Plant Cell Tiss Organ Cult (2015) 123:455–466
DOI 10.1007/s11240-015-0848-6

ORIGINAL ARTICLE

Genetic transformation of cultivated sesame (*Sesamum indicum* L. cv Rama) through particle bombardment using 5-day-old apical, meristematic tissues of germinating seedlings

Jagannath Bhattacharyya[1] · Anirban Chakraborty[1] · Joy Mitra[1] · Saikat Chakraborty[1] · Subrata Pradhan[1] · Anulina Manna[1] · Narattam Sikdar[1] · Soumitra Kumar Sen[1]

Received: 10 March 2015 / Accepted: 7 August 2015 / Published online: 18 August 2015

Abstract An in vitro plant generation and genetic transformation protocol was established in sesame (*Sesamum indicum* L. cv Rama) through biolistic particle gun bombardment. 5-day-old apical, meristematic tissues of in vitro-germinating seedlings were used as explants. 10–15 Multiple shoots were generated from each explant using Murashige and Skoog basal medium containing 18.0 μM benzylamino purine and 5.37 μM naphthalene acetic acid. Four independent sets of transformation were carried out and each set consisted of three independent experiments each comprising three replications with 30 explants per replication. A synthetically designed bialaphos resistance gene (*bar*) was used for transformation. The positive transformants containing the *bar* gene were selected in growth medium containing 2.5 mg/L bialaphos. Green shoots recovered from bombarded explants were subjected to root development on Murashige and Skoog basal medium containing 5.37 μM naphthalene acetic acid. The rooted shoots were established in soil and grown to maturity in greenhouse. Polymerase chain reaction (PCR), Southern and reverse-transcription PCR, real-time quantitative PCR, western blot and enzymatic assay of four putative transformants from independent sets provided evidence for full-length gene integration as well as high level expression of the transgene. Analysis of the T_1 plants revealed a stable inheritance of the transgene through the progenies. This is the first report of biolistic mediated stable transformation of sesame and should pave the way for future genetic engineering strategies to be employed for improvement of this very important oil-seed crop.

✉ Soumitra Kumar Sen
soumitrakumar.sen@gmail.com;
jagannathb357@gmail.com

[1] Advanced Laboratory for Plant Genetic Engineering, Indian Institute of Technology, Kharagpur 721302, India

Keywords *Bar* gene · Bialaphos · Particle bombardment · Sesame transformation · Transgenic plants

Introduction

Sesame (*Sesamum indicum* L.) of Pedaliaceae family is an important oil seed crop, cultivated in the tropics and the temperate zone of the world (Biabani and Pakniyat 2008). The major producers include India, China, Korea, Turkey, several countries of Latin America and Africa (FAOSTAT 2008). India ranks first in the area of land covered under its cultivation (www.crn.india) and also in the export of sesame. Sesame is known for its superior oil quality and quantity in comparison to other vegetable oils. The oil content ranges from 35 to 55 % in different cultivars with an average of about 50 % of the seed weight (Ashri 1989). The oil has long shelf life due to the presence of a variety of lignans or natural antioxidants, like sesamolin, sesamin and sesamol with medicinal and pharmaceutical value (Kamal-Eldin and Appleqvist 1994). Despite these nutritional and medicinal merits, the sesame oil contains substantially low amount (<1 %) of α-Linolenic acid (Mondal et al. 2010), which is an important health-beneficial ω-3 polyunsaturated fatty acid. Also, yield loss due to different biotic (including fungal diseases) and abiotic stress factors poses major challenge to sesame cultivation (Silme and Cagirgan 2010; Chowdhury et al. 2014a). Introgression of desired trait from wild varieties into the cultivars through conventional breeding approach is rather limited due to post fertilization barrier (Tiwari et al. 2011). Genetic engineering through plant transformation can serve as an alternative in this regard.

Sesame is known for its recalcitrance to in vitro regeneration due to the production of various secondary

metabolites (Bhaskaran and Jayabalan 2006). There were early efforts towards successful in vitro multiplication of sesame (George et al. 1987). This led to establishment of tissue culture system from seven different cultivars of sesame using seedling explants. Multiple shoot buds were obtained from shoot tip cultures and rooted plantlets obtained from shoot buds were established in soil (George et al. 1989). High-frequency in vitro plant regeneration without transformation has been reported from seedling cotyledon, hypocotyl explants (Were et al. 2006; Younghee 2007), internodal transverse thin layer culture (Chattopadhyaya et al. 2010) and through adventitious shoot formation from de-embryonated cotyledon of mature sesame seeds (Seo et al. 2007). Somatic embryogenesis from zygotic embryo (Ram et al. 1990), hypocotyl segments (Mary and Jayabalan 1997; Xu et al. 1997), cotyledons, root and subapical hypocotyl parts of young seedlings (Shashidhara et al. 2011) has also been reported, however, without successful plant regeneration. In this context, hairy root cultures using *Agrobacterium rhizogenes* have also been established (Ogasawara et al. 1993; Jin et al. 2005). Despite of susceptibility of sesame to *Agrobacterium tumefaciens*, no transformed shoot or plant was recovered (Taskin et al. 1999). However, in recent years, there have been a few reports of successful *Agrobacterium*-mediated genetic transformation of sesame (Yadav et al. 2010; Al-shafeay et al. 2011; Chowdhury et al. 2014b) with transformation and regeneration of fertile transgenic sesame plants. Among them, Chowdhury et al. (2014b) reported high regeneration and transformation efficiencies of 57.33 and 42.66 %, respectively. However, till date, there is no report of direct gene delivery system in sesame via microprojectile-mediated particle-gun bombardment. This method has been employed successfully in many crops such as rice (Datta et al. 1990), soybean (Christou et al. 1990), maize (Gordon-Kamm et al. 1990) and recalcitrant plants like jute (Ghosh et al. 2002; Bhattacharyya et al. 2015). Shoot tips have been successfully employed for efficient biolistic-based genetic transformation, such as in *Zea mays* (Zhong et al. 1996), *Glycine max* (Aragao et al. 2000) and *Sorghum bicolor* (Devi and Sticklen 2003). Recently, our group reported biolistic-mediated genetic transformation in *Corchorus capsularis* using 1-day-old apical meristematic tissue from germinating seedling as explants (Bhattacharyya et al. 2015). With this background, the present study was undertaken for establishing an efficient and reproducible in vitro plant generation and genetic transformation system in sesame using particle-gun bombardment. The bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* that codes for phosphinothricin acetyl transferase, has been reported to be a useful selectable and screenable marker (Rathore et al. 1993) for the transformation of crop plants. It confers resistance against L-phosphinothricin, the active ingredient in the commercial herbicides, Basta and Liberty (De Block et al. 1987; Christou et al. 1991). Thus, *bar* gene was chosen as the candidate gene for transformation and subsequent selection of the putative transformant lines. We report on molecular level analysis of the transformants in T_0 and T_1 generations and also analyzed transgene expression level in quantitative terms.

Materials and methods

Plant materials and seed germination conditions

Seeds of *Sesamum indicum* cv Rama strain were used as the experimental plant material. Mature seeds were soaked in 0.1 % (v/v) Tween 20 for 25 min, followed by rigorous rinsing with sterile water. Seeds were then surface disinfected with 0.2 % (w/v) $HgCl_2$ for 10 min and rinsed five times in sterile water. Aseptic seeds were germinated in 90 mm × 20 mm petri plates (30 seeds per plate) in the dark at 25 °C for 5 days on hormone-free seed germination medium consisting of Murashige and Skoog basal salt mixture (MS medium, Murashige and Skoog 1962) supplemented with 3 % (w/v) sucrose and 0.80 % agar (as a gelling agent) (Table 1). All the tissue culture media components were purchased from HiMedia, India.

Construction of chimeric gene cassette for biolistic gene delivery

The 555 bp fragment of the *bar* gene coding DNA sequence (CDS) was synthetically reconstructed (Genscript Corporation Inc. USA) using plant preferred codons for optimal expression using the protocols of Grantham et al. (1986) and Murray et al. (1989). It was fused with the CaMV35S promoter at the 5′ end and the nopaline synthase (*nos*) gene termination sequence at the 3′ end. The chimeric gene cassette was cloned in pUC18 vector at the *Eco*RI/*Hin*dIII sites to generate the chimeric gene construct pBAR (Bhattacharyya et al. 2015), subsequently used for plant transformation (Fig. 1).

Explant preparation, biolistic gene delivery, multiple shoot development and plant generation

The apical meristematic region was isolated from in vitro seedlings grown on seed germination medium (Table 1) after elimination of the cotyledon and the root. Apical meristematic tissues from 5-day-old germinating seedlings were found to be best suited for multiple shoot generation. 3 mm long shoot tips were dissected out from apical meristem and were used as explants. Around 30, 5-day-old apical meristems from germinating seedlings were arranged in a circle (1.0–1.5 cm in diameter) on multiple shoot

Table 1 Composition of culture media for *Sesamum indicum* (cv Rama) tissue culture

Medium	Composition	Application
Media for seed germination	MS salts (Macro + Micro, without plant growth regulator) + vitamins + 3 % sucrose (pure) + 0.05 % (w/v) myo-inositol, agar 0.8 % (w/v)	Seed germination
MSGM (multiple shoot generation medium)	MS (Macro + Micro salts) + 18 μM BA + 5.37 μM NAA + 27 μM Glycine, 4.06 μM Nicotinic acid, 1.18 μM Thiamine-HCl, 4.06 μM Pyridoxine, 555 μM Myoinositol, Sucrose-3 %, agar 0.8 % (w/v)	Multiple shoot formation
SM (selection medium)	MSGM + Bialaphos (2.5 mg/L) + agar 0.8 % (w/v)	Selection of transformed cells/tissues
RIM (root inducing medium)	MSGM + 5.37 μM NAA + Sucrose-3 % + agar 0.8 % (w/v)	Rooting from screened shoots
PM (potting medium)	½ Soilrite + ½ clay Soil (autoclaved)	Transplantation of regenerated plants in pots

MS Murashige and Skoog, *BA* benzylamino purine, *NAA* naphthalene acetic acid

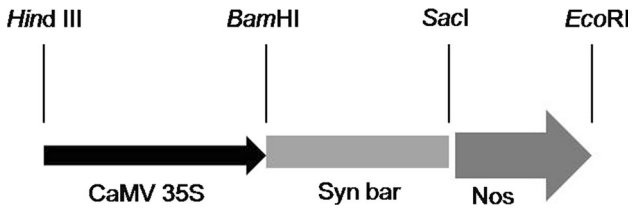

Fig. 1 Schematic map of synthetic *bar* gene cassette (pBAR) with the reconstructed *bar* gene under CaMV35S promoter

generation medium (MSGM) (30 ml per 90 mm × 20 mm petri plate) (Table 1) at the centre of each petri plate. Coating of the plasmid DNA on gold particles (1 μM) was carried out following the method of Cao et al. (1992). Particles were coated with DNA of pBAR (2.5 μg per 30 μL of particle containing 1.5 mg suspension). Particle bombardment was carried out at 1100 psi at a target distance of 11 cm using a Bio-Rad 1000/He Biolistic gun (Bio-Rad, USA) according to the manufacturer's protocol. Four sets of bombardment experiments were carried out (Table 2). Each experimental set comprised of three independent experiments each comprising three replications with 30 explants per replication. Thus, three plates were bombarded per individual experiment and approx. 250–275 Explants were subjected to particle bombardment in each set. The bombarded explants were kept overnight in the dark at 28 °C in the same petri plate. The next day, 15–20 explants each were transferred to fresh MSGM with 5.37 μM naphthalene acetic acid (NAA) and 18.0 μM benzylamino purine (BA) (Table 1). Multiple shoots (10–15) were produced from each explant in about 6 weeks after the bombardment experiment. Transfer to fresh MSGM was carried out at 15 day intervals for these 6 weeks, until multiple shoots developed. Putative transgenic shoots from bombarded explants were separated, with shoots generated from individual explant maintained in a separate cluster with proper marking and placed on selection medium (SM) in 90 mm petri plates containing 2.5 mg/L bialaphos (Table 1) for an additional 2 weeks at 28 °C under a 16:8 h photoperiod (light: dark) with light intensity of 150–200 μE m^{-2} s^{-1} in a Percival plant growth chamber with periodic sub culture every 1 week. Surviving shoots were subjected to further screening for another 2 weeks following similar protocol. Surviving green shoots (after attaining height of at least 3 cm and bearing at least two–three leaves) were finally transferred to root inducing medium (RIM; containing 5.37 μM NAA as auxin supplement) (in plain top round bottom hard glass culture tubes of dimension 25 mm × 150 mm containing 20 mL medium) (Table 1) for 2 weeks. Plantlets were transferred to potting medium (PM) for hardening in a mixture of ½ soilrite (Keltech Energies Ltd) and ½ clay soil (autoclaved) for 2–3 weeks (pot size: 20 cm × 19 cm). Putative transformed plants were transferred to the greenhouse, replanted in sand:peat:soil (1:1:1) and covered with a transparent sheet for 5 days. The covers were removed as the plants acclimatized to the greenhouse conditions. All plants were normal fertile and produced seeds upon maturity (approx. 2 months after transfer to greenhouse). Similar acclimatization conditions were followed when T_1 plantlets were transferred to greenhouse. The entire protocol of biolistic gene delivery to transgenic plant generation is schematically represented in Fig. 2.

Isolation of genomic DNA from sesame plants

Genomic DNA was isolated from young, tender leaves (3–4 cm long) of transgenic (T_0 and T_1 progeny) and non-transgenic sesame plants grown in the greenhouse, following the hexadecyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990; Bhattacharyya et al. 2015).

Table 2 Summary of results of four independent sets of particle bombardment experiments with pBAR

Set	Explants bombarded	Bialaphos resistant shoots	PCR^+ shoots	Trans. efficiency (%)
I	260	40	40	15.38
II	275	45	45	16.36
III	250	46	46	18.4
IV	265	35	35	13.2
Control	250	0	0	Mean = 15.835

Each set represents combined data of three independent experiments, each of which comprised three replications with 30 explants per replication. Trans. Efficiency (%) = percentage of explants transformed into PCR^+ shoots

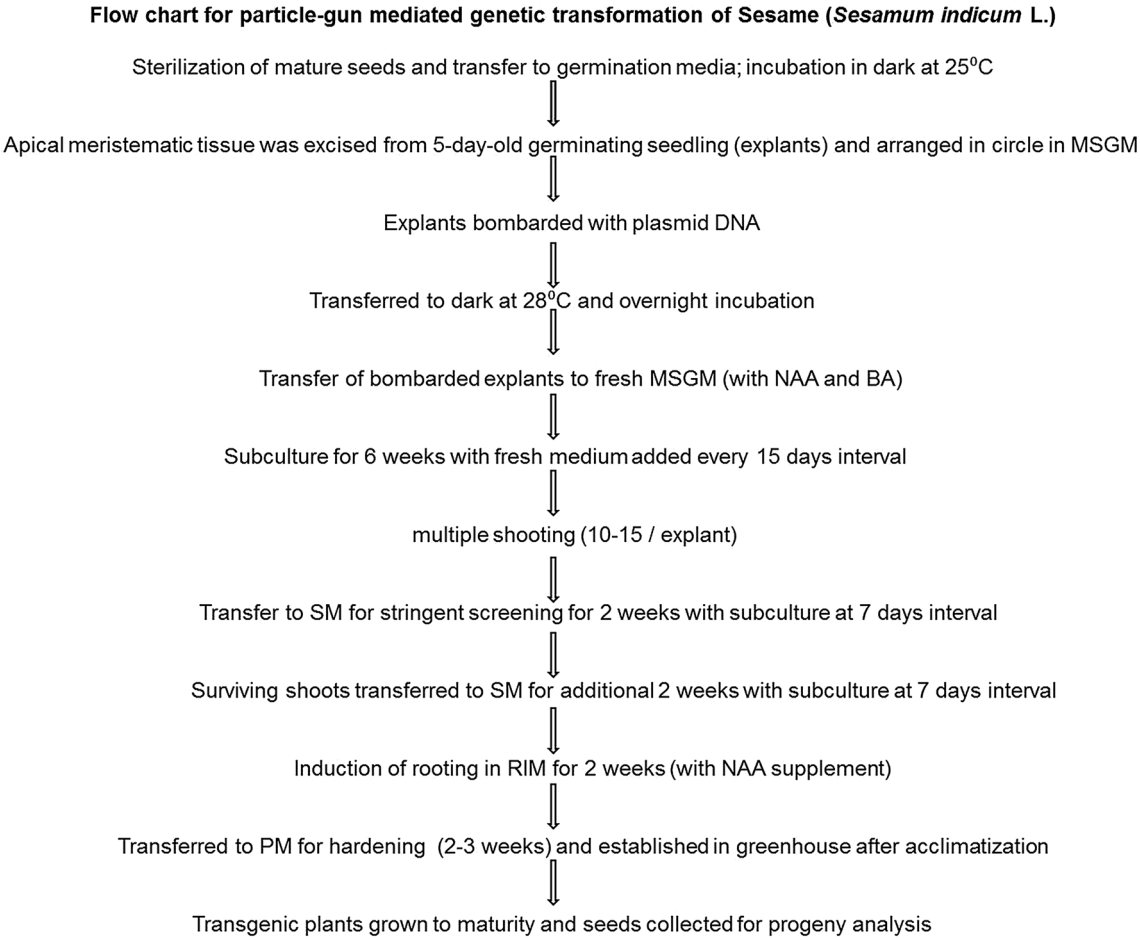

Fig. 2 Schematic overview of the protocol for biolistic mediated sesame transformation and transgenic plant generation

Polymerase chain reaction (PCR) of T_0 sesame plants

PCR was performed with synthetic *bar* gene CDS specific primers (SynBar FP and synBar RP, 0.5 µM each; Table 3) using 100–200 ng of template DNA, according to the protocol described in Bhattacharyya et al. (2015).

Southern blot analysis of T_0 and T_1 sesame plants

Southern blot analysis was performed in accordance with the standard protocol described in Bhattacharyya et al. (2015). 10-µg aliquots of total genomic DNA were digested with *Eco*RI. The transgene cassette contained only one *Eco*RI site and thus, digestion by *Eco*RI helped in identification of the number of transgene integration sites in

Table 3 Primers used in the study

Name and sequence	Purpose	T_m (°)
Syn bar FP: ATAAGGATCCATGAGCCCAGAACGACGCCC	Forward primer for PCR screening of syn *bar* gene and RT-PCR	65.7
Syn bar RP: ATCGGAGCTCTTATCAGATCTCGGTGACGG	Reverse primer for PCR screening of syn *bar* gene and RT-PCR	64.4
Syn bar RT FP: GGTCAACTTCCGTACCGAGC	Forward primer for real time qPCR analysis of syn *bar* gene	60.74
Syn bar RT RP: CAGTCGTAGGCGTTGCGTG	Reverse primer for real time qPCR analysis of syn *bar* gene	61.72
Si actin FP: GTAAAGCAGATCGAAGCGCAA	Forward primer for real time qPCR analysis of *actin* gene and RT-PCR	59.61
Si actin RP: CCGACCCACTATGCTAGGGA	Reverse primer for real time qPCR analysis of *actin* gene and RT-PCR	60.47

sesame genome. α-[^{32}P]-dCTP-labeled synthetic *bar* gene was used as probe.

Isolation of RNA from sesame plants

RNA was isolated using a hot-phenol extraction method (Verwoerd et al. 1989; Bhattacharyya et al. 2015).

Reverse-transcription PCR (RT-PCR) analysis of T_0 sesame plants

RNA was isolated from transgenic and non-transgenic sesame plants according to the protocol described above. First strand cDNA was synthesized with Syn Bar RP using transcriptor 1st strand cDNA synthesis kit (Roche). RT-PCR was carried out with synthetic *bar* gene CDS specific primers (SynBar FP and synBar RP, 0.5 µM each, Table 3) using 2 µL cDNA using Taq DNA polymerase with the following thermal cycle profile: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min in a Veriti gradient thermocycler (Applied Biosystems). In another case, first strand cDNA was synthesized with Si actin RP from the same stock of RNA samples and RT-PCR was carried out with *actin* gene CDS specific primers (Si actin FP and Si actin RP, 0.5 µM each, Table 3) using 2 µL cDNA using Taq DNA polymerase with the following thermal cycle profile: initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min in a Veriti gradient thermocycler (Applied Biosystems).

Real-time quantitative PCR (qRT-PCR) analysis of T_0 sesame plants

Real-time PCR was carried out in an Eppendorf Realplex^2 Master Cycler using SYBR green based relative quantification method with the 5 prime kit (Eppendorf) according to Wang et al. (2006) in 20 µL reaction volume. First strand cDNA (using Syn Bar RT RP) was synthesized from 1 µg of total RNA according to the protocol mentioned previously. Thermal cycling conditions were 2 min at 94 °C followed by 40 cycles of 94 °C for 30 s, 58 °C for 15 s, and 68 °C for 30 s using oligo pair Syn Bar RT FP and Syn Bar RT RP (0.5 µM each, Table 3). For each reading, triplicate C_T values were averaged. Melting curve analysis in each case confirmed the amplification of specific product.

Western blot analysis of T_0 sesame plants

Western blot was performed with 60 µg of leaf protein, according to a protocol described in Bhattacharyya et al. (2015). Total protein was isolated from 100 mg leaf tissue of transgenic and untransformed sesame plants and was quantified according to Bradford (1976). Affinity-purified rabbit polyclonal antibody against Bar (Sigma, P0374) was used as the primary antibody (1:1000 dilution). Mouse monoclonal plant Actin antibody (Sigma, mabGPa) was used as loading control (1:500 dilution).

Phosphinothricin acetyl transferase (PAT) activity assay of *bar* gene in T_0 sesame plants

The protein extraction from leaf tissue (250–500 mg) and PAT activity assay were carried out according to protocol described in Shaw (1975) and Bhattacharyya et al. (2015).

Analysis of stable inheritance of the *bar* transgene in T_1 generation

Seeds were collected from the T_0 plants and were subjected to germination (in 90 mm × 20 mm petri plates with 30 seeds per plate) in seed germination medium (Table 1) containing 2.5 mg/L bialaphos for 4 weeks with periodic sub-culture every 7 days. Generated resistant plantlets were transferred to the greenhouse after proper acclimatization as described before. The representative plants were subjected to Southern blot analysis for assessment of stable inheritance of transgene. All plants were normal fertile and produced seeds upon maturity.

Oligonucleotides used in the study

The oligos used in the study were listed in Table 3.

Results

Transformation of sesame by microprojectile bombardment, multiple shoot formation and recovery of transgenic plants

Microprojectile bombardment was carried out in replicate petri plates containing 30 explants arranged in the centre (Fig. 3a). Following microprojectile bombardment, use of MS medium (macro and micro salts) supplemented with 18.0 μM BA and 5.37 μM NAA promoted multiple shoot formation after 6 weeks with transfer to fresh medium every 2 weeks (Figs. 2, 3b, c). BA (25 μM) (Yadav et al. 2010) and a combination of BA (30 μM) + Indole-3-acetic acid (IAA; 5.7 μM) (Chowdhury et al. 2014b) were also successfully used for multiple shoot regeneration following *Agrobacterium*-mediated transformation. However, in our initial trials, a combination of BA + NAA in the aforesaid concentrations was found to be most suitable for multiple shoot development (10–15 per explant). Explants were transferred to MSGM containing 2.5 mg/L bialaphos for 2 weeks for screening. After 2 weeks of screening, surviving green shoots (Figs. 2, 3d, e, indicated by arrow) along with some yellowish-white or white shoots were transferred to fresh MSGM containing 2.5 mg/L bialaphos and further screened for another 2 weeks (Figs. 2, 3f). Most of the shoots turned brownish to black indicating elimination of the untransformed tissues in the stringent selection process. Elimination of all chimeric untransformed shoots was accomplished by repeated separation. Green shoots (Fig. 3f) were subsequently cultured in RIM for root development for 2 weeks (Figs. 2, 3g). The roots appeared normal and continued to grow in culture in the same medium. Rooting was successfully obtained for 85 % of the developed shoots. The putative transformed plantlets, after selection, were hardened and finally grown under greenhouse conditions to maturity (Figs. 2, 3h). 5 Transgenic plants from each of the four sets of experiments were grown to maturity after transformation and stringent selection. The plants showed no apparent morphological change and were fully fertile (Fig. 3i, j).

PCR screening and Southern blot analysis of the T_0 sesame plants

Four independent sets of biolistic bombardment experiments were carried out and Table 2 summarizes the results of the experiments. PCR was performed to screen for putative transformants after bialaphos screening (Fig. 4). No amplification was visible in the untransformed control line. Four putative transformants (one each from each experimental set) were chosen for further molecular level analysis. The four plant lines (#ST1–ST4) were subjected to Southern blot analysis to confirm the integration of the transgene and the number of transgene integration sites. Three plant lines (ST2, ST3, and ST4) were found to have single site of insertion whereas one (ST1) had two sites of insertion of the *bar* gene in T_0 progeny (Fig. 5a). All bands were of different size indicating different genomic sites of

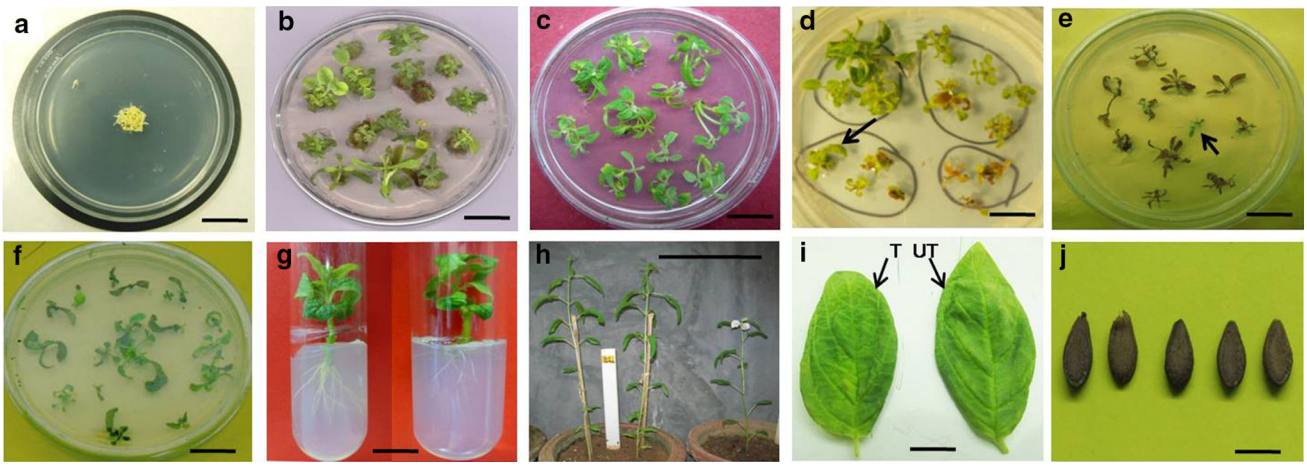

Fig. 3 Different stages of tissue culture to generate mature sesame transgenic plants. **a** Explants arranged for biolistic bombardment (*bar* 0.5 cm). **b–c** Stages showing multiple shoot formation after the bombardment experiment (*bar* 0.5 cm). **d–f** Stages showing selection of shoots against bialaphos (2.5 mg/L) after culture in MSGM. *Arrows* indicate the shoots that can survive the selection pressure (*bar* 0.5 cm). **g** Generation of root from putative transformants in rooting medium (*bar* 0.75 cm). **h** Surviving plantlets growing under greenhouse condition to maturity (*bar* 2.5 cm). **i** Leaves of the transformed (T) and non-transformed (UT) sesame plants (*bar* 0.75 cm). **j** Mature seeds from transformed sesame plants (*bar* 0.75 cm)

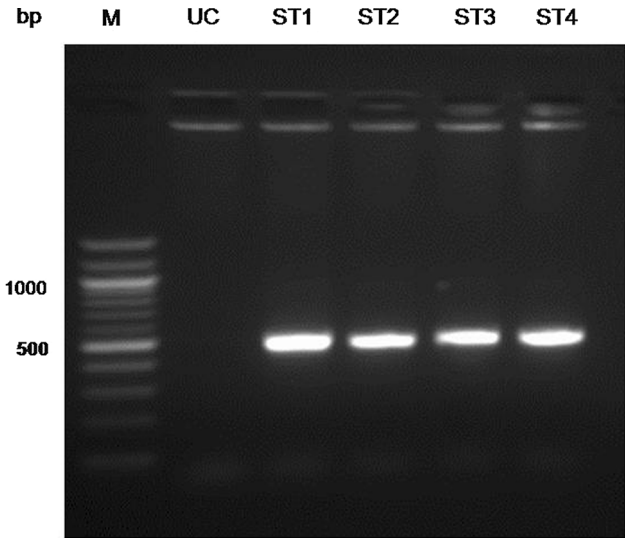

Fig. 4 PCR screening for the *bar* transgene in T_0 putative sesame transformants. *UC* Non-transformed control showing no amplification of *bar* gene, *ST1–ST4* sesame transformants ST1–ST4 showing amplification of the synthetic *bar* gene. *Lane M* NEB 100 bp ladder

transgene integration in each case, and thus all the primary transformant lines were independent. No visible band could be detected in the untransformed control line.

Expression analysis of the synthetic *bar* transgene in T_0 sesame plants

RNA level

Reverse-transcription (RT) PCR

The T_0 plants were subjected to RT-PCR with the synthetic *bar* gene specific SynBar FP and synBar RP oligos. All the four plants (ST1–ST4) revealed a detectable level of generation of the transcripts (Fig. 5b). However, we could not detect much difference in the transcript level between individual transgenic plants, probably due to saturation of the end product. No band could be detected in the untransformed control line. Amplification of *beta-actin* gene specific product (using oligos Si actin FP and Si actin RP, Accession number for *beta-actin* gene of *Sesamum indicum*: AB158612) in different transgenic and untransformed control plants served as loading control, indicating integrity of RNA from each sample.

Real time qPCR analysis

To obtain a quantitative estimate of the level of transcript generation, qPCR was performed using *bar* gene CDS specific primers (Syn Bar RTFP and Syn Bar RTRP, Table 3) which amplified a product of 150 bp. A relative quantification approach was followed, with sesame *actin* gene being used as an internal control (using oligos Si actin FP and Si actin RP). The relative level of generation of the *bar* gene transcript was represented in terms of fold change of the transcript generation (Fig. 5c). No amplification was detected in the untransformed control line. The T_0 progenies with single site of transgene integration (ST2, ST3, and ST4) were found to contain a higher level of transgene expression and the normalized expression level of the *bar* gene in the plant line ST1 (containing two transgene integration sites) was set at unity.

Protein level

Western blot analysis

To confirm expression of the full-length *bar* gene, the translated product in each of the individual transgenic lines was monitored by western blot analysis (Fig. 6a). The transgenic lines revealed distinct band in the 21 kDa region. No band could be found in the untransformed control line. Also, no detectable difference could be obtained in the protein level among individual transgenic lines with single site of transgene integration (ST2-ST4) in terms of band intensity. This may be due to saturation of the band intensity with the amount of total protein used for the study. In each case equal loading of the protein was detected by plant *actin* antibody. This also confirmed specific expression of the *bar* transgene in the transgenic sesame plants.

Activity assay of the bar gene

In order to get a quantitative estimate of the transgenic protein level, spectrophotometric PAT activity assays of the T_0 transgenic plants were carried out. The activity was represented in terms of micromoles of substrate acetylated per min. In order to determine the specific activity, the protein concentration was determined by Bradford protein assay using BSA as the standard. The activity assay (Fig. 6b) correlated well with the relative transcript level of the *bar* transgene as obtained by real time qPCR. Plants with high level of *bar* gene transcript showed high PAT activities (Table 4). Incidentally, the plants with high PAT activities were found to contain a single site of transgene integration. The untransformed control plants had no detectable enzyme activity. The activity assay further confirmed the integration and expression of the full-length transgene, apart from western blot.

Analysis of stable inheritance of the bar transgene in T_1 generation after bialaphos selection

Seeds were harvested from the T_0 sesame plants (ST2-ST4) and they were subjected to germination in 2.5 mg/L

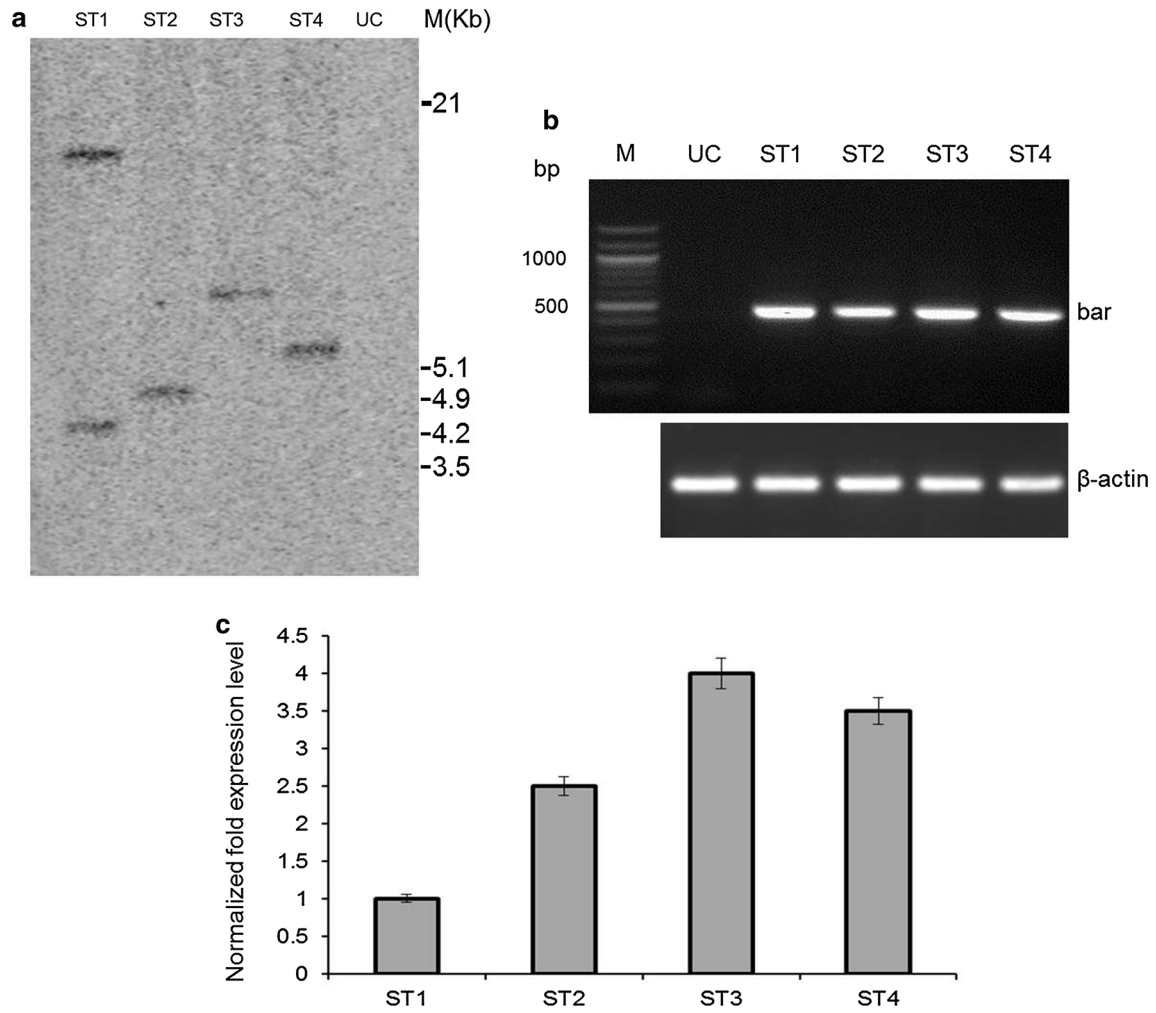

Fig. 5 Analysis of T_0 sesame plants **a** Southern blot analysis of T_0 sesame lines using synthetic *bar* gene as radiolabeled probe. *UC* Untransformed control line. *ST1–ST4* hybridization pattern for sesame transformants ST1–ST4. *Lane M EcoRI/HindIII* digested λ DNA marker. **b** RT-PCR analysis of T_0 sesame lines using *bar* gene specific oligo (*Upper Panel*). *UC* Untransformed control, *ST1–ST4* Lanes showing *bar* gene CDS specific transcript in sesame transgenics ST1, ST2, ST3 and ST4, respectively (*Lower panel*) amplification of *beta-actin* transcript from each RNA sample representing equal loading of RNA in each case. *M* NEB 100 bp ladder. **c** Real time qPCR analysis of the T_0 transgenic lines. *Bar diagram* represents the expression level of synthetic *bar* gene in various transgenic lines in terms of fold change of relative expression level taking the normalized expression level in ST1 as unity. Results are expressed as mean ± SD, for triplicate readings

bialaphos containing seed germination medium. To maintain the selection pressure, the surviving seedlings were sub-cultured in fresh medium with bialaphos every 7 days. The ratio of surviving: etiolated plantlets germinating from the seeds were scored after 4 weeks and it was found to be 3:1 in each case, indicating Mendelian mode of inheritance for the monohybrid cross (Table 5). The surviving plantlets were finally established in the greenhouse after proper acclimatization and grown to maturity. Two representative T_1 plants from each category were analyzed further by Southern hybridization (Fig. 7). All the T_1 plants revealed a similar transgene integration pattern as the T_0 plant.

Discussion

Success of plant genetic engineering largely depends on development of efficient, reproducible and genotype-independent plant transformation techniques. Microprojectile bombardment was developed in 1980s as a direct gene transfer method for development of transgenic plants. Over the years, the technique became widely popular, beside *Agrobacterium* mediated transformation and has been employed for transgene delivery in a variety of agronomically important crop species (Christou 1995; Breitler et al. 2002). The present study, however, stands out as the first report of an efficient and reproducible genetic

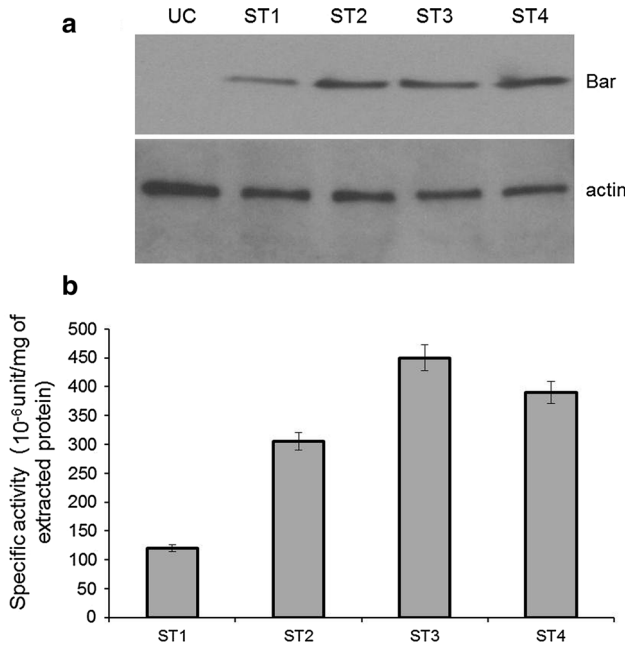

Fig. 6 Analysis of the translated product of the *bar* transgene and its activity. **a** (*Upper panel*) ST1–ST4: Lanes showing *bar* gene translated product in sesame transgenics ST1, ST2, ST3 and ST4, respectively; *UC* Untransformed control; (*Lower panel*) equal loading of proteins shown by stripping and probing the same blot with plant actin antibody. **b** In vitro PAT activity assay of the transgenic sesame plants. *Bar diagram* represents the specific activity of the synthetic *bar* gene in various transgenic lines with one unit representing µmol of PPT acetylated per min at 37 °C. Results expressed as mean ± SD, for triplicate readings

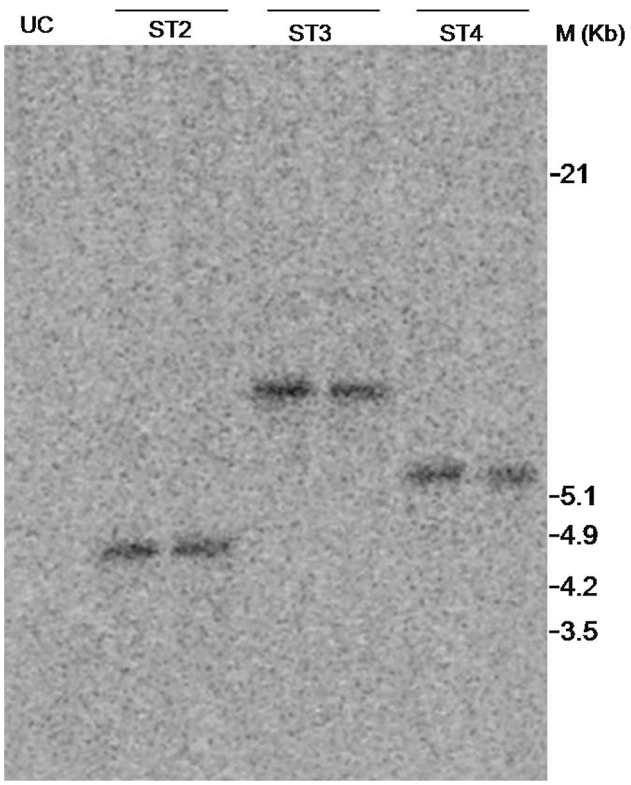

Fig. 7 Southern blot analysis of two randomly chosen T_1 progenies from each of the transgenic sesame plants with single site of transgenic insertion. *Lane 1* Untransformed control; *lanes 2, 3* progeny from ST2; *lanes 4, 5* progeny from ST3; *lanes 6, 7* progeny from ST4; *lane M Eco*RI/*Hin*dIII digested λ DNA marker. Synthetic *bar* gene CDS was used as radiolabeled probe

Table 4 Phosphinothricin acetyl transferase (PAT) activity in crude leaf extracts of transgenic sesame plants

Plant ID	Specific activity (10^{-6} unit/mg of extracted protein)
Control	N.D
ST1	120
ST2	305
ST3	450
ST4	390

N.D Not detected

Unit µmol of PPT acetylated per min at 37 °C

transformation system of *Sesamum indicum* (cv Rama) by particle gun bombardment. The study gains more significance in the backdrop of recent availability of genome sequence of sesame (Zhang et al. 2013) that has further opened up possibilities of development of genetically modified sesame line with improved nutritional and disease resistant traits using endogenous genetic elements. Particle bombardment has the advantage of transferring foreign DNA independent of cell or tissue type, provided the cell wall and plasma membrane can be penetrated. Also expression of multiple transgenes is possible in the target tissue by fusion of genes within the same plasmid that is bombarded into the target tissues. This is desirable sometimes, keeping in mind, the complexity of particular trait to be conferred in target crop and similar strategies have been successful in recent years in both monocot as well as in dicot plants such as wheat, rice, and soybean (Campbell et al. 2000; Agrawal et al. 2005; Schmidt et al. 2008)

Table 5 Segregation analysis of *bar* gene in T_1 progeny of the transgenic sesame lines with single site of synthetic *bar* transgene

Plant ID	Total seeds tested	Bialaphos$^+$	Bialaphos$^-$	Bar$^+$: bar$^-$	Segregation ratio	χ^2
ST2	52	38	14	2.71	3:1	0.101
ST3	48	35	13	2.69	3:1	0.110
ST4	55	40	15	2.66	3:1	0.150

thereby enhancing agronomic traits. In the present study, the apical meristem tissue from the 5-day-old germinating seedling was used as an explant. An earlier study on sesame showed the development of multiple shoot bud (7–12 per explant) from shoot tip as explant in a media supplemented with BA and zeatin with NAA used for subsequent rooting (Chakraborti and Ghosh 2009). Following a similar way, our protocol led to direct plant generation utilizing the morphogenic potential of the apical meristem and thus, intervening steps of dedifferentiation and redifferentiation could be avoided (Sticklen and Oraby 2005). This also reduced chances of somaclonal variation and chromosomal abnormalities. The results were reproducible as the four independent sets of experiments suggest (Table 2). Frequency of multiple shoot formation and plant generation was moderate to high. From the range of 250–275 starting explants, up to 46 transformed shoots could be generated after stringent screening. There are a few reports of stable transformation of sesame via *Agrobacterium*-mediated genetic transformation. In a recent comprehensive study (Chowdhury et al. 2014b), a mean transformation efficiency of 42.66 % was reported. Except only this, other reports (Yadav et al. 2010; Al-shafeay et al. 2011) showed transformation efficiency of <2 %. Our method was based on direct gene delivery to explant followed by tissue culture mediated multiple shoot development and generation of stable transgenic plants after stringent screening. Transformation efficiency is calculated on the basis of frequency of explants transformed. Stable transformation was substantiated by molecular evidences in DNA, RNA and protein level. The mean transformation frequency (15.835 %) was significant when compared to various monocot and dicot plants, where foreign gene(s) could be transferred by particle-bombardment. Though certain monocots, such as rice, showed transformation frequency up to 79.5 %, among the dicot plants the transformation frequency was found to be moderate as in *Arachis hypogea* (12.3 %), in *Brassica oleracea* (11.1 %) except in *Glycine max* (60 %) (Barampuram and Zhang 2011). Considering the overall recalcitrant nature of sesame, our results showed a significant advancement in developing a stable and reproducible transformation system in sesame with moderate transformation efficiency. This is also significant compared to some of the previous reports in sesame (Yadav et al. 2010; Al-shafeay et al. 2011). Efforts are already in progress to reproduce the results in some other indigenous sesame genotypes, coming from distant geographical locations, to ensure genotype independence of the transformation system.

Efficient selection of transformed cells and tissues is a crucial step in any plant genetic transformation protocol. In most attempts, antibiotic resistance gene(s), viz., neomycin phosphotransferase II and hygromycin B resistance genes, in combination with antibiotics had been used as selectable markers. This holds true for the earlier reports of sesame transformation also (Yadav et al. 2010; Chowdhury et al. 2014b), where neomycin phosphotransferase II has been used as the selection marker. In the present study, we have successfully utilized the *bar* gene that gives resistance to phosphinothricin, the active ingredient in the broad spectrum herbicide bialaphos, to produce transgenic sesame plants. This selection marker had been used earlier in genetic transformation of several crop species including rice (Rathore et al. 1993) and maize (Gordon-Kamm et al. 1990). However, this is the first report of use of *bar* as selection marker in case of sesame. The development of *bar* gene as selection marker carries significant agronomic importance because it can provide resistance to herbicides such as Basta or Ignite (or Liberty). Dose optimization for selection pressure is important in any transgenic experiment, as a suboptimal dose results in high frequency of escapes. On the other hand, unnecessarily high doses not only kill untransformed tissues, but also inhibit normal growth of transformed cells, thereby delaying the regeneration process (Wilmink and Dons 1993). Kill-curve experiments revealed an optimal dose of 2 mg/L with two repeated passages of 15 days. However, our recent study in jute (Bhattacharyya et al. 2015) revealed that a selection of higher stringency at 2.5 mg/L could help us to eliminate most of the non-transformed shoots. Following the same strategy, we used bialaphos at an optimal dose (2.5 mg/L) which led to a very stringent selection and eliminated most of the untransformed tissue as was evident in Table 2. In replicate experiments, resistant shoots after stringent screening led to generation of PCR-positive transgenic plantlets. The use of a synthetic *bar* gene reconstructed with codons optimized for high level of plant expression has enabled us to conduct such stringent screening. Stable transformation of the host and transmission of the synthetic *bar* transgene to its progeny could be detected for representative transgenic events analyzed. All the transgenic plants were fertile and showed moderate to high level of transgene expression. Segregation of *bar* into the T_1 progeny plants followed Mendelian inheritance pattern for a single dominant locus, i.e. 3:1. All the progenies were phenotypically normal and fertile. Thus the selection process was reproducible and stringent, signifying feasibility of the synthetic *bar* gene as a potential selectable and screenable marker to be used in future genetic engineering strategies to be employed in sesame. This carries further significance, because bialaphos spray can be developed as a handy and useful method for selection of T_1 plants eliminating the need of labor and cost intensive PCR mediated screening. We have already used a similar approach in case of jute (Bhattacharyya et al. 2015) and would like to extend the same to sesame.

A moderate to high level of target gene expression is the most important attribute in any transgenic approach to

achieve the required agronomic or commercial gains. Thus, evidence for genetic transformation and stable inheritance of the transgene to the next progeny should be supplemented by expression level analysis which allows us to select out the most suitable transgenic event for our purpose. In the present study a detailed expression analysis of the *bar* transgene was carried out in both RNA and protein level in qualitative (RT-PCR and western blot) and quantitative (qPCR and enzyme activity assay) terms. All the results were found to be consistent with each other. Among the four transgenic plants tested, three plants (ST2-ST4) showed higher level of transgene expression level as compared to other lines. Incidentally, all the plant lines with higher level of transgene expression showed integration of transgene at a single site. This was in accordance with some other earlier reports (DeBuck et al. 2001, 2004). The variation in the transgene expression level among individual transformants could be due to "position effect" (Matzke and Matzke 1998).

Despite its usefulness, the particle bombardment technology has several drawbacks, such as the integration of multiple copies of transgene as well as integration of superfluous DNA sequences associated with the plasmid vector. This could be overcome by transferring the desired coding region only with its control elements into the target cells of the plant genome (Lowe et al. 2009). The transgene copy number can also be determined as has been shown in Chowdhury et al. (2014b). Also the transformation efficiency needs to be improved if we intend to employ this technique as a regular molecular tool in genetic engineering approaches involving sesame. Our future research will be directed towards making this microprojectile bombardment-mediated sesame transformation more accurate, efficient and handy for conferring desired traits into sesame cultivars.

Acknowledgments The authors extend their sincere appreciation for Shila Bhattacharjee for her extensive assistance in the tissue culture. The authors are also thankful to Sudarshan Maity, Uttam Dogra and Subhash Ghosh for maintenance and rearing of transgenic sesame plants under containment conditions. Grant support to the laboratory from Indian Council of Agricultural Research (ICAR) is thankfully acknowledged. Finally, the authors express their sincere gratitude to the distinguished editor and the anonymous reviewers, whose incisive comments were of immense help for improvement of the quality of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Agrawal PK, Kohli A, Twyman RM, Christou P (2005) Transformation of plants with multiple cassettes generates simple transgene integration patterns and high expression levels. Mol Breed 16:247–260

Al-Shafeay AF, Ibrahim AS, Nesiem MR, Tawfik MS (2011) Establishment of regeneration and transformation system in Egyptian sesame (*Sesamum indicum* L.) cv Sohag1. GM Crops 2:182–192

Aragao FJL, Sarokin L, Vianna GR, Rech EL (2000) Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean (*Glycine max* L. Merril) plants at high frequency. Theor Appl Genet 101:1–6

Ashri A (1989) Sesame. In: Robbelen G, Downey RK, Ashri A (eds) Oil crops of the world: their breeding and utilization. McGraw Hill, New York, pp 375–387

Barampuram S, Zhang ZJ (2011) Recent advances in plant transformation. Birchler JA (ed) Plant chromosome engineering: methods and protocols. Methods Mol Biol 701:1–32. doi:10.1007/978-1-61737-957-4_1

Bhaskaran P, Jayabalan N (2006) In vitro mass propagation and diverse callus orientation on *Sesamum indicum* L.—an important oil plant. J Agric Tech 2:259–269

Bhattacharyya J, Chakraborty A, Roy S, Pradhan S, Mitra J, Chakraborty M, Manna A, Sikdar N, Chakraborty S, Sen SK (2015) Genetic transformation of cultivated jute (*Corchorus capsularis* L.) by particle bombardment using apical meristem tissue and development of stable transgenic plant. Plant Cell Tiss Organ Cult 121:311–324

Biabani AR, Pakniyat H (2008) Evaluation of seed yield-related characters in sesame (*Sesamum indicum* L.) using factor and path analysis. Pak J Biol Sci 11(8):1157–1160

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Breitler JC, Labeyrie A, Meynard D, Legavre T, Guiderdoni E (2002) Efficient microprojectile bombardment mediated transformation of rice using gene cassettes. Theor Appl Genet 104:709–719

Campbell BT, Baeziger PS, Mitra A, Sato S, Clemente T (2000) Inheritance of multiple genes in wheat. Crop Sci 40:1133–1141

Cao J, Duan X, McElroy D, Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile mediated transformation of suspension culture cells. Plant Cell Rep 11:586–591

Chakraborti P, Ghosh A (2009) Variation in callus induction and root-shoot bud formation depend on seed coat of sesame genotypes. Res J Bot 5:14–19

Chattopadhyaya B, Banerjee J, Basu A, Sen SK, Maiti MK (2010) Shoot induction and regeneration using internodal transverse thin cell layer culture in *Sesamum indicum* L. Plant Biotechnol Rep 4:173–178

Chowdhury S, Basu A, Ray Chaudhuri T, Kundu S (2014a) In-vitro characterization of the behaviour of *Macrophomina phaseolina* (Tassi) Goid at the rhizosphere and during early infection of roots of resistant and susceptible varieties of sesame. Eur J Plant Pathol 138:361–375

Chowdhury S, Basu A, Kundu S (2014b) A new high-frequency *Agrobacterium*-mediated transformation technique for *Sesamum indicum* L. using de-embryonated cotyledon as explant. Protoplasma. doi:10.1007/s00709-014-0625-0)

Christou P (1995) Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. Euphytica 85:13–27

Christou P, McCabe DE, Martinell BJ, Swain WF (1990) Soybean genetic engineering—commercial production of transgenic plants. Trends Biotechnol 8:145–151

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryzae sativa* L.) plant from agronomically important *Indica* and *Japonica* varieties via electrically discharged particle

acceleration of exogenous DNA into immature zygotic embryo. Biotechnology 9:957–962
Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile *Indica* rice recovered from protoplasts. Nat Biotechnol 8:736–740
De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Movva NR, Thompson C, Van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513–2518
DeBuck S, Van Montagu M, Depicker A (2001) Transgene silencing of invertedly repeated transgenes is released upon deletion of one of the transgenes involved. Plant Mol Biol 46:433–445
DeBuck S, Windels P, De-Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable beta-glucuronidase accumulation levels. Cell Mol Life Sci 61:2632–2645
Devi PB, Sticklen MB (2003) In vitro culture and genetic transformation of sorghum by microprojectile bombardment. Plant Biosyst 137:249–254
Doyle J, Doyle J (1990) Isolation of plant DNA from fresh tissue. BRL FOCUS 12:13–15
FAOSTAT (2008) http://faostat.fao.org
George L, Bapat VA, Rao PS (1987) *In vitro* multiplication of sesame (*Sesamum indicum* L. CV. PT) through tissue culture. Ann Bot 60:17–22
George L, Bapat VA, Rao PS (1989) Plant regeneration in vitro in different cultivars of sesame (*Sesamum indicum* L.). Proc Indian Acad Sci (Plant Sci.) 99(2):135–137
Ghosh M, Saha T, Nayak P, Sen SK (2002) Genetic transformation by particle bombardment of cultivated jute, *Corchorus capsularis* L. Plant Cell Rep 20:936–942
Gordon-Kamm WJ, Spencer TM, Mangano ML et al (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618
Grantham R, Perrin P, Mouchiroud D (1986) Pattern of codon usage of different kinds of species. Oxford Surv Evol Biol 3:48–81
Jin UH, Chun JA, Han MO, Lee JW, Yi YB, Lee SW, Chung CH (2005) Sesame hairy root cultures for extra-cellular production of a recombinant fungal phytase. Prog Biochem 40:3754–3762
Kamal-Eldin A, Appleqvist LÅ (1994) Variation in the compositions of sterols, tocopherols and lignans in seed oils from four *Sesamum* species. J Am Oil Chem Soc 71:149–156
Lowe BA, Prakash NS, Melissa W, Mann MT, Spencer TM, Boddupalli RS (2009) Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. Transgenic Res 18:831–840. doi:10.1007/s11248-009-9265-0
Mary RJ, Jayabalan N (1997) Influence of growth regulators on somatic embryogenesis in sesame. Plant Cell Tissue Organ Cult 49:67–70
Matzke AJM, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1:142–148
Mondal N, Bhat VK, Srivastava SP (2010) Variation in fatty acid composition in Indian germplasm of sesame. J Am Oil Chem Soc 87:1263–1269
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Phys Plant 15:437–497
Murray EE, Lotzer J, Eberle M (1989) Codon use in plant genes. Nucl Acids Res 17:477–498
Ogasawara T, Chiba K, Tada M (1993) Production and high yield of napthoquinone by a hairy root culture of *Sesamum indicum*. Phytochemistry 33:1095–1098
Ram R, Catlin D, Romero J, Cowley C (1990) Sesame: new approaches for crop improvement. In: Janick J, Simon JE (eds) Advances in new crops. Timber Press, Portland, pp 225–228
Rathore KS, Chowdhury VK, Hodges TK (1993) Use of *bar* as a selectable marker gene and for the production of herbicide resistant rice plants from protoplast. Plant Mol Biol 21:871–884
Schmidt MA, Lafayette PR, Artelt BA, Parrott WA (2008) A comparison of strategies for transformation with multiple genes via microprojectile-mediated bombardment. In Vitro Cell Dev Biol Plant 44:162–168
Seo HY, Kim YJ, Park TI, Kim HS, Yun SJ, Park KH, Oh MK, ChoiMY PaikCH, Lee YS, Choi YE (2007) High-frequency plant regeneration via adventitious shoot formation from de-embryonated cotyledon explants of *Sesamum indicum* L. In Vitro Cell Dev Biol Plant 43:209–214
Shashidhara N, Ravikumar H, Ashoka N, Santosh DT, Pawar P, Lokesha R, Janagoudar BS (2011) Callus induction and sub-culturing in sesame (*Sesamum indicum* L.): a basic strategy. Int J Agric Env Biotechnol 42:153–156
Shaw WV (1975) Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol 43:737–755
Silme RS, Cagirgan MI (2010) Screening for resistance to Fusarium wilt in induced mutants and world collection of sesame under intensive management. Turk J Field Crops 15:89–93
Sticklen MB, Oraby HF (2005) Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. In Vitro Cell Dev Biol Plant 41:187–200
Taskin K, Ercan A, Turgut K (1999) *Agrobacterium tumefaciens*-mediated transformation of sesame (*Sesamum indicum* L.). Tr J Bot 23:291–295
Tiwari S, Kumar S, Gontia I (2011) Biotechnological approaches for sesame (*Sesamum indicum* L.) and Niger (*Guizotia abyssinica* L.f. Cass.). As Pac J Mol Biol Biotechnol 19(1):2–9
Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucl Acids Res 17:2362
Wang Y, Zhu W, Levy DE (2006) Nuclear and cytoplasmic mRNA quantification by SYBR green based real-time RT-PCR. Methods 39:356–362
Were BA, Gudu S, Onkware AO, Carlson AS, Welander M (2006) In vitro regeneration of sesame (*Sesamum indicum* L.) from seedling cotyledon and hypocotyl explants. Plant Cell Tissue Organ Cult 85:235–239
Wilmink A, Dons JJM (1993) Selective agents and marker genes for use in transformation of monocotyledonous plant. Plant Mol Biol Rep 11:165–185
Xu ZQ, Jia JF, Hu ZD (1997) Somatic embryogenesis in *Sesamum indicum* L. cv. Nigrum. J. Plant Physiol 150:755–758
Yadav M, Chaudhary D, Sainger M, Jaiwal PK (2010) *Agrobacterium tumefaciens* mediated genetic transformation of sesame (*Sesamum indicum* L.). Plant Cell Tissue Organ Cult 103:377–386
Younghee K (2007) Effects of BA, NAA, 2, 4-D and $AgNO_3$ treatments on the callus induction and shoot regeneration from hypocotyl and cotyledon of sesame (*Sesamum indicum* L.). J Korean Soc Hort Sci 142:70–74
Zhang H, Miao H, Wang L, Qu L, Liu H, Wang Q, Yue M (2013) Genome sequencing of important oilseed crop *Sesamum indicum* L. Genome Biol 14:401
Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996) The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol 110:1097–1107