

***Agrobacterium*-mediated Genetic Transformation of Two Varieties of Jute (*Corchorus capsularis* L.)**

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Abstract

Transformation experiments were carried out using different explants of two varieties of white jute (*Corchorus capsularis* L.), namely, CVL-1 and CVE-3 with *Agrobacterium tumefaciens* strain (LBA4404/pBI121) containing the GUS and *nptII* genes. Maximum transformation ability was obtained from petiole-attached cotyledons and mature embryo explants. Kanamycin at a concentration of 200 mg/l was found optimum for selection of transformed shoots developed from mature embryos. Histochemical assay revealed the stable expression of the GUS gene within the various tissues of transformed plantlets. Stable integration of GUS and *nptII* genes were confirmed by PCR analysis of genomic DNA isolated from these transformed shoots.

Introduction

Bangladesh is the home land of quality jute production and as a result it is considered to be a very important cash crop of Bangladesh. However, the production, quality and yield of this crop are affected by various biotic and abiotic stresses. It has been estimated that about 60% yield of jute fiber is lost due to the incidence of fungal diseases alone every year. Major fungal diseases of jute are: stem rot caused by *Macrophomina phaseoli*, anthracnose by *Colletotrichum corchori*, soft rot by *Sclerotium rolfsii*, black band by *Botryodiplodia theobromae*, die-back by *Gloeosporium* sp., powdery mildew by *Oidium* sp. In addition to the damage caused by fungal pathogens, every year about 7, 67,000 bales of jute is lost due to infestation of a number of insects and pests in Bangladesh (BBS 2004). Apart from these, jute productivity is also affected by root-knot caused by a nematode (*Meloidogyne arenaria*) and by leaf yellow mosaic virus (Ghosh 1983). On the other hand, salinity and flood are the major abiotic factors affecting the production of quality jute fiber. Under the circumstances, the higher production of jute fiber can be obtained through the development of improved varieties of jute resistant to biotic and abiotic stresses.

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In the past conventional breeding such as hybridization and selection did not yield desired results in producing jute varieties resistant to diseases and pests (Islam and Rashid 1960, Haque and Islam 1969). It may be mentioned here that there are a number of wild germplasms of jute available in the Gene Bank of Bangladesh Jute Research Institute (BJRI). These germplasms can be utilized for the variety improvement program. But interspecific cross-incompatibility stands in the way of utilizing these wild jute germplasms for transfer of characters of agronomic importance.

Modern biotechnological techniques such as plant genetic transformation can accelerate the development of new crop varieties unattainable through conventional breeding. Under the circumstances, *Agrobacterium*-mediated gene transfer can be one of the methods of choice to introduce gene/s of interest in developing transgenic crop (Gustavo et al. 1998). This technology is regarded as a powerful tool 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 of desirable characters. A number of crop varieties have already been transformed through *Agrobacterium*-mediated system such as cotton, maize, potato, tobacco, rapeseed, raspberry, soybean, pea, tomato, rice (Fisk and Dandekar 1993, Wambugu 1999). A reproducible reliable transformation system would enable us to insert genes of interest in jute cultivars that may be available in germplasm collections of BJRI. An efficient transformation protocol requires an effective plant regeneration system and there is a room for improvement of the regeneration protocols that have so far been published (Das et al. 1986; Ahmed et al. 1989; Seraj et al. 1992; Saha et al. 1999; Khalekuzzaman et al. 2000 and Sarker et al. 2007) including those on transformation of jute (Hossain et al. 1998; Islam et al. 1999; Ghosh et al. 2002). Preliminary studies on *Agrobacterium*-mediated genetic transformation have shown that the GUS gene can be successfully transferred to infected explants of jute varieties (Hossain et al. 1998; Islam et al. 1999), but there are limited numbers of reports on the recovery of *Agrobacterium*-mediated transformed (transgenic) jute plants.

Considering the importance of jute in Bangladesh and limitation in improving its yield and quality, it is necessary to incorporate genes of desired traits in the local varieties. It was with this background the present set of experiments on transformation was carried out using two local varieties of jute.

Materials and Method

Seeds of the two varieties, namely, CVL-1 and CVE-3 of white jute (*Corchorus capsularis* L.), collected from BJRI, Dhaka were used in the present investigation. A series of tissue culture based transformation experiments were conducted using the explants of petiole-attached cotyledons (PAC), cotyledonary nodes

(CN), mature embryos (ME) with and without the cotyledon. Except for mature embryos MS supplemented with different concentrations and combinations of BAP, IAA, IBA, NAA, Kn etc. were used for *in vitro* regeneration and co-cultivation. The methods of explant preparation, culture condition, media composition and hormonal supplements applied during *in vitro* plantlet regeneration were carried out following Sarker et al. (2007).

Agrobacterium tumefaciens strain LBA4404 with the binary plasmid pBI121 was used for transformation of the above mentioned two varieties of jute. The binary vector pBI121 has the background of pBIN19. It contains a reporter gene GUS (β -glucuronidase) driven by a CaMV35S promoter and NOS terminator. In addition it has a selectable marker gene *nptII* fused between NOS promoter and NOS terminator. It encodes for neomycin phosphotransferase that confers kanamycin resistance (Herrera-Estrella et al. 1983). For *Agrobacterium* growth 50 ml of liquid YMB (Hooykaas 1988) containing 50 mg/l kanamycin was inoculated with freshly grown *Agrobacterium* culture kept at 200 rpm on a rotary shaker at 28°C for 16 h. The culture was subsequently centrifuged at 5000 rpm at 15°C for 10 min and the pellet was resuspended in liquid MS having the optical density of 1.0 - 1.72 for the infection of explants.

For infection the explants were dipped in the *Agrobacterium* suspension in a small Petri dish for 30 min for both petiole-attached cotyledons (PAC) and cotyledonary nodes (CN), whereas the incubation period was 60 min for mature embryos (ME). They were then blotted dry on a sterilized Whatman filter paper and co-cultured in Petri plates on MS with 0.2 mg/l BAP + 1.0 mg/l IAA for petiole-attached cotyledon and cotyledonary nodes in case of CVL-1, whereas 2.5 mg/l BAP and 0.5 mg/l NAA-fortified MS was used as a co-culture medium for CVE-3 keeping them in the dark for three days. In mature embryos, only MS was used as a co-culture medium.

Following co-culture the explants were washed several times in sterile distilled water with gentle shaking until no opaque suspension was seen. The infected explants were finally washed for 3 min in sterile distilled water with 300 mg/l ticarcillin (Duchefa, Netherlands), blotted with a sterile Whatman filter paper and finally placed on the regeneration medium containing 100 mg/l ticarcillin to check the overgrowth of bacteria and to obtain initiation of regeneration. The explants were maintained in the growth room for regeneration under 16/8 h light/dark cycle at 25 \pm 2°C.

To eliminate untransformed tissues, the regenerating explants were subcultured after two-three weeks on a fresh regeneration medium, initially with 25 mg/l kanamycin. The concentration of kanamycin (25 - 200 mg/l) was increased every fortnight when fresh subculture was made until its level reached 200 mg/l. During each subculture the dead and deep brown tissues were

discarded and green shoots and shoot buds were subcultured in a fresh medium containing the next higher concentration of kanamycin.

Transformation ability of the explants was monitored through GUS histochemical assay (Jefferson et al. 1987) by submerging them in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl α -D glucuronide) and incubating them at 37°C for two-three days. X-gluc was prepared according to the standard methods (Jefferson et al 1987, Gallagher 1992). The explants were then kept in 70% alcohol at least for two days and scored for GUS expression. Anatomical studies of different parts of transformed plantlets were also carried out to observe the expression of GUS gene. Shoots developing from uninfected jute explants were used as negative control. Shoots obtained from 2-month-old transformants were examined for stable GUS expression.

The presence of the GUS and *nptII* genes in the jute genomic DNA was analyzed by PCR. Genomic DNA was isolated from non-transformed plant and transformants (particularly from leaf tissues) using the CTAB method (Doyle and Doyle 1990). For the detection of the *nptII* coding sequence, DNA was subjected to PCR using forward and reverse primer, comprising 5'-CAT TAG TCC ATG CAA GTT T- 3' and 5'-AAG ATT ATA CCG AGG TAT G- 3', respectively. On the other hand, for GUS gene the primers were: forward 5'-CCT GTA GAA ACC CCA ACC CG- 3' and reverse 5'-TGG CTG TGA CGC ACA GTT CA- 3' (MGW-Biotech, AG, Germany). All primers were used at a concentration of 100 pmol/ μ l. Master mix for PCR was prepared using standard protocol except that the temperature and time for annealing were adjusted, the annealing temperature being 65°C for a minute in case of GUS gene and 55°C for *nptII* gene for the same length of time. The numbers of cycles used for GUS and *nptII* gene were 30 and 29 cycles, respectively. The amplified DNA was run on 1.0% agarose gel and stained with ethidium bromide (0.05 μ g/ml).

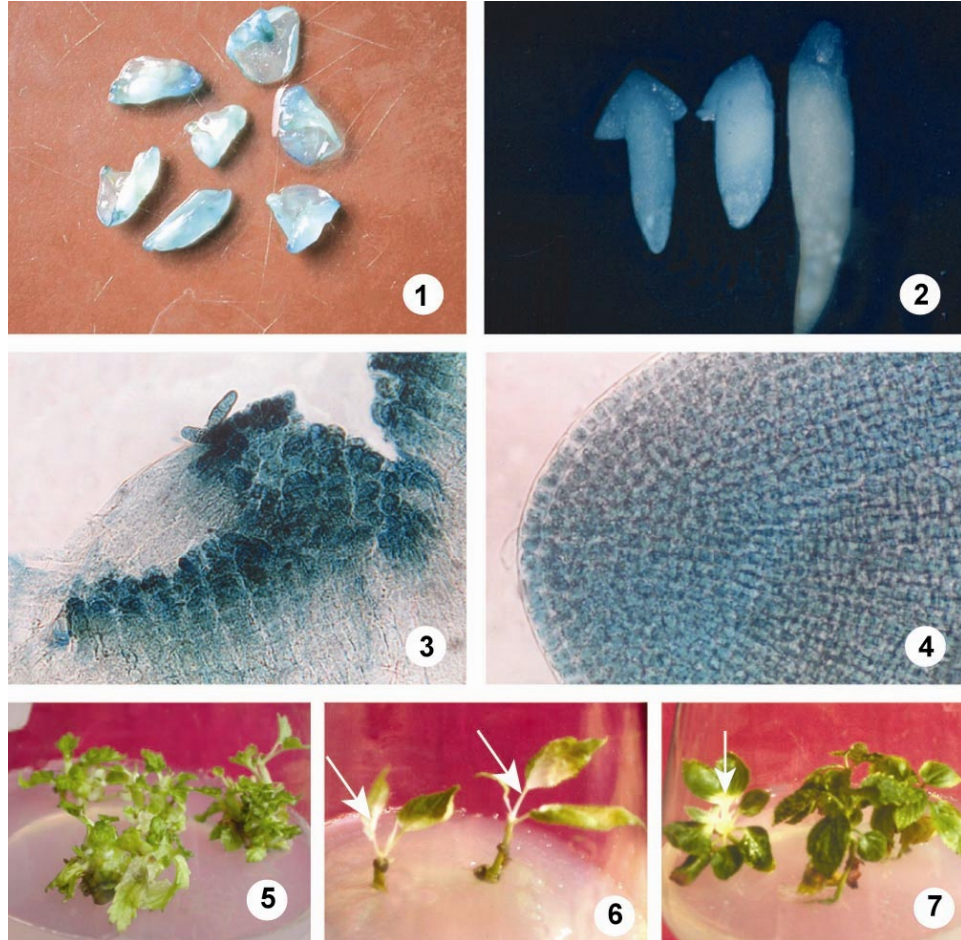
The genomic DNA isolated from transformed tobacco plants used as a positive control.

Results and Discussion

Results of the transient GUS assay for various explants are presented in Table 1. It is evident that petiole-attached cotyledon explants exhibited the highest expression of GUS which was followed by mature embryo explants. Some of the explants co-cultured with *Agrobacterium* showing positive to GUS staining are presented in Figs. 1-4. Microscopic observation confirmed the presence of GUS activity within the internal tissues of such explants (Figs. 3 and 4).

Because of low transformation efficiency of cotyledon-attached mature embryos and cotyledonary nodes, further transformation continued with petiole-attached cotyledons and mature embryos. Several workers (Hossain et al. 1998,

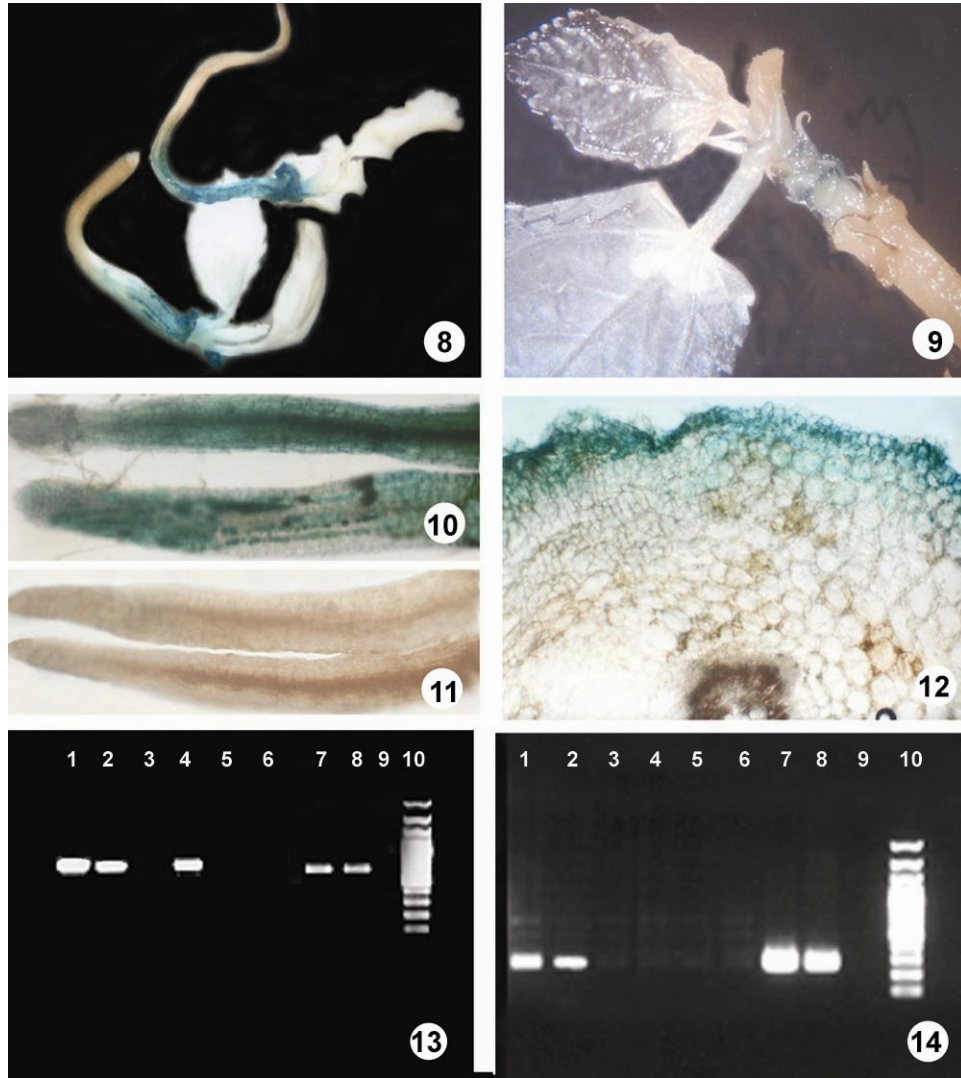
Islam et al. 1999) used hypocotyle, epicotyle and petiole-attached cotyledons, with limited success. However, by means of biolistics system Ghosh et al. (2002)



Figs. 1 - 4: Expression of GUS gene in various explants and tissues. 1. Petiole-attached cotyledon of CVL-1. 2. Stereomicroscopic view of GUS positive mature embryos ($\times 5$). 3. Same as in Fig. 1 but showing a magnified view of a part of the explant ($\times 65$). 4. Magnified view of root primordia from mature embryo explant showing the blue coloured cells ($\times 139$). 5. Initiation of multiple shoots of CVE-3 following co-cultivation before exposing them to selection medium. 6. Non transformed shoots of CVL-1 showing albinism (arrows) on regeneration medium containing 50 mg/l kanamycin. 7. Non transformed shoot developed from mature embryos showing the albinism (arrow) at the leaf base as well as in the part of the leaves. Also note that some healthy shoots (hs) survived through such selection pressure.

obtained transformants using apical meristems of germinating jute seedling. In the present study it was observed that in the case of petiole-attached cotyledon explants regeneration was found to coincide with the transformed cells. There is no information available on the application of mature embryo explants in jute transformation. However, the use of mature embryo explants has previously

been reported in *Hibiscus sabdariffa* leading to the recovery of transgenic plants (Gassama-Dia et al. 2004).



Figs. 8-14: 8. Developing plantlets from mature embryo explants showing partial GUS expression ($\times 7$). 9. Shoot part of transformed plantlet showing expression of GUS gene ($\times 7$). 10. Expression of GUS gene in the roots from transformed plantlets ($\times 60$). 11. Non transformed control roots. ($\times 68$). 12. Transverse section of roots showing the presence of blue colour due to expression of GUS gene in the epidermal and cortical cells of the root. ($\times 156$). 13. PCR amplification of transformed shoots for GUS gene (Lanes 1 - 6 genomic DNA of jute, Lanes 7 and 8 positive control, Lane 9 water control, Lane 10 100 bp ladder (Lanes 1, 2 and 4 indicates the position of GUS positive band). 14. Same as in Fig. 13 for the *nptII* gene (Lanes 1 and 2 indicate the position of *nptII* positive band).

Following co-cultivation, the petiole-attached cotyledons were transferred to suitable medium where regeneration of shoots was obtained through organogenesis. In case of CVL-1 the best regeneration was observed on MS supple-

mented with 0.2 mg/l BAP and 1.0 mg/l IAA, while MS with 2.5 mg/l BAP and 0.5 mg/l NAA was found to be the best in regenerating shoots of CVE-3.

Only agar solidified hormone free MS was used for developing plantlets from mature embryo explants of CVL-1 and CVE-3. It may be mentioned here that the significant difference between the two types of explants lies in the production of single and multiple shoots. Mature embryo explants produced single shoots while those of petiole-attached cotyledons yielded multiple shoots.

Table 1. Responses of various explants of CVL-1 and CVE-3 towards histochemical GUS assay following three - four days of co-cultivation.

Variety	Explant	No. of explants assayed for GUS	No. of GUS +ve explants	% of GUS +ve explants
CVL-1	PAC	95	87	92
	CN	53	24	45
	ME	91	51	56
	CAME	67	21	31
CVE-3	PAC	98	83	85
	CN	64	34	53
	ME	88	59	67
	CAME	46	9	20

PAC = Petiole-attached cotyledons, CN = Cotyledonary nodes, ME = Mature embryo and CAME = Cotyledon-attached mature embryo.

During selection of transformants regeneration experiments were carried out with and without the selection pressure of kanamycin in the medium. Initiation of multiple shoots was found to be retarded in the presence of kanamycin. Therefore, following their initiation in kanamycin-free medium, regenerating shoots were transferred to kanamycin containing medium. Similar observations were reported in other plant species including alfalfa, chickpea, peanut and lentil (Pezzotti et al. 1991, Kar et al. 1996, McHughen et al. 1989, Sarker et al. 2000, 2003), where a preculture period intervened together with a delayed selection in kanamycin-fortified medium.

Initially 25 mg/l kanamycin was used for creating selection pressure. Subsequently, the concentration of kanamycin was increased up to 200 mg/l (Table 2). The infected petiole-attached cotyledon explants produced multiple shoots when subjected to selection pressure (Fig. 5). Due to the effect of kanamycin, the *in vitro* grown shoots first became albino indicating that the elimination of non-transformed tissue (Figs. 6 and 7). With the increase of kanamycin concentration the number of surviving shoots decreased and most of the non-transformed shoots failed to survive in presence of 200 mg/l kanamycin.

None of the petiole-attached cotyledon-derived shoots was able to continue its growth in presence of higher concentration of kanamycin (100 mg/l), whereas only mature embryo-derived shoots were recovered after selection (Table 2). Almost identical observations were made for both CVL-1 and CVE-3. Fully developed shoots of CVL-1 survived in presence of 200 mg/l kanamycin (Fig. 7).

Table 2. Effect of kanamycin in selecting transformed shoots.

Variety	Explant	No. of explants infected	No. of shoots survived on kanamycin containing medium (mg/l)				% of survived shoots
			25	50	100	200	
CVL-1	PAC	1841	225	2	-	-	-
	ME	1256	214	156	102	4	0.32
CVE-3	PAC	1625	19	1	-	-	-
	ME	1322	501	183	112	6	0.45

PAC and ME same as in Table 1.

It has been observed that *nptII* served as an efficient selectable marker for jute. Similar findings were reported in peanut and chickpea by Eapen and George (1994) and Kar et al. (1996). Stable expression of GUS gene was visualized within various parts of plantlets through histochemical staining (Figs. 8 - 10). Free hand sections were made using stems and roots of putatively transformed plantlets to examine the levels of expression of GUS gene (Fig. 12). The intensity of the GUS expression was not equal in different tissues. GUS staining was not uniform within the leaves, stems and roots of the regenerated plantlets.

The transgenic nature of the shoots was confirmed by PCR amplification of the GUS and *nptII* genes (Figs. 13 and 14) of six randomly selected transformants. Specific primers (as described in the Materials and Methods) were used for this purpose. Observations indicated the presence of transgenes in the host plant shoots. From Figs. 13 and 14 it was evident that samples of 3, 5 and 6 were negative to both GUS and *nptII*. On the other hand, samples 1, 2 and 4 were positive to GUS and *nptII*. However, samples 1 and 2 were positive to both GUS and *nptII*, while sample 4 was only positive to GUS gene.

The transformation experiments conducted in this study indicate that, various explants of locally cultivated jute varieties can be infected by *A. tumefaciens* strain. Petiole-attached cotyledon and mature embryo explants showed better susceptibility towards *Agrobacterium* infection as well as satisfactory regeneration responses. Mature embryo explant is an additional advantage in directly providing transformed plantlet without any manipulation.

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