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Agrobacterium Mediated Genetic Transformation of Eggplant (*Solanum Melongena L.*)

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Research Article

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Abstract

An efficient variety-independent method for producing transgenic eggplants (*Solanum melongena* L.) *via Agrobacterium tumefaciens*-mediated genetic transformation was developed. Two weeks old cotyledon explants were transformed by co-cultivation with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pVDH65 carrying the reporter gene β-glucuronidase intron (*GUS*-INT) and the marker gene neomycin phosphotransferase (*NPTII*). Putative transformed explants were cultured on MS basal medium supplemented with different concentration of growth regulators and antibiotics. The best results were obtained when the explants were onto MS medium containing 0.5 mg/l NAA and 0.5 mg/l BA, 50 mg/l kanamycin, 100 mg/l cefotaxime and 25 mg/l acetosyringone. The putative transgenic shoot buds were allowed to elongate on the selection medium for subsequent histochemical *GUS* assay and PCR analysis. The presence of the *GUS* and *NPTII* genes were confirmed through *GUS* assay and PCR analyses.

Keywords: *Eggplant, Agrobacterium, Transformation, β-glucuronidase (GUS).*

Introduction

Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of sub-tropics and tropics. The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit whereas the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. It is rich in reducing sugars, anthocyanin, phenols, glycoalkaloids (such as solasodine), dry matter, and amide proteins[1]. It is also known to have ayurvedic medicinal properties and is good for diabetic patients and recommended as an excellent remedy for those suffering from liver complaints[2].

Since the advent of recombinant DNA technology in the 1970's, the recent years have shown remarkable advances in plant genetics and the rise of plant transformation. These new advances both biological and mechanical have given researchers the ability to express foreign genes, in desired plant hosts, essentially breaking the species barriers. Plant transformation has been defined as the stable incorporation and expression of foreign genes into host plant. Currently the practical success of plant genetic manipulation can be seen in agricultural products such as Bt cotton, Bt brinjal, Newleaf™ potato and the FlavrSavr tomato. With many more products are on the way, it is clear that plant transformation will continue to have a profound impact on plant genetics and global agricultural practices. However, first successful incorporation of kanamycin resistance genes was reported in tobacco[3]. Genetic transformation of eggplant via *Agrobacterium* was first reported by using leaf explants and a co-integrate vector, although no success was achieved with a binary vector[4].

Later, the transformation of leaf and cotyledon explants were reported using the wild supervirulent strain A281[5]. Transgenic plants were obtained by using organogenic regeneration systems[6-7]. An optimization of factors that influence transformation efficiency, including length of pre and post-coculture

periods, explant type, and genotype was performed using a TDZ-based organogenic system[8]. The efficiency of transformation protocols based organogenesis may be influenced by the antibiotic used to eliminate *A. tumefaciens.* For example, augmentin can cause enhanced shoot proliferation induced by TDZ[9]. Recently, an organogenic system from root explants was applied in a protocol for transformation of variety MEBH 11. These explants demonstrated a high susceptibility to *Agrobacterium* and quick regeneration capacity on selection media, resulting in 82.5% of transgenic calli induction with a means of 24 transgenic shoots per callus[10].

Eggplant (*Solanum melongena L.*) is one of the most ergonomically and economically important plants and it is the most productive, common and multiused horticultural vegetable crop in Bangladesh. Therefore, in the present investigation *Agrobacterium* mediated transformation of eggplant was carried out to establish an efficient transformation protocol.

Material and Method

The experiment was conducted at Plant Breeding and Gene Engineering Laboratory, Department of Botany, Rajshahi University, Rajshahi 6205, Bangladesh.

***Plant Materials*:** Cotyledons of 10-12 days old *in vitro* grown plants were used as explants for *Agrobacterium* mediated genetic transformation.

Bacterial Strain and Plasmid: *Agrobacterium tumefaciens* strain LBA4404 (pVDH65, pTOK47) were used for the infection pf explants. The binary vector pVDH65 based on the pBIN19 derivative pMOG18 carried a T-DNA with the chimeric *nos.nptII nos* and intron-GUS genes. Here the selectable marker neomycin phosphotransferase gene (*nptII*) conferring kanamycin resistance explants. The single colony and the liquid culture of that bacterium *A. tumefaciens* LBA4404 were used for infections.

Tissue culture condition*:* Seeds were sterilized with 0.1% $Hgcl_2$ for 3 minutes inside the laminar airflow cabinet. In order to remove disinfectant the seeds were rinsed with double distilled water for 4 to 5 times. Sterilized seeds were cultured on MS_0 basal medium fortified with 3% sucrose. Cultures were kept at the temperature of 25+2º C and a photo period of 14 hours maintained by cooled white fluorescent light. Cotyledons excised from 10-12 day old seedlings were used for *Agrobacterium* co-cultivation. All plant media were adjusted with 1N NaOH to pH 5.7, solidified with 6gm/l agar and autoclaved at 121^0C for 20 min.

Transformation of explants: Explants were inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing pVDH65 plasmid having the chimeric *nos.nptII nos* and intron-GUS genes in liquid MS medium with 50 mg/l kanamycin for 30-90 sec. The density of *Agrobacterium* inoculums of 0.50-2.00 at 600 nm and co-cultivation for 24-48h on agar gelled MS_0 medium.

After co-cultivation, the explants were transferred and placed on selection and regeneration medium (MS+NAA+BA+50 mg/l kanamycin+100 mg/l cefotaxime). After 5-6 weeks, shoots began to regenerate in selection medium from the cut surface of the explants and transferred on to same fresh medium for shoot induction. Kanamycin resistant shoots were transferred to MS basal medium supplemented with 50 mg/l kanamycin for shooting.

***Analysis of Putative Transgenic Plants*:** The presence of the *GUS* and *NPTII* genes were confirmed through *GUS* assay and PCR analyses.

GUS histochemical Assay: The *GUS* histochemical assay solution (modified) was used[11]. GUS activity was assayed using X-glue *(5-bromo-4-chloro-3-indilyl-β-D-glucuronic acid; Gold Biotechnology, St. Louis, USA).* This substrate gives a blue precipitate which is insoluble and highly colored indigo-dye 5, 5'-dibromo-4' dichloro-indigo (ClBr-indigo) at the site of *gus* enzyme activity. This dye is formed by oxidative dimerization of the colorless indolyl derivative that results for *gus* hydrolysis of X-glue.

PCR analysis: Putative transformants and control (non-transformants) plants were used for PCR to detect the presence of *gus* gene. Plant DNA used for PCR analysis was extracted from young leaves using the protocol as well[12]. PCR analysis to detect the presence of the *ferritin* gene were carried out using the PCR Screening Kit (Sigma Chemicals Ltd., USA) in the presence of following pair of primers forward primer 5′-GTCAT CTCAC CTTGC TCCTG CC -3′ and 5′-GTCGC TTGGT CGGTC ATTTC GG-3′. Amplification condition was Denaturation at 94 °C for 1 min. followed by 35 cycles of annealing at 58°C for 40 min, and polymerization at 72 °C for 2 min 35 cycles. Expected PCR product size was about 365 bps.

Results and Discussion

Standardization of plant regeneration from explants: Though the main objective of this study is to transform eggplant with *Agrobacterium*, but before doing this, plant regeneration media composition was standardized for eggplant explants. In *in vitro* condition, explants were excised and cultured onto MS basal medium supplemented with different concentration of NAA (0.5, 1.0 and 2.5 mg/l) and BA (0.5, 1.0 and 2.5 mg/l**)** and incubated in a growth chamber. This experiment reveals that 0.5mg/l NAA and 0.5mg/l BA was the most effective

formulation where the highest 68% of explants were induced to regeneration of shoots. Similar result has already been reported in peanut *Arachis hypogaea* [13], potato[14] and lettuce (*Lactuca sativa*)[15].

Effect of Kanamycin Concentration: The sensitivity of eggplant cotyledon to kanamycin was assayed by culturing the explants without co-cultivation with *A. tumefaciens* on selection medium contained different concentrations of kanamycin (0, 25, 50, 75, 100 mg/l, Table 1). The explants growth was completely inhibited at the level of kanamycin concentration of 50 mg/l So, 50 mg/l kanamycin was chosen for the selection of kanamycin resistant tissues in subsequent transformation experiments. Similar result have been reported in potato and tomato[16], Indian mulberry[17], grasspea[18] and lettuce[15].

Effect of acetosyringone concentration: In the present study, different concentrations (0, 10, 15, 20, 25 and 30 mg/l) of acetosyringone were added into the co-cultivation medium. The highest percentage of kanamycin resistant explants growth was obtained at the 25 mg/l concentration of acetosyringone. Similar result was obtained in cauliflower[19] and in grapevine[20].

Table 1. Effect of Kanamycin concentration in the selection of kanamycin resistant tissues.

Kanamycin Concentration	Control Explants			Experimental Explants		
	No. of Inoculated Explants	No. of survival Explants	Percentage of survived Explants	No. of Inoculated Explants	No. of survival Explants	Percentage of survived Explants
0 mg/l	25	0	0	25	0	0
25 mg/l	25	0	0	25	7	28
50 mg/l	25	0	0	25	10	40
75 mg/l	25	0	0	25	5	20
100 mg/l	25	0	0	25	2	8

Table 2. Effects of infection period on survival percentage of in vitro grown explants of eggplants.

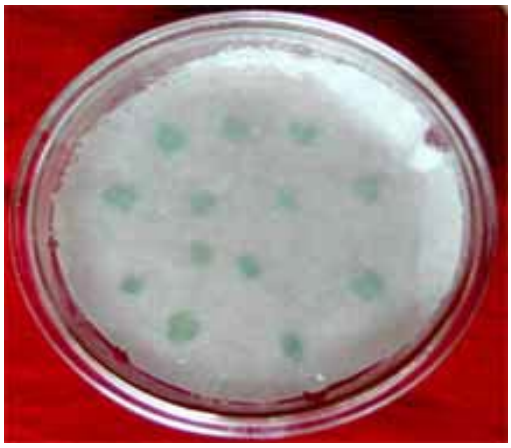
Explants type	Incubation period (sec)	No. of explants inoculated	No. of explants survived	Surviving percentage of explants
In vitro	30	25	7	28
	60		11	44
	90		5	20

Table 3. Effects of co-cultivation period on development of kanamycin resistant tissue with Agrobacterium tumefaciens LBA4404

Explants type	Co-cultivation period (h)	No. of explants inoculated	Number of explants survived	Surviving percentage of explants
In vitro grown cotyledon	24	25	7	28
	48		13	52
	72		3	12

Table 4. Transformation efficiency of explants after co cultivation with *Agrobacterium tumefaciens* LBA4404 carrying *gus* gene.

Explants type	Number of experiment	No. of explants inoculated	Number of kanamycin resistant plantlets recovered	Transformation efficiency (%)
In vitro cotyledon	1. 0.5 mg/l + 0.5 mg/l	25 25 25	8 10 12	40.00
	B. 0.5 mg/l + 1.0 mg/l	25 25 25	6 4 3	17.33
	C. 2.5 mg/l + 1.0 mg/l	25 25 25	2 5 3	13.33
	D. 1.0 mg/l + 2.5 mg/l	25 25 25	4 9 3	20.33



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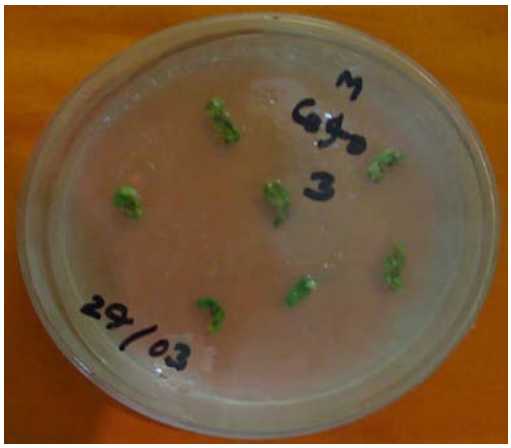

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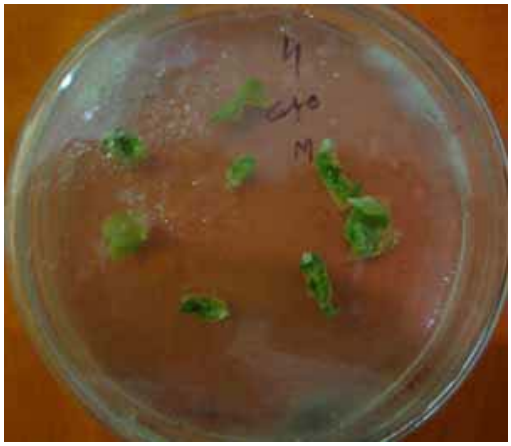

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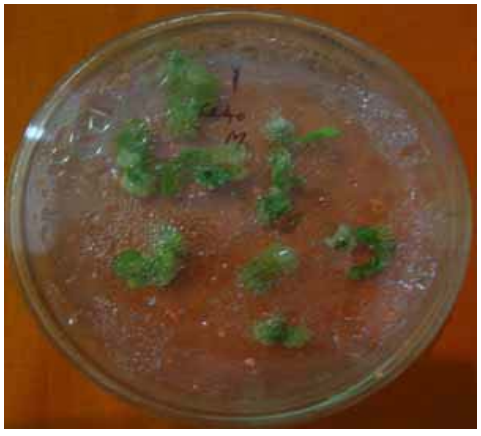

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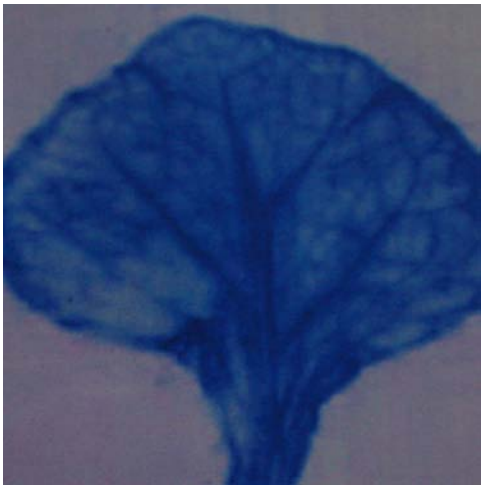
Figure 1 A) Co-cultivation of cotyledon B) Infected explants in regeneration medium C) Regenerated plantlets after 25 days D) Regenerated putative transformed plantlets in shoot induction medium after 4 weeks E) Plantlets in multiplication medium F) Multiplication of transformed plantlets after 6 weeks.

Histochemical *gus* assay of the leaf from putative transformed and non transformed plant.



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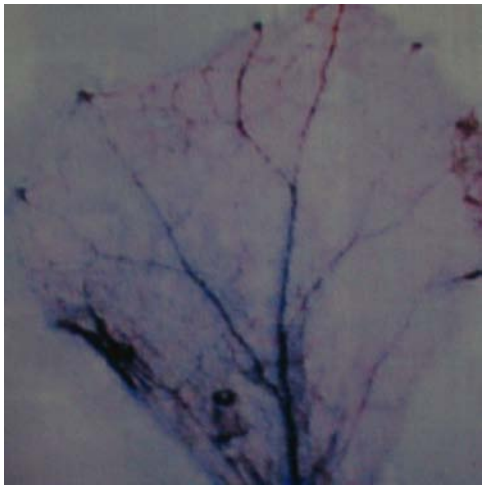

Figure- 2 A) A *gus* stained leaf from kanamycin resistant plant. B) A leaf of non transformed plant

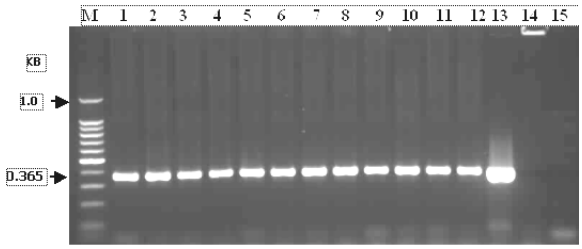

Figure 3. PCR analysis of genomic DNA of transgenic eggplant plants to detect the presence of *nptII* gene in the genomic DNA of the one month old in vitro grown putative transgenic plants. The PCR products were separated on a 1.8 % agarose gel and stained with ethidium bromide. *M*: 1000 bp size marker (Promega). Lanes *1 -12*: plants regenerated from infected leaves showing expected 365 bp band. Lane *13*: plasmid control. Lane *14*: control plant regenerated from uninfected leaf culture. Lane *15*: water (control).

Transformation of explants using optimum parameters: Explants were inoculated with *Agrobacterium tumefaciens* strain LBA4404 chimeric *nos.nptII nos* and intron-GUS genes for different infection period 30-90s (Table-2). Bacterial inoculum density was measured and the highest percentages of kanamycin resistant observed at 1.50 OD 600nm[21]. The post infection process, an infection period 60s (Table-2) and co-cultivation period 48h (Table-3) was optimized for subsequent transformation experiments. After co-cultivation, explants were transferred onto the selection and regeneration medium containing 50 mg/l kanamycin and 50mg/l cefotaxime (Table-4). Similar result was reported in *Arabidopsis* [22-23], in peanut[18], in raspberry[7], in sweet orange[17], in wheat[24], in potato[25] and in lettuce[15]. After 5 weeks, shoots began to regenerate from explants. The experiment was repeated three times. The highest nos. of transgenic plants was recovered from *in vitro* derived cotyledon explants from experiment no-1 that showed the highest transformation efficiency 40% (Table-4). After 5-6 weeks shoots began to regenerate in selection medium from the cut surface of the explants and transferred on to same fresh medium for shoot induction (Figure 1).

GUS Gene Expressions in Kanamycin Resistant Plants: The plantlets regenerated in the presence of kanamycin were tested for gus expression. All kanamycin resistant had the characteristics blue color produced by *gus* activity and was detected in untransformed control plants (**Figure-2).** A positive reaction of the *gus* gene was found in their leaves. The intensity of blue *gus* staining varied between different plants. *GUS* activity was present in all kanamycin resistant plants tested, confirming that they were transgenic.

Molecular Analysis of Putative Transformed Plant through PCR*:* The PCR analysis for *nptII* gene of putative plants of *Solanum melongena* regenerated from kanamycin resistant explants is presented in the **Figure 3**. It was found that putative transformed plants expressed the expected 0.365 kb band of *nptII* gene. The same band also expressed in positive control that confirmed the integration of *npt11* gene in the genome of *Solanum melongena.*

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AUTHORS' CONTRIBUTIONS

Authors contributed equally to all aspects of the study.

PEER REVIEW

Not commissioned; externally peer reviewed

CONFLICTS OF INTEREST

The authors declare that they have no competing interests