Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas*

J. PURKAYASTHA[1], T. SUGLA[1], A. PAUL[1], S.K. SOLLETI[2], P. MAZUMDAR[1], A. BASU[1], A. MOHOMMAD[3], Z. AHMED[3] and L. SAHOO[1,2]*

Center for Energy[1] and Department of Biotechnology[2], Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India
Defence Agricultural Research Laboratory, Haldwani, Uttaranchal Pradesh, India[3]

Abstract

An efficient and reproducible *in vitro* plant regeneration system from shoot apices was developed in *Jatropha curcas*. Benzylaminopurine (BAP; 2.5 μM) was most effective in inducing an average of 6.2 shoots per shoot apex. Incorporation of gibberellic acid (GA_3; 0.5 μM) to basal medium was found essential for elongation of shoots. The BAP-habituated mother explants continuously produced shoots during successive subculture without any loss of morphogenic potential. The shoots rooted efficiently on half-strength MS medium. The rooted plantlets were acclimatized with more than 98 % success and the plants transferred to soil:compost in nursery showed no sign of variation compared to the seed-grown plants. The whole process of culture initiation to plant establishment was accomplished within 5 - 6 weeks. A genetic transformation system in *J. curcas* was established for the first time, using bombardment of particles coated with plasmid pBI426 with a GUS-NPT II fusion protein under the control of a double 35S cauliflower mosaic virus (CaMV) promoter. The β-glucuronidase (GUS) activity in *J. curcas* shoot apices was significantly affected by the gold particle size, bombardment pressure, target distance, macrocarrier travel distance, number of bombardments, and type and duration of osmotic pre-treatment. The proliferating bombarded shoot apices were screened on medium supplemented with 25 mg dm^{-3} kanamycin and surviving shoots were rooted on medium devoid of kanamycin. The integration of the transgene into genomic DNA of transgenic plants was confirmed by PCR and Southern blot hybridization. The transgenic plants showed insertion of single to multiple copies of the transgene.

Additional key words: benzylaminopurine; β-glucuronidase, gibberellic acid, micropropagation, PCR, Southern blot.

Introduction

Jatropha curcas L. is a large shrub or small tree belonging to the *Euphorbiaceae* family. The plant is found in the tropics and subtropics where it is used to reclaim land, fence household land, and produce feedstuff, soap, pesticide, and drugs (*e.g.* Kaushik *et al.* 2007). The seed oil of *J. curcas* can be easily processed to partially or fully replace petroleum based diesel fuel (*e.g.* Tiwari *et al.* 2007). The plant is propagated by direct seeding, planting stem cuttings and stumps. However, conventional propagation is limited by problems associated with poor seed viability, low germination, scanty and delayed rooting of seedlings and vegetative cuttings (Heller 1996). Plants propagated by cuttings show a lower longevity due to pseudo-taproots and possess a lower drought and disease resistance than those propagated by seeds (Heller 1996). The vulnerability of seeds to fungal attack during storage, submergence intolerance of plants in marshy waste lands and a multitude of insect pests restrict its potential yield. Therefore, development of an efficient and reproducible micropropagation system with rapid regeneration of plants may boost mass propagation of this biofuel plant.

Preliminary *in vitro* plant regeneration studies in *Jatropha* are confined to the species, *J. panduraefolia* (Srivastava 1971, Johri and Srivastava 1973, Srivastava and Johri 1974) and *J. integerrima* (Sujatha and Dhingra

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Abbreviations: BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; GUS - β-glucuronidase; NAA - α- naphthaleneacetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog's (1962) medium.
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* Corresponding author; fax: (+91) 361 2582249, e-mail: ls@iitg.ernet.in

1993). Adventitious shoot regeneration through callus derived from vegetative explants reported in *J. curcas* (Sujatha and Mukta 1996) involved a lengthy culture period raising the possibility of incorporating undesirable somaclonal variations. The petiole and hypocotyls explant of seedlings of *J. curcas* have been employed for inducing morphogenesis *in vitro*, however, the plant regeneration suffers from non-reproducibility and low multiplication rates (Sujatha *et al.* 2005). A method for induction of somatic embryogenesis is reported in *J. curcas* (Jha *et al.* 2007) which requires 16 weeks for plant regeneration. However, efforts have not been made to evaluate the regeneration potential of meristematic explants of *J. curcas*. Plant regeneration from meristem explants derived from zygotic embryo has been widely employed for rapid micropropagation, and successfully adapted to gene transfer by both *Agrobacterium*-mediated and direct DNA delivery methods (Sticklen and Oraby 2005). The capacity of shoot apex to induce homogenous and reproducible multiple shoot induction in the apical meristematic region has facilitated generation of transgenic plants by particle bombardment of apical meristematic region (Aragao and Rech 1997). Transgenic plants regenerated through more or less long-term callus phase have an increased risk of somaclonal variation, problems in transgene inheritance and stability of expression (Choi *et al.* 2000, Bregitzer and Tonks 2003).

We report a highly efficient, reproducible and rapid plant regeneration system from shoot apices and demonstrate, for the first time, the feasibility of direct DNA delivery to the shoot apices in *J. curcas* by particle bombardment, and generation of transgenic plants.

Materials and methods

Explant source: Immature seeds of *J. curcas* were collected from North Guwahati, Assam, India. The seeds were surface sterilized with 70 % ethanol for 2 min after removing the outer seed coat, immersed in 0.2 % $HgCl_2$ for 5 min and subsequently rinsed with sterile water for five times. The embryo was carefully exposed by dissecting out the endosperm, and cultured on Murashige and Skoog (1962; MS) medium for 4 d. Subsequently, the shoot apices (3 - 5 mm) were excised (Fig. 1*A*) by removing the cotyledonary leaves.

Plant regeneration from shoot apices: The shoot apices were cultured on MS medium supplemented with various cytokinins [benzylaminopurine (BAP), kinetin, thidiazuron (TDZ) and 2-isopentenyl adenine (2-iP)] individually at different concentrations (1.0, 2.5, 5.0 and 7.5 μM) for multiple shoot induction. The synergistic effect of kinetin with BAP on shoot proliferation was examined by supplementing kinetin (0.5, 1.0, 2.5 and 5.0 μM) to MS medium containing 2.5 μM BAP. The carryover effect of the cytokinins incorporated in the primary medium was assessed by transferring the shoot clusters after 14 d of culture on MS medium supplemented with 0.5 μM BAP. Culture of shoots on the medium supplemented with BAP, or BAP in combination with kinetin occasionally resulted in stunted growth with condensed nodes. Therefore, the effect of gibberellic acid (GA_3) at concentrations of 0.1 - 5.0 μM on elongation of shoots, from primary shoot clusters, was evaluated after 10 d of culture period. The mother explants were repeatedly subcultured on shoot multiplication medium after harvesting elongated shoots.

The culture media were supplemented with 3 % sucrose and 0.7 % agar in all the experiments. The pH of the media was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. Cultures were maintained at 25 ± 2 °C under a 16-h photoperiod (cool white fluorescent tubes, irradiance of 35 μmol m^{-2} s^{-1}).

The elongated shoots (≥ 2 cm) were transferred to half strength MS medium supplemented with various concentrations of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Rooting was scored after 8 - 10 d of culture. Well-rooted plantlets were washed thoroughly in running tap water before being transplanted in plastic pots containing sterilized soil and *Vermiculite* (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture and transferred to the greenhouse for acclimatization. Plantlets were then transferred to soil:compost (1:1) in shade house for two weeks before being transferred to field.

Experiments were set up in a completely randomized design and each treatment had three replicates of 20 explants each. All data are statistically analyzed by *ANOVA* followed by Newman-Keul's multiple range test for mean comparison.

Plasmid constructs: The plasmid pBI426 with a GUS-NPT II fusion gene under the control of a double 35S cauliflower mosaic virus (CaMV) promoter (Datla *et al*. 1991) was used in all experiments. Plasmid DNA was isolated from bacterial cultures using plasmid *Miniprep* kit (*Qiagen*, Hilden, Germany).

Microprojectile bombardments: Prior to bombardment, shoot apices were placed in a circle with a diameter of 18 mm on solid MS medium supplemented with 0.2 M mannitol for osmotic pretreatment. The plasmid coated microparticles were delivered to target explants using a biolistic *PDS-1000/Helium* (*Bio-Rad*, Hercules, CA, USA) as per the manufacturer's instruction. The critical bombardment parameters, *i.e.*, microparticle size (0.6, 1.0 and 1.6 μm in diameter), bombardment pressure (900, 1100 or 1500 psi), target distance (6, 9 or 12 cm between stopping screen and target plate), and osmotic pretreatments (0.2 M sorbitol, or 0.2 M mannitol, or 0.2 M sorbitol + 0.2 M mannitol) and their duration (2, 4

or 6 h) were optimized based on transient *gus* expression. For all experiments, the gap distance (between rupture disc and macrocarrier) was adjusted to 0.9 cm.

GUS histochemical assay: The transient GUS activity was examined 2 d after bombardment using histochemical staining (Jefferson *et al.* 1987). Stable GUS expression was monitored in regenerated shoots. Samples were incubated in GUS substrate solution consisting of 0.5 M $NaPO_4$ (pH 7.0), 50 mM potassium ferricyanide, 50 mM potassium ferrocyanide, 10 mM EDTA (pH 7.0), 0.1 % Triton X-100 with 1 g dm^{-3} of 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-gluc) (*Biosynth AG*, Staad, Switzerland) for 24 h in dark at 37 °C. Following incubation, tissues were bleached with 100 % ethanol to examine and count blue spots. An explant with at least one discrete blue region on the tissue was scored as GUS positive.

Selection of transformants: Two days after bombardment, explants were cultured onto shoot multiplication medium supplemented with kanamycin (25 mg dm^{-3}). Proliferating cultures were transferred to fresh selection medium for 45 d. Later, the kanamycin-resistant elongated shoots were transferred to rooting medium without selective agent.

PCR analysis and Southern blot hybridization: Genomic DNA from young leaves of putative transformants and control plants were extracted using the CTAB method (Rogers and Benedich 1988). Polymerase chain reaction (PCR) was performed to verify the presence of the *gus* gene by amplification of 0.25 kb internal fragment of *gus* gene. The amplification reaction was performed as follows: 38 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C. Southern hybridization analysis was carried out on six PCR positive T_0 plants and control untransformed plants to confirm the stable integration of *npt*II gene in genome of *J. curcas*. Genomic DNA (10 µg) digested with *Bam*HI were separated in a 0.8 % of agarose gel and blotted onto *Zeta-Probe* membrane (*Bio-Rad*). Southern hybridization and detection were performed using the non-radioactive *DIG* labeling and detection system (*Roche*, Manheim, Germany) by probing with *DIG*-labelled coding regions of *npt*II (540 bp) following supplier's instructions.

Results and discussion

Shoot multiplication and plant regeneration: Access to a prolific system of shoot proliferation from explants with pre-existing meristem is a prerequisite for rapid micropropagation and meristem-based transformation. Shoot apices derived from embryos from immature seeds were found to be ideal owing to their extensive proliferative ability. The responding shoot apices showed visible signs of swelling within 4 - 5 d and showed the development of both shoot buds and callus proliferation at the base of explants after 7 d of culture on MS medium supplemented with different cytokinins. Proliferation occurred either by elongation of the main shoot with swollen axillaries at nodes, or suppression of the main shoot with four to six small buds at the basal callus. Shoot apices produced multiple shoots with callusing and/or suppressed the radicular end depending on the type and concentration of the cytokinin in medium. The type of cytokinin used had a profound effect on frequency of shoot multiplication and number of shoots induced from shoot apices (Table 1). The frequency of shoot multiplication and average number of shoots were significantly higher on BAP-supplemented media followed by kinetin and 2-iP, and lowest on media supplemented with TDZ (Table 1). BAP at 2.5 µM induced maximum number of shoots per explant (6.2) in 96 % of the cultured shoot apices within 14 d of culture (Table 1, Fig. 1*B*,*C*). The frequency of regeneration and number of shoots were lowest on the medium supplemented with 7.5 µM TDZ (Table 1). Interestingly, the shoot apices cultured on TDZ-supplemented media showed a drastic reduction in emergence of shoots with regeneration response restricted to stunted bud formation. The BAP has been found to be effective over other cytokinins on multiple shoot induction in various members of *Euphorbiaceae* (Nair *et al.* 1979, Tideman and Hawker 1982, Ripley and Preece 1986). In most of the *Euphorbiaceae* members, the

Table 1. Effect of different cytokinins (CK) on shoot proliferation from shoot apices of *Jatropha curcas* on MS medium after 14 d. Each value represents the mean of three experiments with 20 replicates in each. Means within a column followed by same letters are not significantly different at $P < 0.05$ by Newman-Keul's multiple range test.

CK	Conc. [µM]	Shoot development [%]	Shoot number [explant^{-1}]	Shoot length [cm]
BAP	1.0	96	2.4^{d}	1.6^{a}
	2.5	96	6.2^{a}	1.1^{b}
	5.0	81	4.1^{b}	0.6^{c}
	7.5	72	3.5^{c}	0.4^{d}
Kinetin	1.0	86	1.7^{e}	1.9^{a}
	2.5	82	3.2^{c}	1.4^{ab}
	5.0	75	2.6^{d}	1.1^{b}
	7.5	68	1.3^{e}	0.9^{b}
TDZ	1.0	63	1.2^{e}	1.0^{b}
	2.5	57	1.6^{e}	0.8^{bc}
	5.0	45	1.1^{e}	0.6^{c}
	7.5	28	0.8^{ef}	0.6^{c}
2-iP	1.0	72	1.3^{e}	1.3^{b}
	2.5	68	1.5^{e}	1.1^{b}
	5.0	59	2.7^{d}	0.9^{b}
	7.5	52	1.4^{e}	0.9^{b}

presence of cytokinin alone induced optimal shoot proliferation such as BAP in *Euphorbia lathyrus* (Tideman and Hawker 1982, Ripley and Preece 1986), *E. peplus*, *E. tannensis* (Tideman and Hawker 1982), *Manihot esculenta* (Nair *et al.* 1979), 2-iP in *E. lathyrus* (Lee *et al.* 1982), and TDZ in *R. communis* (Sujatha and Reddy 1998). Although maximal shoot induction was observed at 2.5 μM BAP, the shoots were found compact with shortened internodes and small leaves, which remained stunted till the third subculture, thereby restricting the number of shoots that could be rooted. Reduction of the BAP concentration in subculture medium was not favourable for shoot elongation, furthermore, it lead to the loss of shoot multiplication capacity. The BAP have often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Brassard *et al.* 1996, Figueiredo *et al.* 2001). Shoots regenerated on kinetin-supplemented medium showed

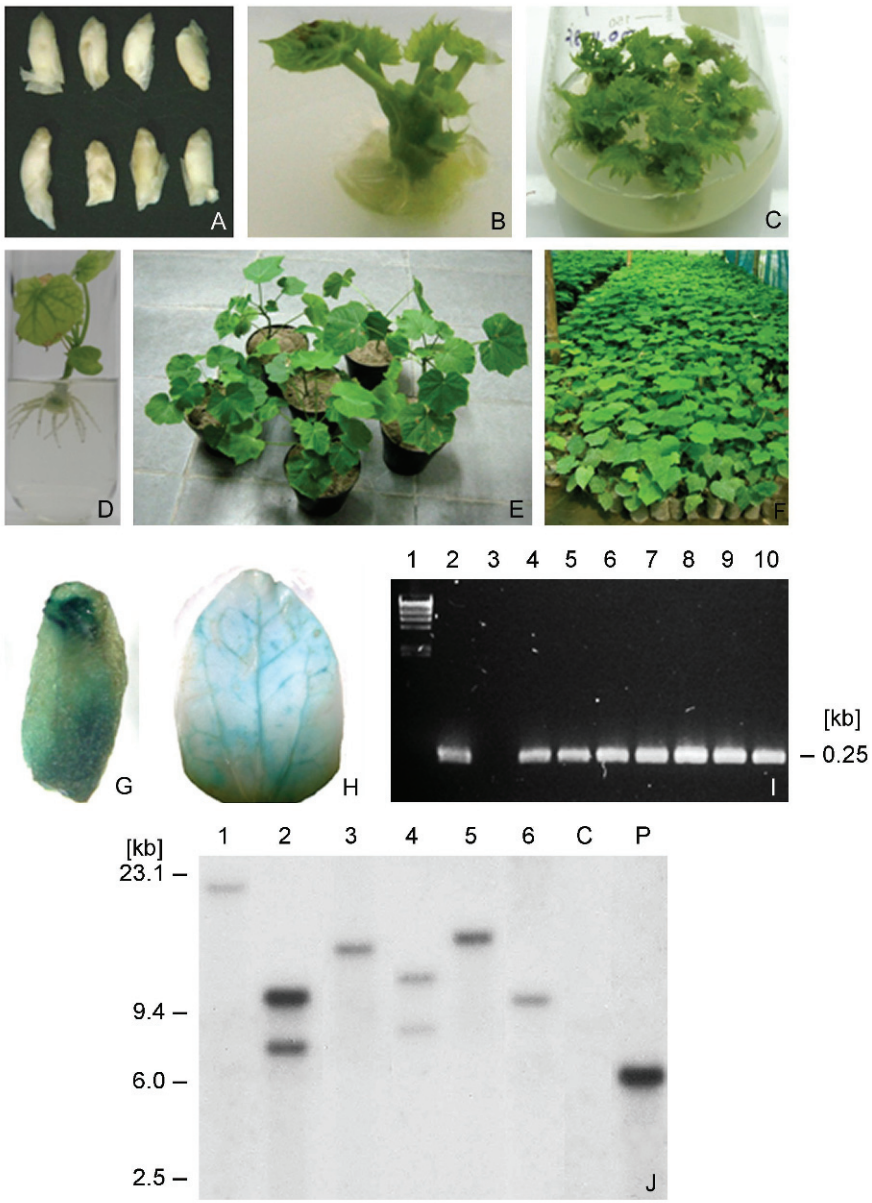

Fig. 1. Plant regeneration, transient and stable transformation of *Jatropha curcas* following particle bombardment of shoot apices with pBI426. *A* - Excised embryos of *J. curcas*. *B*,*C* - Multiple shoot induction from shoot apices on MS medium containing 2.5 μM BAP. *D* - Induction of roots on half strength MS medium without growth regulator within 10 d of culture. *E* - Acclimatized plants in soil:*Vermiculite*. *F* - Plants established in nursery. *G* - Transient GUS activity in bombarded shoot apex. *H* - Stable GUS activity in leaf of putative transformant. *I* - PCR analysis of primary transformants using *gus* primers: *lane 1* - marker DNA, *lane 2* - plasmid DNA, *lane 3* - DNA from untransformed control, *lanes 4 to 10* - putative transformed plant. *J* - Genomic Southern hybridization; 0.1 and 10 μg of genomic DNA digested with *Bam*HI, respectively, from leaves of positive (P) control, un-transformed (C) plant and six transgenic plants were hybridized with *npt*II fragment probe.

Table 2. Synergistic effect of kinetin with BAP (2.5 μM) on multiplication and elongation of shoots of *Jatropha curcas* on MS medium after 2 weeks of culture. Means of three experiments with 20 replicates in each. Means within a column followed by same letters are not significantly different at $P < 0.05$.

Kinetin [μM]	Shoot development [%]	Shoot number [explant^{-1}]	Shoot length [cm]
0.5	84	5.2^{a}	1.0^{a}
1.0	75	4.1^{b}	0.8^{a}
2.5	67	3.2^{c}	0.6^{a}
5.0	43	2.6^{c}	0.6^{a}

Table 3. Effect of gibberellic acid on elongation of shoots from multiple shoot cultures of *Jatropha curcas* in MS medium after 10 d of culture. Means of three experiments with 20 replicates in each. Means within a column followed by same letters are not significantly different at $P < 0.05$.

GA_3 [μM]	Shoot elongation [%]	Shoot length [cm]
0	18^{e}	1.3^{c}
0.1	59^{b}	1.9^{b}
0.5	98^{a}	3.1^{a}
1.0	62^{b}	1.7^{b}
2.5	45^{c}	1.5^{c}
5.0	38^{d}	1.4^{c}

comparatively better elongation than those regenerated on the medium containing other cytokinins. However, kinetin in medium containing BAP (2.5 μM) had no effect on enhancement of proliferation and elongation of shoots from shoot apices (Table 2) suggesting absence of any synergistic role. This is in contrary to the earlier studies wherein media containing kinetin in conjunction with BAP induced higher frequency of shoot multiplication and greater number of shoots in some perennial plants (Figueiredo *et al*. 2001, Baskaran and Jayabalan 2005).

Incorporation of 0.5 μM GA_3 enhanced the shoot elongation by three fold in 98 % of cultures within 10 d while higher concentrations proved ineffective (Table 3). However, prolonged culture on GA_3 containing medium produced thin, lanky shoots unsuitable for rooting. The results suggest exposure to low concentration of GA_3 for a short period was effective for shoot elongation in *J. curcas*. The promotive effect of GA_3 on elongation of stunted shoots, generated on BAP containing medium, has been reported in several other plant species (Jordan and Oyanedel 1992, Hossain *et al*. 1994, Geetha *et al.* 1998, Purohit and Singhvi 1998). The BAP-habituated mother explants continuously produced shoots during successive subculture on MS medium supplemented with 2.5 μM BAP, without losing their shoot forming potential. An average of 24 - 26 shoots was produced from each explant in four consecutive harvests. Repeated transferring of meristematic explants on media containing cytokinins was found to cause rejuvenation of explants tissue, activation, conditioning of meristems and retention of their morphogenetic potential (Shekhawat *et al.* 1993, Naik *et al.* 2000, Vengadesan *et al.* 2002, Gopi *et al.* 2006). Continuous proliferation of BAP habituated shoot apices cultures during successive subcultures indicates persistence of shoot regeneration potential in *Jatropha* tissues. Cultures were maintained for more than two years and those which showed abnormal growth, senescence and leaf fasciation were periodically discarded.

Table 4. Effect of various concentrations of IAA and IBA on rooting of proliferated shoots of *Jatropha curcas* cultured on half-strength MS medium after 10 d of culture. Means of three experiments with 20 replicates in each. Means within a column followed by same letters are not significantly different at $P < 0.05$.

Auxins	Conc. [μM]	Rooting [%]	Callusing [%]
None		100^{a}	0
IAA	0.5	72.4^{c}	17.5
	1.0	68.6^{cd}	29.3
	2.5	58.6^{e}	43.8
IBA	0.5	90.2^{b}	9.6
	1.0	78.4^{c}	21.2
	2.5	66.8^{d}	34.7

Long primary root with numerous lateral roots were formed in all the shoots within 8 - 10 d of culture on half-strength MS medium (Table 4, Fig. 1*D*). Addition of IAA and IBA to half-strength medium induced shorter primary roots with few lateral roots with varied basal callusing (Table 4). Rooted plantlets were transferred to soil:*Vermiculite* in the pots and successfully acclimatized with 98 % survival (Fig. 1*E*). All the potted plants transferred to soil:compost in nursery survived and showed no sign of variation (Fig. 1*F*).

Particle bombardment parameters: To determine the optimal condition for particle bombardment, the transient GUS activity of transformed *J. curcas* shoot apices was examined, comparing the gold particle size, bombardment pressure, and target distance, macrocarrier travel distance, and type of osmotic pretreatment and its duration. Strong transient GUS expression was observed in the shoot apices, at the region where the shoots developed (Fig. 1*G*), after bombardment with plasmid pBI426 carrying the GUS-NPT II fusion gene under the control of a double 35S cauliflower mosaic virus (CaMV) promoter. The highest frequency of transient expression was observed when explants were bombarded at 1100 psi with a target distance 9 cm (Table 5), similar to the observation reported in *M. esculenta* (Schopke *et al.* 1997), rice (Zhang *et al.* 1996, Ramesh and Gupta 2005), cassava (Schopke *et al.* 1997) and oil palm (Parveez *et al.* 1997). Shorter target distance (6 cm) in combination with 1100 psi pressure resulted in lower expression level,

which could be due to tissue damage as tissue dislocation was observed at this closed-up range. Diminished expression at low pressure could be attributed to the poor penetration capability of the microparticles, while at higher pressure, the high penetrating force of the microparticles might have resulted in injury of target tissues. The reduced expression level at 12 cm (data not shown) was possibly due to decreased velocity of the microparticles with the long flight distance resulting in reduced penetration force and few target cells receiving the incoming DNA. GUS expression was not detected in control explants, which were either not subjected to particle bombardment or subjected to bombardment with naked particles. Among the different gold microparticles sizes compared for optimal DNA delivery, particles with intermediate size (1.0 μm) showed significantly higher frequency of transient GUS expression (Table 6). This result is consistent with the observations obtained by Parveez *et al.* (1997), Schopke *et al.* (1997), Xiao and Ha (1997), and Kamo and Blowers (1999) in oil palm, cassava, creeping bent grass and *Gladiolus* plants. Folling and Olsen (2002) reported higher damaging effect with larger microparticles size in wheat transformation. Preculture of shoot apices on 0.2 M mannitol for 4 h prior to bombardment improved transient GUS expression (data not shown). The effect of a short-term osmotic preconditioning (plasmolysis) of target cells or tissues on transient and stable transformation has been reported in several studies (Iglesias *et al.* 1994, Perl *et al.* 1992, Altpeter *et al.* 1996). Short-term high osmotic treatments, typically for a few hours before or after bombardment, are thought to minimize cytoplasm leakage from target cells (Ortiz *et al.* 1996). However, contrasting results have been obtained in *Dendrobium* wherein the GUS activity in protocorm was not significantly affected by size of the target, type of particles, and helium gas pressure (Suwanaketchanatit *et al.* 2007).

Table 5. Effect of bombardment pressure and target distance on transient GUS expression in shoot apices of *Jatropha curcas*. Each value represents the mean of three experiments. Means within the column followed by same letters are not significantly different at $P < 0.05$.

Pressure [psi]	Distance [cm]	GUS expression [%]
900	6	26^{e}
	9	30de
1100	6	34^{d}
	9	58^{a}
1350	6	43^{c}
	9	51^{b}

Table 6. Effect of gold particle size and number of shots on transient GUS expression in shoot apices of *Jatropha curcas*. Means of three experiments. Means within the column followed by same letters are not significantly different at $P < 0.05$.

Gold particles [μm]	Number of shots	GUS expression [%]
0.6	1	18^{fg}
	2	23^{f}
	3	39^{d}
1.0	1	32^{e}
	2	62^{a}
	3	58^{b}
1.6	1	27^{ef}
	2	49^{c}
	3	54^{bc}

Selection and regeneration of transformants: The gold particles of size 1 μm coated with pBI426 were utilized for bombardment at pressure of 1100 psi with 9 cm target distance. Two shots of bombardment were given to target explants precultured for 4 h on medium containing 0.2 M mannitol for all bombardment experiments. The putative transformed shoots were recovered on medium containing 25 mg dm^{-3} kanamycin within 6 - 7 weeks of culture. Stable GUS expression was detected in leaves of transformed shoots (Fig. 1*H*). The concentration and timing for the selection process were found important for recovery of transformed plants. The transformed shoots showed root induction when kanamycin was withdrawn in rooting medium suggesting that root induction step was more sensitive to kanamycin than shoot induction.

Molecular analysis: PCR analysis showed expected amplification of 0.25 kb internal fragment, corresponding to the *gus* gene, indicating the presence of transgene in putative transformants (Fig. 1*I*). No amplification was detected in the control untransformed plants. The plants that did not show amplification were probably the escapes that survived the selection. To confirm *npt*II integration in the genome of the putative transgenic plantlets, genomic DNA from leaves of control untransformed plants and six transformed plants were digested with *Bam*HI and hybridized with a 0.54 bp of *npt*II fragment probe. As this plasmid carries a unique *Bam*HI site, digestion of the genomic DNA of putative transformants with *Bam*HI would generate a different fragment for each integrated copy. Southern hybridization, therefore, provided copy numbers of the *npt*II in the genome of the putative transgenic plantlets. Unique *npt*II specific hybridization signals, indicating independent transformation events, were detected in all putative transformed plants (Fig. 1*J*). A varied hybridization signals revealed single to multiple copy insertion in transgenic plants (Fig. 1*J*). No signal was detected in genomic DNA of plants regenerated from unbombarded explants. The results demonstrated that this strategy could deliver transgene into *J. curcas* shoot apices producing stable transgenic plants. The importance of having access to a prolific regeneration system from shoot apices for direct DNA delivery by particle bombardment technology has lead to successful recovery of transgenics in recalcitrant plants (Christou 1992). However, the inherent constraints associated with bombardment are derivation

of plants with varying copy numbers of transgenes, introduction of vector backbone sequences in conventional plasmid DNA and unnecessary insertion of elements including bacterial origin of replication and a selectable bacterial gene, which are not essential for plant transformation. Persistence of these sequences in field-released commercial transformants is considered undesirable from the stand-point of biosafety. The use of minimum gene cassettes (containing the promoter, coding sequence and polyadenylation signal of the transgene) has been shown to simplify the bombardment protocol and maximize its efficiency and improve its selectivity to regenerate high expressing low copy number plants in rice and bentgrass (Fu *et al.* 2000, Breitler *et al.* 2002; Jayaraj *et al*. 2008).

In conclusion, this work presented a rapid and efficient micropropagation method for *J. curcas* using shoot apices for mass propagation of this potential biofuel plant. The developed protocol would be of immense importance for mass multiplication of elite stocks owing to the fast and high proliferation rates of shoot apices, and for genetic improvement of this valuable plant with desired traits by particle bombardment.

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