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Biolistic Transformation of *Citrullus vulgaris* Schrad (Watermelon)

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Abstract: In this study, DNA-coated gold particles were used to transform cotyledon sections including axillary meristem of *Citrullus vulgaris* using the biolistic method for optimizing the transformation parameters and eventually regeneration of putative transgenic watermelon plants. Initially, five biolistic parameters namely helium pressure, macrocarrier to target tissue distance, explant ages, DNA concentrations and osmotic treatment were optimized using plasmid pRQ6 (carrying both *gusA* and *hpt* genes). The optimized biolistic parameters for cotyledon sections of *Citrullus vulgaris* explants were determined as follow: 1100 psi helium pressure, 6 cm target distance, 4-day-old explants, concentration of DNA at 1.2 µg/bombardment and osmotic treatment for 24 h on medium supplemented with 0.6 M mannitol prior to bombardment. Using the optimized parameters, transformation of watermelon cotyledons was carried out using pAHG11, pCAMBIA 1301, co-transformation (pAHG11+pCAMBIA 1301) and pCambar plasmids. Bombarded tissue's were selected on either 5 mg L^{-1} hygromycin or 2 mg L^{-1} PPT. Finally, the transformants produced were subjected to GUS histochemical assay. Integration of the transgenes into transgenic watermelon genome was confirmed by PCR analysis.

Key words: Biolistics, transgenic watermelon, *Citrullus vulgaris*, *optimization*

INTRODUCTION

Watermelon is one of popular fruits consumed all over the world. Beside its juicy texture, watermelon is rich in useful antioxidant (mainly lycopene) which has been demonstrated to inhibit growth of cancer cells (Hall, 2004). Due to its fruits property and size, genetic manipulation of this plant will have a lot of advantages. Development of a reliable transformation system is important for genetically improving a particular crop. Besides the pioneer method, *Agrobacterium*-mediated, biolistic mediated gene transfer is one of the popular methods to introduce foreign genetic material into plant cells (Potrykus, 1991). Gene transfer by biolistic using high velocity DNA-coated gold microcarrier is a rapid and simple means of transforming intact cells and has been widely used to introduce foreign DNA into the plant cells (Finer and McMullen, 1990).

Optimization of gene transfer using reporter marker genes is a pre-requisite for developing an efficient and reliable transformation system for any crop species. Furthermore, identification of suitable selection agents, such as antibiotic or herbicide, and its minimal concentration is important for isolating and regenerating transformed cells into whole transgenic plants. Successful transfer of foreign DNA into plant cells could be achieved after optimizing physical and biological parameters that effect DNA delivery and biological condition of the receiving cells (Birch and Bower, 1994). Among the parameters commonly optimized for DNA delivery into plants are helium pressure, distance from macrocarrier carrying the DNA to the target tissue, osmotic treatment prior to transformation, explant age and the concentration of DNA used per bombardment (Vain *et al*., 1993; Parveez, 2000; Ingram *et al*., 1999; Vidal *et al*., 2003; Wagiran, 2003; Ivic-Haymes and Smigocki, 2005; Indurker *et al*. 2007).

Currently, there are only two reports on transient expression of transgene in watermelon using biolistic (Compton *et al*., 1993; Yin *et al*., 2009). Stable transformation using this method has not been reported. In this study, we describe the optimization of physical and

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biological parameters affecting DNA delivery into watermelon and followed by regeneration of putative transgenic watermelon plants.

MATERIALS AND METHODS

Explants preparation for biolistic transformation: Explants were obtained from 4-day-old *in vitro* diploid watermelon (yellow watermelon) seedlings. Twenty pieces of cotyledon were removed and placed in the centre (20 mm) of a 9 cm diameter petri dishes containing semi-solidified MS medium with 0.32% (w/v) phytagel, supplemented with 3% (w/v) mannitol, 3% (w/v) sorbitol, 3% (w/v) sucrose and 20 µM BAP and incubated at 25°C. The following day, the cotyledons were immediately subjected to bombardment with the Biolistic Particle Delivery PDS-1000/He (Bio-Rad) device.

Bacterial strains and plasmids: Bacteria strains and plasmids used in this study were given in Table 1. The plasmid pRQ6 containing both *gusA* and *hpt* genes (Fig. 1a-d) was used for optimizion of transient expression using GUS assay.

Preparation of plasmid DNA: *Escherichia coli* strain DH5" carrying the desired plasmid was grown in LB liquid medium (10 mL) containing 100 µ mLG^{1} ampicillin. *Agrobacterium tumefaciens* carrying pCAMBIA 1301 plasmid was grown in YEP broth (10 mL) containing 50 µ mLG^{1} of kanamycin. Bacterial cultures were grown at 37°C (*E. coli*) and 28°C (*A. tumefaciens*) with agitation at 180 rpm for 16 h. The plasmid was harvested using commercially available QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instruction. This method was used to prepare small quantities of plasmid DNA (<10 µg). The DNA was resuspended either in TE buffer or sterile nano-pure water (1 µg µLG^{1} of plasmid). The DNA solution was stored at -20°C for long term storage and 4°C for short term storage.

Table 1: Bacteria strains and plasmids used

Plasmid/strain	Genotype	Source/References
pRQ6:: *E coli* DH5*a*	*gusA*	MARDI
	hpt	
pAHG11:: *E coli* DH5*a*	*hpt*	MARDI
pCamBar:: *E coli* HB 101	*gusA*	FRIM
	bar	
	hpt	
pCAMBIA 1301::	*gusA*	Mahalakshmi *et al*. (2006)
A. tumefaciens EHA 101	*hpt*	

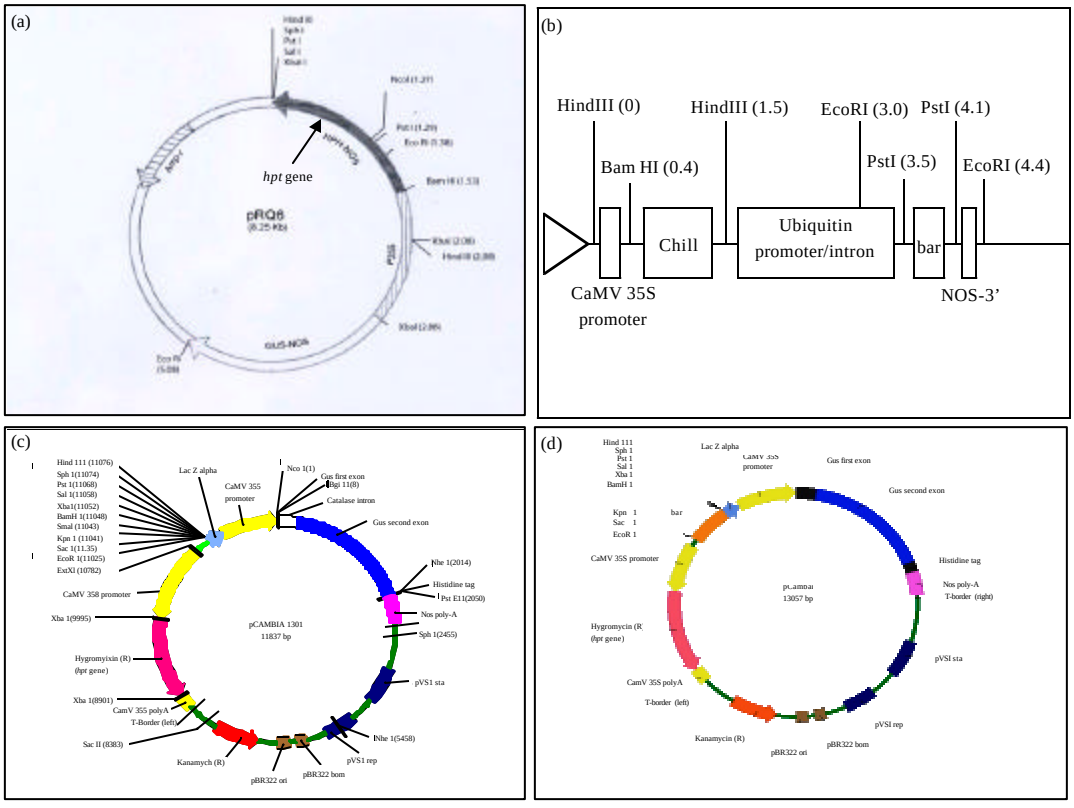

Fig. 1: Restriction maps of transformation vectors (a) pRQ6, (b) pAHG11, (c) pCAMBIA 1301 and (d) pCambar

Gold particle preparation: Twelve mg of 1.0 µm gold particles were washed with 200 µL of 100% ethanol in eppendorf tube. It was vigorously vortexed for 1-2 min followed by 10 sec of centrifugation at 20, 000 g. Ethanol was discarded and the pellet was resuspended with 200 µL of sterile double distilled water, vortexed and centrifuged at 20,000 g. The suspension was discarded and the gold pellet was resuspended in 200 µL of sterile double distilled water and vigorously vortexed for 1-2 min. The gold was then suspended in sterile double distilled water and dispensed into 100 µL aliquots, which were kept frozen at -20°C.

Plasmid DNA coating with gold particles: To precipitate plasmid DNA onto the microcarrier, 100 µL of gold suspensions (0.12 mg µL^{-1}) were mixed with 20 µL of pRQ6 plasmid (1 µg µL^{-1} stock concentration) and 40 µL sterile spermidine. Then 100 µL of sterile $CaCl_2$ was added drop by drop. The particle/DNA suspension was vortexed and left for 10 min at room temperature. The DNA-coated particles were pelleted by centrifugation at 20,000 g for 10 sec. The supernatant was then completely removed. The pellet was resuspended in 100 µL of 100% cold ethanol. A total of 12 µL of the particle/DNA suspension was pipetted in the center of the microcarrier and left to dry for 30 sec.

The target cells (explants) were placed at a distance of 6, 9 and 12 cm from the stopping screen. Explants on each plate were bombarded at 1100 psi helium pressure. After bombardment, the cultures were maintained on the regeneration medium in 16 h photoperiod at 25°C for 24 h.

Histochemical GUS assay: Histochemical assay for glucuronidase (GUS) was conducted on explants 24 h after bombardment as described by Jefferson (1987). The material was incubated in potassium ferricyanide (8.2 mg mL^{-1}), potassium ferrocyanide (10.6 mg mL^{-1}), 5-bromo-4-chloro-3-indolyl-$-D-glucuronide (X-Gluc) (100 mg mL^{-1}), sodium phosphate buffer (0.2M [pH 7]), 20% (v/v) methanol and 0.5% (v/v) Triton X-100 at 37°C overnight. The chlorophyll was removed by washing the tissues with 100% ethanol before GUS detection. The GUS expression was recorded in terms of number of blue spot per explants.

Bombardment parameters: Biological and physical parameters mentioned below for biolistic transformation were optimized using $-glucuronidase (GUS) as the reporter gene using plasmid pRQ6. The biological parameters optimized in this work included the explant ages (4 and 5 days old), osmotic treatment using sorbitol or mannitol at 0.2, 0.4, 0.6 M concentration and DNA concentrations (0.6, 1.2 and 1.8 µg per bombardment). For each parameter, the explants were bombarded at 9 cm target distance with 900 psi helium pressure. The reason for using only two explant ages in this experiment was to reduce the size of explants in order to maximize the number of explants used for bombardment.

Optimization of the physical parameters was carried out under the following conditions; rupture disk pressure (650, 900 and 1100 psi) and distance from stopping plate to target tissue (6, 9 and 12 cm). Gold microcarrier size and vaccum pressure were fixed at 1.0 µm and 26 mmHg, respectively. Each of these treatments was conducted in three replicates and the experiment was repeated twice unless otherwise stated. All bombarded explants were histochemically tested for GUS expression for parameter optimization 24 h after bombardment.

Biolistic transformation using optimized condition: Explants were subsequently bombarded under the optimized conditions at 1100 psi helium pressure, 6 cm target distance, 4-day-old explants, DNA at 1.2 µg/bombardment and osmotic treatment for 24 h on medium supplemented with 0.6 M mannitol prior to bombardment. These parameters have been used to produce stably transformed explants in subsequent experiments using plasmid pAHG11 (*bar* gene driven by maize ubiquitine promoter), pCAMBIA 1301 (*hpt* gene driven by CaMV35S promoter (Mahalakshmi *et al*., 2006) and pCambar (*bar* and *hpt* genes driven by CaMV35S promoter). Each treatment had three replicates and the experiment was repeated two times. Thirty of the bombarded explants were histochemically GUS tested and others were subcultured on MS medium containing 20 µM BAP for 3 weeks before being subcultured onto selective medium (MS + 20 µM BAP + 5 mg L^{-1} hygromycin). The explants were cultured on the selection medium for more than 2 months. Small pieces of shoots surviving on the selection media were isolated and multiplied on antibiotic-free medium for 4 weeks. The GUS assay and PCR analysis were conducted randomly on leaf sections from regenerated plants prior to exposing them onto selection medium for another 4 weeks.

Molecular analysis

DNA isolation: DNA was isolated from young leaves of putative transgenic plants using the Wizard® Genomic Purification Kit (Promega). The young leaves were selected from positive GUS and hygromycin-resistant or PPT-resistant shoots, post-biolistic transformation with plasmid pRQ6 (containing *gusA* and *hpt* genes) and pCAMBIA 1301 (containing *gusA* and *hpt* genes), pAHG11 (containing *bar* gene) and pCambar (containing *gusA*, *hpt* and *bar* genes).

Polymerase Chain Reaction (PCR) analysis: The PCR was used to confirm the presence of the transgene in GUS, hygromycin-resistant and Basta-resistant tissues. DNA from young leaves of putatively transformed and untransformed plants was extracted and used in PCR analysis. Detection of *hpt* gene by PCR was carried out using the *hpt* gene specific primers (*hpt*-forward, GGG GGG TCG GTT TCC ACT A; *hpt* -reverse, ATC GTT ATG TTT ATC GGC ACT TTG {produce 821 bp fragment}) and the PCR conditions as follow: one cycle of 94°C, 2 min; followed by 30 cycles of 94°C, 45 sec (Denaturation); 55°C, 30 sec (Annealing) 72°C, 30 sec (Extension) and finally one cycle of 72°C, 5 min. Detection of *bar* gene was carried out using the *bar* gene specific primers (*bar* -forward, TCA AAT CTC GGT GAC GGG CA; *bar* -reverse, GGT CTG CAC CAT CGT CAA CC {produce 492 bp fragment}) and the PCR conditions are as follow: 35 cycles of 95°C, 30 sec (Denaturation); 62°C, 40 sec (Annealing) 72°C, 60 sec (Extension). The PCR reactions were carried out in 50 µL reaction using MyCyler PCR System 9700 thermal cycle.

Agarose gel electrophoresis: The PCR-amplified products (DNA fragments) were analysed using agarose gel electrophoresis. The DNA fragments on the gel were visualized using a Gene Flash Gel Documentation system (Syngene). The sizes were estimated by comparison with a 1 kb ladder (Gene Craft) or O'GeneRuler™ DNA Ladder Mix (Fermentas) standard DNA marker.

Statistical analysis: Data were collected from the transient expression of GUS assay of yellow watermelon cotyledon explants and sensitivity of leaf explants to hygromycin as media supplement. The result were analysed statistically using the SPSS for windows software (SPSS Windows Version 12, SPSS, Inc). Data was analysed using one way ANOVA and the differences were contrasted using Tukey's Honestly Significant Difference (HSD value) (Richard, 2008). All tests were used to compare the treatments at $p<0.05$.

RESULTS AND DISCUSSION

In the shoot regeneration study of watermelon, multiple shoots from the axillary meristems were obtained instead of adventitious shoot (induced at cut edges of explant) (Suratman *et al*., 2009). Based on the above observation, transformation via biolistic method was the best method for use in the transformation study. The present studies showed that cotyledon sections including axillary meristem are potentially useful targets for biolistic-mediated transformation. In this study, five important parameters for successful biolistic-mediated transformation, namely, helium pressure, macrocarrier to target tissue distance, explant ages, DNA concentrations and osmotic treatment were optimized. Compton *et al.* (1993) has described the parameters for optimizing transient expression of GUS in cotyledon of watermelon following particle bombardment using a Particle Inflow Gun (PIG), a modification of the commercially available Biolistics PDS-1000He devise (Bio-Rad). Recently, another transient expression of GUS gene was demonstrated for watermelon using biolistic transformation (Yin *et al*., 2009). However, evidence of stable integration of transgene/foreign DNA for this method has not been reported in the above report.

Effect of explant age: Watermelon explants were bombarded at 900 psi helium pressure and 9 cm target distance. The results showed that the optimal favorable percentage of cotyledon explants expressing GUS was at 98% (33.13 GUS spots/explant) for 4-day-old seedling as compared to 90% for cotyledons from 5-day-old seedling (29.55 GUS spots/explant) (Table 2). Eventhough the differences were not significant, the cotyledons from 4-day-old seedlings were used in subsequent experiments to maximize the number of explants used. The 4-day-old explant were more suitable for biolistic transformation as the size of cotyledon is smaller and thus, a bigger area of the explants could be covered by the DNA-coated microcarriers.

Effect of osmotic treatment: Osmotic treatment at different concentrations of mannitol and sorbitol was tested to evaluate whether or not the osmotic pressure could help to increase transformation efficiency by maintaining the shape of explants and meristem for direct bombardment. Explants were exposed to different concentrations of mannitol and sorbitol for 24 h prior to bombardment. The results showed that the highest average number of spots per explant was obtained after treatment with 0.6 M mannitol (37.78±4.6). However, it was not significantly different to other concentrations of osmoticum and untreated controls at $p<0.05$ (Fig. 2). Nevertheless, the effect of osmotic treatment on shape of explants after 24 h pre-culture on medium with or without osmotic treatment was observed. The explants on medium without

Table 2: The effect of explants ages on GUS expressions in *Citrullus vulgaris*, 24 h after bombardment

Explant ages	Bombardment explants No.	Average No. of GUS spots/explant±SE	Percentage of explants express GUS +ve
4-day-old	60	33.13±3.54	98
5-day-old	60	29.55±4.76	90

Data were subjected to ANOVA with 20 explants per bombardment. Data presented as Means±SE. Each treatment had three replicates and this experiment was conducted twice. The average number of GUS spots/explant not significantly different at $p<0.05$

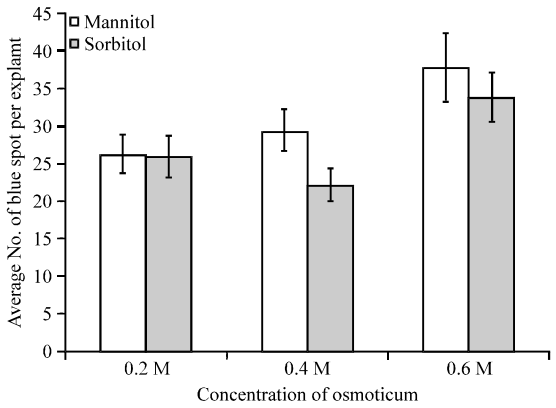

Fig. 2: The effect of osmotic treatment on GUS expression in yellow watermelon cotyledons at 900 psi helium pressure and 9 cm target distance 24 h after bombardment

osmotic treatment was expanded and swelled. This swelling may cause the bombarded microcarriers to travel out of the target area (meristem region). Therefore, 0.6 M mannitol was used in subsequent experiments.

Osmotic treatment prior to particle bombardment is a widely applied procedure to reduce tissue damage and enhanced transient expression of *gusA* gene and subsequently stable transformation of the transgene. The expansion and the changes in shape reduced the probability of bombarded microcarriers penetrating into the meristems due to the possibility of cell damage or death. Osmotic treatment is therefore, crucial to help maintain the explants and meristem in shape for direct bombardment. The use of an osmoticum can facilitate stabilization of cell membranes for faster healing of the lesion and reduce turgor pressure of cells to reduce leakage and cell rupture (Perl *et al*., 1992; Ye *et al*., 1994). Vain *et al*. (1993) reported that osmotic enhancement of transient expression and stable transformation of maize was facilitated through plasmolysis of target cells. Plasmolyzed cells may be less likely to extrude their protoplasm following penetration of the gold particles (Nandadeva *et al*., 1999; Uze *et al*., 1997; Armaleo *et al*., 1990). In this study, the osmotic treatment tested at 0.6 M mannitol resulted in improved number of GUS spots in explants compared to control. In maize cells, 0.4 to 0.6 M osmoticum showed higher GUS expression (Vain *et al*., 1993). However, in pearl millet (*Pennisetum glaucum* L.), castor and garden balsam (*Impatiens balsamina*), 0.2-0.25 M manitol and 0.2-0.25 M sorbitol resulted in the highest *gusA* gene expression (Latha *et al*., 2006; Sailaja *et al*., 2008; Taha *et al*., 2009). In contrast, treatment of mannitol for up to 48 h prior to bombardment did not show any positive effects on biolistic transformation of olive somatic embryos (Perez-Barranco *et al*., 2009).

Table 3: The effect of plasmid DNA concentration on GUS expression in cultivar Hwang Fong Yellow Queen cotyledon on GUS expression 24 h after bombardment

Plasmid (µg/bombardment)	Bombardment explants No.	Percentage of GUS + explants	Average No. of GUS spots/explant±SE
0.60	60	93	11.27±1.15[a]
1.20	60	97	39.83±4.67[b]
1.80	60	93	32.83±3.86[b]

Data were subjected to ANOVA with 20 explants per bombardment. Data shown as Mean±SE. Each treatment had three replicates and the experiment was conducted twice. The different letters showed significant different at $p<0.05$

Effect of DNA concentration: The effect of DNA concentration per bombardment on GUS expression in bombarded watermelon cotyledon was also examined. The quantity of DNA coated with gold particles were varied from 0.6 to 1.8 µg per bombardment. The concentration of DNA at 1.20 µg per bombardment produced the highest number of GUS spots per explant as compared to 0.6 and 1.8 µg per bombardment (Table 3). Aggregation of plasmid coated gold particles was observed under the microscope when the highest DNA concentration was used for bombardment. This observation could explain why transient expression did not increase when high DNA concentration was used (1.8 µg/bombardment). Eventhough the effect of DNA concentration at 1.2 and 1.8 µg per bombardment were not significantly different at $p<0.05$, DNA concentration of 1.2 µg per bombardment was chosen for subsequent experiments as at this concentration gold particle and DNA aggregation did not occur.

The use of the appropriate amount of DNA is important in order to produce efficient DNA-gold particle binding. The precipitation of DNA on gold particles will also determine the potential amount of DNA for delivery. It was found that 1.2 µg DNA per bombardment gave the highest transient GUS expression in this study. At the highest concentration of DNA (1.8 µg per bombardment), lower transient GUS activity was observed, possibly due to aggregation of DNA coated particles. Similar observation was reported for wheat tissues that showed high DNA concentration did not increase transient expression due to aggregation of microcarriers resulting in poor efficiency of cell penetration and cell injury (Rasco-Gaunt *et al*., 1999). Lower concentrations (0.6 µg per bombardment) of DNA also produced lower transient expression. This could be due to a lower number of DNA molecules transferred into the plant cells. Similar observation was reported in olive. It was demonstrated that 1.2 µg DNA per bombardment was better then 1.8 µg and 0.6 when somatic embryos were bombarded

Table 4: The effect of helium pressure (psi) on GUS expression 24 h after bombardment of yellow watermelon cotyledons, at 9 cm target distance

Helium pressure (psi)	Bombardment explants No.	Percentage of GUS + explants	Average No. of GUS spots/explant±SE
650	60	90	27.97±3.62
900	60	92	30.10±3.73
1100	60	95	37.62±3.56

Data was subjected to ANOVA with 20 explants per bombardment. Data presented as Mean±SE. Each treatment had three replicates and was conducted twice. Average number of GUS spots/explant was not significantly different at $p<0.05$. The significance levels (Tukey HSD) between treatments = 0.149

Table 5: The effect of different target distance on GUS expression in yellow watermelon cotyledons at 1100 psi helium pressure 24 h after bombardment

Target distance (%)	Bombardment explants No.	Average No. of GUS spots/explants±SE	Percentage of explants expressed GUS +ve
6	60	54.27±6.25[a]	97
9	60	31.37±4.44[b]	92
12	60	1.10±1.55[c]	55

Data were subjected to ANOVA with 20 explants per bombardment. Data presented as Mean±SE. Each treatment had three replicates and this experiment was conducted twice. Values with different letters (a, b and c) were significantly different at $p<0.05$. The significance levels (Tukey HSD) between treatments = 1.00

(Perez-Barranco *et al*., 2009). However, in a hardwood tree (*Paulownia elongata*) and garden balsam (*Impatiens balsamina)* DNA concentration of 1.66 and 1.0 µg shown to produce the maximum expression for abaxial leaf surface and cotyledonous explants, respectively (Castellanos-Hernandez *et al*., 2009; Taha *et al*., 2009).

Effect of helium pressure: The maximum GUS expression was observed when the DNA-coated gold particles were propelled at 1100 psi helium pressure. However, the differences to the other helium pressures were not significant at $p<0.05$ (Table 4.). The helium pressure at 1100 psi was chosen because a higher number of GUS spots obtained and the GUS spots were spread near the meristematic cells.

The results of the present experiment suggested that microcarrier flight distance of 6 cm was optimal for use at 1100 psi pressure. In another transient expression study on watermelon, 1100 psi helium pressure was also used (Yin *et al*., 2009). Similarly, 1100 psi was also found to be optimum for cowpea, rice, banana, sugarcane, castor and garden balsam (Ikea *et al*., 2003; Ramesh and Gupta, 2005; Sreeramanan *et al*., 2005; Jain *et al*., 2007; Sailaja *et al*, 2008; Taha *et al*., 2009). However, in barley, mothbean and olive, 900 psi helium pressure was found to be optimum (Odake, 2004; Kamble *et al*., 2003; Perez-Barranco *et al*., 2009). In a hardwood tree (*Paulownia elongata*), 450 psi was found to be the best helium pressure as compared to 900 and 1350 psi when bombarded on abaxial leaf surface (Castellanos-Hernandez *et al*., 2009). However, in hop (*Humulus lupulus* L.), a target distance of 12 cm and 1,350 psi helium pressure were found to be optimal condition (Batista *et al*., 2008). Higher pressure, 1550 psi, was demonstrated to cause damage to the target tissues in sorghum (Tadesse *et al*., 2003).

Effect of target distance: Figure 3 shows that the increase in the target distance caused reduction in the average number of GUS spots/explant and percentage of explants expressing GUS. The results showed that the target distance of 6 cm was significantly better than the other

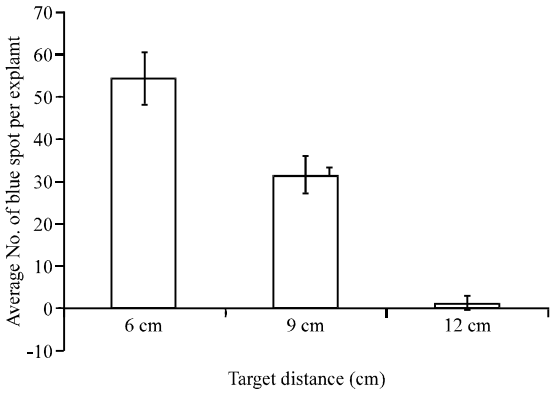

Fig. 3: Effect of target distance from stopping plate to target tissue (cm) on transient GUS expression

distances in terms of the number GUS expressing cells. Target distance of 6 cm exhibited 97% of GUS expressing explants compared to other target distances (Table 5). The GUS expression pattern for cotyledons was shown in Fig. 4a and b. Some of the GUS spots dispersed near the meristematic region. Comparatively, the number of GUS spots at 9 cm target distance was only at 92% and many of the GUS spots spread out of the meristem region. Therefore, in the subsequent experiments, a 6 cm target distance and 1100 psi helium pressure were used.

One of the most important parameters to optimize for biolistic transformation is the target distance between the macrocarrier to the target tissue in the bombardment chamber. This directly affects the distance that the microcarriers travel to the target cells for microcarrier's penetration and gene transfer into plant genome. In this study, 6 cm target distance is optimal in terms of GUS expression as compared to 9 and 12 cm. Increasing the distance causes reduction in the average number of explants expressing GUS. The same distance was found to be significantly better than other distances in mothbean, cowpea, wheat, castor and olive (Kamble *et al*., 2003; Ikea *et al*., 2003; Odake, 2004; Sailaja *et al*., 2008; Perez-Barranco *et al*., 2009). In another report for watermelon biolistic transient expression experiments 7 cm

was used as the target distance (Yin *et al*., 2009). This target distance is similar to the target distance used for sugarcane and hardwood tree (*Paulownia elongata*) (Jain *et al*., 2007; Castellanos-Hernandez *et al*., 2009). In hop (*Humulus lupulus* L.) and garden balsam (*Impatiens balsamina)*, 12 and 9 cm target distances, respectively, were found to be optimum (Batista *et al*., 2008; Taha *et al*., 2009). At 12 cm target distance, the GUS expression levels were significantly reduced. This may be due to decrease in velocity of the microcarriers as a result of increasing flight distance which consequently reduced penetration force and followed by fewer cells receiving the incoming DNA. Similar observation was reported by Sreeramanan *et al*. (2005) that increasing flight distance resulted in reduced transient expression.

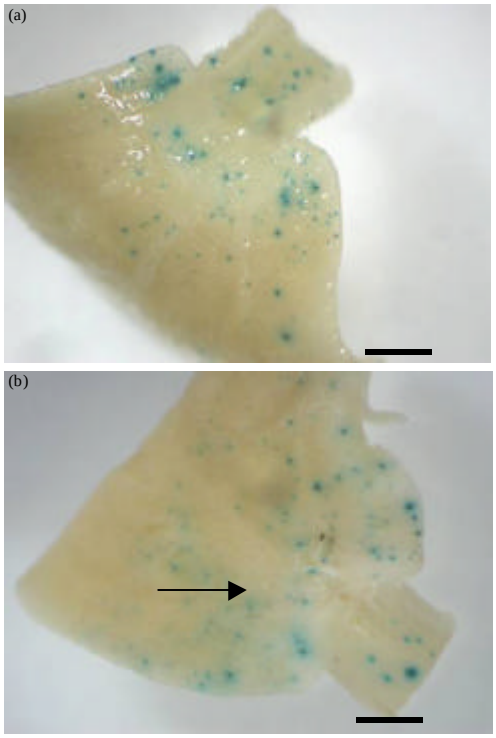

Fig. 4: GUS expression on cotyledon segments of watermelon at 1100 psi and 6 cm target distance 24 h after bombardment: (a) Diverse of GUS expression and (b) GUS spot near to meristem (as shown by arrow) Bar = 1 mm

Effect of optimized biolistic protocol on GUS expression and regeneration in *Citrullus vulgaris* cotyledons: The optimized biolistic parameters for cotyledon sections of *Citrullus vulgaris* explants were determined at 1100 psi helium pressure, 6 cm target distance, 4-day-old explants, concentration of DNA at 1.2 µg/bombardment and osmotic treatment for 24 h on medium supplemented with 0.6 M mannitol prior to bombardment. These parameters have been used to obtain stably transformed explants from subsequent experiments using plasmid pRQ6, pCAMBIA 1301 (Table 6), pAHG11 alone and co-transformation (pAHG11 + pCAMBIA 1301) and pCambar (Table 7).

In this study, the pRQ6 (carrying the *gusA* and *hpt* genes), pCAMBIA 1301 (carrying the *gusA* and *hpt* genes), pAHG11 (carrying *bar* gene) and pCambar (carrying the *gusA*, *hpt* and *bar* gene) were used as transformation vectors. The transformed explants were either selected on medium containing 5 mg L^{-1} hygromycin (pRQ6 and pCAMBIA 1301) or 2 mg L^{-1} PPT (phosphinothricin), an active ingredient of herbicide Basta (pAHG11). The bombarded explants were subjected to selection at 3 weeks post-bombardment. The explants were cultured on the selection medium for more than 2 months. Small pieces of shoots surviving on the selection media were isolated and multiplied on antibiotic- or PPT-free medium for 4 weeks and were recultured back to the selection medium to ensure that there were no escapes.

Results showed that different number of explants survived the hygromycin and PPT selections after

Table 6: The effect of the optimized bombardment protocol on GUS expression and regeneration in *Citrullus vulgaris* cotyledons after 2 months in hygromycin selection medium

Plasmid used	Explants bombarded	Explants tested for GUS expression	Ave. no of GUS spot/explant±SE	Survival (%) on hygromycin (2 months)	% tve GUS per explants	% tve PCR- hpt primer
pRQ6	120	30	54.37±6.31	14 (13)[a]	0	9
pCAMBIA 1301	120	30	67.17±7.56	23 (21)[a]	0	17

Data were subjected to ANOVA with 20 explants per bombardment. Each treatment had six replicates and the experiments were conducted twice. The letter a is a number of plants survive on hygromycin after 2 months

Table 7: The effect of the optimized bombardment protocol on GUS expression and regeneration in *Citrullus vulgaris* cotyledons after 2 months on PPT selection medium

Plasmid used	Explants bombarded	Explants tested for GUS expression	Ave. no of GUS spot/explant±SE	Survival (%) on PPT (2 months)	% tve GUS per explants	% tve PCR- hpt or bar primer
pAHG11	120	NA	NA	19 (17)[a]	NA	1 (bar)
pAHG11+pCAMBIA 1301	120	30	35.83±5.02	22 (20)[a]	0	4 (bar)
pCambar	120	30	72.03±7.95	0	0	0

NA: not available. Data were subjected to ANOVA with 20 explants per bombardment. Each treatment had six replicates and the experiments were conducted twice. The letter a is a number of plants survive on PPT after 2 months

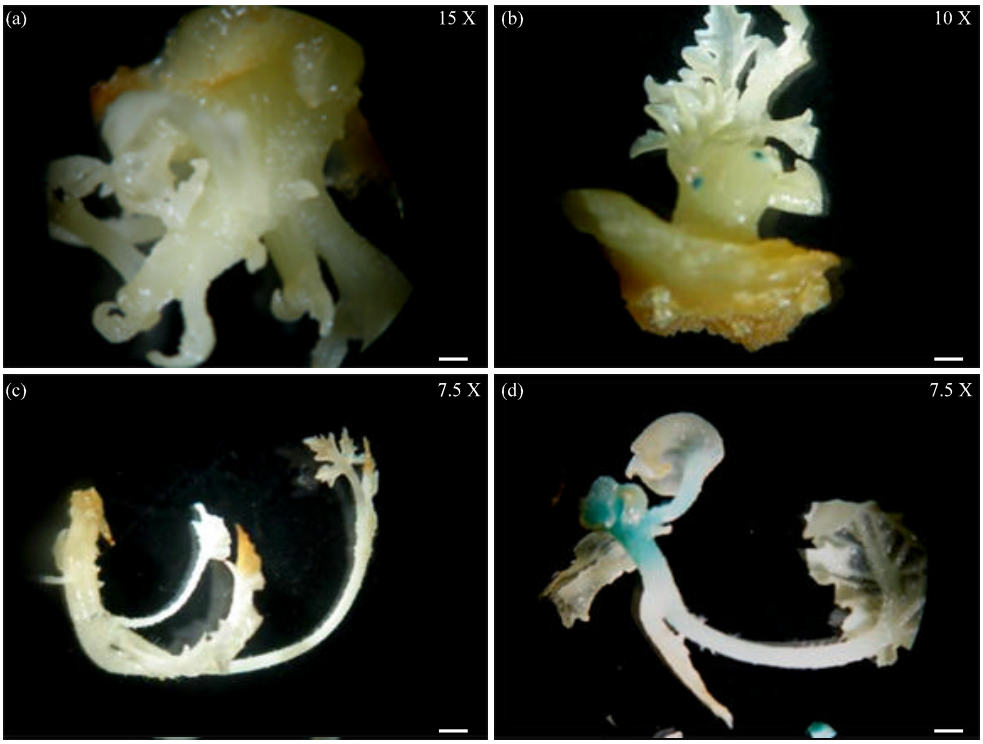

Fig. 5: Histochemical GUS assay, (a) Untransformed plant after 4 weeks in culture, (b) GUS expression of a cotyledon explant 4 weeks after bombarded with pCAMBIA 1301, (c) Untransformed plant after 4 months in culture and (d) GUS expression after 4 months on medium without antibiotic (Control plant after bombarded with pRQ6) Bar = 4 mm

bombardment with different plasmids (Table 6, 7). Initially some of the regenerated shoots were subjected to histochemical GUS analysis Fig. 5a-d. Results show that some of the regenerants exhibited GUS coloration only as small blue spots (Fig. 5b). This regenerants might be escapes or chimeric plantlets (only part of cells successfully transformed). From 60 explants tested, only one shoot was detected to show GUS positive on some parts of the shoot (Fig. 5c, d).

In this study, transformants were selected on either 5 mg L^{-1} hygromycin or 2 mg L^{-1} PPT. This was determined through minimal inhibitory concentration experiment. The selected concentration should be able to kill all the untransformed explants. The hygromycin concentration was much lower as compared to concentration reported for other plants such Kentucky blue grass and garden balsam (*Impatiens balsamina*), where transformants were selected on 100 or 75 mg L^{-1} hygromycin, respectively (Gao *et al*., 2006; Taha *et al*., 2009). On the other hand, 50 mg L^{-1} was used for rice and pepper (Lee *et al*., 2003; Li *et al*., 2003). For the selection using PPT, it was reported that 2 mg L^{-1} PPT was also used for selection of transgenic wheat after bombardment of scutella's derived callus (Tamàs *et al*., 2009). However, selection of bombarded *Gladiolus's* embryogenic callus was carried out using 6 mg L^{-1} PPT (Kamo and Joung, 2009). In this work, transformants were selected 3 weeks after bombardment. The timing of selection has been known to influence the transformation efficiency and there were reports demonstrating that delayed exposure to selection agent resulted in a higher number of transformants obtained (Parveez and Christou, 1998; Ghosh *et al*., 2002; Li *et al*., 2003). However, lower transformation efficiency was observed when delayed selection was carried out in orchid (Men *et al*., 2003). Selection at the 3rd week after bombardment was preferred as it allows transformed cells to divide several times prior to selection.

The putative transformed plants (T_0) from pCAMBIA 1301 and pRQ6 survived in the presence 5 mg L^{-1} hygromycin but showed GUS negative. Similar results were reported for cowpea (Ikea *et al*., 2003). The amplification of DNA samples from GUS negative plants proved that the plants were transformed with the exogenous DNA integrated into the genome. It was possible that *gusA* gene fused with selectable marker

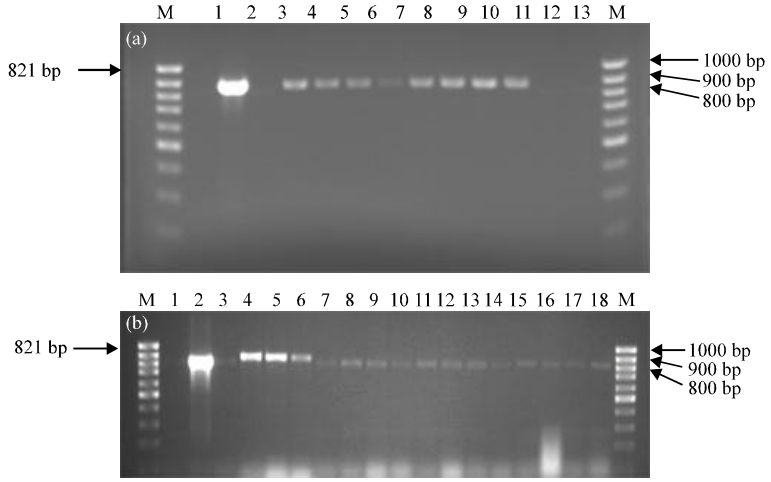

Fig. 6: (a) PCR analysis of T_0 plants. M: 1 kb DNA ladder, Lane 1: is negative control without DNA template; Lane 2: pRQ6 plasmid with the *hpt* gene; Lanes 3, 12 and 13 are untransformed watermelon plants; Lanes 4-11 are transformed plants amplifying 821 bp PCR products showing the presence of *hpt* gene and (b) PCR analysis of T_0 generation of plant cell. Lane M: 1 kb DNA ladder. Lane: 1 negative control without DNA template; Lane 2: pCAMBIA 1301 with *hpt* gene; Lane 3: untransformed plant cell; Lanes 4-18 are transformed plants amplifying showed 821 bp PCR products showing the presence of *hpt* gene

gene (*bar*). As a result, the *gusA* gene was not being expressed even though the selectable markers were expressed (Pereira anqd Erickson, 1995; Russel *et al*., 1993). Ikea *et al*. (2003) also reported that out of 231 T_1 cowpea seedlings raised from the T_0 plants subjected to GUS assay, only eight plants showed positive GUS reaction, even though possible high frequency of transgene inheritance among the progenies.

PCR analysis of putatively transformed tissues: After performing histochemical GUS analysis, PCR analysis was carried out to detect the presence of *hpt* or *bar* gene in the transformed tissues. Transmission of the transgene from shoots surviving on the selection media (hygromycin) was examined by PCR using primers specific for the *hpt* gene. Amplification of the predicted 821 bp fragment of *hpt* gene (Fig. 6a, b), confirmed the successful integration of the *hpt* gene into the explants. However, these regenerated shoots failed to develop further and eventually died 4 weeks after transplantation on MS medium containing 5 mg L^{-1} hygromycin (Fig. 7a, b).

On the other hand, the explants bombarded with single plasmid pAHG11 or co-transformation (pAHG11+ pCAMBIA 1301) was subjected to selection medium containing 2 mg L^{-1} PPT. The regenerated shoots were subjected to PCR using primers for the *hpt* and *bar* genes. PCR results indicated that the number of shoots showing positive PCR using *hpt* primer was higher (11) than *bar* primer (4) for shoots regenerated with co-transformation

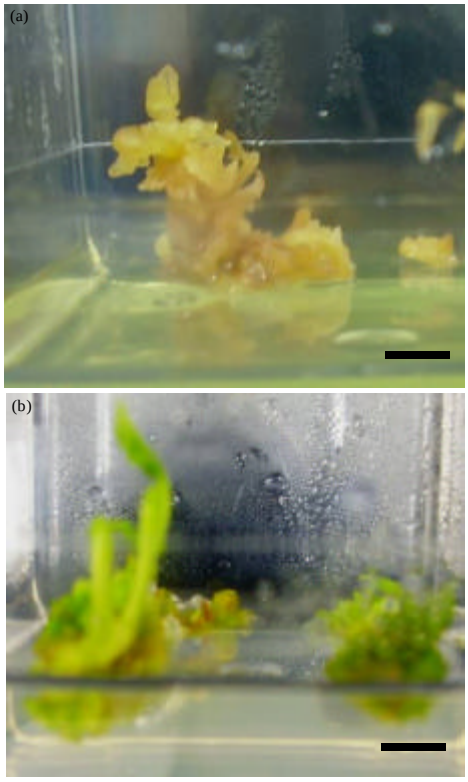

Fig. 7: Putative transformed plant at T_0 generation: (a) Untransformed plant on selection medium and (b) Transformed plant on 5 mg L^{-1} hygromycin after 2 months Bar = 5 mm

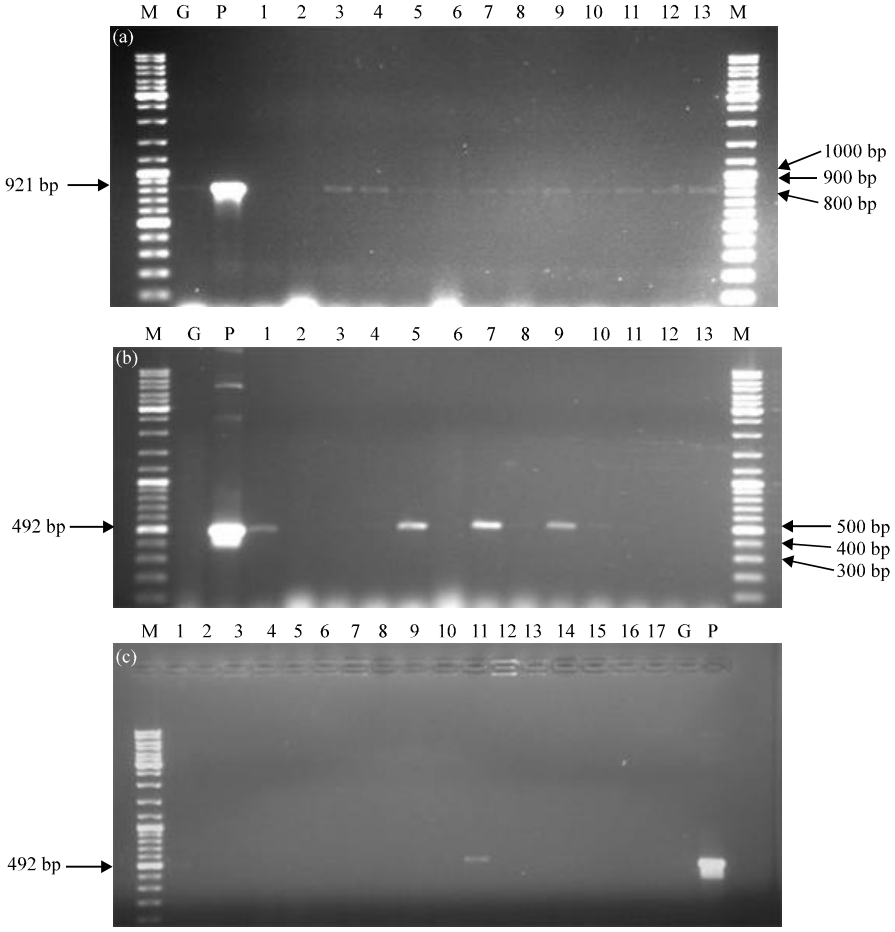

Fig. 8: (a) PCR analysis of T_0 plants. Lane M: DNA marker mix; Lanes G:untransformed plant cell; Lane P: pCAMBIA with *hpt* gene; Lanes 3 - 13 transformed plants showing amplification of 821 bp PCR products using *hpt* primer, (b) PCR analysis of T_0 plants. Lane G: untransformed plant cell; Lane P: pAHG11 plasmid with the *bar* gene; Lanes 1, 5, 7, 9 transformed plants showing amplification of 492 bp PCR products using *bar* primer and (c) PCR analysis of T_0 plants. Lane G: is untransformed plant cell; Lane P: pAHG11 showing *bar* gene DNA fragment, Lane 11: transformed plants showing amplification of 492 bp PCR products using *bar* primer

plasmid (Fig. 8a, b). Figure 8b and c show PCR amplification of 492 bp fragment of *bar* gene, confirming the presence of the *bar* gene in the transformed plants selected on 2 mg L^{-1} PPT.

Based on the GUS and PCR analysis, it is suggested that many chimearic plants have been produced. Generally, it is possible that chimeric plants arose or both genes (*gusA* and *hpt*) became silenced in certain parts of the putative transformed plants. Gene silencing was observed in transgenic soybean and it is common in particle bombardment (Reddy *et al*., 2003). In garden balsam (*Impatiens balsamina*), it was reported that 14 out of 84 plants tested were GUS positive and followed by positive PCR for the presence of the *hpt* gene in the plant genome (Taha *et al*., 2009). This low rate of positive transformants may be due to escapes or chimeric nature of the transformants.

CONCLUSION

In this study, the following biolistic parameters were successfully optimized: 1100 psi helium pressure, 6 cm target distance, 4-day-old explants, concentration of DNA at 1.2 µg/bombardment and osmotic treatment for 24 h on medium supplemented with 0.6 M mannitol prior to bombardment. The optimized condition was used to transform watermelon and followed by selection on either 5 mg L^{-1} hygromycin or 2 mg L^{-1} PPT depending on the plasmid used. Transformants were verified with GUS histochemical assay and followed by PCR analysis.

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