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Full Length Research Paper

Optimization and transformation of *Arundo donax* L. using particle bombardment

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An optimized particle bombardment protocol to introduce DNA into *Arundo donax* L. (giant reed) embryogenic callus cells was developed. The physical and biological parameters tested for optimal transient expression of ß-glucuronidase (GUS) and green fluorescent protein (GFP) genes were: helium pressure, distance from stopping screen to target tissue and vacuum pressure together with other factors such as gold microparticle size, DNA concentration and the number of bombardments. The highest transient GUS and GFP expression was obtained when cells were bombarded twice at 1100 psi, with 9 cm target distance, 24 mm Hg vacuum pressure, 1 mm gold particle size, 1.5 µg DNA per bombardment, three days pre-culture prior to bombardment and six days post bombardment culture. This is the first report of optimization of particle bombardment parameters for high-efficiency DNA delivery combined with minimum damage to target giant reed tissues.

Key words: *Arundo donax*, particle bombardment, ß-glucuronidase (GUS), green fluorescent protein (GFP), transient gene expression, genetic transformation.

INTRODUCTION

Contamination of soils by toxic heavy metal accumulation such as arsenic (As), cadmium (Cd), lead (Pb), nickle (Ni), phosphorous (P) and mercury (Hg) is of widespread occurrence as a result of human, agricultural and industrial activities (Rugh, 2001; Doty et al., 2007). Phytoremediation is an environmentally friendly, cost-effective technology that makes use of green plants to clean up contamination from soil, sediments and water (Cunningham et al., 1997). Presently, this technology is one of the most effective and affordable technological solutions used to extract or remove inactive metals and metal pollutants from contaminated soils (Shah and Nongkynrih, 2007). Plants with exceptional metal-accumulating capacity are known as hyperaccumulators. Many plant species such as *Brassica juncea* and *Thlaspi* spp. have been successfully used to absorb contaminants such as Pb, Cd, As, Ni and various radionuclides from soils (Salt et al., 1995; Kos et al., 2003; Dal Corso et al., 2005).

Arundo donax L. (giant reed), which is also known as wild cane, is a non-food perennial grass resembling bamboo. It is a valuable source of cellulose for rayon and paper pulp. This plant is considered as one of the most cost effective and environmental friendly energy crops (Barbucci et al., 1994; Angelini et al., 2004). Recently, this plant has been used in phytoremediation, in particular, for P, Cd and Ni uptake potential and biomass production for energy purposes in contaminated sites (Han and Hu, 2005; Papazoglou et al., 2005; Papazoglou et al., 2007). Traditional large-scale propagation of giant reed is from rhizomes and stem nodes, or by embryo rescue (does not produce viable seeds) that requires a

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Abbreviations: GUS, ß-Glucuronidase; **DNA,** deoxyribonucleic acid; **GFP,** green fluorescent protein; **MS,** Murashige and Skoog's medium; **2,4-D,** 2,4-dichlorophenoxy acetic acid; **EDTA,** ethylene diamine tetraacetic acid; **TE,** tris- EDTA buffer; **RNase,** ribonuclease.

significant amount of time and effort to successfully establish the plants (Lewandowski et al., 2003). Moreover, conventional methods of propagation provide limited opportunity for genetic manipulation. Conventional propagation also requires large areas for production of propagules to be used for the production of fuel or biomass, or for use in phytoremediation programs (Papazoglou et al., 2007). The opportunities for the use of genetic engineering and biotechnology techniques, for the genetic improvement of plants by inserting metal resistance genes, have tremendous potential (Czako et al., 2006; Doty et al., 2007).

However, there are only few reports on the production of transformed plants by use of biolistics (the gene gun) that can accumulate metal and are being used for removal and detoxification of hazardous compounds. Biolistic-mediated transformation is still preferred over *Agrobacterium*-mediated transformation as it does not require construction of a specialized vector and is host-independent. Considering that the nature of the biolistic delivery system has many potential variables, it is therefore necessary to optimize the system for the most efficient delivery of plasmid DNA into target tissues.

The biolistic method relies on direct transfer of genetic material into plant cells by bombardment of explants with DNA-coated gold or tungsten particles using high-pressure gas. The efficiency of biolistic transformation depends on physical parameters such as particle velocity, size and number, as well as the amount of loaded DNA (Sanford et al., 1993). The most significant biological parameters for high transformation efficiency include the explants type and osmotic pressure, as well as the duration of explant culture prior to bombardment (Klein and Jones, 1999). Reporter genes have played an important role in developing and optimizing transformation protocols for plant species. The aim of these optimization studies is to achieve a high frequency of transiently expressing cells.

However, it is also very important that the target tissue is not damaged significantly from the bombardment (Russell et al., 1992). The degree of tissue damage depends on the type of explant, the particle density and the acceleration pressure. The β–glucuronidase (GUS) and green fluorescent protein (GFP) reporter systems have been most useful for determining the number of transgene expressing cells per bombardment (Elliot et al., 1999; Rajasekaran et al., 2000; Men et al., 2003). GFP holds tremendous advantage not only in studies involving the use of fusion proteins, but also in the optimization of transformation protocols for various plant species (Tee et al., 2003). Its advantage over other reporter proteins is that the formation of a fluorescent chromophore is self-catalyzed and requires only excitation under ultraviolet or blue light to emit a bright green fluorescence, which can be monitored in living cells with a standard fluorescent microscope (Heim and Tsein, 1996; Hraska et al., 2006). In addition, giant reed cells are recalcitrant to tissue culture manipulation and have a low rate of proliferation. Hence, the use of GFP as a non-destructive reporter is more desirable than GUS expression for optimizing genetic transformation in this plant.

In this study, we assessed the biolistic device parameters (helium pressure, distance from stopping screen to target tissue and vacuum pressure) together with other factors such as gold microparticle size and the number of bombardments affecting the delivery and expression of GUS and GFP genes for transient expression in *A. donax.* We also tested the ability of the embryogenic callus to produce secondary embryos and to regenerate after the bombardment.

MATERIALS AND METHODS

Induction and proliferation of embryogenic callus

In this study, young inflorescence segments collected from mature field grown plants (4 - 6 cm long with cover sheath) were surface sterilized by a 1 min immersion in 70% (v/v) ethanol. This was followed by stirring segments for 15 min in a freshly-made solution of 10% (w/v) NaOCl (commercial bleach 4% active chlorine) containing few drops of detergent. Finally, the segments were rinsed with sterile distilled water four times. The explants were cut into 5 - 10 mm long sections and cultured on Murashige and Skoog's (1962) medium containing 3% (w/v) sucrose, 0.3% gel-rite and supplemented with 2.0 mg/L of 2,4-dichlorophenoxy acetic acid (2,4-D). The plates containing the explants were incubated in the dark in a growth chamber set at 25 ± 2°C. Callus proliferation was tested on MS medium with various concentrations of 2, 4 - D (0.5, 1.0, 2.0, 2.5 and 5.0 mg/L). Plates were observed weekly for callus proliferation and embryogenic callus induction. Four to six hours prior to bombardment, 20 - 30 pieces of embryogenic callus (1 - 3 mm) were placed at the center of each Petri dish (100 X 15 mm, Fisher brand).

Plasmid DNA Isolation

The transient expression assay vector pCambia 1304 (CAMBIA, Canberra, Australia) carries a selective marker gene, hygromycin phosphotransferase (*hpt*II), conferring hygromycin resistance and a fusion between the reporter genes coding for β-glucurdinase (GUS, *uid*A) and a green fluorescent protein (GFP, *mgfp5)*, both driven by a 35S promoter from cauliflower mosaic virus (CaMV). Plasmid DNA was transformed into *Escherichia coli* JM109 cells and purified by the cesium chloride gradient method as reported by Sambrook et al. (1989). Supercoiled plasmid DNA was directly employed in transformation experiments or linearized by digestion with *Eco*RI (GIBCO BRL). The DNA was precipitated with two volumes of ethanol at room temperature by vortexing and then air dried at room temperature for 5 – 15 min. The DNA pellet was resuspended in 50 µl TE buffer and then treated with 20 mg/ml RNase. Plasmid DNA was quantified at O.D. A260/A280 using a spectrophotometer.

Preparation of DNA and particle bombardment

The plasmid DNA was precipitated onto gold particles according to the manufacturer's protocol for the Biolistic PDS-1000/He device (Bio-Rad). A 6 µL sample of the aliquot was loaded onto the center of the macrocarrier, air-dried and used for bombardment unless otherwise mentioned. The biolistic device parameters analyzed

were as follows: rupture disc pressure (helium pressure of 450, 650, 900, 1100, 1350 and 1550 psi); distance from stopping plate to target tissue (3, 6, 9 and 12 cm); vacuum pressure (20, 22, 24, 26 and 28 mmHg), number of bombardments (1X, 2X and 3X) per target tissue plate and gold microcarrier size (0.6, 1.0 and 1.6 µm). Distance from rupture to macrocarrier and macrocarrier to stopping screen was fixed at 4 and 11 mm, respectively. The biological parameters included the explant type (embryogenic callus, leaf and root segments and suspension culture cells), pre-culture treatment of tissues prior to bombardment (0, 1, 2, 3, 4 and 7 days), effect of post-bombardment incubation time (3, 6, 9, 12 and 15 days) and DNA concentration (0.5, 1.0, 1.5, 2.0 and 2.5 µg). For each parameter, four replicates were tested containing embryogenic callus cells on a 9 cm filter paper (unless mentioned otherwise).

Histochemical GUS staining and GFP fluorescence microscopy

Bombarded and non-bombarded tissues were placed in individual 1.5 ml Eppendorf tubes and stained overnight at 28°C with 0.5 ml filter-sterilized GUS assay buffer (0.1 M $NaPO_4$ buffer, pH 7.0, 0.5 mM K-Ferricyanide, 0.01 M EDTA, 1 mg/ml X-gluc and 0.3% Triton X-100). The count of transformed cells as blue spots was carried out using 40 x magnifications on day 2 post-bombardment. Non-bombarded tissues of the same age were used as a control.

GFP-expressing cells were detected using a Nikon florescence microscope aided with a GFP 2 filter set to mask chlorophyll fluorescence thereby, permitting visualization of distinctive green fluorescent GFP-expressing cells. The number of GFP positive cells (green fluorescent spots) was recorded.

RESULTS AND DISCUSSION

In the present study, a transformation system for *Arundo donax* was developed by optimizing the physical and biological parameters affecting GFP and GUS gene integration into the genome. It has been reported that for any plant tissue that is used for particle bombardment for the first time, optimal parameters for transient or stable gene expression must be established. Because transient expression of introduced genes in the target tissue can be studied on the first day after bombardment and the conversion rate of transient expression over stable transformation is only 0.1 - 3% (Sanford et al., 1993), it is very useful for optimizing variables affecting the efficiency of DNA transfer through microprojectile bombardment. We investigated several of the factors that are likely to have the highest impact on the efficiency of transient expression, which once optimized will be subsequently used for stable transformation.

Callus induction and proliferation

Embryogenic calli were induced on Murashige and Skoog's (MS) medium supplemented with 2.0 mg/L 2, 4-D from immature inflorescence of mature field grown plants. Callus was observed within a week of incubation. After four weeks of incubation at 26°C in the dark, embryogenic callus proliferation was observed. Two types of embryogenic calli were observed - hard, nodular (embryogenic) and friable (non-embryogenic). After subculturing on MS medium with various concentrations of 2, 4-D (0.5 -5.0 mg/L), developmental stages of embryos were observed (Figures 1A - B).

The effect of helium pressure on transient gene expression

It was observed that 1100 psi helium pressure gave the highest (107 ± 2.9) transient GUS gene expression (blue isolated events) compared to 900 psi (67 ± 3.8) and 1350 psi (77 ± 2.4) in embryogenic cells. GUS expression in embryogenic callus was significantly lower ($p < 0.05$) at different pressures. GUS events were never detected from non-bombarded samples, utilized as controls (Figure 2). As another potential marker to screen for transformation in giant reed, the GFP gene was tested after bombardment into cells. Green fluorescent positive spots were observed and the intensity of fluorescence ranged from barely visible to bright green (Figures 1C -D). This agrees with previous observation on barley (Chernobrovkina et al., 2007). No background fluorescence was detected in non-transformed cells. However, transient GFP gene expression was higher than GUS gene expression in embryogenic callus after bombardment at different pressures. Experimental results showed that low acceleration pressures (450 psi) resulted in a smaller area being covered by particles than higher acceleration pressures (1350 psi). The highest GFP transient expression in embryogenic callus was observed using 1100 psi (137 ± 4.4) followed by 1350 psi (117 ± 2.4) (Figure 2). Hence, 1100 psi was chosen for subsequent experiments as it gave the highest GUS and GFP expressions in embryogenic cells.

Microscopic analysis of bombarded tissue showed that at low helium pressure GUS- and GFP-positive spots were evenly distributed at a relatively low density. At high pressures, a small area of the target tissue was strongly targeted and thus likely to be damaged, especially at the highest acceleration pressure (1550 psi).

The effect of distance of stopping screen to target tissue

Optimization of the distance between the stopping screens to target tissue is necessary to allow even spread of the DNA microcarrier onto the target tissue without causing damage to the tissues (Chernobrovkina et al., 2007). A short distance (3 cm) caused considerable tissue damage and decreased the number of fluorescent spots on the explant surface. As the distance increased to 9 cm, the number of GUS- (149 ± 2.1) and GFP-positive (182 ± 3.5) spots were significantly higher than at other distances (Figure 3). In most cases single transformed cells were seen after the histochemical

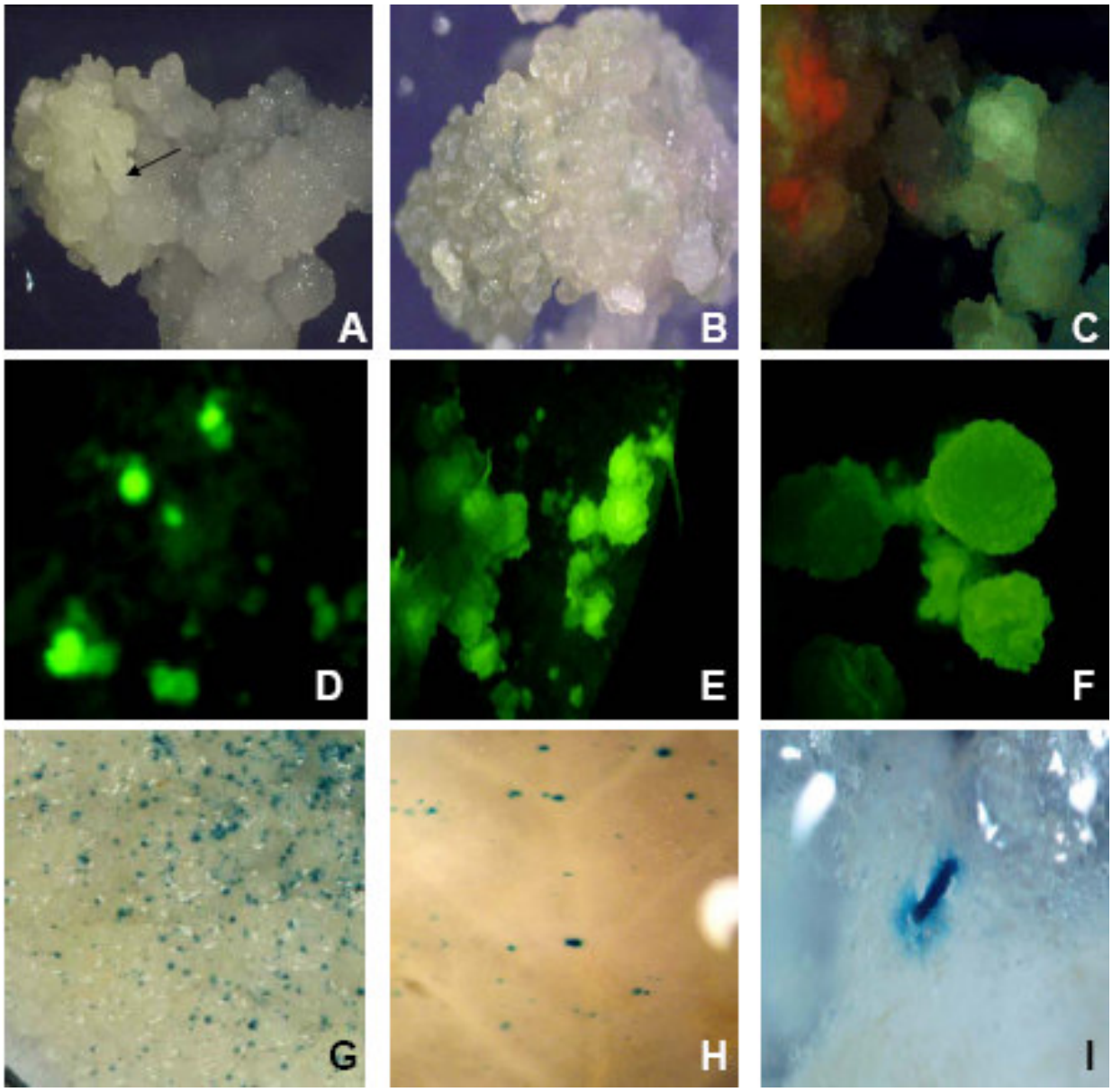

Figure 1. Callus induction, somatic embryogenesis and transient gene expression in *A. donax*. A) Callus induction from immature inflorescence; B) Embryogenic callus utilized for bombardment; C, D, E, F) Transient GFP expression in embryogenic calli 48 h after bombardment; G, H) Transient GUS expression in embryonic calli and leaf segment after 48 h; I) GUS expression 6 days after bombardment.

reaction. These cells varied from intensely blue to slightly blue (Figures 1G and 1H) suggesting a varying level of GUS gene expression.

At 12 cm, the number of GUS- and GFP-positive spots were significantly reduced, 78 ± 3.5 and 113 ± 3.5, respectively due to decreased velocity of the micro-particles with the increasing distance resulting in reduced penetration force and therefore fewer cells receiving DNA. Overall, the results in this experiment suggested that a microcarrier flight distance of 9 cm was optimal to use at 1100 psi in embryogenic callus of *A. donax* (Figure 3). Similar observations were also reported by Parveez et al. (1997).

The effect of vacuum on transient gene expression

Vacuum in the chamber determines acceleration of the microcarrier from stopping screen to the target tissue (Parveez et al., 1997). At low vacuum, the particles cannot reach the target tissue. It was observed that transient GFP expression was higher than GUS expression when the embryogenic callus pieces were bombarded at 24, 26 and 28 mm Hg vacuum (Figure 4). Vacuum

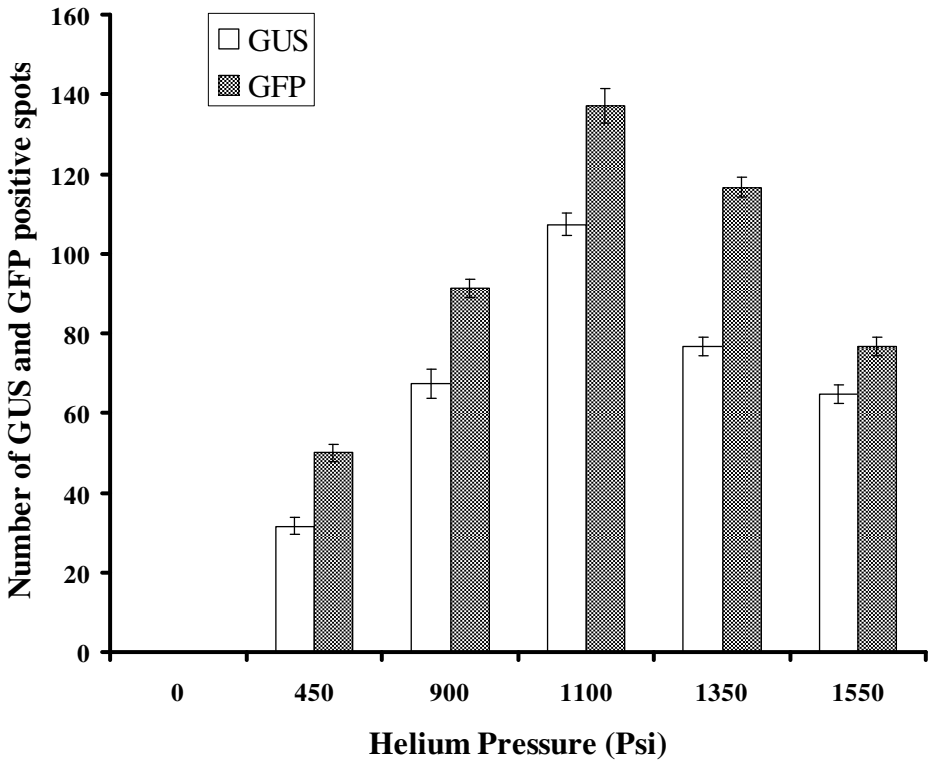

Figure 2. Effect of helium pressure on transient expression of GFP and GUS genes in *A. donax* using a biolistic device.

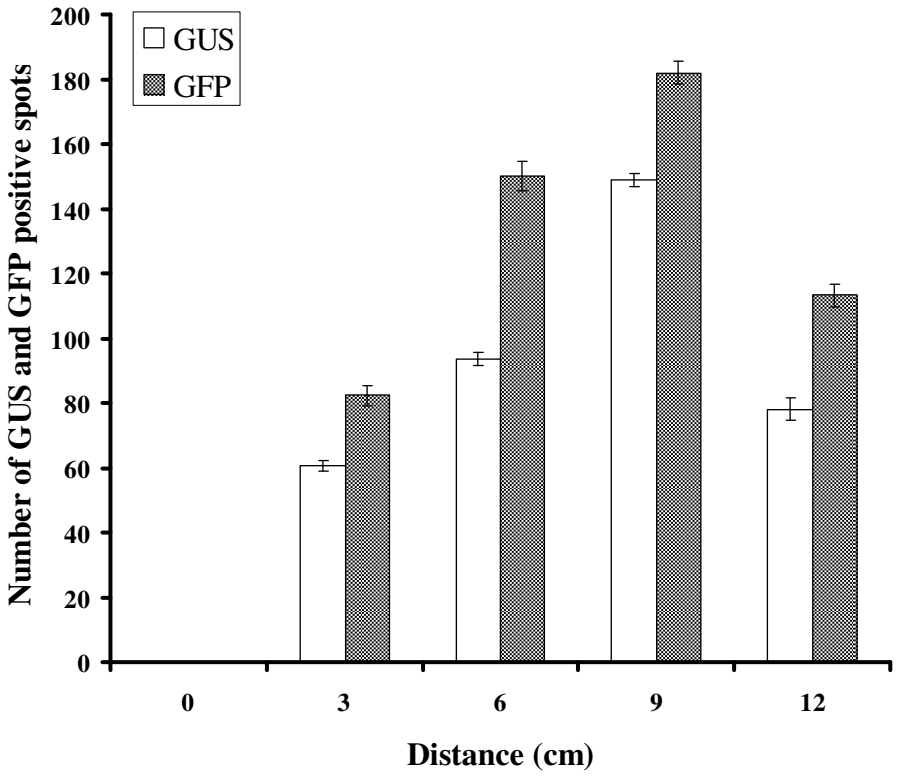

Figure 3. Effect of distance from stopping plate to target tissue on transient gene expression of GFP and GUS genes in *A. donax* using a biolistic device.

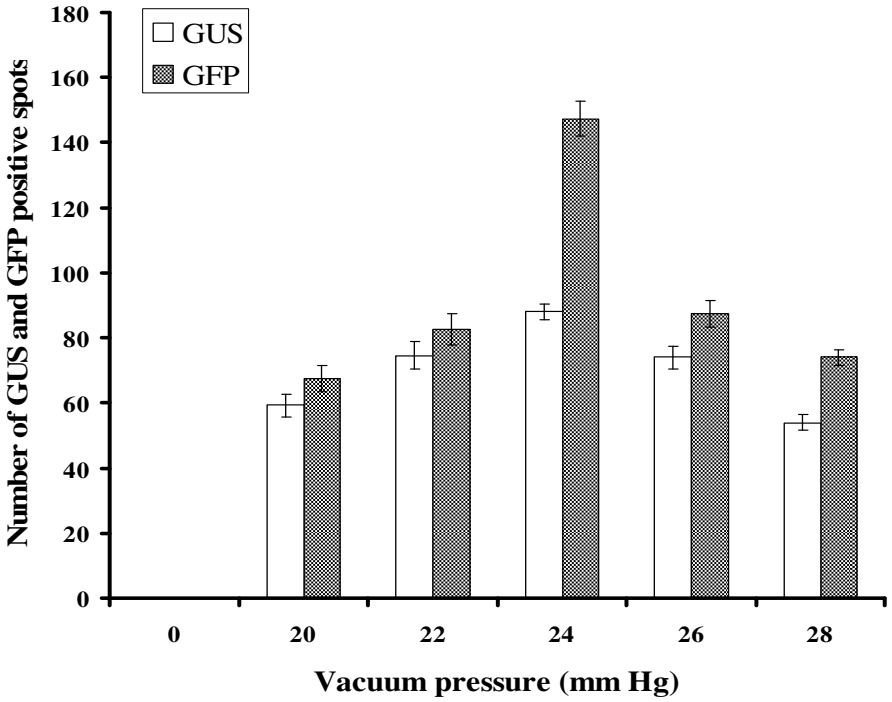

Figure 4. Effect of vacuum pressure on transient gene expression of GFP and GUS genes in *A. donax* using biolistic device-gene gun

pressure of 24 mm Hg was found to be the optimal vacuum condition for bombardment with 1100 psi for high levels of GUS and GFP expression.

The effect of multiple bombardments on transient gene expression

Multiple bombardments are normally carried out with the objective of getting better coverage of targeted areas. It is useful when primary delivery is not efficient. The down side is increased in the amount of existing damage to the target tissue. In this experiment, two consecutive bombardments showed an increase in transient gene expression in embryogenic callus with an average of 110 ± 3.2 GUS-positive and 152 ± 2.5 GFP-positive spots, compared with one or three bombardments (Figure 5). In agreement with our results, double bombardment has been shown to increase transient GUS gene expression proportionally in rice, wheat, cotton and sugarcane (Bower and Birch, 1992). It is recommended to utilize multiple bombardments only after accessing the extent of tissue damage. The extensive tissue damage could result in reduced transient gene expression.

The effect of gold microcarrier size on transient gene expression

A gold microcarrier of 1.0 µm was found to result in significantly higher transient GUS and GFP gene expression than use of 0.6 µm or 1.6 µm micro-carriers (Figure 6). The use of smaller particles would be expected to minimize tissue damage and browning around the surface of the tissue. This result is consistent with the observations obtained in oil palm by Parveez et al. (1997) and by Janna et al. (2006) in *Dendrobium*. Microparticle size of 1.0 µm was used in all subsequent bombardment experiments. Using a gold microcarrier is better than a tungsten microcarrier because it is biologically inert, non-toxic and uniform in size.

Tungsten is highly heterogeneous in size and shape and potentially toxic to some cell types as it acidifies and degrades DNA. Theoretically, small particles provide for a more homogeneous DNA distribution over the explants surface and should have less negative physical affects on explants, which should improve the transient expression level and the frequency of transgenic events.

The effect of pre-culture treatment prior bombardment

Bombarding explants at the right physiological and development stage is important because actively dividing cells are most receptive to the particle bombardment method of transformation. During pre-culture, isolation of the explants and exposure to new medium with nutrient and growth regulators may stimulate cells to actively

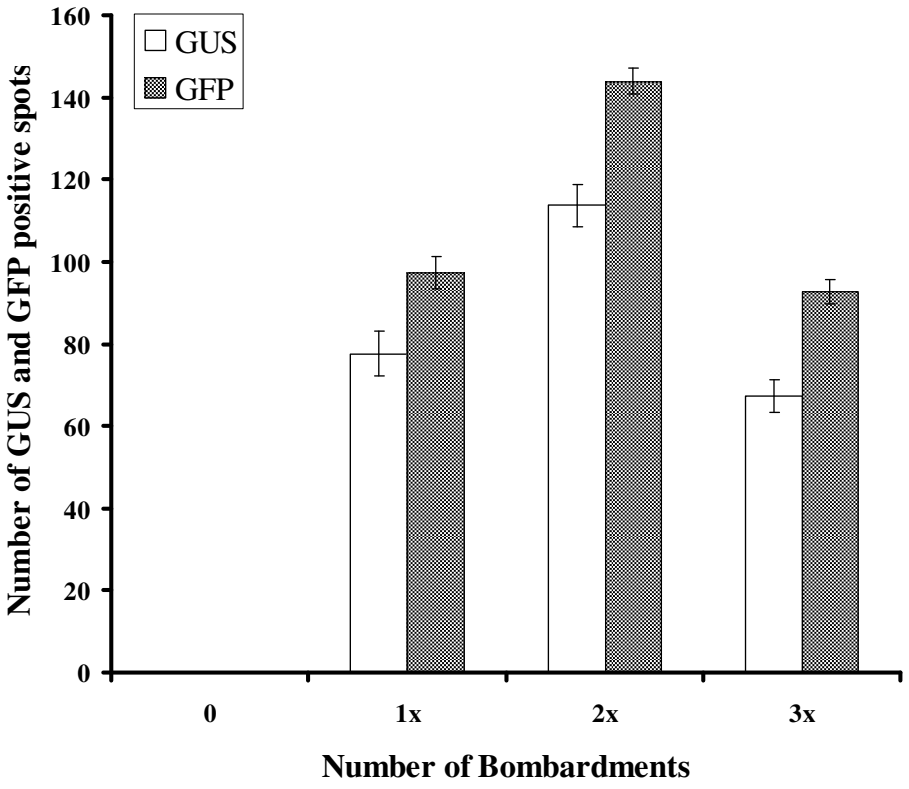

Figure 5. Effect of number of bombardments on transient gene expression of GFP and GUS genes in *A. donax* using biolistic device-gene gun

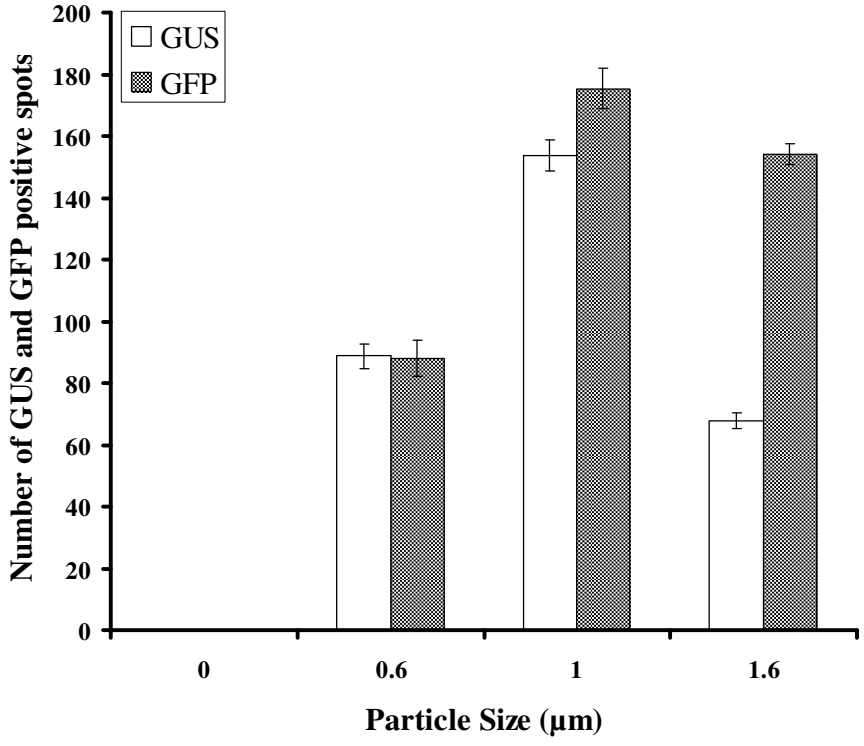

Figure 6. Effect of particle size on transient gene expression of GFP and GUS genes in *A. donax* using biolistic device-gene gun.

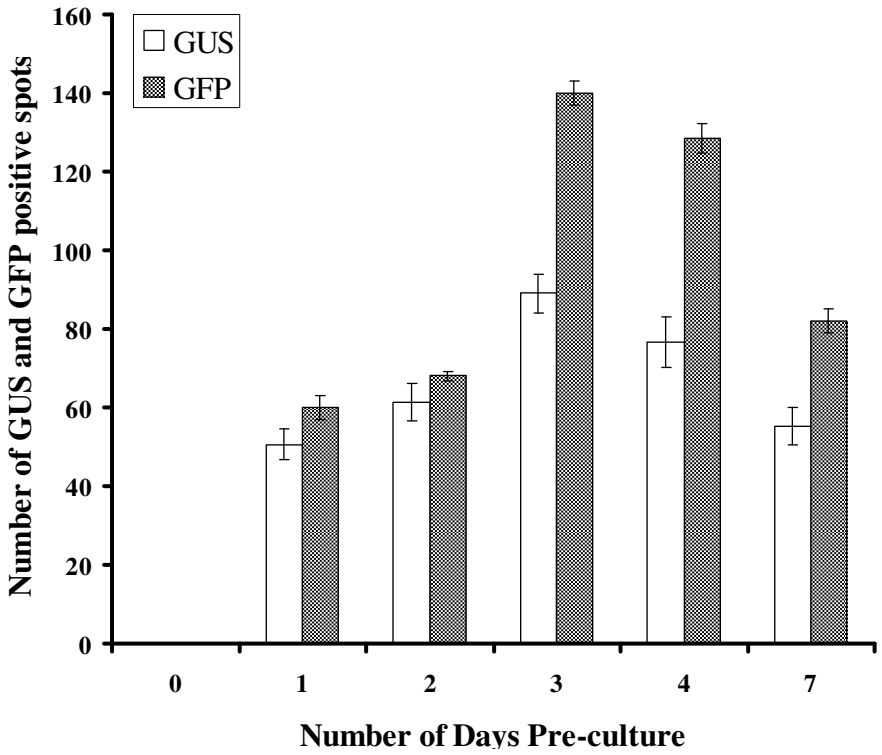

Figure 7. Effect of pre-culture treatment on transient gene expression of GFP and GUS genes in *A. donax* using biolistic device-gene gun.

divide (Janna et al., 2006; Taha et al., 2009). In our experiments, transient GUS and GFP gene expression was monitored from 1 to 4 days after bombardment. The highest numbers of GUS- and GFP-positive spots were observed in embyogenic callus pre-cultured for 3 days before bombardment followed by two, one and four days of pre-culture (Figure 7).

The effect of DNA concentration on transient gene expression

The precipitation of DNA onto gold particles usually determines the potential amount of DNA delivered into tissues. We evaluated the effect of changing the concentration (0.5 - 2.5 µg) of plasmid DNA on gold particles. The results showed that 1.5 µg DNA per bombardment gave the highest transient GUS and GFP gene expression (Figure 8). The higher amounts of DNA resulted in aggregation of DNA coated gold particles as observed by light microscope. This aggregation may reduce transformation efficiency due to higher cell damage. Lower concentrations of DNA were found to give lower transient GUS activity but the differences were not significant ($p < 0.05$). Similar observations were also reported by Rochange et al. (1995) and Parveez et al. (1998) in case of *Eucalyptus globules* zygotic embryos and oil palm immature embryos, respectively.

The effect of post-bombardment incubation time

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 and GFP transient expressions for 3 day intervals up to 18 days. Three and six days after bombarding gave significantly higher transient expressions as compared to other treatment used. The results imply that it takes at least three to six days for the cells or tissues to recover from the injuries caused by bombardment (Figure 1l). On the ninth day, the GFP and GUS transient expression was reduced by about 50% compared to day 6.

To our knowledge, this is the first report of a simple and reliable procedure for microprojectile-mediated transfer of GUS and GFP reporter DNA into embryogenic tissue of *A. donax*. Optimized bombardment conditions were: bombarding twice at 1100 psi, 9 cm target distance, 28 mm Hg vacuum, 1 µM gold particle size, 1.5 µg DNA per bombardment, three days pre-culture prior to bombardment and six days post-bombardment. It was shown that the GFP reporter had higher transient expression in embryogenic callus compared to GUS, with all parameters investigated. Hence, it is suitable to use GFP as the reporter system for transformation of *A. donax*.

A reproducible regeneration system is essential for the genetic transformation of *A. donax*. We have tried to

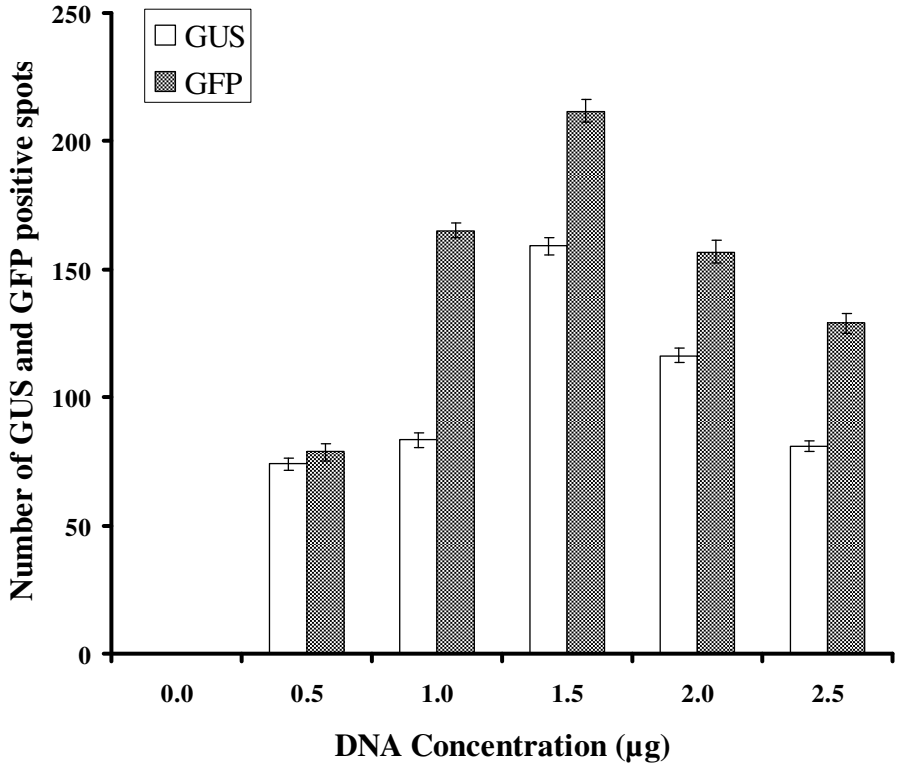

Figure 8. Effect of DNA concentration on transient gene expression of GFP and GUS genes in *A. donax* using biolistic device-gene gun.

establish such a system but we have encountered many difficulties. To date, as far as we know, no system exists for high frequency plant regeneration from embryogenic callus. Microprojectile bombardment-mediated transformation of this plant will be possible when a reliable and reproducible regeneration system become available. In the future, the results from this investigation will serve as simple and reliable technique for DNA transfer for genetic manipulation of *A. donax* either by inserting genes for metal resistance and or for enhanced cellulosic content as a potential energy crop.

Furthermore, this method should allow the development of assays for the transient and homogeneous expression of promoters of various genes in giant reed.

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