus tissue and represent the average of 3 experiments using 15 calluses per bombardment and 2 bombardments per treatment ± S.E.M.

^b The number of regenerable calluses was counted after 6 weeks of culture in the regeneration medium.

^c The number of plants regenerated from calluses was counted after being transferred to soil. Values represent the average of 3 experiments using 15 calluses per bombardments and 2 bombardment per treatment ± S.E.M.

In parallel, the influence of the physical parameters of bombardment on callus regeneration capacity was analysed.

Histochemical GUS activity was assayed as described by DeBlock & Debrouwer (1992), using 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc), as substrate and expressed as number of blue spots per 10 mm² of primary calli.

Results and discussion

Callus formation and plant regeneration from mature rice seeds

Mature dehusked seeds from japonica and indica rice varieties were cultured in a callus induction medium in the dark. After 3–4 days of culture, a swelling of mesocotyl and radicle regions was observed and callus formation was initiated in the area corresponding to the scutellum. Compact callus became visible after one week of culture, especially in indica rice varieties (Figure 1A), which could be directly transferred to a regeneration medium in order to shorten the regeneration process without reducing its efficiency. Japonica rice varieties needed to be maintained for 3–4 weeks in the callus induction medium to proliferate and acquire the regeneration capacity. Reducing this culture time resulted in a regeneration frequency close to zero. Calluses with a dry, compact and nodular appearance from both japonica and indica rice varieties (Figure 1A, B), were cultured on regeneration medium in darkness for 48 h to form organized somatic embryo-like structures, which developed into bipolar embryos (Figure 1C). These embryos germinated after 1–2 days of culture under a 16 h/8 h photoperiod (Figure 1D, E), followed by proliferation of multiple shoots and roots (Figure 1F). After 3–6 weeks, independent small plantlets were transferred to a hormone-depleted medium for 2 weeks, where shoots and roots were developed and finally plantlets were transferred to soil (Figure 1G).

Histological observations revealed that the regeneration process occurs from the peripheral cells of callus (Figure 2). Primary rice calluses consisted of meristematic cells which divide actively, especially on the external regions in contact with the culture medium, where an increased stainability of membranes was detected (Figure 2A,B). Williams & Maheswaran (1986) have described this increase in stain intensity as the indication of the gelification of the middle lamella required for the subsequent isolation of these cells. After 4–7 days of callus growth on the regeneration medium the external embryogenic cells became isolated from each other and displayed the first polarized divisions transforming them into 4, 8, 16-cell-globular structures (Figure 2C–F), which proliferated to form proembryos (Figure 2G, H) and finally developed into somatic embryos with a bipolar structure (Figure 1C, D).

Figure 1. Plant regeneration from primary rice calli. A) Callus formation in the area corresponding to the scutellum from a mature seed of rice variety Perla, after 8 days of culture in a callus induction medium. B) Callus proliferation from mature seed of rice var. Leda, after 4 weeks in a callus induction medium. C) Somatic embryo-like structures on a rice callus after 6 days of culture on the regeneration medium. D, E) Germination of the somatic embryos. F) Proliferation of shoots and roots. G) Regenerated rice plants growing in the greenhouse after being transferred to soil.

Figure 2. (left) Histological sections of the regeneration process from primary rice callus. A, B) Transverse sections of peripheral layers of primary rice callus. C, D) Transverse sections of peripheral layers of primary rice callus. E, F) Transverse sections of peripheral layers of primary rice callus. G, H) Transverse sections of peripheral layers of primary rice callus. (right) Histological GUS staining of primary rice calluses osmotically treated with different concentrations of mannitol. Transverse sections of bombarded calluses treated with 0.4 M (A, B), 0.8 M (C, D) and 1.2 M (E, F) mannitol. Bars: A, C, E: 40 μ m. B, D, F: 20 μ m.

The percentage of callus formation was similar in japonica and indica rice varieties, but the frequency of regenerable calluses was strongly dependent on the genotype (Table 1). Indica rice varieties produced a 65–85% of regenerable calluses and each callus yielded an average of 2.1–4.3 plantlets, while in the japonica varieties tested 25–60% of calluses regenerated into plants with an average of 1.0–2.2 plantlets per callus. These results allowed the classification of the two indica varieties Perla and IAA-Cu-14 and the japonica variety Leda as highly regenerable, when compared to the regeneration capacity data published for other indica and japonica rice varieties (Rueb et al., 1994, Jain et al., 1996a, Seraj et al., 1997). Nevertheless, the number of plants regenerated from calluses could be increased after high osmotic treatments (see results of bombardment).

Optimization of bombardment parameters and their effect on the regeneration capacity

To ascertain that highly regenerable primary calluses of rice varieties Leda and Perla, were suitable targets for microprojectile-mediated transformation, selected calluses were bombarded with gold particles coated with a GUS-encoding plasmid and transient expression of the reporter gene was analysed 48 h after bombardment. The proliferation stage of the calluses to be bombarded was selected according to the regeneration capacity of each variety (Table 1). This stage corresponded to 8 days of culture in the callus induction medium for Perla and 4 weeks for Leda.

The optimization of physical bombardment parameters was performed in order to produce the highest levels of gene expression in primary rice callus without reducing their regeneration capacity. The acceleration of microprojectiles determines the particle penetration and the cell viability of target tissues. The DuPont gene delivery system allows the control of the particle acceleration by modifying the target distance and/or the helium pressure using different rupture discs (Sanford et al., 1993). Adjusting the gas pressure with 1100 psi rupture discs to 7.5 MPa, primary calluses of varieties Perla and Leda were bombarded at distances of 6, 9 and 12 cm from the stopping plate. In both varieties shorter distances produced higher numbers of blue spots per callus area, but reduced their regeneration capacity in relation with the non-bombarded control (Figure 3). After comparing the GUS expression levels and the regeneration capacity in each case, distances of 6 and 9 cm appeared more

suitable for obtaining the highest frequencies of GUS expressing cells per regenerable callus. In similar experiments, Jain et al. (1996b) bombarded rice cell suspensions twice, at 9 cm and 12 cm keeping the gas pressure at 1500 psi, and obtained high levels of transient expression with minimum damage to the cells. In our case, when primary rice calluses of variety Perla were bombarded twice at 6 cm target distance at 1100 psi, no effect was detected in the number of blue spots/callus, but the percentage of regenerable calluses was reduced about 1.5-fold and the number of regenerated plants per callus decreased almost 2-fold.

The histological observations showed that the GUS expressing cells were located in the peripheral cell layers of the callus from which somatic embryos are produced in the regeneration process, when the osmotic concentration used was 0.4M (Figure 4A, B). Rice calluses treated with higher osmotic concentrations tended to show the GUS expressing cells in deeper cell layers (Figure 4C–F). Large blue spots appeared as a result of diffusion from a densely stained central cell, independently of the osmotic treatment. Similar effect of diffusion has been also described by Christou & Ford (1995) in bombarded immature rice embryos using the electric discharge particle acceleration. In particular, concentrations of 1.2 M mannitol, sorbitol or equimolar mixture of both, showed the blue color diffused through the tissue section making it difficult to locate the transformed cell, which could reflect some kind of cell damage. In fact, only a few cells from the peripheral region of calluses treated with 1.2 M osmoticum continued to divide (Figure 4E, F), which is in agreement with the reduced frequency of calluses regenerating plants after treatment with 1.2 M osmoticum (Table 2), although the number of regenerated plants per callus was increased.

The regeneration frequency of bombarded calluses was always lower in comparison to non-bombarded calluses (mean decrease of regenerable calluses from 80 to 50%, Table 2) as also described with bombardment. The number of plants regenerated per callus (from 9.4 ± 0.8 to 4.8 ± 0.3 ; Table 2). However, pre- and post-bombardment osmotic treatment had an opposite effect on the regeneration capacity. Increasing the concentration of the osmo-protectant in the regeneration medium resulted in a decrease in the number of regenerable calluses, but the number of regenerated plants per bombarded callus reached values up to 14.2 ± 1.2 and 16.5 ± 1.3 after treatments of 1.2 M mannitol and 1.2 M sorbitol respectively. This was an increase over the value of 9.4 ± 0.8 obtained for non-

Figure 3. Effect of bombardment target distances on the GUS expression and the regeneration capacity of microbombarded primary rice calluses. GUS activity is expressed as the average number of blue spots per 10 mm² of primary rice callus bombarded at 6, 9, and 12 cm from the stopping screen plate. The regeneration capacity is the % of plants regenerated from bombarded calluses at the different target distances. C: control non-bombarded calluses. Data are means of three experiments using 15 calluses per bombardment and two bombardments per target distance. Bars indicate standard error of the mean. Rice var. Perla (■) and Leda (▨).

Figure 5. GUS expression and regeneration capacity of bombarded primary rice calluses of indica (Perla and IAA-Cu-14) and japonica (Senia, Leda, Thainato, Thaibonnet) varieties. GUS activity is expressed as the average number of blue spots per 10 mm² of primary rice callus treated with 0.4 M mannitol and bombarded at 6 cm target distance with 1100 psi rupture discs. The regeneration capacity indicates the % of plants from bombarded calluses. Data are means of two experiments using 15 calluses per bombardment and three bombardments per variety.

bombarded explants (Table 2). The improvement in the regeneration capacity could be a response to the stress treatment similar to those reported in rice callus after dehydration and high osmolarity shocks (Higuchi & Maeda, 1991; Christou & Ford, 1995; Jain et al., 1996a).

The bombardment parameters (1100 psi rupture discs, 6–9 cm target distance and one shot) which we have shown to target the gene delivery into peripheral cells of primary rice calluses from which somatic embryos can develop, were used to transform primary calluses of six different rice varieties. After treatment with 0.4 M mannitol for 4 h before and 16 h after DNA delivery, bombarded calluses were histochemically analysed for GUS activity, and regeneration capacity. The evaluation of these parameters showed a strong dependence on the genotype (Figure 5). The expression of the reporter gene was detected in all rice varieties studied, but at different frequencies. The regeneration capacity of bombarded callus treated with 0.4 M mannitol was also different for each variety, but a reduction of 25–30% was always observed in comparison to the respective non-bombarded controls. It can be concluded that by manipulating the bombardment parameters, both the frequency of transgenic expression as well as the regeneration capacity of the bombarded cells can be substantially affected.

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