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ORIGINAL PAPER

Tissue culture independent transformation for *Corchorus olitorius*

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Abstract In vitro regeneration is difficult for species of *Corchorus* and several transformation attempts based on tissue culture have failed. We describe a successful transformation protocol for *C. olitorius* using a technique independent of tissue culture. Primary growth of a plant is brought about from the activities of meristematic region and subsequent division and differentiation of the derivative cells into the tissues and organs of the plant. The principal behind the current study was that if some dividing cells in the meristematic region become transformed by *Agrobacterium* and the cells retain the capacity for cell division, then the transgene(s) will be transferred to progeny cells, and if some of these cells later differentiate to form floral buds, seeds generated from these buds will inherit the transgene(s) to next generation. In this study young jute plants were transformed at shoot apical meristematic region using *Agrobacterium tumefaciens*. Heritable transmission of the transgene to progeny from genetically modified plants was confirmed by *gus* gene expression by histochemical analysis, selection on kanamycin containing medium, RT-PCR, PCR amplification and Southern hybridization. Efficiency of transformation was determined by selection on medium containing kanamycin, and inheritance of transgene to T_2 generation plants.

Keywords Jute · Transformation · *Agrobacterium tumefaciens* · *In planta*

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Introduction

Jute is a versatile and environment-friendly natural fibre that generates diversified value-added products. It falls into the bast fibre category along with kenaf, industrial hemp, ramie and banana fibres. Though there are over 100 species of *Corchorus*, only two, *Corchorus capsularis* L. and *C. olitorius* L., are cultivated widely (Sarker and Al-Amin 2007). These species of jute are self-pollinating and contain very limited genetic variability with respect to (i) adaptability to different agronomic environments, (ii) fibre quality, (iii) fibre yield, and (iv) susceptibility to diseases and pests (Basu et al. 2004). For example,

Corchorus capsularis is comparatively more resistant to flood and drought but slightly more susceptible to diseases and pests. It provides white commercial fibre which is not as strong as the fibres of the other cultivated species. *C. olitorius* is relatively tolerant to diseases and pests and produces the stronger commercial fibre (Roy et al. 2006). Therefore, a combination of useful characters of these two species in a single genotype is highly desirable.

Attempts to cross the species have been made with the aim of combining the desirable agronomic characteristics of both species, but all have been unsuccessful (Finlow 1917, 1921, 1923; Datta et al. 1960; Patel and Datta 1960; Islam and Rashid 1961; Chaudhuri and Mia 1961; Isalm 1964; Hoque et al. 1988). Though there a number of reports on in vitro regeneration of jute (Islam et al. 1982; Rahman et al. 1985; Seraj et al. 1992; Khatun et al. 1993; Hossain et al. 1994; Saha et al. 1999), the regeneration protocols are difficult to reproduce (Sarker and Al-Amin 2007). Regeneration in vitro is hard to achieve with *Corchorus capsularis*, and virtually impossible, with *C. olitorius*. Whole plant regeneration following tissue culture and transformation was also not very successful with jute (Sarker and Al-Amin 2007).

There is only one established transformation protocol for jute (Ghosh et al. 2002) involving *Corchorus capsularis*. However, this study involved costly particle bombardment technique and was subjected to tissue culture. Therefore, the aim of this study was to establish tissue culture independent *Agrobacterium tumefaciens* mediated transformation protocol for *C. olitorius*.

A number of laboratories have pursued plant transformation methods that avoid tissue culture or regeneration and multiple plant species have been reported to be successfully transformed using *Agrobacterium* mediated *in planta* approaches (Bent 2000) opening up the possibility of widespread application to crop improvement. *Agrobacterium* or tungsten particles have been used in a number of species to transform cells in or around the apical meristems that are subsequently allowed to grow into plants and produce seeds. Inoculation of naked DNA into ovaries has also been reported to produce transformed progeny. Electroporation-mediated gene transfer into intact meristems *in planta* and a variety of pollen transformation procedures have also been reported (Bent 2000). Techniques based on similar principles have been applied to other plant species, including Arabidopsis (Clough and Bent 1998), *Triticum aestivum* L. (Supartana et al. 2006), *Fagopyrum esculentum* (Kojima et al. 2000), *Morus alba* L. (Ping et al. 2003), *Hibiscus cannabinus* var. *aokawa* No. 3 (Kojima et al. 2004), *Oryza sativa* L. (Supartana et al. 2005), *Notocactus scopa* cv. *Soonjung* (Seol et al. 2008) and *Hibiscus sabdariffa* L. (Gassama-Dia et al. 2004).

Materials and methods

Source of plants

Seeds of *Corchorus olitorius* var. O-72, a popular and high-yielding jute variety cultivated in Bangladesh, were obtained from the Gene Bank of Bangladesh Jute Research Institute (BJRI).

Agrobacterium tumefaciens strain and binary vector

Agrobactarium tumefaciens LBA4404 harbouring the binary vector pBI121 (GenBank accession number: AF485783) containing selectable marker *nptII* gene and screenable marker *gus* gene were grown on YMB (Yeast Mannitol Broth) medium (1.0% Mannitol, 0.04% Yeast extract, 0.01% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4) containing kanamycin as the selective agent at 200 rpm in a shaker at 28°C for overnight. Bacterial concentration was determined by spectrophotometer (SPECORD® 50) using the software WinASPECT® at a wavelength of 600 nm. *Agrobacterium* from these cultures were used for infection of shoot apical meristematic region of young jute plants.

Transformation of jute shoot tips

The shoot tips of young jute plants (15–20 cm in height) were injured with a fine needle. After an hour, the injured regions were infected with a few drops of *A. tumefaciens* suspension in YMB medium with an O.D. of 0.8. This was followed by a second infection at the same regions an hour later. The infected plants were placed in dark for 3 days. Then plants were allowed to grow under normal conditions in light.

Assessment of transformation

For preliminary assessment of transformation, GUS activity was assayed with newly formed leaves of T_0 plants above the infected regions 3 weeks after infection.

Seeds from mature plants were collected and germinated. Leaves from the T_1 plants were tested for GUS activity. PCR was also performed with DNA of these plants using *gus* specific primers. Plants with positive results were identified and next generation seeds (T_2) were collected from them.

Transgenic seedlings (T_2) were confirmed by four lines of evidence: (1) *gus* gene expression was confirmed by RT-PCR, (2) activity of *gus* gene product was confirmed by GUS assay, (3) presence of *gus* gene(s) in the genome of these plants was confirmed by PCR, and (4) southern hybridization.

Histochemical GUS assay

Leaves or seedlings to be tested for GUS activity were washed in 70% ethanol for 30 s followed by three washes with ddH_2O under a laminar flow hood. The plant parts were placed in fixation solution (750 µl of formaldehyde, 2 ml of 0.5 M MES solution at pH 5.6 and 5.46 g of mannitol in 100 ml of ddH_2O) and vacuum infiltrated for 20 min. The tissues were washed thoroughly three times with 50 mM phosphate buffer (pH 7.0; 50 mM solution of $NaH_2PO_4 \cdot 2H_2O$ and 50 mM solution of $Na_2HPO_4 \cdot 2H_2O$). X-gluc solution (0.1% X-gluc and 1% dimethyl formamide in 50 mM phosphate buffer) enough to immerse the leaves/seedlings was added to each 1.5 ml tube. These were then incubated at 37°C. Samples showing blue colour were washed with 70% ethanol solution for at least 24 h. Non-transformed jute plants were used as negative controls and transformed tobacco plants containing *gus* gene were used as positive control (as transformed jute plants containing *gus* gene were not available).

gus specific PCR

DNA was extracted from both transgenic jute plants of the species *C. olitorius* var. O-72 and non-transformed control plants of same species following a procedure modified from Doyle and Doyle (1990) using non-ionic detergent cetyl trimethyl ammonium bromide (CTAB). pBI121 plasmid containing *gus* gene was isolated from *A. tumefaciens* strain LBA4404 for use as positive control. The free software Primer3 (http://frodo.wi.mit.edu/) was used for designing primers (Forward, 5′-ACCTCTCTTTAGGCATTGGTTTC-3′ and Reverse, 5′-GCACACTGATACTCTTCACTCCAC-3′). *gus* gene specific sequence (511 bps) was amplified using a thermal cycler (GeneAmp^R PCR System 9700, Applied Biosystems) following thermal cycling profile: initial denaturation at 94°C for 5 min, 40 cycles, each for 1 min at 94°C (denaturation), 1 min at 60°C (annealing), 1 min at 72°C (elongation) and a final elongation at 72°C for 5 min, followed by cooling at 4°C for infinite period. Amplified products were separated on a 1.5% agarose gel and bands were visualized by ethidium bromide staining.

gus-specific RT-PCR

RNA was isolated from 1.0 g of 3-day-old frozen seedlings using TRIZOL reagent®. Seedlings were ground to powder in liquid nitrogen using mortar and pestle and 1 ml TRIZOL reagent® was added to the ground tissue, homogenized thoroughly and incubated at room temperature for 10 min. The samples were centrifuged at 12,000 × *g* for 10 min at 4°C and supernatant (aqueous phase) was transferred to a new tube and 0.2 ml chloroform was added and mixed thoroughly for 15 s and was incubated at room temperature for 3 min. The sample was again centrifuged at 12,000 × *g* for 15 min at 4°C and supernatant was transferred (aqueous phase) to a new tube. About 0.25 ml of iso-propanol and 0.25 ml of 2 M NaCl was be added to this supernatant and was mixed by inversion before incubating for 10 min at room temperature. It was then centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatant (aqueous phase) was removed and the pellet was washed twice with 75% (v/v) ethanol. The pellet was air-dried and re-suspended in DEPC-treated nanopure water. Isolated RNA was stored at −80°C.

First strand cDNA was prepared from mRNA following the manufacturer's protocol for SuperScript™ III Reverse Transcriptase (Invitrogen). *gus* specific PCR amplification was carried out using these cDNAs as templates. Amplified products were separated on a 1.5% agarose gel and visualized after ethidium bromide staining.

gus specific Southern hybridization

gus specific Southern hybridization was performed following Sambrook and Russell (2001). G*us* sequence in pBI121 was amplified by PCR and labelled with digoxigenin-dUTP following manufacturer's protocol (DIG DNA labeling Kit, Roche). *gus* gene specific sequence blotted on nylon membrane was detected following an immunologic assay according to manufacturer's protocol (DIG Nucleic Acid Detection Kit, Roche).

Determination of efficiency

Seeds from T_0 plants were grown on MS medium containing kanamycin (0.1 mg/ml) as a selective agent for transgenic plants. For comparison seeds from non-transgenic control plants from the same species were grown on the same medium. To avoid ambiguity regarding seed vigour related to germination, seeds from both transformed and non-transformed plants were also grown without antibiotic selection. Efficiency of the transformation process was calculated by dividing the number of seeds that germinated in selection medium with the total number of seeds that were used in the selection assay.

Results

All the wounded plants (T_0) survived and showed normal growth upon infection with *A. tumefaciens* (Fig. 1a). However, at the infection sites the stems became a little thicker than the surrounding regions (Fig. 1b) and some of the plants also showed branching at the site of infection.

New leaves above the infected sites 3 weeks of infection showed GUS activity in some cells (Fig. 1c). Intensity of blue colour (due to GUS activity) was in some cases comparable, or darker than the positive transgenic control tobacco plants (Fig. 1d). Seeds collected from these plants showed normal growth. Leaves from the T_1 generation showed GUS activity all over the leaves (Fig. 1e). About 77 out of 101 T_2 seedlings tested showed positive GUS activity (Fig. 1g).

DNA was isolated from the leaves of the second generation transgenic plants (T_1) that were GUS positive and PCR was performed using *gus* gene specific primers. A band of specific size (511 bps) was amplified only from GUS positive plants. But no band was amplified from non-transformed negative control plants of the same species.

When PCR was carried out for transgenic seedlings an expected size fragment of 511 bps was amplified (Fig. 2a). No amplification was observed from DNA of non-transformed jute plant of the same species. The same size amplified product was detected from *Agrobacterium* plasmid, pBI121, DNA which was used as a positive control.

RNA from T_2 seedlings was isolated and RT-PCR was performed with *gus* specific primers. The specific band for the *gus* gene was found only with transformed seedlings, but not for non-transformed seedlings (Fig. 2b). To confirm that this amplification did not occur due to DNA contamination of the RNA sample, simple PCR was performed using the RNA sample with *gus* specific primers. However, no amplification was observed.

Southern hybridization with *gus* specific probe gave specific signals for *EcoRI* digested genomic DNA from transformed (T_2) plants, pBI121 and *gus* specific PCR product from transformed (T_2) jute plants, whereas non-transformed plants did not give any signal (Fig. 3).

Efficiency of transformation process was determined following selection on kanamycin containing medium (Table 1). Efficiency varied between 13.11% and 48% for different transformation events with an average of 27.23 ± 7.01. None of the seeds from non-transgenic control plants showed germination and growth in selection medium. However, for both transgenic and non-transgenic plants similar growth rate was observed in non-selection medium attesting to the viability of the seeds tested.

Discussion

In this transformation experiment with *C. olitorius*, the apical meristematic regions of young jute plants were infected with *Agrobacterium tumefaciens* harbouring pBI121. Some of the seeds collected from these plants showed stable transformation. Most of the seeds (T_2) obtained from T_1 generation transgenic plants were also transgenic.

Five different lines of evidence attest to the success of the transformation process: (i)

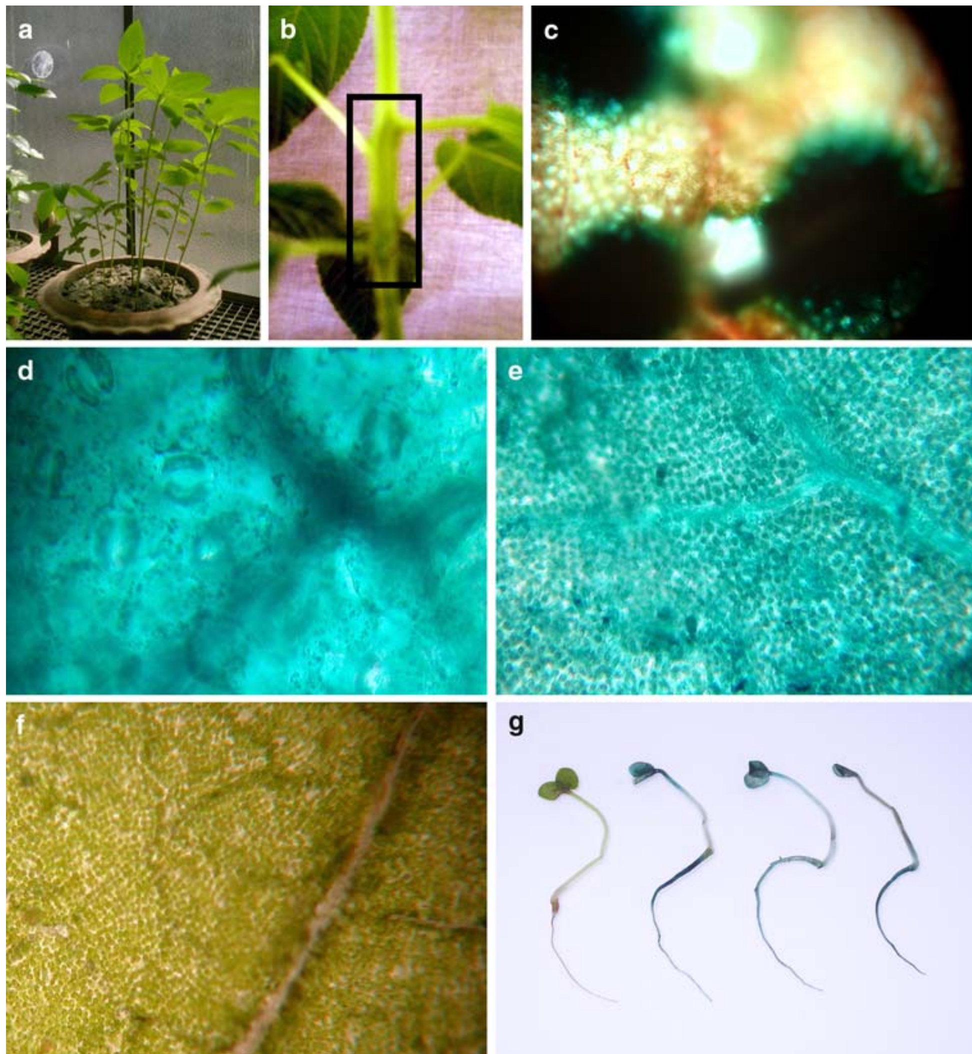

Fig. 1 (**a**) Jute plants (~45–60 cm) after 2 weeks of infection with *A. tumefaciens* at the shoot tips. (**b**) Increased thickness at the site of wounding after 2 weeks of infection with *A. tumefaciens*. (**c**) Sporadic GUS activity observed under microscope (400×) in the new leaves above the infection site after 3 weeks of infection. (**d**) GUS activity observed under microscope (400×) in the leaves of transgenic tobacco plants used as positive control. (**e**) GUS activity observed under microscope (400×) in whole leaves of T_1 transgenic jute plants. (**f**) No GUS activity observed under microscope (400×) in leaves of non-transgenic jute plants of same species. (**g**) The first seedling (~5 cm) in the left is non-transgenic control plant. The other three are transgenic seedlings (T_2)

Histochemical GUS assay was performed in order to assess GUS activity in transgenic plants, (ii) For confirming the presence of *gus* gene in the transgenic plants, PCR with *gus* gene specific primers was performed, (iii) To ascertain the presence of *gus* transcript in the GUS positive plants, *gus* gene specific RT-PCR was conducted, (iv) *gus* specific Southern hybridization, and (v) Transgenic plants were also selected on kanamycin containing medium.

If some of the dividing cells in a meristematic region are transformed by *Agrobacterium*, transgene(s) will be transferred to progeny cells, and if some of these cells later differentiate to floral buds, seeds generated from these buds will inherit the transgene(s). This theoretical consideration was in harmony with our observation of β-glucuronidase (GUS) activity. In leaves of T_0 plants some but not all cells were positive for GUS expression, but whole plants were positive in the T_1 and T_2 generations.

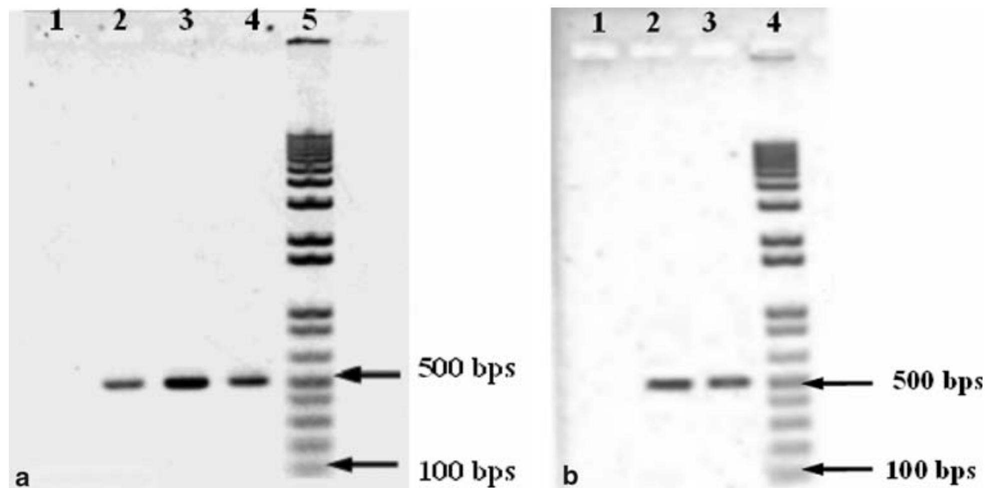

Fig. 2 (**a**) Gel electrophoresis of the amplified gus gene sequence. Lane 1: non-transformed jute; lane 2: pBI121 plasmid containing gus gene, used as positive control; lane 3 and 4: T_2 generation seedlings from transgenic plants; lane 5: 1 kb + ladder. (**b**) Gel electrophoresis of the RT-PCR product specific for gus gene sequence. Lane 1: non-transformed jute; lane 2 and 3: T_2 generation seedlings from transgenic plants; lane 4: 1 kb + ladder

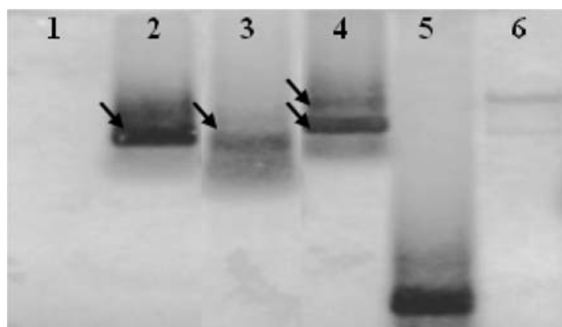

Fig. 3 *gus* specific Southern hybridization; Lane 1: non-transformed jute; lane 2, 3, and 4: *Eco*RI digested transformed (T_2) jute genomic DNA; lane 5: PCR product from transgenic plant using gus specific primers; lane 6: *Eco*RI digested pBI121 plasmid containing gus gene, used as positive control

There is a drawback in using pBI121, as both the reporter and the selectable marker genes, *gus* and *npt II*, were taken from *E. coli*. It would have been easier to determine false positive results, if the vector sequence contained marker genes from more than one organism. However, pBI121 is a popular binary vector and has recently been used by researchers for several plants including peanut (Rohini and Rao 2001; Sharma and Anjaiah 2000), shallot (Zheng et al. 2001), spruce (Le et al. 2001), buckwheat (Kojima et al. 2000), poppy (Park and Facchini 2000) and pine (Tang 2001). pBI121 has also been used for *in planta* transformation of cactus (Seol et al. 2008).

About 77 out of 101 plants assayed for GUS activity in T_2 generation were transgenic. The ratio of transgenic plants vs. non-transgenic plants obtained in T_2 generation was 3.12:1. Some of the GUS positive leaves showed diffusion of blue colour from leaves in the solution during GUS assay, although the positive control plants (transgenic tobacco) had relatively little diffusion of the same in the solution. In an experiment in this lab (data not shown), jute

Table 1 Transformation efficiency based on selection on medium containing kanamycin

Replication	Number of seeds (T_1) that germinated and had subsequent growth	Number of seeds (T_1) that failed to germinate	Percentage of efficiency
R_1	35	154	18.52
R_2	8	53	13.11
R_3	19	96	16.52
R_4	48	52	48.00
R_5	32	48	40.00
Mean ± SE	–	–	27.23 ± 7.01

Each treatment event included at least 15 or more plants

leaves were found to release more carbohydrates and proteins into solution than tobacco leaves which might account for the greater loss of the blue colour.

To ascertain the expression of *gus* genes in transgenic T_2 seedlings, total RNA was isolated and RT-PCR was performed using primers specific for *gus* transcripts. Expected size fragment was visible upon gel electrophoresis. To exclude the chance of amplification from contaminating DNA in the RNA sample, PCR was performed directly with the same sample of RNA without reverse transcription. However, no amplification of sequence was visible. None of the non-transgenic plants of the same species, used as negative control, gave amplified products in RT-PCR using the same primers.

To confirm further the transformation event, PCR was performed with DNA isolated from both T_1 and T_2 plants using *gus* specific primers. To increase the specificity of PCR amplification long primers (>22 bps) with high annealing temperatures (60°C) were designed. Primers that were designed, using one software (Primer3) were crosschecked with another (GeneFisher2: http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). The primers were also searched for homology to other sequences using BLAST to avoid getting multiple spurious bands during PCR amplification. Using these primers the amplified products from transgenic plant gave a single and expected size band of 511 bps, identical to the positive control (pBI121 plasmid containing *gus* gene). DNA from non-transgenic plants of the same species gave no band. This strengthened the fact that the plants that tested positive results for transformation in the previous two assays were true transformants. Southern hybridization with *gus* specific probe further confirmed the success of the transformation process.

None of the transformed plants showed any phenotypic change except for thickening at the infection sites in T_0 plants. This might possibly be the result of plant defense responses. Plants produce signal molecules that induce resistance responses due to wounding or pathogen attack. Induced defense responses include reinforcement of cell wall synthesis among others (Mello-Farias and Chaves 2008).

Selection on kanamycin containing medium also provided proof for transformation as only seeds from transformed plants germinated and showed growth while no germination was observed with seeds of non-transgenic plants. But seeds of non-transgenic plants had normal growth in non-selection medium. This eliminated doubts about the vigour of non-transgenic seeds.

Efficiency of transformation process at an average of 27.23 ± 7.01 was much lower than those obtained with other *in planta* transformation studies obtained with *Notocactus scopa* cv. *Soonjung* (Seol et al. 2008), *Triticum aestivum* L. (Supartana et al. 2006), *Hibiscus sabdariffa* (Gassama-Dia et al. 2004) and *Oryza sativa* L. (Supartana et al. 2005), which had maximum transformation efficiencies up to 100%, 75%, 68% and 43%, respectively. However, the efficiency obtained in this study is sufficiently high for practical purposes.

The simple transformation protocol for *C. olitorius* described here does not use tissue culture and opens up new possibilities for improvement of jute varieties through the introduction of new traits.

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