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Effect of varying concentration of mannitol on transient expression of 2-glucuronidase gene in engineered cells

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ABSTRACT

Particle bombardment has emerged an excellent approach for plant genetic transfer which circumvents the limitations of Agrobacterium-mediated gene transformation. This study aimed to determine appropriate concentration of mannitol for production of clear blue spot or transient expression of 2-glucuronidase gene in genetically transformed cells by particle bombardment. Transient GUS activity was observed using mature rice seed (Genotype LX 278) scutellar derived calli as an explant and treated with different concentrations of mannitol. It was observed that calli exposed to 30 mg/l mannitol concentration before bombardment produced the best embryogenic calli that ultimately yielded more transient GUS expression of β 2-glucuronidase gene in transformed cells. The article provides an enhancement of transformation efficiency using 30 mg/l mannitol which is the crucial to maintain cell targor after wounding with the gold particles. This information will help the researchers for the development of transgenic crop variety development.

Key words: embryogenic callus, osmotic treatment, particle bombardment, rice

INTRODUCTION

Particle bombardment is also called biolistic method or the microprojectile bombardment procedure which was developed for the transformation of embryogenic suspension cells and calli of pusa basmati-1 (Jain *et al*., 1996c; Minshas *et al*., 1996; Balasubramanian *et a*l., 1997) and KDML-105 (Nimlek *et al*., 1997). Khanna *et al*. (1996) transformed mature embryos of Basmati rice cv. Karnal local, via microprojectile bombardment using plasmid pAHC25 carrying GUS and bar for resistance to phosphinothricin (glufosinate) (PPT) gene coding regions. Christou *et al*. (1991, 1992) reported that immature embryos are good targets for biolistic transformation of important cultivars of japonica, javanica and indica rice. With further improvements in technique, the bombardment of rice embryos and cultured cells has become a routine method for transformation of japonica rice (Li *et al*., 1993). The successful transformation of indica rice varieties using cells in suspension culture was described by Jain *et al*. (1996) and Zhang *et al*. (1996). Similarly, gene transfer by particle bombardment into organized tissues such as immature embryos has been used for generating transgenics of several japonica and some indica cultivars (Christou, 1996).

Biolistic transformation offers the advantage that it can deliver any form of DNA, RNA or protein (Svitashev *et al*., 2015; Gil-Humanes *et al*., 2017; Shi *et al*., 2017), a property that has been exploited to facilitate gene editing technologies (Begemann *et al*., 2017; Liang *et al*., 2017). Biolistic transformation is also free of the constraints associated with *Agrobacterium*-host plant interactions. Unaltered bacterial artificial chromosome sequences larger than 100kb and an intact linear 53-kb molecule (Partier *et al*., 2017) have been integrated into plants by biolistic methods (Liu *et al*., 2019).

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A successful case of rice transformation of the Vietnamese aromatic variety, Nang Huong Cho Dao using particle gun was reported by Ho *et al*. (1995). Regenerable calli and embryogenic suspension cultures of Nang Huong Cho Dao (indica rice) were bombarded with pRQ6 containing hpt coding for hygromycin phosphotransferase and GUS A, both driven by CaMV 35S promoter. Regenerated plants were resistant to hygromycin B and expressed the *gus A* gene.

In rice transformation, it is crucial to maintain cell turgor after wounding with the gold particle. Osmotic treatment using osmoticum (Mannitol) in shoot regeneration medium for reducing water content in rice cells was one of the factors discovered to increase the frequency of plant regeneration from suspension-derived cells and seed-derived cells. Lee *et al.* (2003) reported that osmotic treatment of embryogenic rice callus enhanced transient expression of the *gusA* gene. He also mentioned that the sample treated with osmotic stress revealed 3.2-fold higher frequency of GUS expression in callus compared to the sample without osmotic treatment.

The objective of the study was to determine which mannitol concentration is suitable for production of clear blue spot and/or transient expression of 2-glucuronidase gene in transformed cells.

MATERIALS AND METHODS

Preparation of plant material

Calli derived from mature rice seed of genotype LX 278 were allowed to grow in medium with three concentrations: (i) LS + 0 mg/l mannitol, (ii) LS + 30mg/l mannitol and (iii) LS + 50mg/l minnitol). For each of the treatment, 4-Petridishes were used with 30 calli per Petridish. Calli were kept for 2-weeks to observe their growth and transfer the calli in the middle of Petridish a day prior to bombardment. Two Petridishes per treatment were used for bombardment. ACT/GUS/NOS as plasmid and 1-micron gold particles were used for bombardment.

Preparation of gold particle (microcarriers)

Sixty mg of gold was taken in a 1.5ml microcentrifuge tube and then added 1 ml of 70% ethanol (analytical grade). The particles were allowed to soak in 70% ethanol for 15 min. and to have the pellet spinned the microparticles for 5 seconds in a microcentrifuge. Removed and discarded the supernatant. Then 1ml filter sterile water was added and vortex vigorously for 5 seconds. The paricles were allowed to settle for 5 seconds. Spinned the microparticles gently in a microcentrifuge to get the pellet. Again removed and discarded the supernatant. Washed with filter sterile water for 3 times and added 1 ml 100% ethanol and kept at –20^{o}C.

Coating washed microcarriers with DNA

Gold microcarriers prepared in 60 mg/ml d H_2O was vortex for 5 min. to resuspend and disrupt agglomerated particles. Pipette out 60 µl of microcarriers into a 1.5 ml microcentrifuge tube and continuously vortex the tube containing with microcarries. During vortexing the materials were added in order to (a) 10 µl of plasmid DNA (ACT/GUS/NOS)(300mg/l), (b) 60 µl 2.5 M $Cacl_2$ and (c) 30 µl 0.1 M spermidine (free base). Vortex was done completely for 5-10 sec. and the materials were incubated at room temperature for 30 min. in order to settle the microcarriers. The pellet was resuspend and microcentrifuze at 9000 rpm for 5 sec. The supernatant was removed and 170 µl 70% ethanol (160 µl + 10 µl of original solution) was added without disturbing the pellet. 60 µl of 100% ethanol was added and the pellet was resuspended by vortexing and tapping the side of the tube.

Bombardment

Before bombardment, bombardment parameters were adjusted for distance between rupture disk and macrocarrier ¼ inch, macrocarrier and stopping screen, 6 mm. stopping screen and target plate, 6 cm (L_2 level). Stopper and macrocarrier holders were autoclaved. Macrocarrier membrane was sterilized in 100% ethanol for 5 min. The membrane was dried on a piece of sterilized kimwipe. The microcarrier solution was briefly sonicated before loading onto the membrane. A 10 µl aliquot

of microcarrier solution was then evenly dispensed onto the center of each macrocarrier membrane and allowed to dry.

Biolistic device

PDS-1000/He using 1100 psi with a 900 psi rupture disk was used as biolistic device.

Firing the device

Power cord was plug in from main unit to electrical outlet. Chamber walls were sterilized with 70% ethanol. Sterile rupture disk was loaded into sterile retaining cap. Retaining cap was secured to end of gas acceleration tube and tighten with torque wrench. Macrocarrier and stopping screen was loaded into microcarrier launch assembly. Microcarrier launch assembly and target cells were placed in chamber and closed the door. The chamber was evacuated and hold vacuum at desired level. Bombard sample. Fire button was continuously depressed until rupture disk bursts and helium pressure gauge drops to zero then released the fire button. After bombardment, the petridish containing the bombarded calli were removed from the chamber and sealed with parafilm and kept in the dark before staining for GUS activity.

GUS assay

Three days after bombardment, bombarded calli were picked up from all petridishes in all treatment to detect GUS activity for monitoring transient expression. For GUS staining, calli were placed in a corning cell wells^{TM} and incubated in X-gluc solution (Jefferson 1987) at 37 ^{0}C overnight (14-16 h) and scored for evidence and extent of GUS activity. Calli were scored as GUS-positive (GUS^{+}) if any blue spot (Figure 1) was present on the calli or GUS- negative (GUS-) if no blue stained calli were observed.

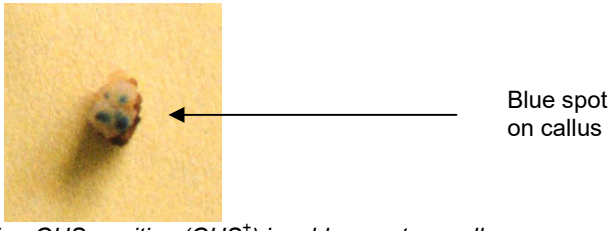

Figure 1. Showing GUS-positive (GUS^{+}) i.e., blue spot on callus

Data analysis

The experiment was designed following completely randomized design (CRD) with four replications. Data on embryogenic, non-embryogenic and dead (necrotic+brown) calli and GUS^{+} calli were recorded and were subjected to analysis of variance and means were separated by LSD using R statistical software program version 4.3.1.

RESULTS AND DISCUSSION

Observation of callus growth plated on different mannitol concentrations after 1 and 2 weeks of culture

Embryogenic calli, non-embryogenic calli and dead or necrotic calli were recorded and the mean values were shown in Table 1. Results showed that after one week of culture in LS medium with different concentrations of mannitol, percent embryogenic calli were observed more in without mannitol concentration than 30 mg/l and 50 mg/l mannitol concentration but not significantly different. While observation was made after 2 weeks of culture, it was found that 30 mg/l mannitol concentration gave the higher percent of embryogenic calli than 0 and 50 mg/l mannitol. On the other hand, considering non-embryogenic calli 1 week after observation, 0 mg/l mannitol showed lower percentage of non-embryogenic calli than the other two treatments. But after 2 weeks observation, 30 mg/l mannitol concentration produced lower non-embryogenic calli than 0 and 50 mg/l mannitol concentration. Results also showed that after 2 weeks of culture 0 mg/l mannitol concentration produces more dead (necrotic+brown) calli than the other two treatments. In overall

observation, it was found that 30 mg/l mannitol concentration found better for embryogenic callus growth. Similar results were also reported by Suprasanna et al. (1994). They mentioned that more embryogenic calli were obtained in 30 mg/l mannitol concentration and better revival and retention capacity for regeneration. In this report we used scutellar derived calli rather than suspension-derived cells, seed-derived cells or immature embryos for biolistic transformation of rice with osmotic treatment.

Table 1. Mean values of embryogenic calli (compact, whitish), dead calli (brown + necrotic), and non-embryogenic calli (loose, watery, yellow) observed 1 week and 2 week of culture in different mannitol concentration

Treatments	1 Week			2 Week		
	Embryo genic calli (%)	Non-embryo genic calli (%)	Dead (brown + necrotic) calli (%)	Embryo genic calli (%)	Non-embryo genic calli (%)	Dead (brown+ necrotic) calli (%)
LS+0 mg/l mannitol	50.84	13.34	35.84	32.50	15.00	52.50
LS+30 mg/l mannitol	48.33	20.00	31.67	38.34	11.67	50.00
LS+50 mg/l mannitol	48.33	22.50	29.17	30.00	20.00	50.00
LSD (0.05)	11.06	9.19	13.59	10.62	8.71	17.48

Least significant difference at 5% level of significance

Observation of GUS positive calli after bombardment with plasmid ACT/GUS/NOS

Three days after bombardment, GUS activity was observed from transformed calli which was stained with X-gluc solution. A significant difference was found on GUS positive calli among the treatments. Result showed that calli derived from 30 mg/l mannitol concentration gave the highest percentage (63.75%) of GUS activity followed by 50 mg/l mannitol concentration (57.50%) (Table 2).

Table 2. Mean values of GUS positive calli obtained from different mannitol concentration after bombardment of calli using plasmid ACT/GUS/NOS

Treatment	Percentage of GUS^+ Calli
LS+0 mg/l mannitol	46.67 b
LS+30 mg/l mannitol	63.33 a
LS+50 mg/l mannitol	56.67 ab
CV(%)	11.31
LSD (0.05)	10.05

Means bearing same letters do not differ significantly at 5% level of probability

Among the treatment, the lowest GUS activity was observed when there was no mannitol concentration. The result obtained from this experiment suggested that calli treated with mannitol before bombardment allowed to yield more transient GUS expression than without mannitol concentration. These results are similar with the results of Lee et al. (2003) and Naveeda (2001). They reported that transient GUS expression increased four times when calli were treated with high osmoticum medium (0.2M-0.4 M) 4 hrs before and 16 hrs after bombardment. Result also suggested that if increased the mannitol concentration above 30 mg/l, it showed lower percentage of GUS activity than 30 mg/l but not lower than 30 mg/l mannitol concentration. Based on the results of the study, it may conclude that before bombardment, calli treated with 30 mg/l mannitol concentration are better for more transient GUS expression.

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