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GENETIC TRANSFORMATION AND HYBRIDIZATION

Stable genetic transformation of castor (*Ricinus communis* L.) via particle gun-mediated gene transfer using embryo axes from mature seeds

M. Sailaja · M. Tarakeswari · M. Sujatha

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Abstract The first successful attempt to produce stably transformed castor plants through direct gene transfer using particle gun (BioRad) is described. Decotyledonated embryos from mature seeds were germinated and the embryonic axis was induced to proliferate on Murashige and Skoog (MS) medium supplemented with 0.5 mg l^{-1} thidiazuron (TDZ) and subjected to bombardment after 5–7 days of pre-incubation. The physical parameters for transient transformation were optimized using the *UidA* gene encoding β-glucuronidase (GUS) as the reporter gene and with hygromycin-phosphotransferase (*hptII*) gene as selectable marker. Statistical analysis revealed that helium pressure, target distance, osmoticum, microcarrier type and size, DNA quantity, explant type and number of bombardments had significant influence on transformation efficiency, while the effect of genotype was non-significant. Of the different variables evaluated, embryonic axes from mature seeds, a target distance of 6.0 cm, helium pressure of 1,100 psi, 0.6 μm gold microcarriers, single time bombardment and with both pre- and post-osmoticum were found ideal. Selection of putative transformants was done on MS medium supplemented with 0.5 mg l^{-1} BA and hygromycin (20, 40 and 60 mg l^{-1}) for 3 cycles. The stable integration of the incorporated gene into castor genome was confirmed with PCR and Southern analysis of T_0 and T_1 plants. Transformation frequency in terms of plants grown to maturity and showing the presence of the introduced genes was 1.4%. The present results demonstrate the possibility of transformation of embryonic meristematic tissues of castor through particle delivery system.

Keywords Biolistics · Castor · Meristem-based transformation · *Ricinus communis* · Transgenics

Abbreviations

BA	N^6-Benzyladenine
DMRT	Duncan's multiple range test
GUS	β-Glucuronidase
HPT	Hygromycin-phosphotransferase
MS	Murashige and Skoog basal salt media
NAA	∝-Naphthaleneacetic acid
NPT	Neomycin phosphotransferase
PCR	Polymerase chain reaction
TDZ	1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron)
X-Gluc	5-Bromo, 4-chloro, 3-indolyl, β-D-glucuronide

Introduction

Castor (*Ricinus communis* L.) is one of the most important non-edible oilseed crop and is cultivated on 1.26 mha with an annual seed production of 1.14 m tons and an average seed yield of 902 kg/ha (FAOStat 2006). More than 95% of the castor cultivation in the world is concentrated in India, China and Brazil. Castor owes its importance to the uniqueness of its oil which is rich (80–85%) in the hydroxy fatty acid, ricinoleic acid. Castor oil was primarily used for medicinal purposes and as high-quality lubricants for heavy equipment or jet engines. Later, the utility of castor oil and its derivatives were found to have wide range of

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M. Sailaja · M. Tarakeswari · M. Sujatha (✉)
Crop Improvement Section, Directorate of Oilseeds Research, Rajendranagar, Hyderabad 500-030, India
e-mail: mulpurisujata@yahoo.com

uses in agriculture, textile chemicals, paper, plastics and rubber, perfumeries, cosmetics, electronics and telecommunication industry, pharmaceuticals, paint industry, lubricants, folk medicine, etc. (Atsmon 1989; Vignolo and Naughton 1991). In the recent past, the potential of castor oil as a biofuel has been investigated (http://www.castoroil.in).

A major production constraint for profitable production of the crop is the vulnerability of the released varieties and hybrids to several biotic stresses. Another major concern is the presence of the toxic protein ricin in the seed and the problem is being approached through conventional breeding, mutagenesis (TILLING) and transgenic technology (Auld et al. 2001; http://www.arcadiabio.com). Among the biotic stresses, insect pests (foliage feeders and capsule borer) and diseases (Fusarial wilt and *Botrytis* grey rot) are of a serious concern. Castor belongs to a monotypic genus and the genetic variability available in the germplasm is not at acceptable levels to breed for varieties/hybrids with in built tolerance to biotic threats. Hence, novel approaches to introgress genes from alien sources are envisaged. However, the major limitation is lack of efficient tissue culture and transformation protocols for castor.

Castor proved to be highly recalcitrant to in vitro manipulations and successful regeneration through direct as well as callus-mediated methods is yet to be reported. Regeneration is reported mainly from explants with meristematic explants such as, shoot apices and embryonic axes (Athma and Reddy 1983; Reddy et al. 1987; Molina and Schobert 1995; Sujatha and Reddy 1998; Ahn et al. 2007). Shoot regeneration from explants devoid of meristematic tissues was sporadic and frequency of morphogenesis was low and genotype dependent (Reddy and Bahadur 1989; Sarvesh et al. 1992). Keeping in view the need for genetic transformation of castor for genetic improvement of the crop for agronomical characteristics, studies have been undertaken in India and USA for development of transgenic castor. The most widely used methods of transformation are the direct gene method using particle gun and the vector-mediated method using *Agrobacterium tumefaciens.* Since both the methods have their own advantages and limitations (Potrykus 1991; Sharma et al. 2005), attempts were made to optimize conditions for transformation of castor using the two methods. McKeon and Chen (2003) obtained genetically engineered plants by employing the method of *Agrobacterium*-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6,620,986). Owing to the difficulties with shoot regeneration, genetic transformation studies in India relied on meristem-based proliferation system developed by Sujatha and Reddy (1998). Meristem explants have been successfully transformed in several crops (Bilang et al. 1993; Saeed et al. 1997; Sticklen and Oraby 2005). The first success at stable transformation of meristematic tissues of castor through *Agrobacterium*-mediated transformation has been reported (Sujatha and Sailaja 2005). Subsequently, transgenic castor resistant to castor semilooper through deployment of *Cry1Ab* gene has been developed (Malathi et al. 2006). In this investigation, stable transformation of castor through optimization of physical and biological parameters for direct gene transfer using particle gun is presented.

Materials and methods

Plant material and culture conditions

Seeds of the cultivar DCS-9 (Jyoti) were used in all the experiments unless specified. This variety was specifically selected as it is cultivated as a variety under rainfed conditions and as a pollen parent for the hybrid, DCH 177. To assess genotypic differences, the genotypes DCS-9, 48-1, VP-1 and Bhagya were used for particle bombardment. Decoated seeds were surface sterilized with 0.1% (w/v) of mercuric chloride for 8.0 min and rinsed thoroughly four times in sterile distilled water. Whole embryos with papery cotyledons were carefully dissected from the endospermic seeds. Immature embryos collected at 20 days after pollination and embryo axes from mature seeds were aseptically isolated and pre-incubated on semi-solid Murashige and Skoog's (1962) medium containing 3% sucrose and 0.5 mg l^{-1} TDZ (thidiazuron) for 5–7 days in the dark. All media were prepared according to the standard procedures and pH was adjusted to 5.6 ± 0.2 prior to autoclaving at 121°C for 20 min. Cultures were maintained at 26 ± 2°C under a 16/8-h light/dark photoperiod provided by cool white fluorescent lamps at an intensity of 30 μmol m^{-2} s^{-1}.

Transformation with particle gun

Following pre-incubation, germinating embryo axes were selected for bombardment with the biolistic PDS-1000 He system (BioRad Hercules, CA). About 50–60 embryo axes were arranged on Whatman No.1 filter paper in a circle of 1.5 cm diameter in the centre of a 9.0 cm petriplate with osmoticum (0.2 M each of sorbitol and mannitol) and plasmolysed for 2 h prior to bombardment. The plasmid DNA, pCAMBIA 1305.1 containing genes encoding *hpt* (hygromycin phosphotransferase) and gusplus in the T-DNA region was obtained from the Center for the Application of Molecular Biology in International Agriculture (CAMBIA), Australia and used for most of the experiments. Few experiments were carried out with pCAMBIA 2301 with plant selection on kanamycin.

Preparation of microcarriers

Microcarriers were coated with 6 μg of plasmid DNA per 50 μl particle preparation (3 mg microcarrier/50 μl) using the $CaCl_2$ (50 μl, 2.5 M) and spermidine (20 μl, 0.1 M) precipitation method according to the manufacturer's instructions to give a DNA concentration of 2 μg/mg of microcarriers. Following precipitation, the supernatant was removed and pellet was washed with 300 μl of absolute ethanol. After washing, the particle DNA pellet was resuspended in 50 μl of absolute ethanol for 6 bombardments. Care was taken to ensure uniform particle distribution and minimize agglomeration. Bombardments were done under a vacuum of 27 inches of Hg, a 25 mm distance from rupture disc to macrocarrier and a 10 mm macrocarrier flight distance for all the bombardments. The variables tested included rupture disc pressures (450–1,350 psi), three microprojectile travel distances (6, 9, and 12 cm) and microcarrier type (gold particle size 0.6, 1.0, 1.6 μm and tungsten 1.1 and 1.7 μm). To investigate the effect of DNA concentration, bombardments were carried with 1, 2 and 5 μg of DNA per mg of microcarriers. Likewise, the concentration of gold microcarriers was tested at 1.5, 3.0 and 6.0 mg per bombardment mix of 50 μl. In addition to these, pre- and post-bombardment treatments with MS medium containing 0.2 M each of mannitol and sorbitol were optimised (2 h pre- and 2 h post-osmoticum). Non-bombarded embryo axes and embryo axes bombarded with uncoated microcarriers were used as controls. Following bombardment, the explants were kept in dark at 25°C for 2 h and then transferred to shoot proliferation on MS medium containing 0.5 mg l^{-1} BA and after 15 days the explants were transferred to selection medium. For effective selection, the explants were transferred at 15 days interval to shoot multiplication medium with increasing concentrations (20, 40 and 60 mg l^{-1}) of hygromycin. Embryo axes bombarded with pCAMBIA 2301 were selected on kanamycin (50, 100 and 200 mg l^{-1}). After three cycles of selection, the putative transformed shoots were transferred to MS medium supplemented with 0.5 mg l^{-1} BA for further multiplication and 0.2 mg l^{-1} BA for elongation. For rooting, elongated shoots were transferred to medium containing half-strength MS basal salts, 1.0 mg l^{-1} NAA and 3% sucrose. Rooted shoots were transferred to sterile vermiculite and maintained under high humidity for 7–10 days and established plantlets were transferred to pots for hardening.

Histochemical GUS assay

Transient GUS assays were performed 48 h after bombardment by incubating the tissues in assay buffer (0.05 M NaH_2PO_4 with 500 mg l^{-1} X-Gluc and 30% Triton X-100) for 12 h at 37°C, after which they were destained and stored in 70% alcohol (Jefferson et al. 1987).

Analysis of transgenic plants

Molecular analysis

To test for the presence of *UidA* and *hpt* genes, genomic DNA was isolated from leaf tissues of putative, independently transformed lines using the CTAB method (Doyle and Doyle 1987). PCR amplification for detection of the presence of *hpt* gene was according to Sujatha and Sailaja (2005). For amplification of *UidA* and *nptII* genes, the primer sets used were *UidAF*-GGTGGGAAAGCGCGTTACAAG; *UidAR*-GGTTACGCGTTGCTTCCGCCA and *nptF*-GAGGCTATTCGGCTATGACTG; *nptR*-TCGGGAGCGGCGATACCGTA to amplify fragments of 1,200 and 700 bp, respectively. The PCR amplification reaction (10 μl) consisted of 50 ng of DNA, 1× PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 150 μM of each of the four dNTPs, 1 μl of each of the forward and reverse primers to give a final concentration of 0.25 μM and 0.3 U of *Taq* DNA polymerase (Bangalore, Genei, India). The annealing temperature for *UidA* and *nptII* genes was 60°C but the time for annealing was 60 s and 30 s for *UidA* and *nptII* genes, respectively. Multiplex PCR was done using primer sets specific to *UidA* and *hpt* genes. PCR amplifications were done in a thermocycler (PE 9700, Perkin Elmer, Foster City, CA, USA). The expected sizes of the amplified DNA products are 520, 700 and 1,200 bp, respectively, for *hpt, nptII* and *UidA* genes.

For Southern blot analysis, 10 μg of genomic DNA was digested with *Xho*1, separated on 0.8% agarose, blotted onto a nylon (N^+) membrane (Amersham) and hybridized with a probe prepared from *hpt* gene fragment as described earlier (Sujatha and Sailaja 2005).

Data analysis

Each treatment consisted of at least three plates and was replicated thrice. Frequency of GUS activity was calculated as the number of explants showing intense GUS expression to the total number of explants stained following bombardment and is expressed as percentage. Data on GUS foci was not recorded as in most of the explants the foci coalesced which probably could be due to the very soft tissues that were used for bombardment. Data on the number of embryo axes showing transient GUS expression and the number of explants producing multiple shoots after the third cycle of selection was subjected to analysis of variance (ANOVA) using MSTATC statistical program (Anonymous 1988). Treatment differences were compared using Duncan's multiple range test ($\alpha = 0.05$).

Results

Transformation in castor was based on proliferation of embryonic meristematic tissues. In the procedure followed, decotyledonated embryos elongated up to 0.5 cm with distinct swelling of the meristematic region within 5–7 days of incubation on medium supplemented with 0.5 mg l^{-1} TDZ. The elongated embryos with their radicular regions excised, showed green shoot bud-like protrusions within 15 days on transfer to medium supplemented with 0.5 mg l^{-1} BA. The shoot bud-like structures formed multiple shoots on subculture to the same medium. Subsequent transfer to the same medium resulted in differentiation of well-developed shoots with two distinct nodes. After excision of elongated shoots, the original explants continued to proliferate on the multiplication medium. In transformation experiments, the same procedure was followed and bombarded explants were transferred from 0.5 mg l^{-1} TDZ to 0.5 mg l^{-1} BA with the selection agent for 3 cycles. The bombarded explants continued to produce shoot bud-like structures from transformed sectors during selection and formation of well-developed shoots occurred only after removal from the selection medium.

Placement of embryos

One advantage with the use of decotyledonated embryos precultured on cytokinin medium for 5 days is the swelling of the embryonic axis region. When such embryos are placed on filter paper for bombardment, the swollen embryo axis portions are much closer to the particle accelerator as compared to the radicular region. This enables bombardment and particle penetration only in the embryonic axis region, which is in an active state of division due to the influence of TDZ. Embryos placed randomly revealed GUS spots all over the explants, while explants implanted carefully with the swollen embryonic axis upright showed particle concentration in the embryonic axis region only (Fig. 1a, b). Four genotypes (DCS-9, Bhagya, VP-1 and 48-1) were subjected to bombardment. Maximum transient GUS expression was recorded in Bhagya (48.0%), while the lowest was in VP-1 (33.0%) but differences were not significantly different (data not shown).

Optimization of bombardment parameters

Preliminary investigations were carried out with concentrations of DNA and gold particles. Among the quantity of DNA adsorbed to the microprojectiles (1, 2 and 5 μg/mg of microcarriers), a concentration of 1 μg gave significantly higher frequency of GUS expression (79.6%) as compared to the higher concentrations of DNA tested (42 and 50%, respectively). Differences in transient GUS expression due to 2 and 5 μg were not significant. Likewise, the microcarrier concentrations tested at 1.5, 3.0 and 6.0 mg per bombardment mix indicated that lower concentrations of particles were optimal (33–39%), while a threefold reduction (11.5%) in transient GUS expression was observed with 6.0 mg of microcarriers. Explants with meristematic centers viz., embryo axes from mature seeds, very young embryos (from capsules 10–15 days after pollination), immature embryos (from capsules 25–30 days after pollination), proliferating shoots from shoot apices, cotyledonary nodes and shoot tips were bombarded. Transient GUS expression was evident only in the embryo explants and was maximum in embryo axes from mature seeds (100%) followed by immature embryos (40%) and very young embryos (22.2%).

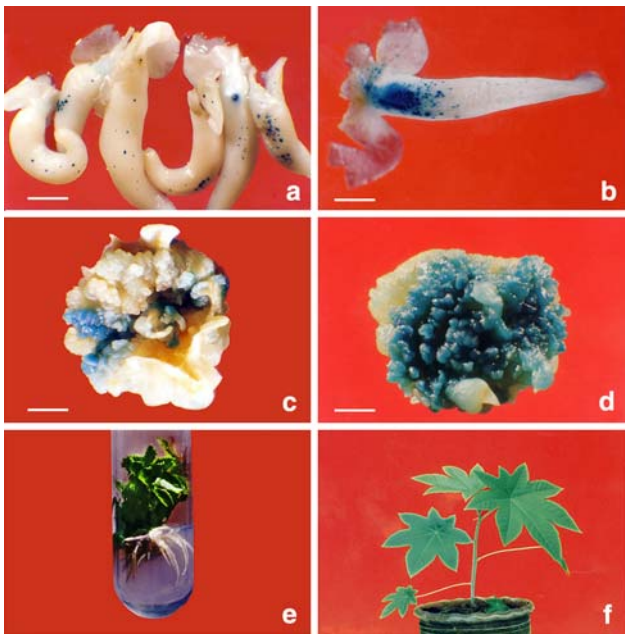

Fig. 1 Transformation and whole plant regeneration of transformed embryo axes. **a** GUS expression all over the embryo axes that were placed randomly (*bar* = 0.4 mm). **b** GUS expression localized in the embryonic axis region in embryo axes placed with the meristematic tip region upwards (*bar* = 0.4 mm). **c** Transformed and untransformed sectors after the third cycle of selection (*bar* = 0.4 mm). **d** GUS expression in all the proliferating shoot buds after the third cycle of selection (*bar* = 0.4 mm). **e** Rooted shoots on half-strength MS medium with 1.0 mg l^{-1} NAA and 3% sucrose. **f** Acclimated plants

Osmoticum

This experiment was carried out without and with either pre-, post- or both pre- and post-osmoticum. Transient GUS expression results revealed that post-osmoticum improved transformation efficiency of castor embryos.

Table 1 Effect of osmoticum on transformation efficiency

Helium pressure (psi)	Transformation frequency (%)								Mean transformation frequency over different osmoticum treatments
	Osmoticum								
	Without osmoticum		Pre-osmoticum		Post-osmoticum		Pre- + post-osmoticum		
	No. of explants	Transient GUS expression (%)	No. of explants	Transient GUS expression (%)	No. of explants	Transient GUS expression (%)	No. of explants	Transient GUS expression (%)	
450	130	85.0	113	89.0	90	100	130	100	93.5 ab
650	139	100	112	52.7	150	100	105	89.0	85.4 b
900	122	66.6	110	100	155	100	116	100	91.5 ab
1100	120	100	106	100	126	100	135	100	100 a
1350	110	92.0	118	83.1	135	100	110	100	93.7 ab
Mean transformation frequency over different helium pressures		88.6 b		84.8 b		100 a		97.8 a	

Coefficient of variation (%) = 12.1

Means followed by same letters are not significantly different according to DMRT at $\alpha = 0.05$

Bombardments were performed at a target distance of 6.0 cm

Embryo axes subjected to post-osmoticum either singly or in combination with pre-osmoticum revealed significantly higher frequency of transient GUS expression (97.8–100%) across different pressures (Table 1). The individual effects due to helium pressure, osmoticum treatments and their interaction effects were found to be highly significant.

Pressure vrs microcarrier type and size

Averaged over all other parameters, the mean transient GUS expression was 52.1, 25.4 and 8.0% for target distances of 6, 9, and 12 cm, respectively (Table 2). In general, frequency of transient GUS expression decreased with increase in the particle size. Comparison of similar particle sizes (1.0 μm gold to 1.1 μm tungsten, and 1.6 μm gold to 1.7 μm tungsten) shows higher transient GUS expression with tungsten particles than with gold. However, the frequency of putative transformants following three cycles of selection was significantly low in embryo axes bombarded with tungsten as compared to gold microcarriers. Averaged over microcarrier type and size, differences due to different helium pressures were found significant with the lowest frequency of transient expression in explants bombarded at helium pressure of 450 psi. The individual effects due to microcarrier type and size, the target distance and their interaction effects were highly significant.

Helium pressure vrs target distance

For assessment of the effect of helium pressure and target distance during bombardment, the frequency of transient GUS expression and recovery of putative transformants after the third cycle of selection were taken into consideration. The frequency of transient GUS expression increased with increase in helium pressure and was maximum at a helium pressure of 1,350 psi (Table 3). On the contrary, the frequency of GUS expression declined with increase in the target distance and was maximum at a target distance of 6 cm. ANOVA revealed highly significant effects due to helium pressure, target distance and their interaction on transient GUS expression. Despite increase in frequency of GUS expression with increase in helium pressure, there was a drastic reduction in the frequency of surviving shoots and shoots failed to survive after the third cycle of selection in explants bombarded with helium pressure of 1,350 psi. The effects due to helium pressure and its interaction with target distance on the frequency of shoot recovery were highly significant. Correlations of helium pressure and target distance on frequency of GUS expression and recovery of putative transformants were made. Highly significant and positive correlation was found between helium pressure and GUS expression, while

Table 2 Effect of microcarrier type and size, helium pressure and target distance on transient GUS expression

Microcarrier type and size	Helium pressure (psi)															Mean
	450			650			900			1,100			1,350			
	Distance (cm)															
	6	9	12	6	9	12	6	9	12	6	9	12	6	9	12	
0.6 μm (Gold)	14.4	0	0	79.4	26.7	29.6	100	61.2	8.9	74.6	10.3	100	100	44.8	27.5	44.0 a
1.0 μm (Gold)	49.0	0	0	86.3	43.4	0	34.7	43.4	0	61.5	74.7	38.7	90.2	19.5	40.1	33.2 b
1.6 μm (Gold)	28.3	11.1	0	40	13.3	0	41.2	12.5	0	52.4	31.1	0	32.5	17.9	0	11.3 d
1.1 μm (Tungsten)	68.4	61.1	63.1	58.8	70.6	31.3	21.1	65	0	29.9	25.9	11.7	41.2	27.8	0	32.3 b
1.7 μm (Tungsten)	57.9	25	7.1	17.6	41.2	31.2	25	27.8	6.7	20	17.6	0	16.7	15.8	4.8	16.3 c
Mean	17.0 c			32.1 a			24.0 b			32.0 a			28.3 ab			

Coefficient of variation (%) = 18.2

About 150–230 embryo axes were bombarded per each treatment

Means in each column followed by same letters are not significantly different according to DMRT at $\alpha = 0.05$

Experimental design: three factor completely randomized design with three replicates

the correlations were negative and highly significant between helium pressure *vrs* shoot recovery ($r = -0.543$), target distance *vrs* GUS expression ($r = -0.680$) and target distance *vrs* shoot recovery ($r = -0.025$).

Number of bombardments

In this experiment, data was recorded on the frequency of surviving shoots at each of the three cycles of selection on hygromycin as valid inferences cannot be drawn with transient GUS expression. There were no differences in the transient GUS expression as the same explants after first bombardment were subjected to the second round of bombardment by leaving the explants as such or reversing the surface of the explants by reversing the filter paper (Table 4). In this experiment, shoot survival rates were significantly different from the selection cycle I itself unlike in other experiments where differences in the number of putative transformed shoots were not significantly different in selection cycles I and II. The number of

Table 3 Effect of helium pressure and target distance on transformation efficiency

Helium pressure (psi)	Distance (cm)	Number of explants bombarded	Frequency of transient GUS expression (%)	Frequency of shoot survival (%)		
				Selection I	Selection II	Selection III
450	6	198	15.7	86.3	85.0	40.7
	9	216	8.6	62.9	49.6	20.2
	12	223	0	77.2	44.6	21.5
650	6	273	44.2	99.4	44.9	33.2
	9	184	10.0	91.3	22.7	18.0
	12	163	8.1	93.5	27.9	8.6
900	6	145	32.7	89.1	42.1	26.8
	9	184	34.9	81.6	45.1	33.2
	12	161	0	77.8	53.8	37.4
1,100	6	201	59.6	96.9	54.1	22.8
	9	137	20.5	90.3	37.7	17.6
	12	172	2.7	94.6	45	17.5
1,350	6	91	97.4	81.3	5.3	0
	9	107	17.1	75.4	12.7	0
	12	128	35.2	89.1	41.7	0
Coefficient of variation (%)			15.5	6.4	13.5	14.9

Experimental design: two factor completely randomized design with three replicates; subculture was done at 2-week interval onto medium with 0.5 mg l^{-1} BA and 20, 40 and 60 mg l^{-1} hygromycin for selections I, II and III, respectively

shoots surviving selection was significantly higher with explants subjected to single bombardment as compared to double (two times bombardment) and reverse bombardment (both sides of embryo axis). This probably could be due to the explant injury during bombardment. The effect due the number of bombardments and method of bombardment on the frequency of shoot survival at all the three selection cycles was found to be highly significant.

Histochemical assay and whole plantlet recovery

During selection on medium with 40 and 60 mg l^{-1} hygromycin, non-transgenic tissues gradually turned brown, while putative transformed sectors remained green and showed slow growth. After the third cycle of selection, few of the shoot cultures showed both transformed and non-transformed sectors (Fig. 1c), while others showed GUS activity in all the proliferating buds (Fig. 1d). GUS expression was observed only in the newly proliferated shoots and not in the original explant which indicates origin of the buds from transformed meristematic zones. The proliferating bud cultures on transfer to medium devoid of the selection agent grew vigorously and produced 4–6 healthy shoots. Elongated shoots rooted well on the rooting medium (Fig. 1e) and could be successfully acclimatized with more than 70% success (Fig. 1f). Sexually mature plants were studied for variations in reproductive traits. All plants were fertile and produced good seed set.

Molecular analysis of transgenic plants

The presence of the reporter gene, *UidA* and the selectable markers, *hpt* and *nptII* was confirmed by PCR analysis based on amplification of expected bands of sizes 1,200, 520 and 700 bp, respectively. PCR analysis of primary transformants for *hpt* gene showed the presence of the gene in 16 of 19 putative transformant plants tested (Fig. 2a). Multiplex PCR analysis of one each of the plants from five independent events for presence of the reporter and selectable marker genes revealed presence of both the genes in four transformants and only the selectable marker in one of the transformants (Fig. 2b). In order to check the fidelity of the PCR amplification products, the PCR gel with amplified bands of *hpt, nptII* and *UidA* genes was probed with *Xho*1 restricted *hpt* fragment of the plasmid pCAMBIA 1305.1. Hybridization signals only in the *hpt* amplified bands confirm amplification of the specific gene product (Fig. 2c, d). Southern analysis revealed detectable signals in four of eight PCR positive plants (Fig. 2e). The transformants showed 1–2 copies of the introduced genes. Two plants (lanes marked 2 and 3) derived from the same shoot cluster showed similar hybridization signals. The T_1 plants derived from transformation event harboring *nptII* gene as selectable marker showed the presence of the gene in the progeny (Fig. 2f).

Averaged over several experiments involving bombardment of 22,800 embryo axes, the frequency of transient GUS expression was 47.4%; frequency of shoot recovery was 22.0%; frequency of transient GUS expression to stable transformation was 13.8% and frequency of plants grown to maturity and showing presence of the introduced genes was 1.4%.

Discussion

Gene delivery by particle bombardment is regarded as the most efficient and consistent genotype-independent method for transfer of foreign DNA. Unlike *Agrobacterium*-mediated method which is a biological process, particle bombardment is a physical process of gene delivery and theoretically any plant tissue can be transformed by this method (Christou 1996; Sharma et al. 2005). In particle bombardment experiments, the different physical and biological parameters such as the mechanism of delivery, osmoticum, the velocity of particle delivery, DNA particle concentration and precipitation procedures, amenability of target tissues for transformation need to be carefully

Table 4 Effect of number of bombardments on recovery of putative transformed shoots

Method of bombardment	Number of explants bombarded	Selection 1	Selection II	Selection III
Single	375	94.2 a	83.5 a	34.3 a
Double (reversing the filter paper after first bombardment)	320	70.5 b	47.9 b	9.7 b
Double	424	75.4 b	50.3 b	11.3 b
Coefficient of variation (%)		11.2	11.6	13.1

A helium pressure of 1,100 psi and a target distance of 6 cm were used for bombardment of embryo axes of DCS-9

Means in each column followed by same letters are not significantly different according to DMRT at $\alpha = 0.05$

Experimental design: single factor completely randomized design with three replicates

Subculture was done at 2-week interval onto medium with 0.5 mg l^{-1} BA and 20, 40 and 60 mg l^{-1} hygromycin for selections I, II and III, respectively

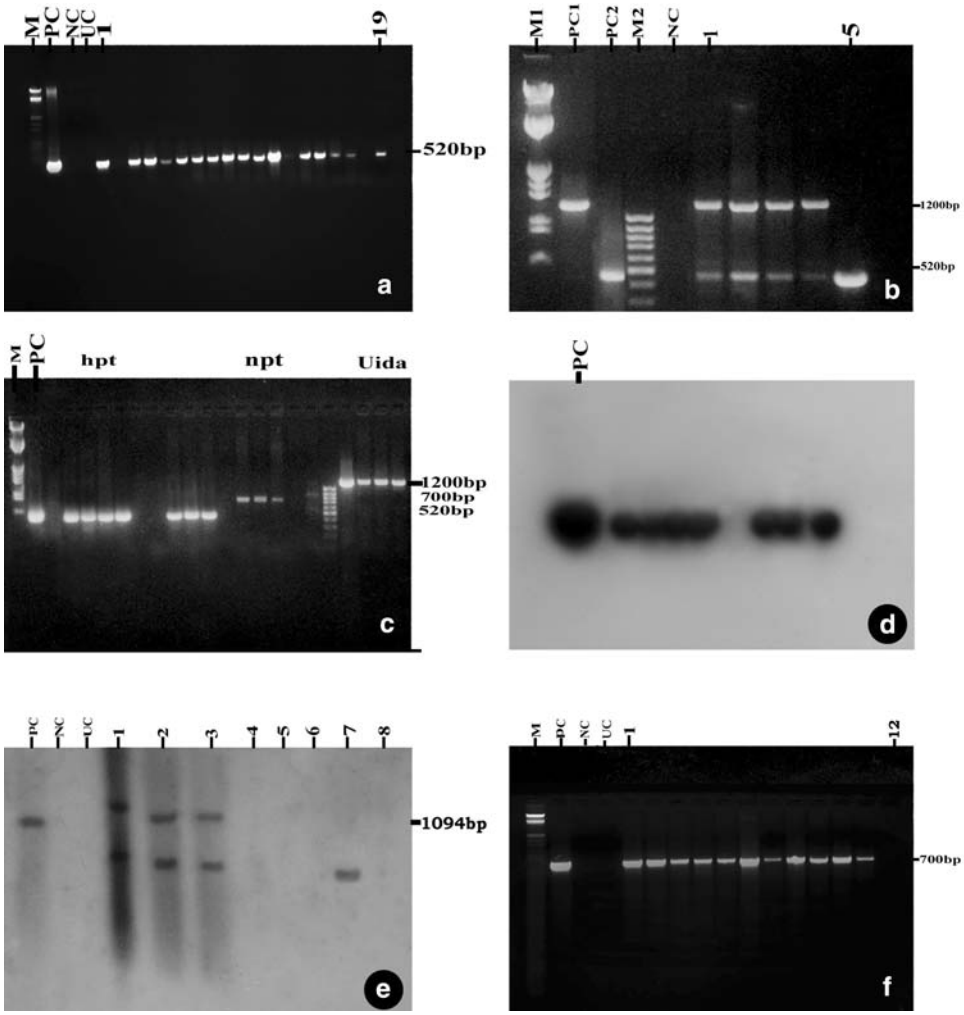

Fig. 2 Molecular analysis of transformed shoots. (The numbers indicating the transformants do not represent the same transformants as these results are obtained from different experiments.) **a** PCR analysis of primary transformants showing amplification of a 520-bp fragment of the *hpt* gene. Lanes: *M* λ DNA double digest with *EcoR1/HindIII*, *PC* pCAMBIA 1305.1 DNA, *NC* No DNA control; *UC* DNA from untransformed plant (control), *1–19* DNA from putative transformants. **b** Multiplex PCR of primary transformants showing amplification of a 520-bp fragment of *hpt* gene and 1200-bp fragment of *UidA* gene. Lanes: *M1* λ DNA double digest with *EcoR1/HindIII*, *PC1* pCAMBIA 1305.1 DNA amplified with *UidA* specific primers, *PC2* pCAMBIA 1305.1 DNA amplified with *hpt* specific primers, *M2* 100 bp ladder, *NC* No DNA control, *1–5* DNA from putative transformants. **c** PCR amplification of primary transformants for different genes. Lanes: *M* λ DNA double digest with *EcoR1/HindIII*, *PC* pCAMBIA 1305.1 DNA amplified with *hpt* gene-specific primers, lanes over marked with hpt, npt and UidA represent amplified products with the respective primer sets. **d** PCR-Southern of gel described at **c** with *hpt* fragment showing signal only in the *hpt* amplified bands. **e** Southern analysis using *hpt* amplified fragment as the probe. Lanes: *PC* pCAMBIA 1305.1 DNA, *NC* No DNA control; *UC* DNA from untransformed plant (control) digested with *Xho*1, *1–8* DNA from putative transformants digested with *Xho*1. pCAMBIA 1305.1 DNA is a 11.8 kb vector with *Xho*1 restriction sites flanking the *hpt* gene at 8,910 and 10,004 bp and digestion with *Xho*1 releases the 1,094 bp fragment of the *hpt* gene. **f** PCR analysis of T_1 plants showing amplification of a 700-bp fragment of the *npt* gene. Lanes: *M* λ DNA double digest with *EcoR1/HindIII*, *PC* pCAMBIA 2301 DNA, *NC* No DNA control, *UC* DNA from untransformed plant (control), *1–12* DNA from progeny plants

considered. This study examines various factors in particle bombardment that could enhance transient GUS expression and lead to stable integration of the introduced genes in castor.

Genotypic differences in tissue culture experiments are known to influence gene expression as well as the transformation efficiency. In case of castor, there were no significant differences in terms of transformation efficiency among the genotypes which probably could be due to the use of meristem-based transformation system. Particle gun bombardment with embryonic axis as target tissues has been used as a method for production of transgenic plants in soybean (McCabe et al. 1988) and peanut (Brar et al. 1994). Particle bombardment through PDS-1000/He gene gun is known to be gentle to target cells and is more consistent from bombardment to bombardment (Hagio

1998). Our studies are well in agreement with these observations but variation in frequency of GUS expression between different experiments was observed. It could be due to several factors such as, the preparation of microcarriers, DNA quality, spermidine, etc., for which particle gun bombardment is known to be sensitive.

Gene transfer through particle gun bombardment is reported to be applicable to any kind of target tissue but in this study, embryo axes subjected to 5–7 days of pre-induction appeared to be the most suitable target for bombardment. Embryo axes are usually used as the explant of choice for production of transgenic plants because of their excessive proliferative ability on TDZ supplemented media (Sujatha and Reddy 1998; Sujatha and Sailaja 2005; Malathi et al. 2006). Differential tissue response could probably be due to the penetrative ability of the microprojectiles in different meristematic regions which in turn depends on the physical resistance of the tissue to particle penetration and the receptivity of the target cells to receive and express DNA.

The probability of stable transformants can be maximized if young, actively dividing cells are subjected to transformation as these have the ability to survive and grow under stress imposed during the bombardment process. Studies of Gheysen et al. (1987) suggest that host plant DNA synthesis is required for integration of T-DNA into the plant genome. In castor, embryo axes pre-incubated on TDZ supplemented media have extensive expansion of the meristematic zone and provides a suitable substrate of actively dividing cells (Sujatha and Reddy 1998). In general, meristems are deep seated and are located several layers inside. GUS expression in the proliferated shoots of castor clearly demonstrates the penetrative ability of the microprojectiles through different layers. Penetration of several cell layers of an intact tissue by microprojectiles has been observed in *Allium cepa* bulb tissue (Klein et al. 1987).

There was a drastic decline in transient GUS expression with increase in target distance. Reduced frequency of transient GUS expression with increased target distance could be due to the deceleration of the microprojectiles caused by air resistance that increases as distance is increased. The transformation efficiency was determined at various pressures and distances both in terms of transient GUS expression and frequency of shoot recovery. The results revealed that transient GUS expression is not an index for the survival of the transformed tissues. In spite of a very high frequency of GUS expression in explants bombarded at 1,350 psi, the frequency of surviving explants drastically declined by the second and third selections. Increasing helium pressure will increase particle acceleration and subsequent target tissue penetration by the DNA coated microcarriers. Despite the high transient expression at high-helium pressure, shoot recovery following selection was low, which could probably be as a consequence of the acoustic shock wave accompanying the helium pressure. Increased quantity of DNA had no improvement in transient GUS expression. Studies of Klein et al. (1988) indicated that relatively high DNA concentrations resulted in severe aggregation of the microprojectiles which apparently were not effective for DNA delivery.

The transformation efficiency was analyzed using different types and size of microcarriers (gold and tungsten). The optimal helium pressure was different for each microcarrier type and size. Similar observations were made by Hunold et al. (1994). Although the transient GUS expression was higher with tungsten particles than gold particles of comparable size, the shoot survival in embryos bombarded with tungsten particles was lower compared to those bombarded with gold microcarriers. The differences in transformation due to particle size could be due to nearly spherical and uniform size of gold particles as against the heterogeneous size and irregular surface of tungsten particles, which leads to the formation of agglomerates (Russell et al. 1992). In the studies of Russell et al. (1992), a higher toxicity of tungsten as compared to gold was observed with a concomitant decrease in the ratio of transient-to-stable transformants following bombardment with tungsten rather than gold particles. Studies of Hunold et al. (1994) revealed that gold particles penetrated into deep cell layers due to their higher density, whereas the tungsten particles did not pass beyond the epidermal layers in tobacco leaves. This probably could be the reason for the low recovery of shoots following bombardment with tungsten particles. Smallest particles were found to be most efficient in delivering the genes through particle bombardment (Yang et al. 1999). In castor, transformation is meristem-based and the particles should be targeted to these deep-seated regions.

Studies of Vain et al. (1993) showed that addition of an osmoticum to the bombardment medium increases the rate of transient and stable transformation. In the present study, explants subjected to post-osmoticum treatment alone or in combination with pre-osmoticum resulted in a higher frequency of transient as well as stable transformation frequencies which indicates the importance of post-osmoticum in achieving higher transformation frequency in castor. Osmotic treatments are known to enhance the efficiency of transient and/or stable transformation and also the survival of tissues from damage incurred during transgene delivery in maize, rice and peanut through particle bombardment (Vain et al. 1993; Deng et al. 2001). Increased osmoticum protects the cells from leakage and bursting and also improves particle penetration itself.

The method of bombardment plays an important role in transformation. Of the three methods of bombardment

tested, single bombardment resulted in a higher frequency of transformation as compared to two bombardments on the same side or on either side of the embryo axis. This clearly indicates that heavier bombardment is lethal to the treated cells. On the contrary, increasing the number of bombardments on the same tissue from one to three bombardments enhanced the expression level fourfold in maize (Weissinger et al. 1988) and *Larix* (Lin et al. 2005). This probably could be due to the differences in the target tissues used in the experiments and the study indicates susceptibility of castor embryo axes to repeated bombardments.

GUS staining during the third cycle of selection showed presence of some untransformed sectors. Non-amplification of specific bands in some of the transformants could be due to recovery of shoots from such sectors. Generally in case of transformation via particle bombardment, multiple copies of the inserted genes are reported. However, in the present study only one to two copies are observed which could be due to the restriction enzyme used. The *hpt* gene has *Xho*1 restriction sites on either side of the gene which cleaves the *hpt* fragment of 1,094 bp intact. Hence, a signal at 1.1 kb will be invariably detected. Fragments of smaller size than the expected size in plant samples 1, 2, 3 and 7 represent a second insertion event, which could have undergone a rearrangement/deletion. Probably, use of a different restriction enzyme could have been more informative. Nevertheless, the main purpose of the investigation is to optimize variables for both transient and stable expression of the introduced gene; and Southern analysis confirmed stable integration of the introduced gene.

Despite a very high frequency of transient GUS expression (47.4%), the frequency of its conversion to stable transformation was low (13.8%). Low conversion rates of transient-to-stable expression could be due to several factors such as, low efficiency of integration of the introduced DNA, low survival of the bombarded cells, toxicity of the heavy metals used for bombardment, mechanical damage due to particle penetration in the cell wall (Hunold et al. 1994).

The frequency of transformation obtained through particle gun gene transfer in the present study is 1.4% and higher than those reported earlier for castor. McKeon and Chen (2003) obtained 12 genetically engineered plants through flower bud transformation. The transformation frequencies through *Agrobacterium*-mediated transformation were 0.08% (Sujatha and Sailaja 2005) and 0.42% (Malathi et al. 2006). Higher frequency of transformation through particle gun bombardment could probably be due to the higher number of explants tried for transformation. The transformation efficiency through particle gun bombardment was derived from experiments using 22,400 embryo axes. Sujatha and Sailaja (2005) used about 13,000 explants while Malathi et al. (2006) tried transformation with 5,000 embryos. Despite using higher number of explants, the frequency of transformation was low in the study of Sujatha and Sailaja (2005) than that reported by Malathi et al. (2006). While Sujatha and Sailaja (2005) optimized several parameters for transformation, Malathi et al. (2006) used the optimized protocol with minor modifications for transformation of castor with *Cry1Ab* gene. McKeon and Chen (2003) used inflorescences and the flower buds in castor racemes will be at different developmental stages and receptivity for DNA imbibition varies with the growth stage of flower buds.

The results of the present investigation amply demonstrate the possibility of stable transformation of castor through direct gene transfer method as well. To the best of our knowledge, this is the first report of generation of stable transformants through particle gun bombardment method in castor. In our laboratory, both *Agrobacterium*-mediated and particle gun bombardment methods are being used to transform castor with constructs harboring insect resistance (*Cry 1Aa, Cry1Ac* and *Cry 1Ec*) genes.

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