Plant Cell Tiss Organ Cult (2015) 121:311–324
DOI 10.1007/s11240-014-0702-2

ORIGINAL PAPER

Genetic transformation of cultivated jute (*Corchorus capsularis* L.) by particle bombardment using apical meristem tissue and development of stable transgenic plant

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Received: 15 June 2014 / Accepted: 24 December 2014 / Published online: 3 January 2015

Abstract An in vitro plant regeneration and genetic transformation protocol was established in jute (*Corchorus capsularis* L. var JRC321). One-day-old apical, meristematic tissues of germinating seedlings were used as explants. Multiple shoots were regenerated from each explant using Murashige and Skoog basal medium containing 1.78 μM benzylamino purine and 4.92 μM indole-3-butyric acid. Transformation was carried out in three independent sets (each set comprising of three independent experiments each comprising three replications with 35 explants per replication) using the bialaphos resistance gene (*bar*), synthetically designed for high level plant expression. The positive transformants containing the *bar* gene were selected in growth medium containing 2.5 mg/l bialaphos. Polymerase chain reaction (PCR), Southern and northern blots, real-time quantitative PCR, western blot and enzymatic assay of five putative transformants from three independent sets provided evidence for full-length gene integration into the genomic DNA of transformed jute, 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. The data presented in this report showed considerable advancement in jute transformation and should improve future genetic engineering strategies to be employed for improvement of this very important fibre crop.

Keywords *Bar* gene · Bialaphos · Herbicide resistance · Jute transformation · Particle bombardment · Transgenic plants

Introduction

The genus *Corchorus* consists of more than 50 species (Edmonds 1990). Among them only two species are cultivated, *C. capsularis* and *C. olitorius*, because of the biodegradable ligno-cellulosic fibre material commonly called jute. *C. capsularis*, white jute, is widely cultivated across southeastern Asia and the Indian subcontinent over the gangetic plains of northeastern India. Jute is one of the most important and environmental-friendly natural fibre crop after cotton, and it is used to produce diversified products of economic importance worldwide. Until recently, the biodegradable bast fibre produced from the stem of jute served as an excellent raw material for production of eco-friendly packaging material. With increasing global awareness for a sustainable environment and with expanding diversified applications of jute fibres, the demand for jute is increasing. However, significant progress in genomic and biotechnological study of this crop has yet to be achieved. Jute is a self-pollinating dicot plant that lacks genetic variability, and is susceptible to huge yield loss as a result of biotic and abiotic stresses (Sarker et al. 2008). As agriculture lands decrease gradually because of high population pressure, jute cultivation is being pushed constantly to marginal and sub-marginal lands. Under these circumstances, improved genetic

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Electronic supplementary material The online version of this article (doi:10.1007/s11240-014-0702-2) contains supplementary material, which is available to authorized users.

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variability of jute with higher yield is in dire need. However, the existence of a strong sexual incompatibility barrier (Patel and Datta 1960; Swaminathan et al. 1961) between jute species limits the application of conventional breeding approaches to develop improved varieties possessing characteristics of different species. Plant genetic transformation can serve as an alternative. It can accelerate the development and add source of variability to this crop species. Fine fibre quality is an essential attribute of any jute variety. In this regard, *C. capsularis* (JRC321, Sonali) is better than many other released varieties. This variety can withstand soil moisture stress and is harvested early. Thus, any further improvement of this variety through genetic engineering is a most welcome proposition.

In the absence of suitable plant breeding techniques, particle-gun bombardment and *Agrobacterium*-mediated gene transfer were the preferred methods of choice to introduce gene(s) of interest into crop species (Barampuram and Zhang 2011). These are regarded as powerful tools that can provide a solution to certain constraints that limit crop production and can be used to widen the genetic base of a crop by incorporating specific genes with desirable traits. A reproducible and reliable transformation system would enable insertion of suitable gene(s) of interest into jute cultivars for further improvement. Direct DNA delivery to various explants followed by regeneration from callus has been employed successfully in many crops such as rice (Datta et al. 1990), soybean (Christou et al. 1990), and maize (Gordon-Kamm et al. 1990). Similar procedures will not be suitable for *Corchorus* as most of its cultivars are recalcitrant to in vitro regeneration and genetic manipulation. In vitro regeneration was found to be genotype dependent in *C. capsularis* (Naher et al. 2003) and *C. olitorius* (Khatun et al. 2003). Hence, the focus shifted towards transformation strategies utilizing the organogenic potential of preformed regenerable cotyledonary nodes, cotyledonary petioles, and mature embryos (Ahmed et al. 1989; Seraj et al. 1992; Hossain et al. 1998; Islam et al. 1999; Sarker et al. 2008; Bharadwaj et al. 2011; Amin et al. 2012; Saha et al. 2014). Studies on *Agrobacterium*-mediated genetic transformation have shown that the *gusA* (β-glucuronidase; Gus) gene can be successfully transferred to explants of jute varieties (Hossain et al. 1998, Islam et al. 1999; Bharadwaj et al. 2011; Saha et al. 2014). Somatic embryogenesis of protoplast-derived calli of cultivated jute was reported (Saha and Sen 1992), however, plant regeneration potential was found to be exceedingly low. Efforts continued for regeneration of plants from cotyledonary explants (Saha et al. 1999). One efficient protocol for stable transformation of cultivated jute via microprojectile-mediated particle-gun bombardment of shoot tips of *C. capsularis* was reported (Ghosh et al. 2002). Shoot-tip culture offers the advantage of regenerating plants directly without an intervening callus phase, thereby reducing the chances of somaclonal variation and chromosomal abnormalities. Shoot tips have been successfully employed for efficient biolistic-based genetic transformation of cereals, such as *Zea mays* (Zhong et al. 1996), *Glycine max* (Aragao et al. 2000), and *Sorghum bicolor* (Devi and Sticklen 2003). This could also be beneficial in terms of genotype-independent applicability. Thus, the present study was undertaken for establishing an efficient and reproducible in vitro plant regeneration and genetic transformation system in jute using particle-gun bombardment. The bialaphos resistance gene (*bar*) of *Streptomyces hygroscopicus* that codes for phosphinothricin acetyl transferase, has proven to be a useful selectable and screenable marker (Rathore et al. 1993) for the transformation of crop plants and for the production of herbicide-resistant 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 the transformation and for subsequent selection of the putative transformant lines. We also report on molecular level analysis of the transformants in terms of transgene expression level in quantitative terms.

Materials and methods

Plant materials and seed germination conditions

Seeds of *C. capsularis* (JRC321) were obtained from the Central Research Institute for Jute and Allied Fibres, Indian Council of Agricultural Research, Barrackpore, India. 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 30 °C overnight on seed germination medium (SGM), which contained half-strength plant growth regulator-free Murashige and Skoog (1962) (MS) salts with vitamins, 1 % (v/v) sucrose and 0.8 % (w/v) agar as the gelling agent (Table 1). Seeds were thereafter maintained at 25 °C with 16 h light/8 h dark in a growth chamber (150–200 μE m^{-2} s^{-1}).

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 following the protocols of Murray et al. (1989) and Grantham et al. (1986). It was fused with the CaMV35S promoter at the 5′ end and the nopaline synthase

Table 1 Composition of culture media for *C. capsularis* JRC 321 tissue culture

Medium	Composition
SGM (seed germination medium)	Half strength MS salt (plant growth regulator free) + vitamins + 1 % sucrose (pure) + 0.05 % (w/v) myo-inositol, agar 0.8 % (w/v)
MSRM (multiple shoot regeneration medium)	MS (macro + micro salts) + 1.78 μM BA + 4.92 μM IBA, 3.42 mM glutamine + new organics: 27 μM glycine, 4.06 μM nicotinic acid, 1.18 μM thiamine-HCl, 2.95 μM pyridoxine, 555 μM myoinositol, 0.5 μM Ca-pantothenate, 250 mg/l casamino acid, 1.5 % sucrose-, agar 0.8 % (w/v)
SM (selection medium)	MSRM + bialaphos (2.5 mg/l) + agar 0.8 % (w/v)
RIM (root induction medium)	MSRM + sucrose-2 % + agar 0.8 % (w/v)
PM (potting medium)	½ Soilrite + ½ clay soil (autoclaved)

Plant tissue culture tested agar powder was obtained from Sigma-Aldrich. Soilrite was from Keltech Energies Ltd

(*nos*) gene termination sequence at the 3′ end. The entire chimeric gene cassette was cloned in pUC18 vector at the *Eco*RI/*Hin*dIII sites to generate the chimeric gene construct pBAR, subsequently used for plant transformation (Fig. 1).

Biolistic gene delivery, plant regeneration, and multiple shoot development

Around 35 1-day-old apical meristems were arranged in a circle (1.2–1.5 cm in diameter) on multiple shoot regeneration medium (MSRM) (30 ml per 90 mm × 20 mm petri plate; Table 1) at the centre of each petri plate. The apical meristematic region was isolated from in vitro seedlings grown on SGM, eliminating the cotyledons and the root. 1.5–3 mm long shoot tips were dissected out from apical meristem. Shoot tips were longitudinally sliced and the longer half was used as the explant. 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 1,100 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. Three sets of bombardment experiments were carried out (Table 2). Each experimental set comprised of three independent experiments each comprising three such replications with 35 explants per replication. Thus, three plates were bombarded per individual experiment and approx. Three hundred explants were subjected to particle bombardment in each experimental 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 MSRM for 8 weeks with 4.92 μM indole-3-butyric acid (IBA) and 1.78 μM benzylamino purine (BA). Multiple shoots (30–35) were produced from each explant in about 8 weeks after the bombardment experiment. Transfer to fresh MSRM was carried out at 15 day intervals for these 8 weeks, until multiple shoots developed. Putative transgenic shoots from bombarded explants were separated and placed on selection medium (SM) in 90 mm petri plates containing 2.5 mg/l bialaphos (Table 1) for an additional 3 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. Surviving green shoots (after attaining height of at least 2.5 cm and bearing at least two leaves) were finally transferred to root induction medium (RIM; containing 4.92 μM IBA 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 and replanted in sand:peat:soil (1:1:1) and covered with a transparent sheet for 3–4 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). Similar acclimatization conditions were followed when T_1 plantlets were grown in fields for bialaphos screening.

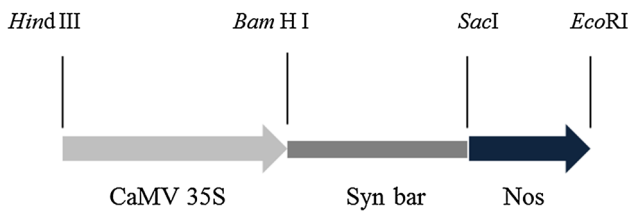

Fig. 1 Schematic map of synthetic *bar* gene cassette (pBAR) carrying the reconstructed *bar* gene under CaMV35S promoter

Isolation of genomic DNA from jute plants

Genomic DNA was isolated from 45-day-old leaves of putative transgenic and non-transgenic jute plants grown in the greenhouse, following the hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). For progeny analysis, 30-day-old field grown progenies were used for genomic DNA isolation. Briefly, tender leaves (approx.

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

Set	Explants bombarded	Bialaphos resistant shoots	PCR^+ shoots	Trans. efficiency (%)
I	308	50	50	16.23
II	290	45	45	15.5
III	280	35	35	12.5
Control	215	0	0	Mean = 14.7

Each set represents combined data of three independent experiments, each of which comprised three replications with 35 explants per replication
Trans. efficiency (%), transformation efficiency in terms of percentage of explants transformed into PCR^+ shoots

5–6 cm long) were ground in a pre-chilled mortar with liquid nitrogen. The ground material was mixed with equal volume of extraction buffer and incubated at 60 °C for 50 min in a water bath with occasional gentle swirling and mixing. The material was extracted with equal volume of 24:1 chloroform–isoamyl alcohol. Precipitation of DNA was done for 5 min at room temperature by adding an equal volume of isopropanol followed by centrifugation at 8,000 rpm for 10 min in a Corex tube (DuPont). The supernatant was discarded and the pellet dissolved in 500 µl of Tris–EDTA (10:1 mM, pH 8). It was then extracted with equal volume phenol–$CHCl_3$ followed by 24:1 $CHCl_3$-isoamyl alcohol and DNA was precipitated with 1/10th volume of 3 M sodium acetate (pH 7) and double volume of chilled ethanol for 30 min at −20 °C. The DNA pellet was washed with 70 % ethanol and dried in vacuum. It was finally dissolved in Tris–EDTA (10:1 mM, pH 8).

Polymerase chain reaction (PCR) of T_0 jute plants

PCR was carried out with synthetic *bar* gene CDS specific primers (SynBar FP and synBar RP, 0.5 µM each; Table 3) using 50–100 ng of template DNA 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).

Southern blot analysis of jute transformants

Southern blot analysis was performed in accordance with the standard protocol described by Ghosh et al. (2002). Briefly, 10-µg aliquots of total genomic DNA were digested with *Eco*RI. *Eco*RI would digest the entire expression cassette only at one end and thus, *EcoRI* digestion would serve as an efficient strategy to ascertain the number of transgene integration site(s) in the genome. The digested DNA was electrophoresed on an 0.8 % agarose gel and blotted onto nylon membrane (Hybond N + , GE Healthcare). Hybridization was carried out separately with α-[^{32}P]-dCTP-labeled synthetic *bar* gene as probe, for 16–18 h at 42 °C. The probes were prepared using the Rediprime kit (GE Healthcare). The highest stringency wash was carried out in 0.1 × SSC, 0.1 % SDS at 60 °C, and autoradiograms were developed by exposing the membrane to Multisensitive (MS) screen (PerkinElmer) inside a Hyper cassette (Amersham Inc.) for 5–30 min depending on radioactive count, following which the MS screen was scanned in Storage Phosphor system (Cyclone Plus, PerkinElmer) at 300 dpi resolution to generate the autoradiographic image.

Isolation of RNA from jute plants

RNA was isolated using a hot-phenol extraction method of Verwoerd et al. (1989). In brief, plant tissue was homogenized in liquid Nitrogen. Then equal volumes of saturated phenol and RNA extraction buffer (pre equilibrated at 85 °C) were added to the crushed plant material and mixed well. The mixture was incubated at 85 °C for 10 min with intermittent vigorous shaking. It was extracted with half volume of $CHCl_3$. The upper aqueous layer was collected and RNA was precipitated with 1/10th volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of chilled ethanol at −70 °C overnight. The RNA pellet was dissolved in RNase-free water, extracted with an equal volume

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	65.7
*Syn bar RP: ATCGGAGCTCTTATCAGATCTCGGTGACGG	Reverse primer for PCR screening of syn *bar* gene	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

*Syn: synthetic

saturated phenol and chloroform, finally precipitated with 1/10th volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of chilled ethanol at −70 °C overnight. The RNA pellet was washed twice with 70 % alcohol, air-dried, and dissolved in deionized formamide.

Northern blot analysis of jute transformants

Northern blot analysis was performed with the standard protocol described by Ghosh et al. (2002). The 555 bp synthetic *bar* gene fragment was used as radiolabeled probe. Briefly, northern analysis was performed on 10 µg of total RNA fractionated under denaturing condition in a 1.2 % agarose gel containing 6 % formaldehyde. RNA was transferred to solid support (Hybond N^+, GE Healthcare) and hybridized with radiolabeled probe similarly as in Southern hybridization. Washing and blot development conditions were similar to Southern hybridization except the wash was performed at 42 °C.

Real-time quantitative PCR (qRT-PCR) analysis of transgenic jute plants

Real-time PCR was carried out in an Eppendorf Realplex^2 Master Cycler using SYBR green based absolute quantification method using the 5 prime kit (Eppendorf) according to Wang et al. (2006) in 20 µl reaction volume. Total RNA was isolated according to the protocol mentioned previously. First strand cDNA was synthesized using primer Syn Bar RT RP using transcriptor 1st strand cDNA synthesis kit (Roche). 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. 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 transgenic jute plants

Total protein was isolated from 100 mg leaf tissue from transgenic and untransformed jute plants using extraction buffer [50 mM Tris pH 8, 3 mM EDTA, 1 % β-mercaptoethanol, 0.1 % bovine serum albumin (BSA), 0.1 % ascorbic acid, 1 % polyvinylpyorrolidone (PVP-40), 1 mM phenylmethanesulfonyl fluoride (PMSF)] and then quantified according to Bradford (1976). Western blotting was carried out using a Lumi-Light^{PLUS}Western Blotting Kit (Roche Molecular Biochemicals), in accordance with the manufacturer's protocol using an affinity-purified rabbit polyclonal antibody against Bar (Sigma, P0374) as the primary antibody (1:1,000 dilution). Mouse monoclonal plant Actin antibody (Sigma, mabGPa) was used as loading control (1:500 dilution). Briefly, an aliquot of 60 µg of extracted protein was loaded onto 15 % SDS-PAGE, electrophoresed and blotted onto Hybond C nylon membrane (Amersham Pharmacia Biotech) by using the wet transfer method. Blocking was carried out in 5 % non-fat dry milk prepared in 1X Tris-buffered saline containing 0.1 % tween-20 (TBST) for 2 h. Incubations with primary and secondary antibody (rabbit or mouse; 1:2,000 dilution) were for 2 and 1 h, respectively. Three stringency washes (15 min each) by 1× TBST were carried out following each antibody incubation. Antibody dilutions were carried out in blocking buffer.

Phosphinothricin acetyl transferase (PAT) activity assay of *bar* gene

Leaf tissue (250 mg) was ground in 500 µl of half-concentration extraction buffer [50 mM Tris–HCl, pH7.5, 2 mM Na_2EDTA, 0.15 mg/ml leupeptine, 0.15 mg/ml PMSF, 0.3 mg/ml BSA]. The extract was enriched for the PAT enzyme by a differential $(NH_4)_2SO_4$ precipitation (30–60 %). The protein pellet was dissolved in 200 µl buffer (50 mM Tris–HCl pH 7.5, 2 mM Na_2EDTA). Protein concentration was measured according to Bradford (1976). The rest of the procedure was as described for chloramphenicol acetyl transferase assay (Shaw 1975). The final concentrations of each component in the assay mix were as follows: Tris.HC1 (100 mM), acetyl-CoA (0.1 mM), and DTNB (0.4 mg/ml). Ten micrograms partially purified protein was used for each reaction. The reaction was started by adding phosphinothricin (PPT) at a final concentration of 0.1 mM. The rate of phosphinothricin (PPT) acetylation was quantified by measuring the free CoA sulfhydryl group coincident with transfer of the acetyl group to PPT. The reaction of the reduced CoA with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) yielded a molar equivalent of free 5-thio-2-nitrobenzoic acid with a molar extinction coefficient of 13,600 at 412 nm. The net change in extinction per min was divided by 13.6 to give the result in µmol/min of PPT-dependent DTNB generated. One unit of PAT activity was defined as 1 µmol PPT acetylated per min at 37 °C. The assay was carried out in 1-mL individual cuvettes and the data was recorded in an UV–visible spectrophotometer (Hitachi Inc.).

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 SGM containing 2.5 mg/l bialaphos for 6 weeks with periodic sub-culture in the same medium every 2 weeks. Regenerated resistant plantlets were transferred to the field after proper acclimatization. The field-grown plants were subjected to another round of screening

using 2.5 mg/l bialaphos spray along with some randomly chosen control non-transgenic plants. The spray was applied for three consecutive weeks and each alternate day (twice a day: morning and afternoon, from all sides) using a standard solo one hand sprayer (Niagara machine Inc.) with proper calibration. The control plants did not survive the stringent bialaphos screening and started withering after 1 week of spray regimen itself. The resistant plants were grown for one more week before they were established in the greenhouse after proper acclimatization as previously described and representative plants (30-day-old) 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

Regeneration through multiple shoot formation

Use of MS medium (macro and micro salts) with 1.78 μM BA and 4.92 μM IBA promoted multiple shoot formation after 8 weeks with transfer to fresh medium every 2 weeks. Indole-3-acetic acid (IAA) (Saha et al. 2014) and naphthalene acetic acid (NAA) (Bharadwaj et al. 2011) were also successfully used for multiple shoot regeneration individually in combination with BA. However, our previous experience with a combination of BA and IBA (Ghosh et al. 2002) prompted us to use IBA as the auxin supplement. One-day-old apical meristems of cultivar JRC-321 yielded more than 30 shoots per explant. Some explants produced fleshy, abnormal shoots. Roots were induced on regenerated shoots after 2 weeks in RIM. The roots appeared normal and continued to grow in culture in the same medium. Rooting was successfully obtained for 90 % of the regenerated shoots.

Transformation by microprojectile bombardment and transgenic plant regeneration

Following microprojectile bombardment, regenerating explants were transferred to MSRM containing 2.5 mg/l bialaphos for 2 weeks for screening. After 2 weeks of screening, surviving green shoots (Fig. 2a, indicated by arrow) along with some yellowish-white or white shoots were transferred to fresh MSRM containing 2.5 mg/l bialaphos and further screened for another 2 weeks. Most of the shoots turned white or yellowish-white indicating elimination of the untransformed tissues in the stringent selection process. Finally, green shoots and partial white shoots were separated out and subjected to a final round of selection for another 2 weeks in the MSRM containing 2.5 mg/l bialaphos. Elimination of all chimeric untransformed shoots was accomplished by repeated separation. Green shoots (Fig. 2b) were subsequently cultured in RIM for root development for 2 weeks (Fig. 2c). The putative transformed plantlets, after selection, were hardened and finally grown under greenhouse conditions to maturity (Fig. 2d). Ten transgenic plants from each of the three sets of experiments were grown to maturity after regeneration and stringent selection. The plants showed no apparent morphological change and were fully fertile (Fig. 2d, e).

PCR screening and Southern blot analysis of the T_0 transformant lines

Three independent sets of biolistic bombardment experiments were carried out and Table 2 summarizes the results of these experiments. PCR was carried out to screen for putative transformants after bialaphos screening. Among them, five putative transformants (two each from first two sets and one from the third) were chosen for further molecular level analysis. All of them contained the full-length *bar* gene (Fig. 3). No amplification was visible in the untransformed control line. The five plant lines (#JT1–JT5) were subjected to southern blot analysis to confirm the integration of the transgene and the number of transgene integration sites. Three plant lines (JT2, JT3, and JT4) were found to have single site of insertion whereas two (JT1 and JT5) had two sites of insertion of the *bar* gene in T_0 progeny (Fig. 4a). All bands were of different size indicating different genomic sites of 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 the T_0 progenies of primary transformant lines

RNA level

Northern blot analysis The T_0 plants were subjected to northern blot analysis with the synthetic *bar* gene CDS as the radiolabeled probe. Among the five plants, three (JT2, JT3 and JT4) revealed a relatively high level of generation of the transcripts and the level of transcription varied between individual plant lines (Fig. 4b). The plant lines showing a higher level of expression of the *bar* gene, were found to contain a single site of transgene integration. No band could be detected in the untransformed control line.

Real time qPCR analysis To obtain a quantitative estimate of the level of transcript generation, qPCR was

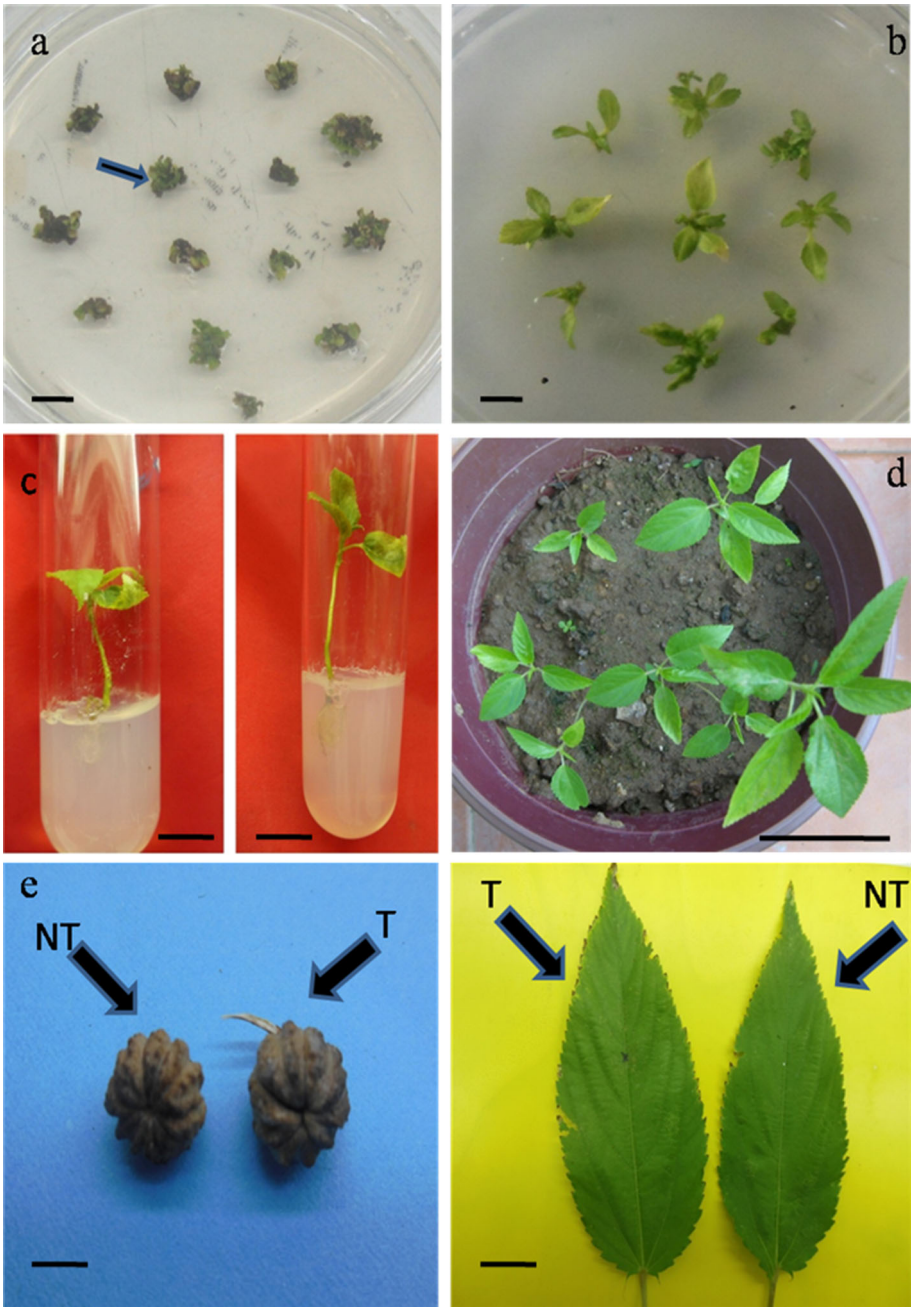

Fig. 2 Different stages of tissue culture to generate mature jute transgenics. **a** Selection of shoots for bialaphos resistance (2.5 mg/l) after culture for 2 weeks in MSRM. *Arrow* indicates the tissues that can survive the selection pressure (*bar* 0.5 cm). **b** Surviving shoots with axillary branches after stringent bialaphos screening (2.5 mg/l) for 6 weeks (*bar* 0.5 cm). **c** Generation of root for putative transformants in rooting medium (*bar* 0.75 cm). **d** Surviving plantlets growing under greenhouse condition to maturity (*bar* 2.5 cm). **e** Fruits (*left panel*) and leaves (*right panel*) of the transformed (*T*) and non-transformed (*NT*) jute plants (*bar* 0.75 cm)

performed using *bar* gene CDS specific primers (Syn Bar RTFP and RTRP, Table 3) which amplified a product of 150 bp. An absolute quantification approach was followed using a standard curve (Supplementary Fig. 1) generated from the serial dilution of the gene expression cassette harboring synthetic *bar* gene CDS. The generation level of the *bar* gene transcript could be detected in terms of absolute value of mRNA copy number from the standard curve generated (Fig. 4c, Table 4). No amplification was detected in the untransformed control line. The T_0 progenies with single site of transgene integration (JT2, JT3, and JT4) were found to contain a higher level of transgene expression. This result was in accordance with the northern analysis.

Protein level

Western blot analysis of the T_0 transformants To confirm the integration and expression of the full-length *bar* gene, the extent of generation of translation product in each of the individual transgenic lines was monitored by western blot analysis (Fig. 5a). The transgenic lines showed distinct

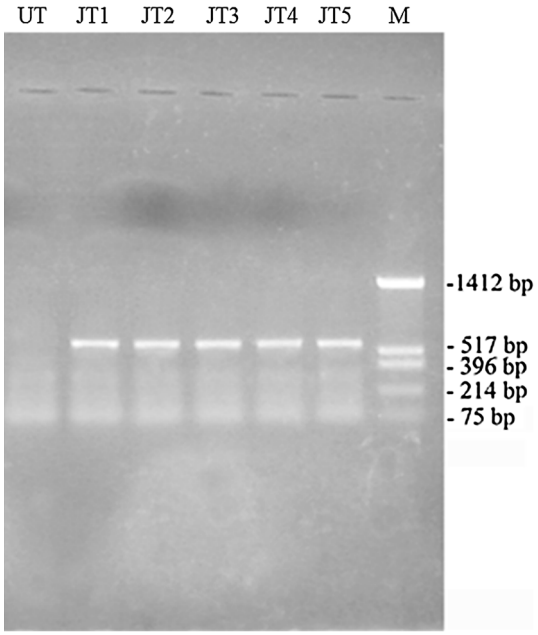

Fig. 3 PCR of the *bar* transgene in T_0 putative jute transformants. UT: non-transformed control showing no amplification of *bar* gene, JT1–JT5: jute transformants JT1–JT5 showing amplification of the synthetic *bar* gene. Lane M: pUC18/*Hin*fI marker

band in the 21 kDa region. No detectable band could be found in the untransformed control line. The expression level was found to be higher for transgenic lines (JT2–JT4) with a single site of transgene integration. In each case equal loading of the protein could be detected by plant *actin* antibody.

Activity assay of the bar gene in the T_0 transgenic lines Spectrophotometric PAT activity assays of the T_0 transgenic plants were carried out in order to monitor the quantitative estimate of the protein level of the transgene. The activity data was calculated 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 data (Fig. 5b) correlated well with the transcript level of the *bar* transgene as obtained by real time qPCR. Plants with high PAT activities (Table 5) were found to contain a high level of transcript generation of the *bar* gene and 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.

Analysis of stable inheritance of the bar transgene in T_1 generation after selection using bialaphos spray Seeds were collected from the T_0 progenies and screened by germination in 2.5 mg/l bialaphos containing SGM. To maintain the selection pressure, the surviving seedlings were sub-cultured in the same medium containing the same concentration of bialaphos every 2 weeks. The ratio of surviving: etiolated plantlet germinating from the seeds was scored after 6 weeks and it was found to be 3:1 in each case, indicating a Mendelian mode of inheritance for the monohybrid cross (Table 6). The resistant plantlets were subjected to a second round of screening using 2.5 mg/l bialaphos spray after transfer to the field (Fig. 6) under acclimatized condition. The resistant plants survived the screening confirming the stable inheritance of the *bar* transgene, whereas the control non-transgenic plants withered even after 1 week of stringent spray regimen and could not survive the stringent selection pressure. The surviving plantlets were finally established in the greenhouse and grown to maturity (Fig. 7). Representative T_1 plants from each category were analyzed further by Southern hybridization (Fig. 8). All the plants revealed a similar transgene integration pattern as the T_0 plant. This also indicated that the multiple insertion events in plant lines JT1 and JT5 was on the same chromosome and thus these segregated with each other.

Discussion

Plant genetic engineering has become one of the most important molecular tools in the modern breeding of crops. Over the last decade, significant progress has been made in the development of new and efficient transformation methods in plants. Despite a variety of available DNA delivery methods, *Agrobacterium*- and biolistic-mediated transformation remain the two predominantly employed approaches. Microprojectile bombardment is one of the direct gene transfer methods for development of transgenics. This method was developed in the 1980s to genetically engineer plants recalcitrant to transformation with *Agrobacterium*. Subsequently, the technique has been widely used to produce transgenic plants in a wide range of plant species including rice, corn, wheat, sugarcane, cotton, and soybean among many others (Christou 1995; Breitler et al. 2002). However, a stable transformation system through direct gene delivery to jute remained elusive, until our group reported such a protocol (Ghosh et al. 2002). In the present study, we have reported an efficient and reproducible method of particle bombardment-mediated genetic transformation of *C. capsularis* cv. JRC 321 using the apical meristem as an explant. This is more relevant for a plant such as *Corchorus*, characterized by its typical recalcitrance to tissue culture. Direct plant regeneration utilizes the morphogenic potential of the apical meristem without any intervening steps of dedifferentiation and redifferentiation (Sticklen and Oraby 2005). Particle bombardment has the ability to transfer foreign DNA into

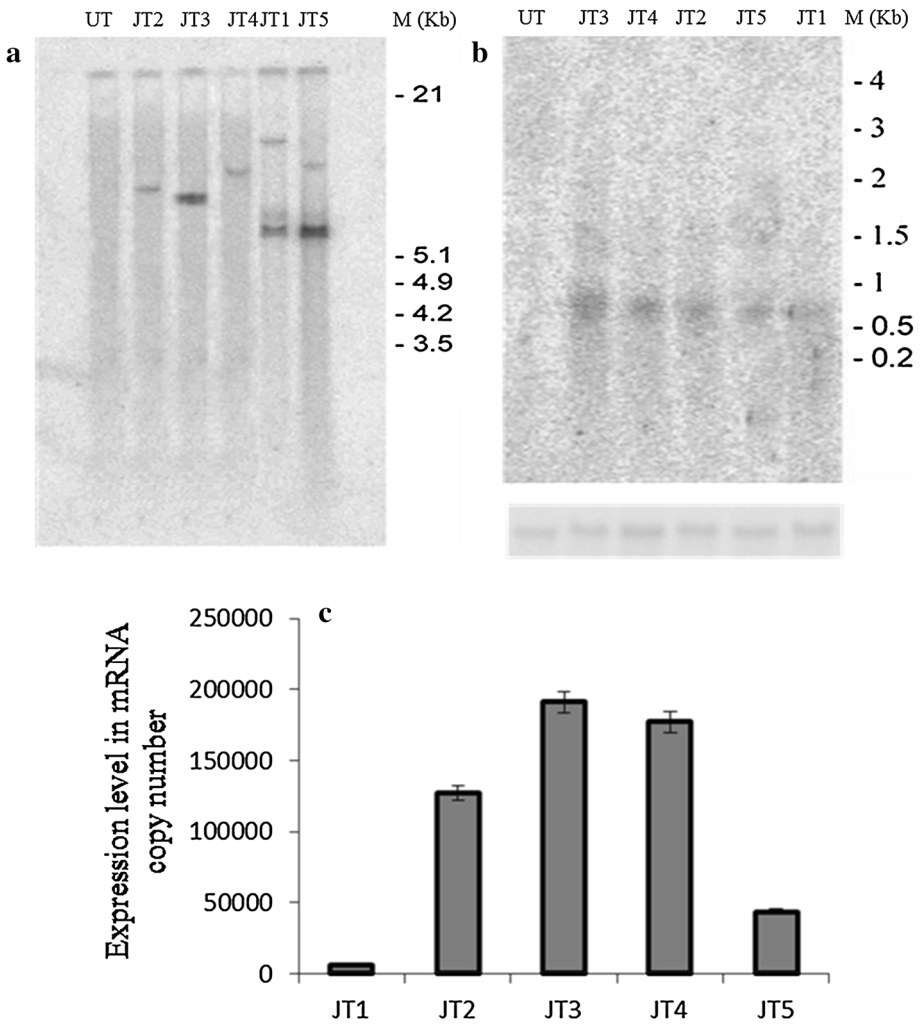

Fig. 4 Analysis of primary transformant jute lines. **a** Southern blot analysis of T_0 transformant lines using synthetic *bar* gene as radiolabeled probe. UT: untransformed control line. JT2–JT4: hybridization pattern for jute transformants JT2–JT4. JT1 and JT5: hybridization pattern for jute transformants JT1 and JT5, respectively. Lane M: *Eco*RI/*Hin*dIII digested λ DNA marker. **b** Northern blot analysis of T_0 transformant lines using *bar* gene as radiolabeled probe. (*Upper Panel*) UT: untransformed control, JT3–JT1: lanes showing *bar* gene transcript profile of various jute transgenics JT3, JT4, JT2, JT5 and JT1, respectively (*Lower panel*) 18S rRNA band from each RNA sample representing equal loading of RNA in each case. The gel was stained with ethidium bromide and image was taken before transfer onto nitrocellulose membrane. **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 mRNA copy number. Results expressed as mean ± SD, for triplicate readings

any cell or tissue type whose cell wall and plasma membrane can be penetrated. Another advantage of particle bombardment is the possible expression of multiple transgenes in the target tissue, which can be achieved by fusion of genes within the same plasmid that is then bombarded into the target tissues. In recent years, multiple, independent gene expression cassettes have been successfully transferred using particle bombardment in both monocot as well as dicot plants such as wheat, rice, and soybean (Campbell et al. 2000; Agrawal et al. 2005; Schmidt et al. 2008) thereby enhancing agronomic traits. The typical recalcitrance of jute to conventional tissue culture as well as our past progress in microprojectile technology (Ghosh et al. 2002) had prompted us to make a convenient and reproducible method of jute transformation. The present study showed reproducibility of our method as the results of three independent set of experiments suggest (Table 2). Frequency of multiple shoot formation and plant regeneration was high. From the range of 280–308 starting explants, we could generate up to 50 shoots after stringent screening. There have been very few reports of stable transformation of jute via *Agrobacterium*-mediated gene delivery. In one of the most recent and comprehensive study (Saha et al. 2014), a mean transformation efficiency of 4.09 % was reported. In our previous report of biolistic-mediated gene delivery to jute (Ghosh et al. 2002), the average transformation frequency was found to be 11.4 % based on the available data. Our present report showed advancement in this respect with an average transformation efficiency of 14.7 % (Table 2). There are limited reports on jute transformation, either by *Agrobacterium*- or biolistic-mediated gene delivery. Among them, a recent report by Sajib et al. (2008) showed some significant advances by using a tissue culture independent *Agrobacterium*-mediated *in planta* transformation of *C. olitorius*. The mean transformation efficiency was determined to be 27.23 % based on selection assay of germinating T_1 seeds. Our method was based on direct gene delivery to explant followed by tissue culture mediated regeneration and generation of stable transformants. Transformation efficiency is based on

Table 4 Absolute quantification of mRNA level of synthetic *bar* gene in transgenic jute plants by real time qPCR analysis

Sample	Mean CtSYBR	Amount SYBR (in mRNA Copies)	Target SYBR
Cassette (1:1,000)	9.79	3.37E+10	Bar
JT1	32.16	5,842	Bar
WOT	–	–	Bar
Cassette (1:2,000)	11	3.37E+09	Bar
JT2	27.55	1.27E+05	Bar
Cassette (1:4,000)	15.51	3.37E+08	Bar
JT3	26.94	1.91E+05	Bar
Cassette (1:8,000)	19.77	3.37E+07	Bar
JT4	27.05	1.77E+05	Bar
JT5	29.14	43,655	Bar
Control	–	–	Bar
Threshold detection parameters			
Threshold		66 (adjusted manually)	
Baseline settings		Auto	
Drift Correction		OFF	
Standard curve parameters			
Slope		−3.448	
Y-intercept		45.14	
Efficiency		0.95	
R^2		0.953	

Different dilutions of the expression cassette used for the standard curve preparation were indicated in the table. The C_t values indicate the mean value from triplicate readings in each case

WOT, without template (negative control); *Control*, untransformed control line

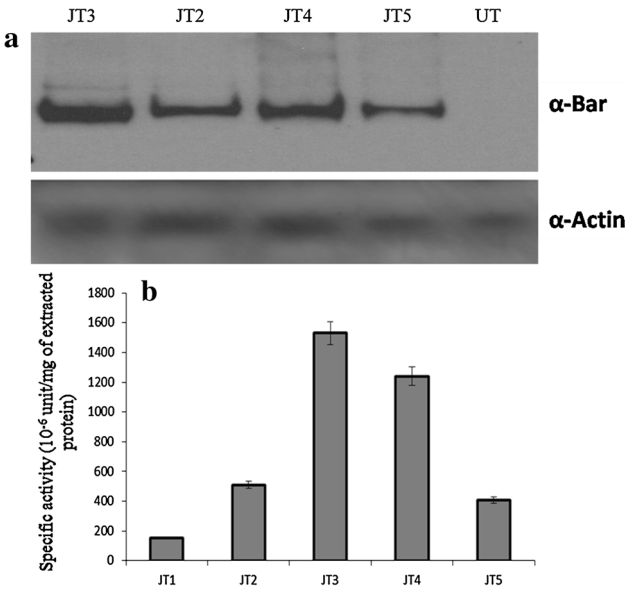

Fig. 5 Analysis of the extent of translated product of the *bar* synthetic transgene and its activity. **a** (*Upper panel*) JT3–JT5: lanes showing *bar* gene translated product in jute transgenics JT3, JT2, JT4 and JT5, respectively; UT: untransformed control; (lower panel) equal loading of proteins shown by stripping and probing the same blot with anti-actin antibody. **b** In vitro PAT activity assay of the transgenic jute 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

Table 5 Phosphinothricin acetyl transferase (PAT) activity in crude leaf extracts of transgenic jute plants

Plant ID	Specific activity (10^{-6} unit/mg of extracted protein)
Control	N.D
JT1	154
JT2	510
JT3	1,530
JT4	1,240
JT5	407

N.D, not detected; *Unit*, µmol of PPT acetylated per min at 37 °C

frequency of explants transformed. Also, transformation experiment was carried out for an economically viable, *C. capsularis* and stable transformation was substantiated by molecular evidences in DNA, RNA and protein level. The transformation frequency (14.7 %) was also significant when compared to various monocot and dicot plants, where foreign gene(s) could be transferred by particle-bombardment. Among the monocots the transformation frequency ranges 79.5 % as in rice (Barampuram and Zhang 2011). However, 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). Jute is a cheap

Table 6 Segregation analysis of *bar* gene in T_1 progeny of the transgenic jute lines transformed with pBAR

Plant ID	Total seeds tested	Bialaphos$^+$	Bialaphos$^-$	bar$^+$:bar$^-$	Segregation ratio	χ^2
JT1	55	40	15	2.66	3:1	0.148
JT2	62	45	17	2.64	3:1	0.193
JT3	58	42	16	2.62	3:1	0.205
JT4	52	38	14	2.71	3:1	0.102
JT5	46	34	12	2.83	3:1	0.224

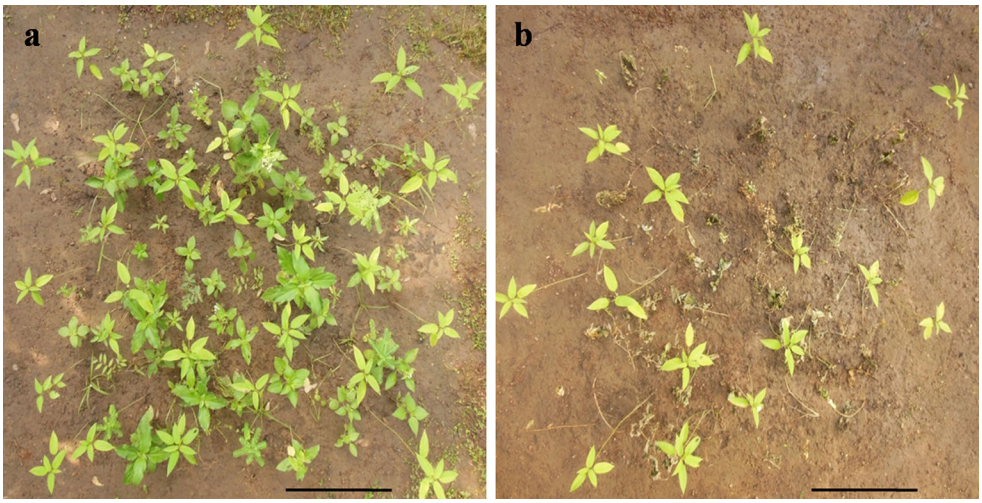

Fig. 6 Representation of small scale field level bialaphos screening of T_1 progenies of transgenic jute plants. **a** Acclimatized T_1 jute plants in field along with a number of non transgenic control plants. Weed flora can be seen in the field (*bar* 2 cm). **b** Surviving T_1 jute plants after extensive bialaphos spray (2.5 mg/l) for 3 weeks (*bar* 2 cm). All control plants withered and did not survive. The weed flora of the field was under control

Fig. 7 Surviving T_1 progenies of the transgenic jute plants established in the greenhouse after bialaphos spray at 2.5 mg/l

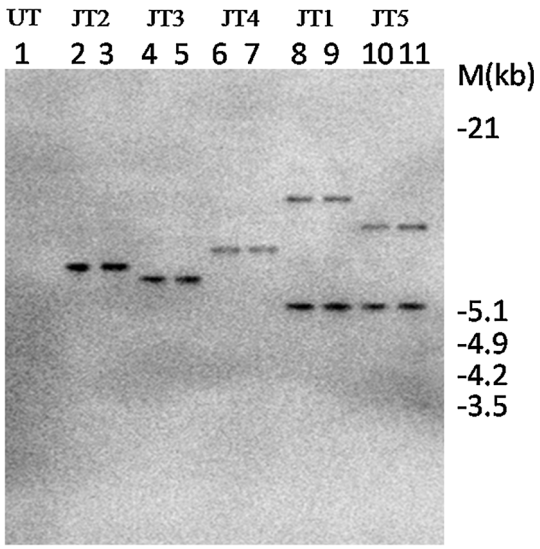

Fig. 8 Southern blot analysis of two randomly chosen T_1 progenies from each of the transgenic jute plants. Lane 1: untransformed control; lanes 2, 3: JT 2; lanes 4, 5: JT 3; lanes 6, 7: JT 4; lanes 8, 9: JT 1; lanes 10, 11: JT 5; lane M: *Eco*RI/*Hin*dIII digested λ DNA marker. Synthetic *bar* gene CDS was used as radiolabeled probe

source of good quality bast fibre, and genetic improvement through efficient plant transformation can produce various new possibilities. Considering the overall recalcitrant nature of jute, our results showed a significant step forward in this regard.

Efficient selection of transformed cells and tissues are crucial for any plant genetic transformation protocol. The choice of selectable marker to produce transgenic plants at high efficiency is rather limited. 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 jute also, as evident from some recent reports (Bharadwaj et al. 2011; Amin et al. 2012; Saha et al. 2014). 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 jute plants. This selection marker had been successfully used in transformation of many crop species including rice (Rathore et al. 1993) and maize (Gordon-Kamm et al. 1990). However, for jute there has been no report of its use except in our earlier study (Ghosh et al. 2002). The development of *bar* gene as selection marker is important agronomically because it can provide resistance to herbicides such as Basta or Ignite (or Liberty). Optimization of the dose for selection pressure is important in any transgenic experiment, as a suboptimal dose results in high frequency of escapes. Unnecessarily high doses not only kill untransformed tissues, but also inhibit growth of transformed cells, leading to delay in the regeneration process (Wilmink and Dons 1993). Based on kill-curve experiments, selection against bialaphos at a dose of 2 mg/l was found to be best with three repeated passages with each passage lasting for 15 days. However, with the newly synthesized codon-optimized *bar* gene with plant optimized codons, selection stringency was increased up to 2.5 mg/l, which in turn helped us to eliminate the non-transformed shoots. In this study, we have efficiently devised a strategy to use 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 all the 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 the transgenic events analyzed. Integration of *bar* into the genome of the transformed plants and inheritance and expression of the transgene by their progenies was confirmed by Southern blot analysis for T_0 and T_1 plants and seed germination test alongside control at 2.5 mg/l bialaphos supplemented medium. All of the transgenic plants developed in this experiment were fertile and have stably expressed the transgenes. Segregation of *bar* into the T_1 progeny plants have occurred following Mendelian inheritance for a single dominant locus, i.e. 3:1. Moreover, the progeny plants inherited the transgene without exhibiting any morphological aberrations. All these point towards a reproducible and stringent selection process, which was made feasible by the use of the synthetic *bar* gene. Our study had another significant observation. The hot and humid climate coupled with intermittent rainfall during the jute-growing season encourages weed growth resulting in severe crop-weed competition (Saraswat 1999); yield losses may be up to 75–80 % (Sahoo and Saraswat 1988), implying the need for judicious weed management. Grasses constitute the dominant weed flora in jute fields and its management using herbicides such as Trifluralin, Fenoxaprop-p-ethyl and Quizalofop ethyl was possible as shown by some earlier reports (Sarkar et al. 2005; Sarkar 2006). Routine screening approach was followed for T_1 plants with inherited synthetic *bar* gene using bialaphos sprayed at a highly stringent dose of 2.5 mg/l. This could 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, but it can also help in weed management at the field level. Our preliminary small-scale observations suggest low to negligible level of weed infestation after the herbicide spray for consecutive 3 weeks (Fig. 6), however, further large scale field level analyses are required before it can be adopted as a suitable method of weed control and progeny selection.

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. Convincing evidence for gene transformation and stable inheritance of the transgene to the next progeny should be accompanied by expression level analysis that allows us to select out the transgenic event most suitable for our purpose. Our study, to the best of our knowledge, was a significant progress in particle bombardment transformation of jute, as detailed expression analysis of the *bar* transgene was carried out in both RNA and protein level in qualitative (northern blot and western blot) and quantitative (qPCR and enzyme activity assay) terms. All the results were found to be internally consistent with each other. Among the five transgenic plants which were subjected to molecular analysis, three plants (JT2–JT4) showed higher level of transgene expression level as compared to other lines. All the plant lines with higher level of transgene expression were single-copy transformants. This was in agreement with some other earlier reports (DeBuck et al. 2001, 2004). The variation in the transgene expression

level among individual transformants could be explained in terms of "position effect" (Matzke and Matzke 1998).

Although biolistic gene delivery could be a preferred method of choice for recalcitrant plants such as jute, this technology is limited because of several drawbacks, such as the integration of multiple copies of the desired transgene, in addition to superfluous DNA sequences that were associated with the plasmid vector. Multicopy integrations and superfluous DNA can lead to silencing of the gene of interest in the transformed plant. This problem 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). Our next line of research will be directed towards making this microprojectile bombardment-mediated jute transformation more accurate and handy for conferring desired traits into jute cultivars for agronomic improvement.

Acknowledgments The authors extend their sincere appreciation to Shila Bhattacharjee for her extensive assistance with the tissue culture, Sudarshan Maity and Uttam Dogra for maintenance and rearing of transgenic jute lines, and Meghnath Prasad for assistance in preparing this manuscript. Finally, the authors would like to extend their sincere thanks and gratitude to the distinguished associate editor for her extensive and incisive comments to improve the clarity of the manuscript. The authors are also thankful to the distinguished reviewers for their critical comments which helped to improve the quality of the manuscript.

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

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