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Enhanced Regeneration Through *ex vitro* Rooting and *Agrobacterium*-mediated Genetic Transformation of Eggplant (*Solanum melongena* L.)

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Key words: Eggplant, *Ex vitro* rooting, Transformation, *Agrobacterium*, *nptII* and *GUS* gene

Abstract

Regeneration of *in vitro* multiple shoots was achieved through organogenesis on MS supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn from cotyledonary leaf explants of two local varieties of eggplant (*Solanum melongena* L.). Elongation of regenerated shoots was obtained on growth regulator free MS. *In vitro* root induction from excised regenerated shoots was less effective on MS with or without plant growth regulators. On the other hand regenerated shoots treated with 10 mM IBA for 5 min were found to be effective for *ex vitro* rooting in sterilized soil. Following sufficient development of roots, the *ex vitro* rooted plantlets were acclimatized in growth room condition, and were transferred to the field having 100% survival rate. The regeneration system developed was utilized for *Agrobacterium*-mediated genetic transformation using *Agrobacterium tumefaciens* strain LBA4404/pBI121 containing *GUS* and *nptII* genes. Adequate transformation response was obtained from cotyledonary leaf segments with bacterial suspension having an optical density of 0.50 at 600 nm with 30 min incubation followed by co-cultivation period of 72 hrs in Nayantara (BARI Begun-5) variety of eggplant. Selection of transformed shoots was carried out on MS supplemented with 2.0 mg/l BAP, 0.5 mg/l Kn, 300 mg/l carbenicillin and 100 mg/l kanamycin. Stable integration of *GUS* and *nptII* genes in Nayantara were confirmed through PCR analysis using the genomic DNA isolated from transformed shoots.

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Introduction

Eggplant or brinjal (*Solanum melongena* L., 2n = 2x = 24) is a popular vegetable crop belonging to the family Solanaceae. This plant is a native to the Indian subcontinent and geographically distributed in different parts of Asia with the secondary center of origin in China (Zeven and Zhukovsky 1975). Eggplant is considered as the second major vegetable crop after potato in terms of acreage and production and the most important indigenous vegetable in Bangladesh (Saifullah 2012).

The fruits of eggplant are good source of vitamins and minerals as well as rich in antioxidant compounds and have hepato protective properties (Concellon et al. 2012). In traditional medicine eggplant is used for the treatment of many ailments like diabetes, arthritis, cholera, asthma and bronchitis (Magioli and Mansur 2005). Moreover, the texture of the fruit of different varieties of eggplant makes it more popular vegetable food item in wide range of dishes.

Eggplant is an important source of income for the small, marginal and resource poor farmers in Bangladesh. It is grown by about 1,50,000 very small resource poor farmers in Bangladesh.This crop is cultivated in about 25% of the total land under vegetable cultivation in Bangladesh. For most of the time, except peak production period, market price of eggplant remains high compared to other vegetables. Therefore, it plays a vital role to boost the national economy (Hossain et al. 2013) in Bangladesh.

It is understood that eggplant is an important vegetable crop of Bangladesh and several different indigenous varieties of eggplant cultivated in different areas of Bangladesh. However, the yield potentials of different varieties of eggplant is found to be variable and the yield of this important vegetable crop is appeared to be affected significantly by various kinds of biotic and abiotic stresses. The biotic stresses includes the infestation of diseases and pests while the abiotic stresses that hampering its productivity includes the effects of drought, salinity, flood, cold and heat stress (Prabhavathi and Rajam 2007).

In the past, several attempts have been made through conventional breeding for the development of stress tolerance in eggplant and to transfer of desired traits into its cultivated species. Conventional breeding approaches through hybridization for developing desired stress tolerance in eggplant is limited due to sexual incompatibilities, prevalence of sterility in the progeny and lack of natural sources of resistance (Shivraj and Rao 2011, Magioli and Mansur 2005). Under these circumstances the techniques of plant genetic engineering can be exploited for the development of desired stress tolerance in eggplant and thus the difficulties associated with conventional method of breeding for the transfer of desired traits can be avoided.

Among the various techniques *Agrobacterium*-mediated genetic transformation has been considered as a method of choice for the transfer of desired genes (Zambryski 1982). In the present investigation, efforts were made to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation in popular eggplant varieties of

Bangladesh using both screenable and selectable marker genes as an aid to incorporate suitable stress tolerant genes in these varieties of eggplant in the future research program. Apart from these *in vitro* regeneration of plantlets was carried out to facilitate *Agrobacterium*-mediated genetic transformation in popular eggplant varieties of Bangladesh.

Materials and Methods

Two local varieties of eggplant (*Solanum melongena* L.) such as, Nayantara (BARI begun-5) and Kazla (BARI begun-4) were used as experimental materials for this investigation. Seeds of these varieties were obtained from the Vegetable Seed Division of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

The procedures for the sterilization of seeds, germination of seeds, and preparation of explants, *in vitro* regeneration as well as rooting were carried out following in protocols developed by Sarker et al. (2006). The cotyledonary leaf explants were cultured on MS with various concentrations and combinations of BAP and Kn for *in vitro* regeneration of shoots through organogenesis. The pH of the regeneration medium was adjusted to 5.8 before autoclaving. Cultures were incubated and maintained in growth room with a fixed photoperiod of 16/8 hrs dark/light cycle at 25 ± 1°C. For *ex vitro* rooting, the *in vitro* raised shoots were separated out from the culture vessels and cut obliquely at the base. Then the base of excised shoots was dipped into 10 mM IBA solution for five minutes and transferred to small plastic pots containing moist sterilized soil. These pots were then covered with transparent perforated polythene bags and were kept in growth room for two weeks. After two weeks the cover was removed and the plantlets were hardened during the next week.

Genetic transformation was performed using Agrobacterium tumefaciens strain LBA4404 harboring binary plasmid pBI121 containing a scoreable reporter gene GUS (β-glucuronidase) driven by CaMV promoter and a selectable marker gene, nptII encoding for the enzyme neomycin phosphotransferase. This reporter gene was used in assessing the efficiency of transformation. A small amount of glycerol stock (preserved in -80°C refrigerator) of Agrobacterium-strain was streaked on solid YEP medium and incubated at 28°C for 48 hrs. After 48 hrs a single colony of Agrobacterium tumefaciens was picked from plate and again streaked on a new YEP medium. After 48 hrs the bacteria were taken from the plate and dissolved in 30 ml of liquid MS containing 9% (w/v) sucrose. Bacterial suspension having optical density (OD) of 0.3, 0.5 and 0.8 were chosen for transformation. The wave length of optical density (OD) of this suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). 100 μmol/l acetosyringone was added to the *Agrobacterium* suspension before infection.

The cotyledonary leaf explants were precultured on regeneration medium (RM) containing 2.0 mg/l BAP + 0.5 mg/l Kn for 48 hrs. After 48 hrs the explants were dipped in the bacterial suspension and incubated for variable time periods (20, 30 and 40 min).

After incubation the explants were transferred to co-cultivation medium (MS + 2.0 mg/l BAP + 0.5 mg/l Kn + 100 μmol/l acetosyringone) and incubated for 2, 3 and 4 days at 25 ± 2°C in an incubator in the dark. After co-cultivation the explants were thoroughly washed with sterilized distilled water for 4 - 5 times and finally washed for 10 mins with distilled water containing 300 mg/l carbenicillin. After that the explants were transferred to selection medium containing MS supplemented with 2.0 mg/l BAP, 0.5 mg/l Kn, 100 mg/l Kanamycin and 300 mg/l carbenicillin. Explants were sub-cultured on the same selection medium at a regular interval of three to four weeks. After 45 to 50 days of infection, the green compact callus was found to develop at the cut ends of explants. These green calli were further sub-cultured and maintained on same selection medium until the shoots were developed. After 100 -110 days small shoots and shoot buds were subcultured to MS basal medium for proliferation and elongation of shoots. Elongated (2.5 - 4 cm long) shoots were excised and used for *ex vitro* root induction. After 3 to 4 weeks, plantlets with well-developed root system were transplanted to large earthen pots containing soil and organic manure (5 : 1) for further growth and development in the greenhouse condition.

Following each transformation experiment, randomly selected ten co-cultured explants were exposed for GUS histochemical assay to determine the efficiency of transformation using X-gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronide) solution and incubated at 37°C for 24 - 48 hrs. After treatment, explants and plant parts were bleached with 70% ethanol to remove chlorophyll before scoring GUS expression. Leaves, shoots, roots, flower, anther, pollen grain and germinated seedlings were assayed from randomly selected transformed and control (wild) plants.

The genomic DNA was extracted from the young leaves of matured transformed and one non-transformed (control) plants using CTAB method (Doyle and Doyle 1990). Plasmid DNA of pBI121 was used as positive control. Presence of introduced GUS and *nptII* genes were detected by PCR analysis (eppendorf Master Cycler Gradient, Germany). The gene-specific primers for GUS gene 5´CATGAAGATGCGGACTTACG-3´ and 3´-ATCCACGCCGTATTCGGCGT-5´ were used as forward and reverse at a concentration of 10 pmol/μl. For the confirmation of the *nptII* gene, DNA was subjected to PCR analysis using 5´TAGCTTCTTGGG TATCTTTAAAATA-3´and 3´-CCAGTTA CCTTCGGAAAAAGAGTT5´, as forward and reverse primer respectively. For 25 μl PCR reaction 0.2 μl Taq DNA polymerase, 10 × 2.5 μl buffer and 0.5 μl dNTPs were used. For GUS gene DNA was denatured at 95°C for 5.0 min, followed by 30 amplification cycles. Each cycle was programmed with three different thermal periods: at 95°C for 1.0 min to denature DNA, at 56°C for 30 sec to anneal the primer and at 72°C for 1.0 min for the extension or elongation of DNA by Taq DNA polymerase. For *nptII* gene DNA was denatured at 95°C for 5.0 min, followed by 30 amplification cycles using 95°C for 1.0 min (denaturation), 55°C for 1.0 min (annealing) and 72°C for 1.0 min (extension). The final extension lasted for 10 min at 72°C to allow complete extension of all amplified fragments. After completion of cycling program, reaction was held at 4°C. The amplified

DNA was electrophoresed on 1.0% (w/v) agarose gel containing ethidium bromide (0.08 µl/ml) for 40 min. The gel was photographed under UV light using gel documentation system (Bio.Sci.Tech.Gelscan, 6.0. Professional, Germany).

Results and Discussion

Different concentrations and combinations of BAP and Kn were used in MS to examine their effects on initiation and development of shoots from cotyledonary leaf explant. Transformation compatible high frequency uniform *in vitro* regeneration of shoots was achieved on MS supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn for both the varieties of eggplant studied (Table 1). Almost similar results were reported earlier by Shivaraj and Rao (2011) in different set of varieties of eggplant. Development of shoots from cotyledonary leaf explants from the variety Nayantara has been presented in Fig. 1.

Table 1. Effect of different concentrations and combinations of BAP and Kn on *in vitro* regeneration of multiple shoot from cotyledonary leaf explants of two varieties of eggplant (Kazla Nayantara).

Concentrations (mg/l)		Varieties	% of responsive explants	Days required for initiation of regeneration	% of shoot forming explants	Mean no. of shoots/ explants after 6 - 8 weeks
BAP	Kn		Mean		Mean	Mean ± SE
0.5	0.5	Kazla	73	12 - 13	47	1.87 ± 0.21
		Nayantara	70	13 - 14	45	2.27 ± 0.24
1.0	0.5	Kazla	80	11 - 12	50	2.47 ± 0.29
		Nayantara	80	11 - 12	50	2.8 ± 0.29
1.0	1.0	Kazla	90	10 - 12	60	3.70 ± 0.31
		Nayantara	87	9 - 10	63	3.60 ± 0.37
2.0	**0.5**	**Kazla**	**100**	**9 - 10**	**73**	**4.33 ± 0.50**
		Nayantara	**97**	**8 - 10**	**77**	**4.70 ± 0.43**
2.0	1.0	Kazla	88	10 - 11	45	2.66 ± 0.23
		Nayantara	90	10 - 11	45	2.53 ± 0.23

The best initiation of shoot buds from cotyledonary leaf explants of Nayantara was achieved on MS supplemented with 2.0 mg/l BAP and 0.5 mg/l Kn. Initiation of regeneration from these explants occurred within 10 - 11 days of culture (Fig. 1a) and the formation of multiple shoots was achieved within 20 to 25 days from the initiation (Fig. 1b). Almost all the explants were found to respond (100% in case of Kazla and 97% in Nayantara) in this combination of hormonal supplements. Moreover, 73 and 77% of the

responsive explants produced multiple shoots in case of the varieties Kazla and Nayantara respectively. The mean number of developing shoots recorded after six weeks of culture were 4.33 ± 0.50 and 4.70 ± 0.43 in case of the variety Kazla and Nayantara respectively (Table 1). In this case, further elongation and proliferation of multiple shoots were done in hormone free MS medium (Fig. 1c).

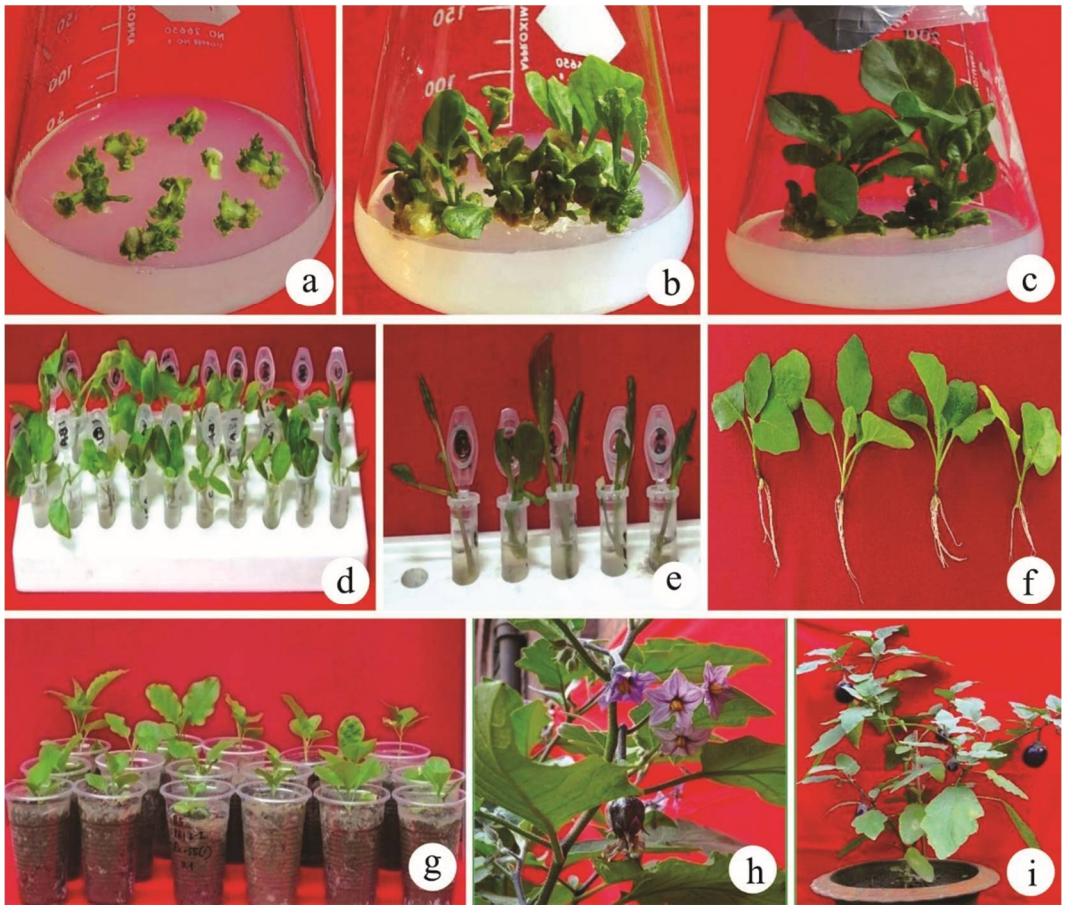

Fig. 1 (a-i). *In vitro* shoot regeneration and *ex vitro* rooting of regenerated shoots of two varieties of eggplant from cotyledonary leaf explants: a. Initiation of shoot buds from cotyledonary leaf explants of Nayantara on MS with 2.0 mg/l BAP & 0.5 mg/l Kn. b. Initiation of multiple shoots on the same media as mentioned in Fig. 1 a. c. Development and elongation of shoots of Nayantara on hormone free MS basal medium. d. Incubation of regenerated shoots in 10 mM IBA solution for *ex vitro* rooting of Nayantara. e. Same as Fig. 1d but in case of regenerated vitrified shoots. f. *Ex vitro* rooted plantlets of Nayantara developed after 21 days of IBA treatment. g. *Ex vitro* rooted plantlets of Nayantara in small plastic pots. h. *In vitro* derived plant of Nayantara flowering and fruit setting after four weeks following transplantation. i. Same as Fig. 1h but showing fully mature plant bearing several fruits.

In the present investigation it was observed that presence of cytokinin in the culture medium for a longer period inhibited the induction of *in vitro* roots from the excised regenerated shoots. Vitrification of shoots were also found to be associated with this event. Such effects of the presence of residual cytokinins in eggplant culture were also reported by Muktadir et al. (2016). In hormone free MS about 70% of the regenerated shoots produced roots but the development of such roots was not synchronized and appeared to be irregular in both the varieties.

However, a number of reports are available where IBA was used for adventitious root formation from regenerated shoots of eggplant (Muktadir et al. 2016, Bhat et al. 2013, Shivraj and Rao 2011). In this study about 85% shoots were found to produce roots on MS supplemented with 2.0 mg/l IBA, but such root formation was found to be ineffective as it was associated with vigorous growth of callus at the base of excised shoot. Further the internode of the cultured shoots for rooting increased rapidly and as a result the shoots became pale green in color and turned fragile in nature. To overcome this problems, *ex vitro* rooting of regenerated shoots of both varieties of eggplant were explored. In a number of medicinal plants IBA treatment was reported to be effective for *ex vitro* root induction (Shekhawat and Manokari 2018, Sharma et al. 2017). *In vitro* regenerated and transformed shoots as well as vitrified shoots of both varieties of eggplant were found to produce roots within 7 to 12 days following 5.0 min incubation of detached shoots in 10 mM IBA solution (Table 2, Figs. 1d-1e). So far there is no previous report on *ex vitro* rooting of *in vitro* regenerated shoots of eggplants. After three weeks of maintenance, 93.33% regenerated shoots of Kazla and 96.66% that of Nayantara produced well developed root system at the base of regenerated shoots (Fig. 1f). In addition, after four weeks of culture, 60 to 67% vitrified shoots of both varieties produced healthy roots and such shoots turned to normal green in color under *ex vitro* condition. *Ex vitro* rooted plantlets were hardened and acclimatized within four weeks (Figs. 1g). After sufficient development of roots plantlets of both varieties were transferred to large earthen pots containing soil and organic manure (5 : 1). In the greenhouse condition 100% plants were survived and these plants flowered after four weeks and producing fruits with viable seeds (Figs. 1h and 1i). These protocols for *in vitro* shoot regeneration as well as *ex vitro* rooting developed for eggplant varieties were utilized for transformation experiments.

To develop an effective transformation system for eggplant, the variety Nayantara was used during this study. Several parameters required for transformation such as optical density of the *Agrobacterium* suspension, incubation period, co-cultivation period of explants were optimized for the variety Nayantara. All the parameters of transformation were optimized through GUS histochemical assay. Transformation efficiency of explants was gradually increased with the increased optical density (OD_{600}) (0.3, 0.5 and 0.8). Cotyledonary leaf segments showed maximum percentage of transformed explants at an optical density of 0.5 (85% *GUS* positive, data not shown). Different incubation periods, such as 10, 20 and 30 minutes were applied using bacterial

suspension with a constant optical density of 0.50. From these results it was evident that transformation efficiency increased with the increase of incubation period of explants. Maximum percentage of the transformed explants (100% *GUS* positive) was observed with an incubation period of 30 minutes. Borna et al. (2010) used 0.8 OD of *Agrobacterium* suspension, 40 minutes of infection period and co-culture period of for 3 days in establishing transformation protocol for potato. Duration of co-cultivation was found to influence the transformation efficiency and 3 days was found to be most suitable. Pratap et al. (2011) reported identical responses for eggplant transformation using cotyledonary leaf explants where 30 minutes of incubation in *Agrobacterium* suspension followed 2-day of co-cultivation was found to be effective.

Table 2. Effects of 10 mM IBA treatment on *ex vitro* root induction from regenerated shoots of two varieties of eggplant on sterilized soil (Data were recorded after three weeks of culture).

Duration of time of IBA treatment (min)	Variety	(%) of rooted shoots	Days required to initiate roots	Days required to obtain well developed roots	No. of roots/ shoot	Length of roots (cm)
		Mean			Mean ± SE	Mean ± SE
5.0	Kazla	93	7-8	20 – 21	8.90 ± 0.66	2.64 ± 0.07
	Nayantara	97	8-10	20 – 21	9.20 ±0.74	2.95 ± 0.08
5.0	Kazla (vitrified shoots)	67	13-15	28 – 30	7.90 ± 0.62	2.52 ± 0.12
	Nayantara(vitrified shoots)	60	13-15	28 -30	8.40 ± 0.56	2.57 ± 0.12

After co-cultivation, the cotyledonary leaf explants were cultured on MS medium containing 2.0 mg/l BAP 0.5 mg/l Kn, 100 mg/l Kanamycin and 300 mg/l carbenicillin for regeneration of shoots and after six to seven weeks from co-cultivation initiation of shoot regeneration was observed. The results of transformation experiments have been presented in Fig. 2. Kanamycin (100 mg/l) was found to be effective in selecting the transformed shoots. About 51% of co-cultured explants were survived and showed initiation of regeneration at the cut ends of explants (Fig. 2a). Due to Kanamycin selection the non-transformed explants as well as a part of several explants gradually turned yellow to brown in colour and such tissue was discarded during the subculture. In this experiment, the effect of kanamycin selection was adequate when it was applied immediately after co-cultivation of infected explants as has been experienced by Franklin and Sita (2003). Following subculture of survived healthy explants the initiation of regeneration was visualized under stereomicroscope (Fig. 2b). Initiation of shoots was observed after 11-12 weeks of co-cultivation of explants (Fig. 2c). It was found that all the responsive explants didn't produce shoots and only 32% of infected explants finally able to produce multiple shoots with efficiency of 2 – 3 shoots per explant. For proliferation and elongation of shoots, the small green shoots along with the shoot buds were further

transferred to fresh MS containing 100 mg/l kanamycin (Fig. 2d). Following elongation the shoots (2.5 – 4.0 cm long) were subjected to *ex vitro* rooting for three to four weeks (Fig. 2e). After four weeks the rooted and acclimatized putatively transformed plantlets were transplanted to large earthen pot. Following transplantation, the plants were flowered after 4 - 5 weeks and found to produce fruits with viable seeds (Fig. 2f).

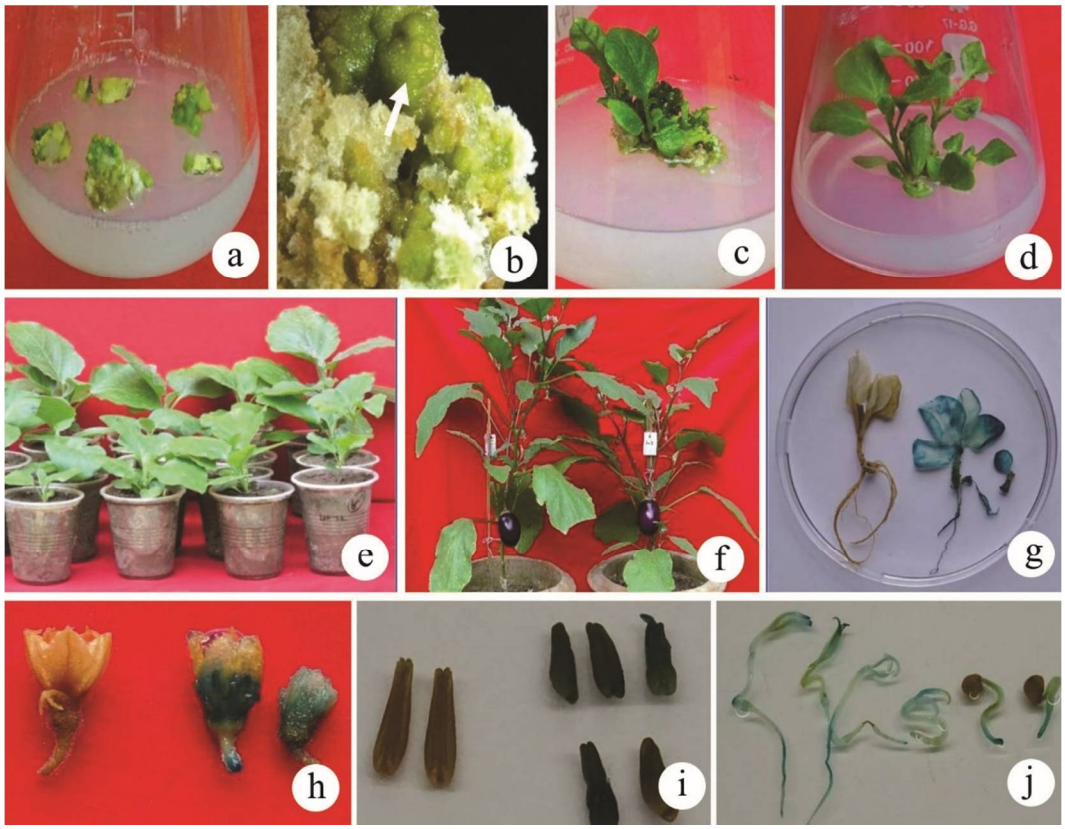

Fig. 2. Various stages of development of transformed plantlets from cotyledonary leaf explants following *Agrobacterium*-mediated transformation in variety Nayantara. a. Initiation of regeneration from cotyledonary leaf explants after 5-6 weeks of infection b. Stereoscopic view of green compact callus of cotyledonary leaf explants after 7 weeks of infection (arrow indicates the formation of shoot buds). c. Initiation of shoots after 10 weeks of infection. d. Fully developed shoots after 14 weeks of infection. e. *Ex vitro* rooted fully developed putatively transformed plantlets on small plastic pot containing sterilized soil. f. Fruits set in putatively transformed plantlets on large earthen pots. g. GUS expression of transgenic plantlets (blue in colour) with a control one (white in colour). h. Flowers from T_0 plant showing GUS expression with a control. i. Anthers of putative T_0 plant showing GUS expression with control. j. Seedlings from T_1 plants showing GUS expression (blue colour). Note that the control materials in all Figs. did not produce characteristic blue colour of GUS expression.

GUS histochemical assay was carried out at every steps of shoot development during the transformation to monitor the efficiency of transformation. Stable expression of *GUS* gene was visualized through histochemical staining of the transformed rooted shoots (Fig. 2g). The floral parts (petals, anthers and stigma) also exhibited the characteristic blue colour indicating the stable integration of *GUS* gene in the transformed plants (Figs. 2h – 2i). Moreover, the T_1 seedlings showed *GUS* positive expressions which confirms the successful insertion of *GUS* genes into the advanced progeny obtained from the transformed (T_0) plants (Fig. 2j).

The transgenic nature of the transformed plants was confirmed through PCR analysis using the primers of *GUS* and *nptII* genes respectively (Figs. 3a, b). Genomic DNA from 18 randomly selected transformed plants, one wild type plant and pBI121 plasmid DNA were used as template for the PCR. From the gel it was evident that both *GUS* and *nptII* genes were inserted in the genomic DNA of 18 transformed plantlets exhibiting very clear and prominent band identical to positive control (pBI121) used for *GUS* and *nptII* genes.

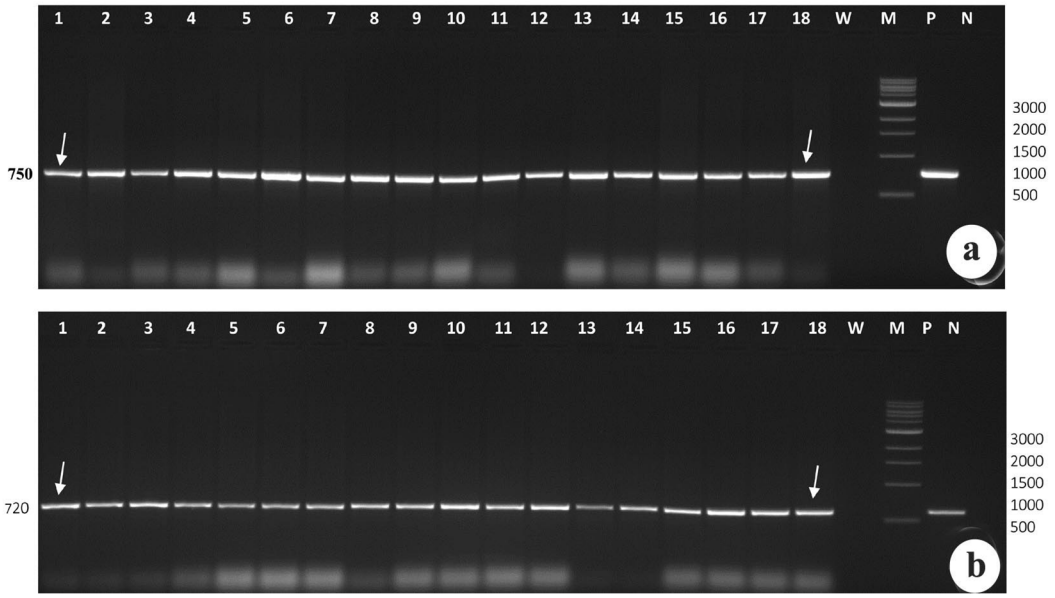

Fig. 3 (a - b): Agarose gel showing the PCR amplified bands for *GUS* and *nptII* genes. a. Molecular characterization of the putative transformants of Nayantara variety through PCR amplification of *GUS* genes (lanes 1-18 genomic DNA of transformed shoots; lane W- wild type; lane M- 1kb ladder; lane P- plasmid DNA of pBI121 as positive control; lane N- negative control). b. Same as Fig. a but for *nptII* gene.

In conclusion, it may be mentioned that an efficient and reproducible *Agrobacterium tumefaciens*- mediated transformation protocol was established for eggplant varieties of Bangladesh using cotyledonary leaf as explants using screenable (*GUS*) and selectable

(*nptII*) marker genes. Following this standardized protocol various other agronomically important gene/genes can be transferred to the locally grown eggplant varieties. Particularly this technique of transformation can be utilized for the development of salinity and drought tolerant eggplant varieties which will certainly contribute significantly in future agriculture of Bangladesh.

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