

***Agrobacterium*- Mediated Transformation of Eggplant (*Solanum Melongena* L.) Using Cotyledon Explants**

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Abstract

Agrobacterium- mediated genetic transformation system was developed for two varieties of eggplant (*Solanum melongena* L.) namely BARI Begun-4 (Kazla) and Singhnath. Transformation ability of cotyledonary leaves explants were tested with *Agrobacterium tumefaciens* strain LBA4404 harboring binary plasmid pBI121, containing the GUS and *nptII* genes. Transformation efficiency of cotyledonary leaves explants in both varieties was found to be maximum (95% in Kazla and 90% in Singhnath) with bacterial suspension having optical density of about 1.00 at 600 nm, 40 minutes incubation period and three days co-cultivation period. These cotyledonary leaves explants showed high percentage of GUS positive expression by transient assays. Cotyledonary leaves explants were also found to be more effective in formation of multiple shoots on MS medium supplemented with 1.0 mg/l BAP, 1.0 mg/l Kn and 100 mg/l ticarcillin following *Agrobacterium* infections and selection. Selection of the transformed shoots was carried out by gradual increasing in concentration of kanamycin up to 200 mg/l. Stable expression of the GUS gene was observed in various parts of the transformed shoots such as leaf, stem, root, corolla, anther, and pollen grains. The seedlings developed from the two varieties also see the expression of GUS gene.

Keywords: *Agrobacterium*, transformation, regeneration, selection, *Solanum melongena* L. (space)

1. Introduction

Eggplant covers about 15% of the total land under vegetable cultivation in Bangladesh. It is grown year round and is one of the most popular vegetables in the country. Farmers mainly grow it in small plots as a cash crop. The area under cultivation is estimated to be around 32000 hectares. The total production stands at about 404000 tons and the average yields are reported to be around 5.6 tons per hectare [1]. There are many local varieties in Bangladesh in addition to improved varieties and hybrids. Several factors are responsible for the lower production of this important crop, which include susceptibility to disease and insect-pests [2]. The productivity and quality of this crop are low mainly as it suffers from a number of viral, bacterial, fungal and nematode diseases. Eggplant is extensively damaged by the Lepidopteran insect, *Leucinodes orbonalis* (shoot and fruit borer) which is specific to this crop. The adults lay eggs on the buds and growing fruits. The larvae, upon hatching bore into the fruits and shoots causing severe damage and rendering the fruits inedible. It is estimated that the damage caused by the shoot and fruit borer in eggplant ranges from 5-20% in shoots and 10-70% in fruits [3].

The production of eggplant in Bangladesh is needed to be increased manifold through the development of disease/insect-pest resistant varieties [3]. The farmers use high doses of toxic and carcinogenic fungicides and pesticides to protect eggplant from these disease and insect-pests [2]. As a result fungicide and pesticides residues enter the human food chain and may cause serious health hazard. Moreover, these fungicides and pesticides are costly and also have an adverse effect on our environment. So it is better to use disease and insect-pest resistant varieties instead of

chemical control. Plant breeding and tissue culture techniques can play a vital role in the development of disease free planting material, but these particular techniques contributed a little in the production of insect-pest resistant plant in eggplant. Genetic engineering techniques like transformation can accelerate the development of eggplant varieties, which is not possible through traditional breeding and tissue culture alone. Stable transformation of eggplant using different *Agrobacterium*-vectors has been achieved using various explants of *in vitro* plantlets [4, 5-6] (space). Considerable work has been done on the transformation as well as on the development of insect-resistant varieties of eggplant [7, 8]. The present investigation has been carried out for the development of a protocol for *Agrobacterium*-mediated genetic transformation of locally grown eggplant and to establish a method for the regeneration of plantlets from the transformed tissues.

2. Materials and methods

Two varieties of eggplant (*Solanum melongena* L.), namely BARI Begun-4 (Kazla) and Singhnath were used in this study. Seeds of BARI Begun-4 (Kazla) were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur while seeds of Singhnath were collected from the local market marketed by Lal Teer Seed (Bangladesh) Ltd.

For the preparation of the explants, the seeds were washed with detergent under running tap water for 3-5 mins. The seeds were then surface sterilized in the laminar air flow with 0.1% HgCl₂ solution for 5-7 minutes. The seeds were then washed five times with sterilized distilled water. The surface sterilized seeds were inoculated into conical flask containing 50 ml of agar solidified MS [9] with 3% sucrose to support germination and seedlings development.

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Seedlings and all *in vitro* cultures were maintained in a growth room under fluorescent illumination on a 16 h photoperiod at $25 \pm 2^\circ\text{C}$. Cotyledonary leaves were collected from 15-17 days old *in vitro* grown seedlings. Each cotyledonary leaf was transversely cut into two- three segments and each segment was used as explant for the purpose of *Agrobacterium* infection.

In the present study *Agrobacterium tumefaciens* strain LBA4404 with the binary plasmid pBI121 was used for transformation. It contained a scoreable reporter gene GUS and a selectable marker gene *nptII* encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance [10]. Fifty millilitre of liquid YMB [11] containing 50 mg/l kanamycin were inoculated with *Agrobacterium* from a fresh bacterial plate and grown at 200 rpm on a rotary shaker at 28°C for 19 h. The culture was subsequently centrifuged at 5000 rpm for 10 mins at 20°C . The supernatant was discarded and the bacterial precipitate was resuspended in an appropriate volume of liquid MS, so that a suitable bacterial density for infection was obtained. The cotyledonary leaves were cut into two pieces and then dipped in the bacterial suspension for different incubation periods. The explants were then blotted dry with filter paper (Whatman no. 1) to remove excess bacteria. Following infection and incubation, the explants were co-cultured in petri plates on MS with 1.0 mg/l BAP and 1.0 mg/l Kn (best regeneration medium selected during regeneration experiments) without selection pressure. All the explants were maintained in co-culture medium for 3 days in dark. Following co-culture, the explants were washed several times in distilled water with gentle shaking until no opaque suspension was seen. The infected explants was finally washed with 300 mg/l ticarcillin (Duchefa, Netherlands) and dried with a sterile filter paper (Whatman no.1) and placed on the regeneration medium. To control bacterial overgrowth 100 mg/l ticarcillin was applied for 7-8 weeks following co-cultivation. To eliminate non-transformed tissues, the regenerating explants were sub-cultured on a fresh regeneration medium initially with 50 mg/l kanamycin after four weeks. Selection of the transformants was carried out using kanamycin. Here, kanamycin concentration were gradually increased (e.g. 50, 100, 150, 200 mg/l) following two subsequent subculture at 10-13 days intervals.

Transformation events were monitored through GUS histochemical assay [12]. For this reason co-cultivated explants and different parts of putatively transformed plantlets were incubated for two to three days at 37°C in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl-glucuronide) and subsequently bleached with 70% ethanol before scoring GUS expression. Shoots developing from uninfected eggplant explants were used as negative control and transgenic tobacco tissues maintained in the laboratory were used as positive control. X-gluc was prepared by dissolving it in 100 μl dimethylformamide and made up to final concentrations of 10 mg/ml with 50 mM phosphate buffer at pH 7.0. Cultured explants were subjected to transient GUS assay after antibiotic wash. Tissues, shoots and roots under selection pressure and different parts (Leaves, flower, anther and pollen grains) of putative

transformed plants grown under net house in confined environment were monitored for stable GUS expression. Histochemical assays were carried out to observe the expression of GUS gene inside the explant tissues as well as in different parts of putatively transformed plantlets.

3. Results and discussion

High regeneration frequency (Singhnath 4.4 shoots/explant and Kazla 3.5 shoots/explant) was achieved from cotyledonary leaves explants through the use of 1.0 mg/l BAP and 1.0 mg/l Kn supplemented MS medium for both varieties of eggplant.

Cotyledonary leaves explants of both varieties of eggplant (Singhnath and Kazla) were found to be compatible with *Agrobacterium tumefaciens* strain LBA4404. Factors influencing successful transformation namely optical density of the bacterial suspension, infection period and co-cultivation period were optimized during this investigation (Figs. 1, 2 & 3).

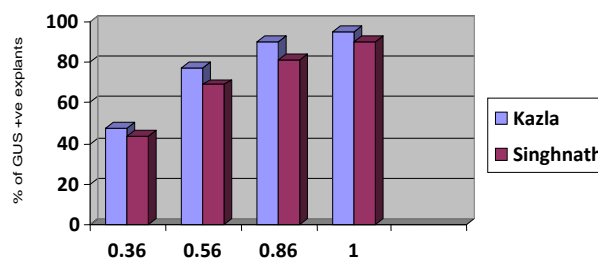


Fig. 1. Optical Density at 600 nm

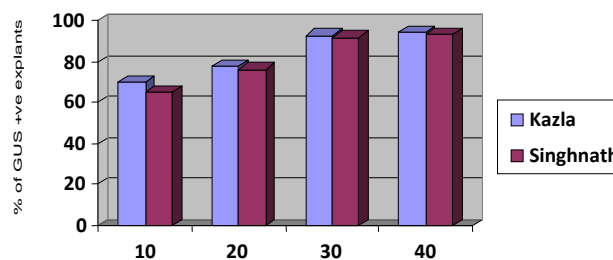


Fig. 2. Incubation period (min.)

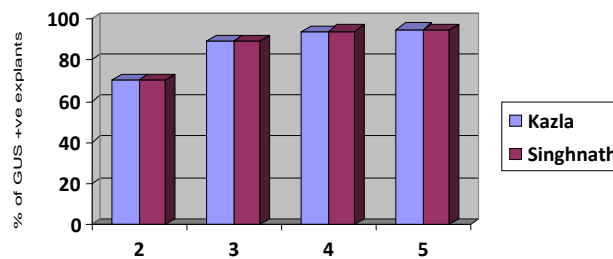


Fig. 3. Co-cultivation period (days)

Figs. 1-3. Effect of optical density (measured at 600 nm) (Fig. 1), different incubation period (Fig. 2) and co-cultivation period (Fig. 3) of cotyledonary leaves explants of eggplant using *Agrobacterium* strain LBA 4404

containing the binary plasmid pBI121 in varieties : Kazla and Singhnath. (Replace it under the graph)

Transformation efficiency of cotyledonary leaves explants in both varieties was found to be maximum (95% in Kazla and 90% in Singhnath) with bacterial suspension having optical density of about 1.00 at 600 nm, 40 minutes incubation period and three days co-cultivation period. In the present investigation it was observed that the transformation efficiency increased with the increased of co-cultivation period but during long co-cultivation period (more than three days) bacteria was found to grow heavily on the co-culture medium which was not suitable for growth and survival of co-cultured explants. Hence, co-cultivation period of three days was found to be most suitable when transformation experiment was performed under optimum condition. Influence of regulatory factors on the transformation efficiency of various explants was also observed by Filippone and Lurquin [5], Franklin and Sita [6].

A total of 785 explants from both varieties of eggplant (Singhnath 425 and Kazla 360) were infected with *Agrobacterium* strain LBA4404/pBI121 and co-cultivated for three days in dark condition. After co-cultivation some explants of each varieties of eggplant were subjected to transient GUS assay and other explants were transferred to regeneration medium (MS + 1.0 mg/l BAP + 1.0 mg/Kn + 100 mg/l T icarcillin) without kanamycin. However, it was found that presence of kanamycin after co-cultivation seriously hampered the growth of the explants. For this reason immediately after co-cultivation kanamycin was not applied for selection. Rather, regeneration was encouraged from the co-cultivated explants. The infected cotyledonary leaves explants with small shoots and shoot buds were transferred to MS basal medium with 50 mg/l kanamycin after 22-25 days of inoculation. Shoots were isolated from the explants and transferred to next higher concentration of kanamycin (100 mg/l) after 10-13 days of subculture. Following two subsequent sub-cultures the kanamycin

concentration of the medium was changed into next higher kanamycin concentration (Table 1). Higher concentrations of kanamycin gradually decreased the number of green shoots. In each subculture white and brown shoots were discarded and green shoots were transferred to fresh medium. The non-transformed shoots showing albinism under selection pressure (Fig. 4). All the control shoots died in the selection medium with 200 mg/l kanamycin (Fig. 5). Therefore, the shoots survived in the medium containing 200 mg/l kanamycin were considered as transformed. Fully developed shoots regenerated from infected cells is shown in Fig. 6. It was also observed that selection medium containing 100 and 150 mg/l kanamycin was effective resulting highest percentage of albino shoots. However, the shoots survived in presence of 150 mg/l kanamycin were subjected to higher selection pressure of 200 mg/l kanamycin to ensure better selection procedure. For selection, 100-200 mg/l kanamycin was reported to be suitable in obtaining transformed eggplant shoots [5- 6] and [13- 14]. Thus following such selection procedure the transformed shoots were recovered and these shoots were allowed to develop roots.

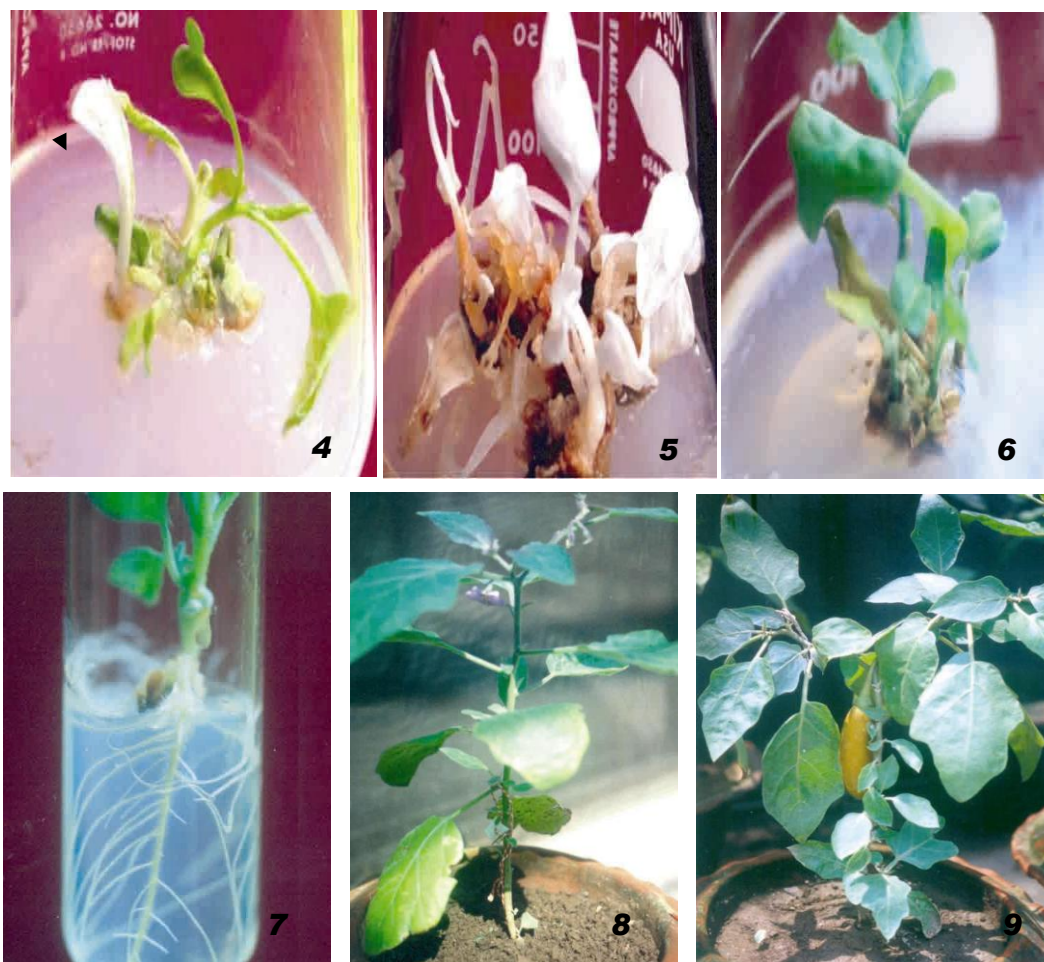
In the present study, 19 survived shoots (10 Singhnath and 9 Kazla) were subjected to rooting medium (MS basal medium) without kanamycin, where almost 76% of shoots developed roots properly in both the varieties (Fig. 7). In lower concentration of kanamycin (50 mg/l) no root formation was observed (Table 2). Kumar *et al.* [8] applied 50 mg/l kanamycin in the rooting medium for root induction from the transformed shoots survived in 100 mg/l kanamycin in different eggplant varieties. After sufficient development of roots from the selected shoots, the plantlets were successfully transplanted to soil. All plantlets (8 Singhnath and 6 Kazla) produced flower after 45-50 days of subsequent transplantation (Fig. 8). Moreover, two of the six transformants in variety Kazla produced mature fruits with viable seeds (Fig. 9).

Table 1. The effect of gradual increase in kanamycin selection pressure on the regenerating shoots from infected cotyledonary leaves explants of two varieties of eggplant.

Varieties	No. of infected explants	No. of shoots & shoot buds in selection (50 mg/l kanamycin)		No. of shoots in selection (100 mg/l kanamycin)		No. of shoots in selection (150 mg/l kanamycin)		No. of shoots in selection (200 mg/l kanamycin)	
		Inoculated	Survived	Inoculated	Survived	Inoculated	Survived	Inoculated	Survived
Singhnath	425	920	440	440	45	45	10	10	10
Kazla	360	775	320	320	34	34	9	9	9

Table 2. Response of root induction from the transformed shoots on the MS basal medium and MS basal medium supplemented with 50 mg/l kanamycin.

Concentration of kanamycin in rooting medium (mg/l)	No. of shoots cultured	No. of shoots rooted	% of root induction	Root induction time (days)
50.0	26	0	0	0
Without kanamycin	19	15	76	12-16

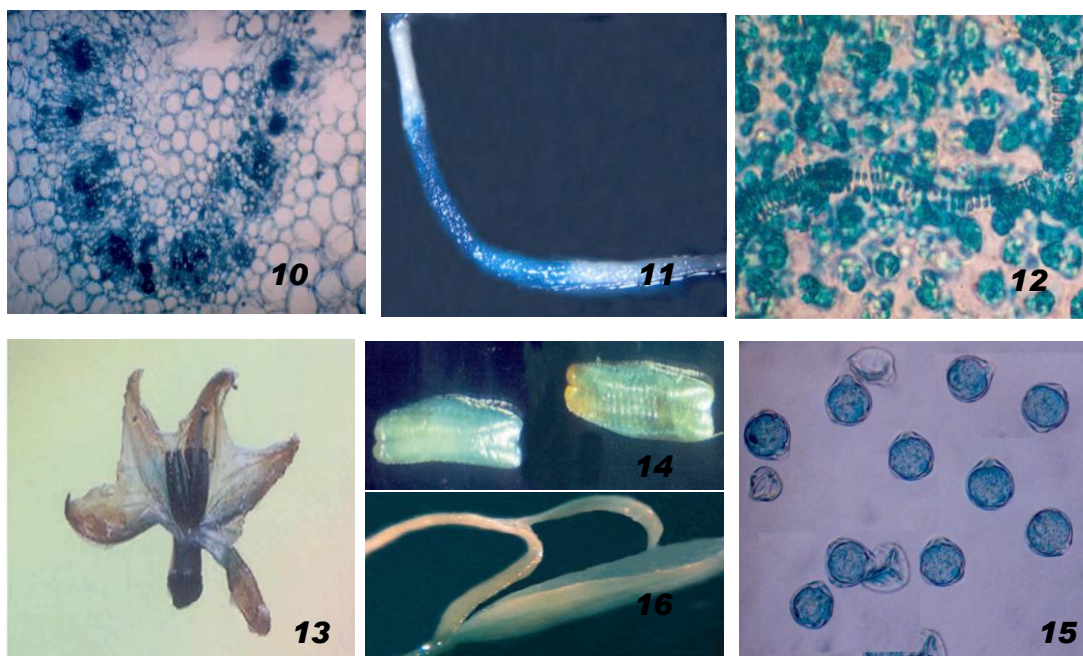


Figs. 4-9. (4) Nontransformed shoots showing albinism (arrow) on regeneration medium containing 100 mg/l kanamycin, (5) Control shoots showing albinism (leading to death) on selection medium (150 mg/l kanamycin), (6) Fully developed shoots of Singhnath on selection medium (200 mg/lkanamycin), (7) Roots developed at the base of regenerated shoot of Singhnath on MS medium (8) A putative plant (T_0) of Kazla flowered after 45 days following transplantation and (9) Kanamycin resistant plant of Kazla (T_0) with mature fruit.

Histochemical GUS assay [12] was used to detect the expression of GUS gene in the infected explants and plantlets that survived kanamycin selection. In the present investigation most of the plant parts (leaf, shoots, stem, roots and flowers) of the putatively transformed plantlets showed GUS positive blue colour in their respective tissue which indicates the stable integration of GUS gene. High GUS expression was observed in the vascular bundle of stem under selection pressure (Fig. 10), whereas the epidermis and cortical cells showed little activity. Roots of developed plantlets were also showed GUS expression (Fig. 11). About 80% of the fully developed transplanted plants (T_0) growing in soil showed GUS positive expression. The expression of GUS gene was observed in the leaves and floral parts from transplanted plants for the both varieties. There were, however, variations in the intensity of the blue coloration in the different organs and also variations as the plant matured. It was found that GUS expression was observed in the phloem tissues and in the veins of leaf whereas the mesophyll and epidermis cells showed high GUS expression (Fig. 12). This is not in agreement with

Fari *et al.* [13]. They found a strong cell-type preference for GUS expression in the phloem tissues and in the veins of leaf, whereas the mesophyll and epidermis cells showed very little activity. This variation in gene expression may be due to the use of different promoters in the present investigation and the previous study of Fari *et al.* [13].

GUS activity was observed in the basal part of corolla, anthers and most of the pollen grains (Figs. 13, 14 & 15). The expression of GUS gene in the flowers indicates the efficient insertion of this gene. Moreover, as the pollens are single cell structures and the expression of the integrated foreign gene within these pollen grains supports the possible expression of inserted gene in the advanced progenies (Fig. 15). Forty seeds of each plant were subjected to germination medium containing 150 mg/l kanamycin. More than 85% seeds were germinated in kanamycin containing medium. Nine T_1 seedlings out of 10 showed GUS positive expressions, which confirm the successful insertion of foreign genes into the advanced progeny obtained from the transformed (T_0) plants (Fig. 16).



Figs. 10-16. (10) T.S. of stem showing conspicuous blue colored region within epidermal and cortical cells (x 210), (11) Root developed from kanamycin resistant shoot showing GUS expression. (x 35), (12) Magnified view of a leaf segment showing conspicuous blue colour cells as well as xylem vessels (x 840), (13) A flower from T₀ plant showing GUS expression, (14) Anthers of T₀ plant showing GUS expression (x 36), (15) Histochemical localization of GUS activity (blue colour) in pollen grains (x 469) and (16) Germinated seedlings (T₁) obtained from kanamycin resistant (T₀) plants showing GUS expression (blue colour) (x 36)

4. Conclusion

In the present investigation the transgenic nature of putatively transformed plants was confirmed through GUS histochemical assay only. However, for confirmation of stable integration of transgenes into plants, specific molecular techniques like PCR analysis and southern hybridization are also necessary. This protocol was primarily developed using screenable marker gene such as GUS and selectable marker gene (*npt11*). The selection procedure of *Agrobacterium*-mediated genetic transformation developed through the present investigation can be used for the production of transgenic eggplant for specific purposes.

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