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Research Article

OPTIMIZATION OF SOME INFLUENTIAL FACTORS IN MAIZE GENETIC TRANSFORMATION THROUGH MICROPROJECTILE BOMBARDMENT

Sreenu P[1,3]., Sridevi M[1,3]., Sateesh Kumar P[1]., Reddy MK[2]., Sailaja D[3] and *Pavan Kumar G[1]

[1]Nuziveedu Seeds Limited, Hyderabad
[2]International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi
[3]Jawaharlal Nehru Technological University, Hyderabad

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ABSTRACT

Transient expression of induced gene by microprojectile bombardment was standardized with embryogenic callus in maize using PDS 1000/He biolistic gun (Bio-Rad, Hercules, CA). Plasmid *pCAMBIA* 1391Z, harboring hygromycin phosphotransferase (*hpt II*) as selectable marker gene and *β-glucuronidase* (*gus*) as reporter gene, was used. E. Callus segments, plain medium without osmoticum, 20 µg of plasmid DNA for coating the gold particles were found to be suitable than the direct usage of immature embryos, medium with osmoticum and 10 µg of DNA for coating. In this method, apart from the genotype, explant, hormonal concentrations and other *in vitro* conditions, certain physical conditions (instrument related parameters) also play a crucial role in delivering the gene into the cell for getting high frequency of transformation. Effect of gold particles size, distance of tissue material from the macro carrier (Slot) and rupture disk pressures were studied on frequency of transient gene expression. Two particle sizes (0.6 and 1.0 µm gold particles), three distances (Slot I - 3 cm., Slot II - 6 cm. and Slot III - 9 cm.) and pressures (650, 900 and 1100 psi) were used. 0.6 µm gold particles, slot II (6 cm.) and 900 psi pressure resulted in maximum frequency of transient expression. ANOVA revealed that among these three parameters, gold particle size showed significant effect on delivering the DNA into the cells.

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INTRODUCTION

Maize is one of the staple food crops globally next to rice and wheat. It is also an important monocot plant model in genetics, genomics and molecular biology studies (Vega *et al.*, 2001). Maize production is limiting by both biotic and abiotic stresses. These ailments can be overcome by conventional breeding and biotechnological approaches. Breeding programme would take long time to get desirable characters. Biotechnological advancement of crop plants through genetic engineering takes less time to get desirable traits. Transgenic plants can be obtained in two ways *viz*., *Agrobacterium* mediated transformation and particle bombardment. Transformation mediated with *Agrobacterium* has its own limitations. Moreover, cereals were not susceptible for agrobacterial cells. Particle bombardment offers a rapid method for DNA delivery into plant cells (Rasco-Gaunt *et al.*, 1999) and suitable method for monocot transgenic development. Biolistic transformation of maize has become routine. Recovery of fertile transgenic plants by this method was first reported by Gordon-Kamm *et al*. (1990). This method has been used to transform various maize target tissues (Gordon-Kamm *et al.*, 1999), including immature embryos from inbred lines (Koziel *et al.*, 1993, Dunder *et al.*, 1995 and Brettschneider *et al.*, 1997) and Hi II germplasm (Songstad *et al.*, 1996 and Pareddy *et al.*, 1997). Immature zygotic embryos provide an excellent transformation target because they give good somatic embryogenic callus than other explants (Brettschneider *et al.*, 1997; Songstad *et al.*, 1996 and Frame *et al.*, 2000).

In bombardment experiments, even though suitable plant material and other *in vitro* conditions resulting in high frequency of regeneration are available, certain instrument related physical conditions which play crucial role in delivering the gene into cell must be optimized. Hence in this article, we embarked on studying the effect of different physical parameters *viz*., gold particles size, distance of tissue material from the macro carrier and rupture disk pressures on frequency of transient gene expression.

*Corresponding author: **Pavan Kumar G**
Nuziveedu Seeds Limited, Hyderabad

MATERIALS AND METHODS

Plant materials

Maize ears obtained from NM5884, a proprietary inbred line of Nuziveedu Seeds Limited (NSL), harvested 12 to 15 days after pollination (DAP) from the field grown plants of NSL, Hyderabad, were used as source material. Isolated Immature Embryos (IEs) and Embryogenic Callus (E. callus, Type I) grown from IEs were used as material for bombardment studies. Isolation and inoculation of IEs and selection of E. Callus for bombardment studies were done according to Ishida *et al.* (2007).

Culture conditions before and after transformation

Immature Embryos isolated from young kernels were directly used for bombardment and few of them have also been inoculated on LS (Linsmaier and Skoog, 1965) medium by keeping scutellum-side touching the medium for callus induction. Cultures were kept in dark for two weeks at 26±1 °C. After two weeks, elongated scutellum was removed and kept again in dark for two more weeks and then, developed E. callus (Type I) was separated and used for bombardment studies (Fig. 1A). After bombardment, IEs as well as calli were kept in dark for 16 hours as they are and then transferred onto fresh medium for callus induction and maintenance respectively. All media compositions and concentrations reported by Ishida *et al.,* 2007 were adopted.

Plasmid

The plasmid vector *pCAMBIA* 1391Z harbouring with hygromycin phospho transferase (*hpt II*) gene and β-glucuronidase (uidA) (*gus*) reporter gene was used in this work. Both genes are driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminated by the *nos*.

Bombardment plates preparation and microprojectile bombardment

PDS 1000/He biolistic gun (Bio-Rad, Hercules, CA) was used for this study. E. callus (Type I) masses were evenly distributed within 3-5 cm diameter target area in the middle of the petriplate having LS medium and bombarded with gold particles pre-coated with plasmid DNA. Gold particles of two sizes *viz*., 0.6 and 1.0 µm were used. Effect of distance between target and macro carrier was tested by keeping the plate in Slot-I (3 cm.), Slot-II (6 cm.) and Slot-III (9 cm.). Three different RDPs (Rupture Disk Pressures) were used at 650, 900, 1100 psi. The target materials were bombarded once with delivery System. All hardware and disposables for the biolistic gun were obtained from Bio-Rad. After bombarding the cultures, plates were wrapped with clinwrap and maintained at 26±1 °C in dark.

Gold particle preparation

15 mg of gold particles (0.6 µm or 1.0 µm) were washed with 500 µl of cold, 100% ethanol in a 1.5 ml sterile eppendorf tube by sonicating for 15 seconds in an 18 OZ ultrasonic cleaner water bath. The particles were allowed to settle for 30 minutes and then centrifuged at 3000 rpm for 60 seconds. The supernatant was discarded and the pellet was washed with 1 ml cold, sterile distilled water by disturbing the pellet by tapping with the finger and allowed to settle down. This was repeated for three times and finally centrifuged at 3000 rpm for 60 seconds. After the final rinse, 250 µl of sterile water was added to gold pellet and ultra-sonicated for 15 seconds. This ultra sonicated suspension was aliquoted to five sterile eppendorf tubes, each of with 50 µl suspension having 3 mg gold particles, was frozen at -20 °C and used within two months (Sanford *et al.,* 1993).

Coating the gold particles with DNA

One hour prior to bombardment, aliquot of sterile gold particles (3 mg) was thawed and sonicated for 15 seconds, then 20 µg of plasmid DNA (pCAMBIA 1391Z) was added. After gentle tapping with finger, 50 µl of $CaCl_2$ (2.5 M) followed by 20 µl of spermidine (0.1 M) were added to the sample while continuously mixing on the vortex mixer. After 5-10 min of mixing, the gold particles were allowed to settle and the sample was centrifuged at 5000 rpm for 15 seconds. The supernatant was discarded and pellet was re-suspended in 250 µl of cold, 100% ethanol by gently rocking the tube. This was done twice and centrifuged at 5000 rpm, 15 seconds. Finally, supernatant was discarded and pellet was re-suspended in 120 µl cold, 100% ethanol, from which 10 µl of the DNA-coated gold was pipetted onto each macrocarrier while shaking the suspension continuously. Now the macrocarrier coated with DNA was placed in sterile petriplate to maintain maximum dryness prior to bombardment (Sanford *et al.*, 1993).

Selection of transformed callus

Selection was made on LS medium supplemented with 10 mg/L Hygromycin after 10 days of bombardment. Calli were transferred every 2 week to fresh selection media. After two or three subcultures, putative transformed calli (Hygromycin-resistant calli) were able to identify as clusters of white, rapidly growing callus masses. Selected callus was transferred onto fresh regeneration medium for shoot regeneration. All the cultures were maintained in the growth chamber at 26±1 °C with a 16: 8 light: dark photoperiod.

Histochemical GUS assay and assessment of transient expression

Transient GUS expression was tested by immersing the segments in X-Gluc buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc), 50 mM Sodium Phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide and potassium ferrocyanide, 10 mM Na_2 EDTA and 0.1% TritonX-100. After 16 hrs of incubation at 37 ºC, blue staining was assessed (Jefferson, 1987). Data were collected from the calli subjected to different variable parameters *viz*., particle size, rupture disc pressure and distance of explant from the pressure source.

Molecular analysis

Genomic DNA was isolated from the selected callus tissues by following Doyle and Doyle (1990). PCR analyses were performed using selectable marker (*hpt II*) specific HPTF: 5’CACAATCCCACTATCCTTCGC3’, HPTR: 5’GCAGTTC GGTTTCAGGCAGGT3’ and reporter gene (GUS) specific primers, GUSF: 5’GGTGGGAAAGCGCGTTACAAG3’, GUSR: 5’GGTTAC GCGTTGCTTCCGCCA3’, which amplify

480 and 1200 bp fragments for *hpt II* and GUS genes respectively.

Statistical analysis

Data obtained on the effects of gold article size, distance and rupture disc pressure were subjected to ANOVA following Snedecor and Cochran (1968).

RESULTS AND DISCUSSION

Initially, both IEs (direct explants) as well as Embryogenic Callus (Type I callus) were used to evaluate the suitable material for getting high frequency of transient expression of induced gene. Immature Embryos and E. Callus (Type I) without bombardment served as controls in this experiment. No callus initiation was observed in bombarded IEs in any of the various parameters used *viz*., gold particle size, distance and pressure, moreover, explant turned to brown and finally dead due to physical damage of the embryo. Hence, E. Callus derived from the IEs was used throughout the experiments for getting the data. Explants directly used for bombardments are rare to survive and die even before they were transferred onto selection medium, which is in support with the reports of Saker *et al*. (2006, 2007) in rice and date palm. In contrary to this, in sorghum, immature embryos were bombarded (Tadesse *et al*., 2003). And also, both materials were incubated in osmoticum medium having mannitol and sorbitol in a concentration of 0.2 M each, which facilitates stable transformation (Vain *et al*., 1993). However, in present study, osmoticum medium has no effect on frequency of transient GUS expression, moreover browning was observed in explants (IEs) and calli, hence, bombardment has been taken place on plain LS medium. In preliminary studies, two concentrations of plasmid DNA *viz*., 10 µg and 20 µg were used for coating the gold particles. GUS expression was very thick on calli when particles were coated with 20 µg of plasmid DNA whereas, it was spotty at a concentration of 10 µg. Hence 20 µg of plasmid DNA was used for coating the gold particles.

Transient expression of GUS in the calli, subjected to different variable parameters *viz*., particle size, rupture disc pressure and distance of explants from the pressure source, showed variation in terms of intensity of colour (stain) as well as extent of expression spread over the callus. Therefore, expression in the explants and/or calli was represented basing on intensity of expression over the segments and four categories were identified *viz*., +, ++, +++ and ++++ which were assigned arbitrary values of 0.25, 0.5, 0.75 and 1 units respectively (Fig. 1. B & C). Control calli did not show signal in GUS assay (Fig. 1 D). The total GUS intensity obtained in a particular parameter or combination of parameters was calculated as:

$$\text{Total Units of GUS intensity} = \sum \left[\text{Number of segments in a category} \times \text{Value assigned to the category} \right]$$

The frequency of transient GUS expression and total units of GUS intensity varied from 40 to 86.67% and 5.75 to 23.25 respectively. The maximum frequency and intensity of GUS expression were observed when the bombardment was done using 0.6 µm gold particles by keeping the material in Slot II at 900 psi pressure, whereas, minimum frequency and intensity were observed with 1.0 µm gold particles in Slot-III at 1100 psi pressure (Table - 1). When the particle size is considered, bombardments done using 0.6 µm particle size resulted in relatively higher frequencies of gene expression than the 1.0 µm particle size in all slots as well as in pressures used.

Table 1 Effect of particle size, distance and pressure variations on frequency, intensity of GUS expression in Embryogenic Calli of maize

Particle Size	SLOT	Pressure (psi)	No. of Calli Shooted	No. of Calli with GUS expression	Percentage of calli with GUS Expression	No. calli in different intensities				Total Units of GUS Intensity
						+ (0.25)	++ (0.50)	+++ (0.75)	++++ (1.00)	
0.6 µM	Slot-I (3 cm)	650	30	23	76.67	0	3	6	14	20.00
		900	30	21	70.00	1	2	2	16	18.75
		1100	30	15	50.00	0	2	1	12	13.75
	Slot-II (6 cm)	650	30	24	80.00	0	2	5	17	21.75
		900	30	26	86.67	1	1	6	18	23.25
		1100	30	22	73.33	1	2	4	15	19.25
	Slot-III (9 cm)	650	30	19	63.33	1	3	6	9	15.25
		900	30	23	76.67	0	1	6	16	21.00
		1100	30	18	60.00	1	2	5	10	15.00
1.0 µM	Slot-I (3 cm)	650	30	16	53.33	1	2	7	6	12.50
		900	30	18	60.00	1	2	8	7	14.25
		1100	30	15	50.00	2	3	5	5	10.75
	Slot-II (6 cm)	650	30	17	56.67	2	3	7	5	12.25
		900	30	19	63.33	3	2	6	8	14.25
		1100	30	16	53.33	2	2	7	5	11.75
	Slot-III (9 cm)	650	30	15	50.00	2	4	8	1	9.50
		900	30	17	56.67	3	6	7	1	10.00
		1100	30	12	40.00	4	5	3	0	5.75

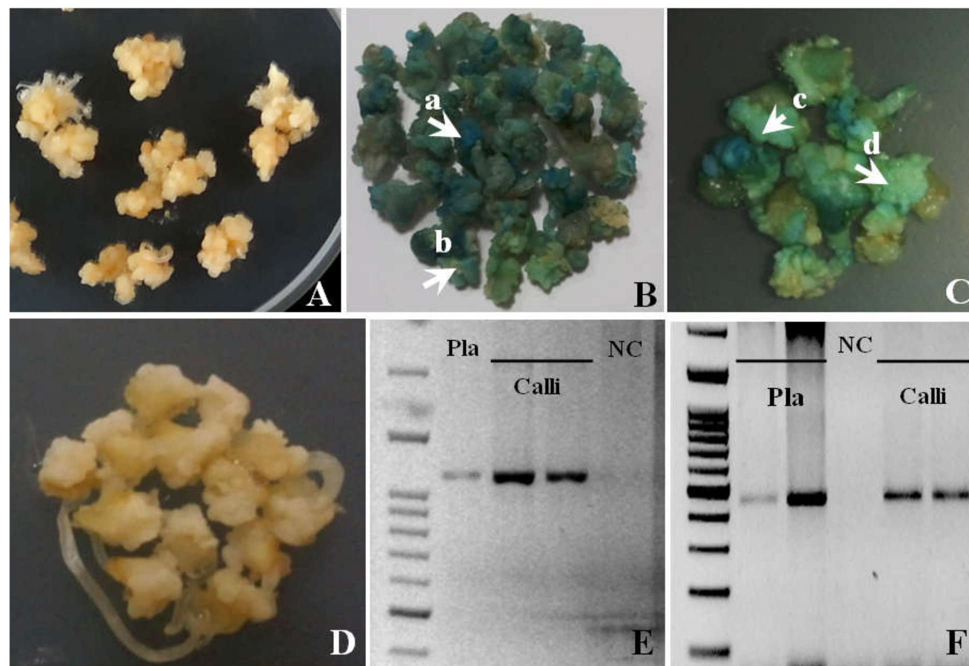

Figure - 1 Differential Transient GUS expression in maize through particle bombardment in different conditions *viz.,* particle sizes, different distances and pressures (A - F). Embryogenic callus segments derived from Immature Embryos used for bombardment experiments (A), showing high intensity of stain on entire segment (a of B) represented by ++++; medium intensity of stain on entire segment (b of B) represented by +++; low intensity of stain in part of the segment (c of C) represented by ++ and very low intensity of stain in part of the segment (d of C) represented by + in gus assay after bombardment. Control segments without bombarded did not show GUS expression after staining (D). PCR with GUS and hptII specific primers resulted in 1200bp (E) and 480bp fragments (F) in plasmid (Pla) and calli, negative control (NC) did not show amplification.

This kind of results with high frequency of expression with less tissue damage using 0.6 µm has also been reported in previous reports (Randolph-Anderson *et al*., 1995 and Frame *et al*., 2000). Slot-I and II resulted in relatively higher frequencies than Slot-III. Even though Slot-I results in good amount of expression, material get shattered, hence Slot-II (6 cm.) is the optimum distance for getting high frequencies at all pressures used (Table - 1).

Pressure and distance seems to be interrelated as the distance increases, pressure get decreased and results in low expression, hence both are inter dependent and have to be studied. The distance which can be changed between the material and macro carrier are the three different slots *viz*., Slot-I, II and III, which measures 3, 6 and 9 cm. distance from the source of pressure. In this study, Slot-II (6 cm) was found to be suitable for maximum GUS expression with less tissue damage (Table - 1). This result is in support with the results of Saker *et al*. (2007).

At lower pressures (650 and 900 psi), even distribution of DNA and maximum transient GUS expression in all calli were observed (Fig. 1), which indicating that the bombardment shock at low pressures resulted in high induction and low tissue injury. In contrary, at higher pressures (1100 psi) less GUS frequency and high tissue damage and browning was appeared in bombarded calli. Among the three pressures used (even though pressures at 1300 and 1550 psi were used, data has been not given due to negligible expression), 900 psi was found to be the effective acceleration pressure for transient GUS expression at high frequency in all three slots and two particle sizes used (Table - 1).

At low pressures, penetration of particles is low and at high pressures, tissues get shattered and may get damaged. In general 900-1000 psi was found to be optimum (Sanford *et al*., 1993 and Saker *et al*., 2006). High frequencies of expression at high pressures were reported at 1100 psi (Swati *et al.*, 2012, Sreeramanan *et al.,* 2005, Petrillo *et al.,* 2008, Kim *et al.,* 2012 and Anoop and Gupta, 2004). Whereas, maximum GUS expression was reported at 1,300 psi rupture disk pressure (Tadesse *et al*., 2003), Saker *et al.* (2007) reported drastic reduction at 1500 psi pressure. It was observed that the tissue type and variation in distance of bombardment also play the role in influencing this parameter.

When this differential response of frequency of gene expression and intensity of GUS staining to particle size, distance and pressure were analyzed, the ANOVA revealed significant variation among the size of the gold particles not only for the frequency of gene expression but also for intensity of GUS staining (Table - 2). In contrary, no significant variation was observed between the distances (Slots) and pressures used neither for the frequency of gene expression nor for the intensity of GUS staining (Table - 2).

E. calli proliferated on selection medium which showed GUS expression was used for testing the presence of induced genes by using specific primers designed for *GUS* and *hpt II* genes. Expected amplicons of 1200 bp and 480 bp sizes were observed in putative transformed calli and the same were not observed in non-transformed control calli (Fig. 1, E and F), which confirm the integration of genes into the genomic DNA of recipient tissues.

Table 2 ANOVA for the effect of gold particle size, bombardment distance and rupture disc pressure on frequency and total units of intensity of GUS expression in E. Callus segments of maize.

Source of Variation	DF	SS	MS	F - value
Frequency (%) of GUS expression				
Between Particle sizes	1	1306.2	1306.28	15.11*
Total Variation	17	2689.2		
Between Slots	2	414.7	207.37	1.36
Total Variation	17	2689.2		
Between Pressures	2	637.2	318.61	2.32
Total Variation	17	2689.2		
Intensity of GUS staining				
Between Particle size	1	249.4	249.38	27.84*
Total Variation	17	392.7		
Between Slots	2	56.4	28.18	1.25
Total Variation	17	392.7		
Between Pressures	2	53.7	26.87	1.18
Total Variation	17	392.6		

* - significant at p=0.05

CONCLUSION

Microprojectile particle bombardment is the suitable and easy method in maize to obtain fertile transgenics in less time. Transient expression of the gene (*GUS*) has been taken into consideration to standardize the optimal physical conditions, as it corresponds to stable fertile transformation (Yao *et al*., 1996). In some cases, even though the transient expression of gene is not directly correlated with the stable transformation (Nandaveda *et al*., 1999), the optimal conditions which results in high frequencies of former one provides the basic protocol for the latter. In the present study, it has been concluded that the particle size of 0.6 µm, distance of 6 cm. (Slot II) and 900 psi pressure were found to be optimal physical conditions. E. Callus segments, plain medium without osmoticum, 20 µg of plasmid DNA for coating the gold particles were suitable rather than the direct usage of immature embryos, medium with osmoticum and 10 µg of DNA for coating.

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