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Bio-physical parameters affecting transient *GUS* expression in *Indica* rice variety PAU 201 via particle bombardment

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ABSTRACT

In present study, calli of commercial indica rice variety PAU 201 were transformed following particle gun methods with the *GUS* gene (encoding β-glucuronidase) for its transient expression. Various biological and physical parameters like age of calli (weeks), preculture treatment prior to bombardment (hrs), post-bombardment incubation time (hrs), helium pressure (psi), vacuum pressure (mm Hg), distance from stopping plate to target tissue (cm), number of bombardments per Petri dish were studied. Optimized conditions include 4 weeks old fresh calli bombarded twice at helium and vacuum pressure of 1100 psi & 28mm Hg, keeping target distance 9cm with 12 hours pre-culture prior to bombardment and 72 hours post bombardment incubation duration gave a transient *GUS* expression of 32-39%. The parameters of this study could be used to improve the transient expression, enabling stable expression of introduced genes using callus as target tissue.

Introduction

Rice (*Oryza sativa* L.) is an important cereal crop, consumed by more than 700 million people as their main food (IRRI, 2006). The productivity of this crop is severely affected by number of biotic and abiotic stresses. The opportunities for improvement to these stresses using genetic engineering and biotechnological techniques have increased tremendously during last one and half decade. However, *indica* rice varieties are often considered to be sensitive to tissue culture and poorly responsive to genetic transformation, exhibiting culture-specific genotype differences that render them recalcitrant to transformation (Lin and Zhang, 2005). It has been a constant endeavour to identify suitable genotype and explants to produce embryogenic calli and their transformation. Recent advances in genetic engineering have emerged as an essential tool for improving agronomic characteristics of plants and supplementing the traditional breeding approaches (Morrish et al., 1993; Folling and Olsen, 2002; Ramesh and Gupta, 2005; Fadeev et al., 2006). A number of laboratories in the world are involved in producing and improving transgenic rice, but the transformation efficiencies achieved are variable (Dutta et al., 2001).

Among the various direct gene transfer methods, 'particle gun' or shot gun bombardment is the most commonly and widely used method for plant transformation. This method of gene transfer is rapid, efficient, tissue non-specific and can be employed for simultaneously bombarding more than one gene (co-transformation) in a host plant. The genotype independent biolistic particle delivery system developed for rice (Christou et al., 1991) has shown reproducibility of results in different laboratories overcoming constraints related to other methods and remains the method of choice for introducing useful genes into rice crop (Christou,1997). As many as 14 genes have been co-introduced in rice by this method (Chen et al., 1998). However, reports on successful transformation of *indica* rice varieties using 'particle gun' for developing resistance against various biotic and abiotic stresses are scanty (Li et al., 1993; Sivamani et al., 1996; Zhang et al., 1996; Jain et al., 1996; Ghosh Biswas et al., 1998; Grewal et al., 2006; Rehman et al., 2010). Since, particle bombardment is a physical method of transformation in which DNA is coated on microscopic (0.2-0.7 µm) heavy metal (gold/tungsten) particles. These particles are then accelerated to high velocity and bombarded into cells.

Several parameters affecting particle bombardment transformation frequency (Target distance, rupture disk pressure, gap distance, tissue pre-culture treatments, and choice of genotype) would also be more easily and effectively performed if transformed shoots could be identified and recovered as soon as possible after bombardment. The most convenient measure of efficiency for DNA delivery into intact cells is the number of cells which transiently express an incoming reporter gene (Hunold et al., 1994). Reporter gene or reporter proteins have played an important role in developing and optimizing transformation protocols for plant species. The GUS (β–glucuronidase) reporter system has been most useful for determining the number of expressing cells per bombardment (Rajasekaran et al., 2000; Men et al., 2003).

In this study, the genetic transformation of indica rice variety PAU 201 was accomplished via particle bombardment using gusA gene for transient expression.

The objective of this study is to optimize the physical and biological parameters using *gusA* transient gene expression in the calli derived from mature seeds using particle bombardment. The aim of these optimization studies is to achieve a high frequency of transiently expressing cells.

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Materials and Methods

Plant material and *in vitro* callus induction

Mature seeds of indica rice variety PAU 201 were collected from Rice section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India. The research work was carried out in the Tissue Culture and Transformation Laboratories, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India during 2006-2009. Dehusked seeds were surface sterilized in 70% (v/v) ethanol for 30 s, followed by 0.1% (w/v) mercuric chloride for 4 min. Seeds were thoroughly washed in sterile distilled water. For callus induction, seeds were plated on Petri dishes (6 cm diameter) containing MS (Murashige and Skoog, 1962) supplemented with 2.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} Kin, 560 mg l^{-1} Proline and 30 g l^{-1} Sucrose (pH 5.8) (Wani et al., 2011). After 4 weeks of culturing at 25 ± 2°C, friable loosely arranged embryogenic calli were separated and sub cultured onto fresh medium for proliferation.

Plasmid

Calli of PAU 201 variety were bombarded with *GUS* gene construct (pWRG 1515) encoding β -glucuronidase driven by CaMV 35S promoter and NOS terminator, using PDS-1000 He gun (Bio-Rad, Richmond, California). The LB medium with bacterial inoculum (*E. coli* strain plasmid containing *GUS* gene construct) was left overnight in incubator shaker at 37°C with constant shaking at 120 rpm (rotations per minute) and bacterial broth was obtained after 12-14 hours. Plasmid DNA was isolated from overnight grown bacterial broth of *GUS* gene constructs by following Qiagen plasmid purification midi kit protocol and quantification of DNA was done by Agarose gel electrophoresis (0.8%).

Particle gun mediated transformation conditions

All DNA introductions into rice calli were done using a Biolistic PDS- 1000/He Particle Delivery System (BioRad, USA). DNA preparation for bombardment was carried out by adding in order, 25µl of gold suspensions, 2.5µg DNA, 10µl spermidine, 50µl CaCl2 and left at room temperature for 10 min. A 7.5µl pf the particle DNA mixture was distributed on the microcarrier (Sivamani et al., 1996). Approximately, 20-25 pieces of fresh embryogenic calli (4-5mm) were excised and placed evenly at centre of Petri plate on CO medium sealed with parafilm and kept in dark in incubation room.

The DNA delivery was used according to the manufacturer's instructions. Optimization of physical parameters was carried out under the following conditions; rupture disc pressure (450, 900 and 1100 psi); distance from stopping plate to target tissue (6, 9 and 12 cm); vacuum pressure (27, 28 and 29 mm Hg), number of bombardments (1, 2 and 3 times) per target plate. The biological parameters included the age of calli (4, 6 and 8 weeks), preculture treatment prior to bombardment (0, 12 and 24 hours) and effect of post-bombardment incubation time (24, 48 and 72 hrs). For each parameter, three replicates were used containing 20-25 calli of 4-5mm size.

Histochemical GUS assay

Transient gene expression experiments with rice calli for PAU 201 variety was done 2 days after bombardment in a GUS substrate mixture (Jefferson, 1987). The percentages of calli were recorded after being incubated in staining solution containing 1mM of 5-bromo-4-chloro-3- indolyl glucuronide (X-Gluc) (Research Organics, Cleveland,Ohio, USA) in 50mM phosphate buffer, pH 7.0 at 37 ^{0}C overnight and examined for the presence of GUS activity (blue colour).

Statistical Analysis

Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. All statistical analyses were performed at the level 5% using statistical software CPCS-1 package developed by Cheema and Singh (1990).

Results and Discussion

Primary calli were formed after 4 weeks of seed culture on callus induction media (Fig 1a).

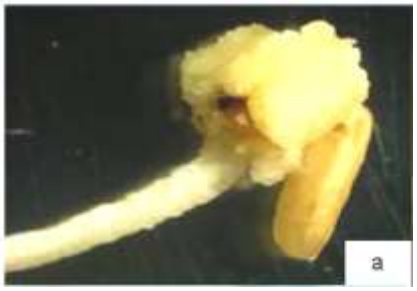

Fig.1a

They were characterized by having an intense yellow colour, small size and compact appearance. These calli were further subcultured on multiplication medium to get sufficient calli for transformation experiments. Small clusters of globular and translucent somatic embryos produced friable yellowish calli (Fig. 1b) derived from the scutellum after four weeks of culture on callus multiplication medium. Transformation experiments were performed with the helium driven Biolistic™ PDS-1000 particle delivery system (BioRad, Richmond, CA, USA). Ultra pure plasmid DNA, pWRG 1515 taken in 1:1 molar ratio were adsorbed on 1.0 m tungsten particles (Bio-Rad) according to Sanford et al., (1993) method and the results regarding various physical and biological parameters optimized in this study are detailed in subsequent section.

Fig.1b

Age of calli

Effect of age of callus tissue on transient expression of *gus* gene after post bombardment are presented in Table 1. Approximately 50% of the calli were tested for *gus* expression after 72 h of bombardment. Activity was always higher in 4 weeks old calli tissues followed by 6 and 8 weeks. Maximum activity (35.93%) was found with mature seed derived calli while its expression decreased with increase in callus age. Highest level of *gus* gene expression were found if calli were selected for transformation after 4 weeks (35.93%) of maintenance and was reduced to 25.80% when the age of calli was 8 weeks. In most of the samples *gus* gene expression exhibiting blue stains were unevenly distributed on the surfaces (Fig 1c). In present study, 4 weeks old calli were chosen for subsequent experiment as it gave the highest GUS expressions. Similar observations have been previously reported for Gus expression in embryogenic calli after bombardment (Li et al., 1993, Sivamani et al., 1996, Sreeramanan et al., 2005).

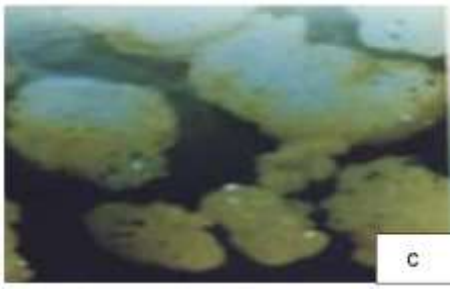

Fig.1c

Helium pressure

It was observed that 1100 psi helium pressure gave the highest (32.75%) transient gusA gene expression compared to 1300 psi (26.66 + 4.39) and 900 psi (24.07%) in calli of indica rice variety PAU 201 transformed via Biolistic particle gun. Similarly in sorghum, a helium pressure of 1100 or 1300 psi resulted in the highest transient gusA gene expression in shoot tips and mature embryos (Tadesse et al., 2003). Experiment results showed that low acceleration pressures (900 psi) resulted in lower area being covered by particles than higher acceleration pressures (1300 psi). This result is in agreement with the published data on wheat tissue (Rasco-Gaunt et al., 1999). Hence, in this study 1100 psi rupture disk pressure was chosen for subsequent experiment as it gave the highest GUS expressions (Table 2). Sanford et al., (1993) suggested that for most plant applications, 1000 psi is optimal or nearly optimal (Ramesh and Gupta, 2005). Microscopic analysis on wheat tissue showed that at low pressure expression events were evenly distributed and at a relatively low density (Rasco-Gaunt et al., 1999). At high pressures, a small area of the target tissues was strongly targeted and thus likely to be damaged, especially at the highest acceleration pressure (1550 psi) (Tadesse et al., 2003).

Fig.1d

Distance between the stopping plate to target tissue

Optimization of the distance between the stopping plate to target tissue is necessary to allow even spreading of the DNA microcarrier onto the target tissue without causing damage to the tissues (Russell et al., 1992; Tadesse et al., 2003). In this study, the 9 cm distance from stopping plate to target tissue was significantly better than the other distances in rice calli using gusA gene expression. At 12 cm, the gusA gene expression levels were significantly reduced in rice calli due to decreased velocity of the micro particle with the long flight distance giving reduced penetration force and thereby fewer cells receiving the oncoming DNA. Similar observations were reported by Parveez et al., (1997) that increasing flight distance resulted in reduced transient expression. Overall, the results in this experiment suggested that micro-carrier flight distance of 9 cm was optimal to use at 1100 psi pressure in 4 week old calli (Table 3). Higher pressure and shorter flight distance were detrimental to the calli as these showed lower transient gene expression levels.

Vacuum pressure

Vacuum plays an important role in the acceleration of the microcarrieer from the stopping plate into the target tissue (Parveez et al., 1997). During this study, it was observed that transient GUS expression was significantly different when the 4 weeks old rice calli were bombarded at different vacuum pressure. The lowest transient expression (27.27%) was observed using GUS at 27 mmHg in calli (Table 4). Lower vacuum pressure did not allow particles to reach the target tissues (Walter et al., 1998). This was expected because the higher the vacuum pressure, the lower the deceleration which resulted in a more consistent velocity of the microcarrier, thus contributing to the lower variability. However, it has also been shown that reducing the vacuum below 27 mmHg resulted in the reduction of the transient GUS expression in wheat (Rasco-Gaunt et al., 1994) and *Pinus radiata* (Walter et al., 1998). Overall results suggested that 28 mmHg was more significant ($p<0.05$) vacuum pressure which could be compensated with 1100 psi for relatively high level of GUS (39.58%) expressions.

Number of bombardments

Multiple bombardments are normally carried out with the objective of getting better coverage of targeted areas. This is similar to the observation with cotton meristems (Chlan et al., 1995, Sanghera et al., 2009). It is useful when primary delivery is not efficient but also increases the damage done to the target tissue. In this experiment, two consecutive of bombardments showed an increase in transient GUS expression in calli compared with one or three times (Table 5). In agreement with our results, double bombardment has been shown to increase transient gusA gene expression proportionally in wheat and rice cultures (Wang et al., 1996; Ramesh and Gupta, 2005), cotton (Rajasekaran et al., 2000) and in sugarcane (Bower and Birch, 1992; Kaur et al., 2005). Although multiple bombardments might allow a better coverage of the target area as indicated by King and Kasha (1994), this is not recommended in view of tissue damage occurring in rice calli. This kind of extensive wounding is probably responsible for the production of polyphenolic compounds following more than one bombardment event, which could reduce the transient gene expression. Nevertheless, in this experiment, double bombardments were sufficient to give a high transient GUS expressions (37.50%) followed by three bombardments(30.00%) in four week old calli of indica rice variety PAU 201 and it was used for all subsequent experiment except for calli which were exposed to single bombardment.

Preculture treatment prior bombardment

Bombarding explants at the right development stage is important because actively dividing cells are most receptive to the particle bombardment method of DNA (Moore et al., 1994). During preculture, isolation of the explant and exposure to new medium with nutrient and growth regulators may have stimulated cells to divide. Transient gusA gene expression was monitored from 1 to 3 days after bombardment. Explants with no preculture period before bombardment were used as controls and for each treatment, three replicates were used. The highest GUS expression (32.50%) was observed for 12 hours preculture in calli followed by 24 hours preculture (Table 6). Similarly, highest GUS expression was observed in tissues with one day of preculture period followed by 2, 3 and 4 days (Jain et al., 1996) in rice. Contrarily, shorter periods of cell pretreatments resulted in higher number of blue spots per gram of *Coffea Arabica* bombarded cells, with the highest transient GUS expression

obtained when treated 4 hours prior to bombardment (Rosillo *et al.* 2003). However, in wheat (Ingram et al., 1999) found better GUS expression when explants were precultured for 8-12 hours prior to bombardment.

Post bombardment incubation duration

Transformation efficiency is also affected by subjecting the bombarded tissue to selection at the right stage of development. In this experiment, the exposure period for post-bombardment was studied based on GUS transient expressions for 24 hours intervals up to 72 hours. Three days (72 hrs) after bombarding gave significantly higher and intense (Fig 1d) transient expressions (36.66%) as compared to other treatment used (Table 7). The results implies that it take at least three days for the cells or tissues to recover from the injuries caused by bombardment. However, in einkorn wheat, highest expression was detected 10-14 days after bombardment (Takumi et al., 1994).

Conclusion

Bombardment conditions were optimized using GUS reporter system for rice calli. The parameters investigated were helium pressure, distance from stopping plate to target tissue, vacuum pressure, bombardment number, calli age, preculture treatment prior bombardment, and post bombardment incubation time. In this study, optimized bombardment conditions was bombarding twice at 1100 psi, 9cm target distance, 28 mmHg, twelve hours preculture prior to bombardment and three days post bombardment. For stable expression of inserted gene for rice callus using Biolistic particle gun that presented in this article would be helpful for producing stable transformation of rice other cereal crops using gene construct of economic importance.

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Table 1 Effect of age of calli on transient expression of gusA gene in indica rice variety PAU 201 transformed via Biolistic article gun

Age of calli (Weeks)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS$^+$ calli	% of calli showing GUS expression
4	126	64	23	35.93^a±1.45
6	112	58	17	29.31^b± 0.95
8	110	62	16	25.80^b±1.30

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 2 Effect of helium pressure (psi) on transient gusA gene expression in calli of indica rice variety PAU 201 transformed via Biolistic article gun

Helium pressure (psi)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS$^+$ calli	% of calli showing GUS expression
900	112	54	13	24.07^a±1.30
1100	100	58	19	32.75^b±1.85
1300	110	60	16	26.66^a±1.05

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 3 Effect of distance from stopping plate to target tissue (cm) on transient gusA gene expression in calli of indica rice variety PAU 201 transformed via Biolistic article gun

Distance from stopping plate to target tissue (cm)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS$^+$ calli	% of calli showing GUS expression
6	90	50	15	30.00^a±1.65
9	86	48	19	39.58^b±2.15
12	92	44	12	27.27^a±1.35

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 4 Effect of vacuum pressure (mmHg) on transient gusA gene expression in calli of indica rice variety PAU 201 transformed via Biolistic article gun

Vacuum pressure (mmHg)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS^+calli	% of calli showing GUS expression
27	80	44	12	27.27^a±0.95
28	86	48	19	39.58^b±1.75
29	90	50	15	30.00^a±1.50

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 5 Effect of number of bombardments on transient gusA gene expression in calli of indica rice variety PAU 201 transformed via Biolistic article gun

No of bombardments	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS^+ calli	% of calli showing GUS expression
1	60	38	9	23.68^a±1.90
2	66	40	15	37.50^b±2.30
3	60	40	12	30.00^c±1.75

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 6 Effect of Preculture duration (hrs) on transient gusA) gene expression in calli of indica rice variety PAU 201 transformed via Biolistic article gun

Preculture duration (hrs)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS^+ calli	% of calli showing GUS expression
0	60	32	5	15.62^a± 1.45
12	58	40	13	32.50^b± 1.60
24	60	36	9	25.00^c±1.55

Values followed by same letter are not significantly different at p>0.05 DMRT

Table 7 Effect of post-bombardment incubation time (hrs) on transient gusA gene expression in calli of indica rice variety PAU 201 transformed via Biolistic particle gun

Post-bombardment incubation time (hrs)	No. of calli bombarded	No. of Calli tested for GUS assay	No. of GUS^+ calli	% of calli showing GUS expression
24	68	36	8	22.22^a±2.15
48	60	30	10	33.33^b±1.90
72	60	30	11	36.66^b±2.45

Values followed by same letter are not significantly different at p>0.05 DMRT