ISSN 1817-3721, E-ISSN 1818-8745

Plant Tissue Cult. & Biotech. **30**(1): 149-160, 2020 (June)

PTC&B

In vitro Regeneration and *Agrobacterium*-mediated Genetic Transformation of a Cultivated Potato Variety Using Marker Genes*

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Key words: Transformation, Potato, *nptII* and *GUS* gene, *Agrobacterium*

Abstract

Agrobacterium-mediated genetic transformation was carried out for Asterix (BARI Alu-25), a popular potato (*Solanum tuberosum* L.) variety cultivated in Bangladesh. For Direct organogenesis of shoots the best response was noted when nodal segments and microtuber discs of Asterix along with Diamant - another popular potato variety were cultured on MS with 4.0 mg/l BAP and 1.0 mg/l IAA. MS without plant growth regulators was most effective for root induction from the excised regenerated shoots. Following optimum root development, the *in vitro* regenerated plantlets were successfully established in soil. *Agrobacterium tumefaciens* strain LBA4404/pBI121 containing *GUS* and *nptII* genes showed maximum transformation response in nodal segment with bacterial suspension having an optical density of 0.6 at 600 nm in Asterix variety. Moreover, 30 min incubation followed by 72 hrs co-cultivation was found most effective for transformation as has been determined by transient GUS histochemical assay. Transformed shoots were selected using MS with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3, 300 mg/l carbenicillin and 200 mg/l kanamycin. Stable integration of *GUS* and *nptII* genes were confirmed by PCR analysis using the genomic DNA isolated from transformed shoots.

Introduction

Potato (*Solanum tuberosum* L.), a solanaceous staple food crop, is ranked fourth among the major crops after maize, rice and wheat. It is a nutritious food for about 1.3 billion people and the tubers are becoming increasingly popular in the developing world (Stokstad 2019).

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DOI: https://doi.org/10.3329/ptcb.v30i1.47800

It produces high yields on small pieces of land, and it can be sold as a cash crop to sustain the livelihoods of small farmers and as such so potato is considered as an important crop in the developing countries of the world (Ramani and Mouille 2019). It also can provide more carbohydrates, proteins, minerals and vitamins per unit area of land and time as compared to other potential food crops (Zaheer and Akhtar 2016). Potatoes contribute key nutrients to the diet including vitamin C, potassium and dietary fiber (McGill et al. 2013). More so, nowadays roots and tubers are the third largest source of carbohydrate in the world, with potatos representing nearly half of the total ~~all~~ consumed root crops-(International Potato Center 2018).

Potato crop is exposed to different kinds of biotic and abiotic stresses including drought, salinity, cold and heat which are considered to be prominent limiting factors for its production (Dangol et al. 2018). Gene stacking in polyploid crops using conventional breeding is a difficult, laborious and time consuming (Weeks 2017). In potato, several breeding efforts have been made for particular trait improvement using wild species but met with limited success (Carputo and Barone 2005). The presence of four copies (alleles) of genes in the tetraploid (2n = 4x = 48) genome of cultivated potato (*S. tuberosum*) makes it difficult for workers to precisely edit the genome using conventional breeding tools (Consortium 2011).

Potato has gone through genetic manipulations with the advent of genetic engineering. These technologies help the workers to introduce the genes of economic importance in the genome of potato. Several studies for abiotic stress tolerances and improvement in nutrient quality have been documented (Hameed et al. 2018). Genetic engineering as a method has the potentiality to clonally propagated plants, as it can bypass the challenges of inbreeding depression, long breeding cycles and sexual incompatibility (Ko et al. 2018). One of the crucial factors that determine the success of genetic transformation and tissue regeneration is the delicate balance between effective negative selection that kills non-transformed cells and supporting the competence of transformed cells to regenerate (Nguyen et al. 2016).

In the light of this information, it should be possible to improve transformation and regeneration efficiency of potato by improving some of the key limiting factors leading to low transformation efficiency. In the present investigation attempts were made to establish efficient methods for *in vitro* plant regeneration and *Agrobacterium*-mediated genetic transformation in cultivated potato variety of Bangladesh using marker genes as an aid to insert salinity and drought tolerant genes in these varieties in any future research endeavor.

Materials and Methods

Tubers of two varieties of potato (*Solanum tuberosum* L.) namely, Asterix and Diamant, used in this investigation were collected from the Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur. Tubers were kept at 4°C refrigerator for 7 days to

break the dormancy. After 7 days, tubers were placed in the dark chamber for 15 days at 25 ± 2°C for the development of sprouts. Sprouts (2.0 cm) were then used as primary explants for the establishment of *in vitro* cultures.

For sterilization the sprouts were first washed three times with distilled water and surface sterilized with 0.1% (w/v) $HgCl_2$ for 7 - 8 min inside a laminar flow cabinet. The surface sterilized sprouts were cultured on MS to obtain shoots. The desired explants of nodal segments were collected from *in vitro* raised shoots. MS with various combinations of BAP and IAA were used for *in vitro* regeneration. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were maintained in growth room with a photoperiod of 16 hrs at 25 ± 1°C with light intensity of 2500 - 3000 lux. For induction of roots, regenerated shoots were excised and transferred to MS without plant growth regulator (PGR).

Glycerol stocks of *Agrobacterium tumefaciens* LBA4404 containing the binary plasmid pBI121 was used to prepare bacterial plates (YEP plates) by streaking. This plasmid contains a scoreable reporter gene *GUS* (β-glucuronidase) driven by CaMV35S promoter and NOS terminator and a selectable marker gene *nptII* fused between NOS promoter and terminator encoding for the enzyme neomycin phosphotransferase. The plates were incubated at 28°C for 48 hrs in incubator. Bacteria was sub-cultured in a new YEP plate. The plate was incubated at 28°C for 48 hrs in incubator.

From the plates, bacterial cells were suspended in 25 ml of bacterial suspension medium (liquid MS containing 9% (w/v) sucrose) using vortex machine (FISONS Whirl Mixer). Then optical density (OD) of this suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). Bacterial suspension of OD 0.6 - 0.8 was preferred for transformation. This bacterial suspension was prepared before 30 min of infection. One hundred μmol acetosyringone was added to the suspension before infection.

The explants were dipped in the bacterial suspension and incubated for 20, 30 and 40 min. Then the explants were soaked in filter papers for a short period of time to remove excess bacterial suspension prior to transfer of all explants to co-cultivation media (MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 100 μmol acetosyringone). All the explants were maintained on co-culture medium for 2, 3 and 4 days at 25 ± 2°C in the dark. Co-cultured explants were washed with distilled water for 3 - 4 times until no opaque suspension was seen. They were then washed for 15 min with distilled water containing 300 mg/l carbenicillin. After that the explants were gently soaked with a sterile filter paper and transferred to selection medium (MS with BAP and IAA) containing 200 mg/l kanamycin and 300 mg/l carbenicillin. Sub-culture of infected explants was done regularly at an interval of 20 - 22 days on selection medium. After 65 - 70 days the green calli were separated and sub-cultured on MS supplemented with BAP, IAA and GA_3. After 95 - 100 days of inoculation the small multiple shoots and shoot - buds were transferred on the same medium for shoot elongation. The elongated shoots

were excised and transferred to root induction medium (PGR free MS). After root formation the plantlets were transplanted to small plastic pots containing soil.

Randomly selected ten co-cultured explants from each of the treatments were examined for GUS histochemical assay to determine the efficiency of transformation. Co-cultured explants were immersed in X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) solution and incubated at 37°C for 24 - 48 hrs. A characteristic blue color would be the expression of GUS gene in the plant tissue. For control treatment, GUS assay was done with the explants from normal plant. Leaves, shoots, roots and germinated micro-tubers were assayed from randomly selected plants. Explants and plant parts were dipped into GUS buffer solution for 24 - 48 hrs at 37°C. After 24 - 48 hrs treatment, explants were bleached with 70% ethanol to remove chlorophyll before scoring GUS expression.

Genomic DNA was isolated from the transformed potato shoots and stable integration of *GUS* and *nptII* genes were confirmed by PCR analysis. CTAB method (Doyle and Doyle 1990) was used for DNA isolation. The primers for *GUS* gene 5´-CATGAAGATGCGGACTTACG-3´ and 3´-ATCCACGCCGTATTCGGCGT-5´ as forward and reverse were used at a concentration of 100 pmol/µl. For the detection of the *nptII* gene, DNA was subjected to PCR using forward and reverse primer comprising 5´-TAGCTTCTTGGGTATCTTTAAAATA-3´and 3´-CCAGTTACCTTCGGAAAAAGAGTT-5´, respectively. Taq DNA polymerase 0.2 µl, 10 × buffer 2.5 µl and dNTPs 1.0 µl were used for PCR reaction. In each reaction, the volume of PCR buffer was used one tenth of the total reaction volume which was 25 µl. For *GUS* gene initial denaturation of PCR at 95°C for 5.0 min followed by 30 cycles of denaturation at 95°C for 1.0 min was performed. The annealing temperature being 56°C for 30 sec and elongation or extension at 72°C for 1.0 min was adjusted for *GUS* gene. After the last cycle, a final step of 10 min at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling program, reaction was held at 4°C. For *nptII* gene the cycling conditions were 5.0 min at 95°C denaturation and 30 amplification cycles using 95°C for 1.0 min, 55°C for 1.0 min (annealing) and 72°C for 1.0 min followed by 10.0 min at 72°C. The amplified DNA was run on 1.0% (w/v) agarose gel and stained with ethidium bromide (0.08 µl/ml).

Results and Discussion

The present investigation was carried out for the establishment of *in vitro* regeneration system and optimization of *Agrobacterium*-mediated genetic transformation parameters in potato using marker genes. Newly developed potato sprouts were used as primary explants for the establishment of *in vitro* cultures. Agar solidified MS with 3% (w/v) sucrose was found to be most effective for the development of shoots from sprout (Fig. 1a). Spontaneous microtuberization was obtained when shoots were cultured on MS containing 3% (w/v) sucrose for 45 - 60 days. However, to increase the number of *in vitro* microtuber formation, 4 - 6 cm long shoots derived from nodal segments were cultured

on MS containing 9% (w/v) sucrose. This high concentration of sucrose was required to enhance the process of microtuber formation. These developing microtubers were green and stout (Fig. 1b) and they were cut vertically into small pieces and were used as explants. From the *in vitro* grown shoots; nodal segments and the microtuber discs were the responsive explants for direct regeneration of shoots. MS containing various concentrations and combinations of BAP and IAA were used for multiple shoot regeneration.

In both Asterix and Diamant varieties, MS with different concentrations and combinations of BAP and IAA were used to observe their effect on induction of multiple shoots and their subsequent development from the nodal segments and microtuber discs explants. Initiation of shoots (Fig. 1c, d) from nodal segments and microtuber discs occurred within 8 - 9 days (Table 1). It is evident from the Table 1 that the maximum number of shoots were developed after 30 days on MS with 4.0 mg/1 BAP and 1.0 mg/1 IAA for Asterix (Fig. 1e). In this case the average number of shoots was 9.88 ± 0.23 per nodal segment and 5.63 ± 0.76 shoots per microtuber discs. Maximum shoot length (an average of 8.90 ± 0.74 cm from nodal segment and 10.30 ± 0.56 cm from microtuber discs developed after six weeks of culture) was observed on the media containing the same PGR supplements.

In case of Diamant, the best proliferation of shoots was observed after 30 days. In this variety average number of shoots was 8.82 ± 0.22 per nodal segment, on the other hand that was 5.31 ± 0.38 shoots per microtuber discs in presence of the same PGR supplements (Table 1, Fig. 1f). Maximum shoot length was recorded as 9.80 ± 0.45 cm from nodal segment and 9.30 ± 0.37 cm from microtuber discs developed after six weeks of culture.

It has been reported earlier that BAP and IAA produced multiple shoots in different potato varieties growing in Bangladesh (Sarker and Mustafa 2002, Borna et al. 2010 and Khatun et al. 2012). Previously, Hussain et al. (2005), reported that nodal explants has high regeneration potential which was followed by shoot apices. Recently, Borna et al. (2019) reported efficient shoot regeneration from the peripheral region of microtuber disc explant on the MS containing 4.0 mg/l BAP and 2.0 mg/l IAA. Sarker and Mustafa (2002) used leaf, nodal segments and inter-nodes of two local potato varieties for *in vitro* regeneration.

Shoots (3 - 5 cm long) were excised and cultured on half and full strength of MS for root induction. The number of healthy roots were more on MS (Fig. 1g). This response was common for both the varieties used here. After enough development of roots, the plantlets raised from two potato varieties were successfully transplanted into small plastic pots containing autoclaved soil. The survival rate of the transplanted plantlets was found to be about 98%. The survived plantlets were transferred to larger clay pots for their further growth (Fig. 1h) and maintained in growth room and 5 - 6 minitubers (Fig. 1i) were collected from each plant after 85 - 90 days.

Table 1. Effect of different concentrations and combinations of BAP and IAA on shoot regeneration from nodal segments and microtuber discs of two varieties of potato.

Varieties	Explants	BAP (mg/l)	IAA (mg/l)	Mean no. of shoots/ explant (±SE)	Mean length of shoots/plant (± SE)
Asterix	Nodal segments	3.0	0.5	3.55 ± 0.98	8.50 ± 0.34
		3.0	1.0	3.68 ± 0.53	7.20 ± 0.67
		4.0	0.5	7.29 ± 0.44	8.00 ± 0.56
		4.0	**1.0**	**9.88 ± 0.23**	**8.90 ± 0.74**
		5.0	0.5	3.29 ± 0.74	7.20 ± 0.55
		5.0	1.0	2.98 ± 0.94	6.90 ± 0.43
	Microtuber discs	3.0	0.5	3.13 ± 0.62	7.10 ± 0.22
		3.0	1.0	3.46 ± 0.80	8.40 ± 0.67
		4.0	0.5	5.93 ± 0.68	9.50 ± 0.32
		4.0	**1.0**	**5.63 ± 0.76**	**10.30 ± 0.56**
		5.0	0.5	3.22 ± 0.49	6.80 ± 0.94
		5.0	1.0	3.40 ± 0.51	7.30 ± 0.43
Diamant	Nodal segments	3.0	0.5	3.40 ± 0.67	6.30 ± 0.52
		3.0	1.0	3.66 ± 0.48	6.30 ± 0.44
		4.0	0.5	7.20 ± 0.34	8.10 ± 0.86
		4.0	**1.0**	**8.82 ± 0.22**	**9.80 ± 0.45**
		5.0	0.5	2.83 ± 0.96	8.90 ± 0.67
		5.0	1.0	2.13 ± 0.81	6.30 ± 0.33
	Microtuber discs	3.0	0.5	3.25 ± 0.35	7.40 ± 0.54
		3.0	1.0	3.43 ± 0.54	6.10 ± 0.12
		4.0	0.5	4.19 ± 0.30	8.10 ± 0.77
		4.0	**1.0**	**5.31 ± 0.38**	**9.30 ± 0.37**
		5.0	0.5	2.88 ± 0.60	7.60 ± 0.49
		5.0	1.0	2.53 ± 0.54	6.70 ± 0.78

In this phase, a series of experiments were carried out to develop a suitable protocol for *Agrobacterium*-mediated genetic transformation of Asterix. The transformation efficiency was significantly influenced by several factors, such as optical density (OD) of *Agrobacterium* suspension, duration of incubation period and that of co-cultivation. By monitoring the histochemical assay of the *GUS* reporter gene in the explant tissue, optimization of these parameters was carried out. The expression of *GUS* gene is associated with the formation of a characteristic blue color within the cell/tissue. Prominent GUS positive (blue colored) regions were detected at the cut surface and in the meristematic part of the nodal segment explants. Several non-infected (treated as control) explants were also examined to compare the expression of GUS genes during this study.

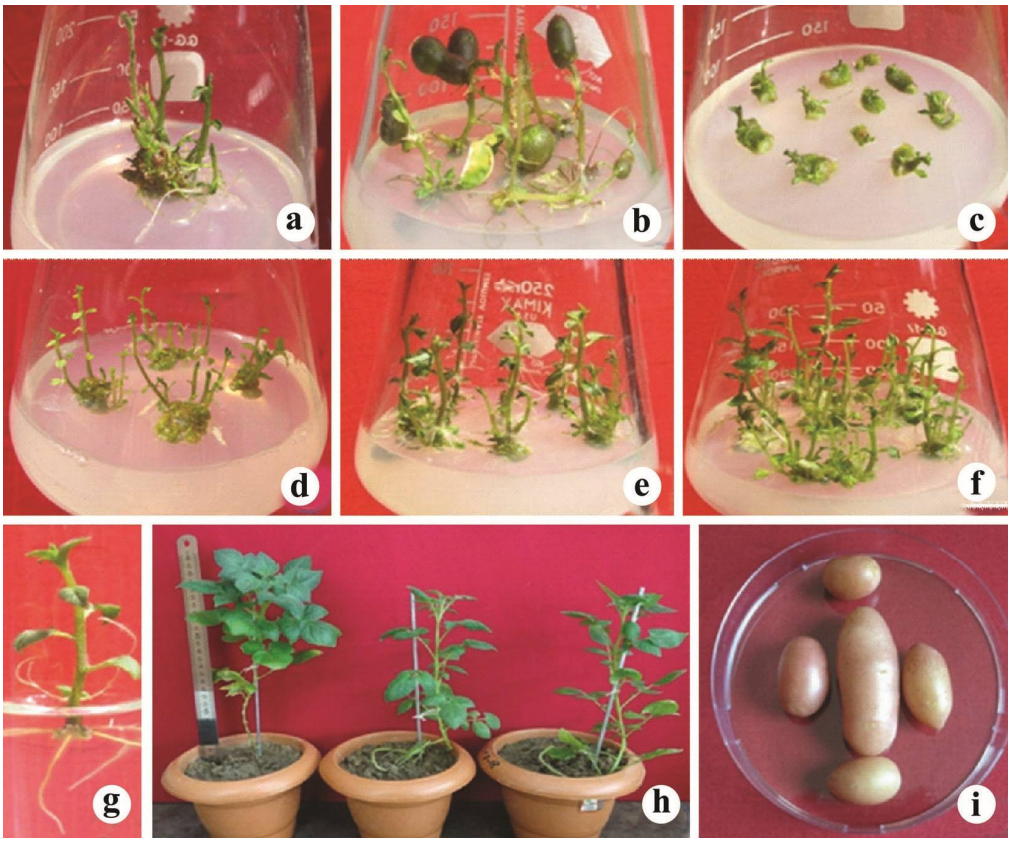

Fig. 1. *In vitro* regeneration of shoots and microtubers directly from the explants of potato: a. Shoots developing from sprouts. b. Microtuber formation in Diamant. c. Initiation of shoots from nodal segment in Asterix. d. Multiple shoot initiated from microtuber disc of Diamant. e. Elongated shoots in Asterix. f. Same as Fig. 1e but in case of Diamant. g. Fully developed rooted plantlet of Asterix. h. Regenerated plants of Asterix established in soil. i. Minitubers of Asterix developed from *in vitro* regenerated plants.

One of the most important influencing factors for transformation efficiency is optical density of *Agrobacterium* suspension. Optical density (OD) was measured at 600 nm. In these experiments optical densities of 0.6, 0.7 and 0.8 were used. Where optical density (OD) 0.6 exhibited the highest GUS +ve nodal explants in Asterix (90%). Another very important parameter is the incubation period where the explants were incubated into bacterial suspension for a definite duration. For this purpose, different incubation periods, such as 15, 30 and 45 min were applied using bacterial suspension with a constant optical density of 0.6. From the result it was evident that, maximum percentage (100%) of the transformed explants was observed with an optical density 0.6 having an incubation period of 30 min. Borna et al. (2010) reported about the various regulatory factors influencing transformation efficiency of potato. In a separate set of experiments Sarker et al. (2009) reported that an incubation period of 40 min was optimum in case of variety Cardinal and Atlas.

Co-cultivation (Fig. 2a) period is another factor that significantly influences the transformation efficiency. The transformed explants were co-cultivated for 2, 3 and 4 days at the constant incubation period. During long co-cultivation period (more than 4 days), bacteria were found to grow heavily on the co-culture medium which was not suitable for the growth and survival of co-cultured explants. Therefore, co-cultivation period of 3 days was found to be the most suitable when transformation experiment was performed under optimum condition. Transformation efficiency in Diamant, Cardinal and Granola was found to be maximum with bacterial suspension having an optical density of about 1.0 at 600 nm, incubation period of 60 min followed by 72 hrs of cocultivation (Borna et al. 2010).

For the selection of transformed tissues, antibiotic selection pressure was applied immediately after co-cultivation. Selection of transformed shoots was done by using kanamycin, since kanamycin resistance *nptII* gene was present in the plasmid of engineered *Agrobacterium*. For such selection, the explants were transferred directly to MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 300 mg/l carbenicillin and 200 mg/l kanamycin. For control, explants were inoculated in selection medium without their infection. Direct selection allows to obtain regeneration of only transformed shoots following co-cultivation. It was observed that when kanamycin was applied immediately after co-cultivation, non-transformed explants turned albino or deep brown in colour (Fig. 2b), while the transformed explants remain green or light brown.

The non-transformed albino explants were discarded. The green and brown explants were sub-cultured regularly after every 20 - 22 days. After 65 - 70 days, the brown explants started greening and formed green callus (Fig. 2c). Regeneration and proliferation of shoots occurred after 95 - 100 days of infection on MS supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3, 300 mg/l carbenicillin and 200 mg/l kanamycin (Fig. 2d). Stereomicroscopic view of initiation of shoots from callus of nodal segments was shown in Fig. 2e.

The transformed shoots (2 - 3 cm in length) survived on the selection medium (Fig. 2f.) were separated and transferred to MS containing 100 mg/l carbenicillin. About 6 - 8 days were required for induction of root (Fig. 2g). These transformed plantlets were transplanted to soil where these plants were found to survive following proper acclimatization (Fig. 2h). These plants were maintained in growth room and after 85 - 90 days several minitubers (Fig. 2i) were collected from these plants.

Stable expression of *GUS* gene was visualized through histochemical staining of the transformed regenerating shoots (Fig. 3a). The roots, leaf, microtuber discs and germinated microtuber from the transformed shoots also exhibited conspicuous GUS gene expression (Fig. 3b-d). This result is also in accordance with Sarker and Mustafa (2009) where histological GUS assay showed the expression of GUS gene in the leaf tissues of transformed shoots.

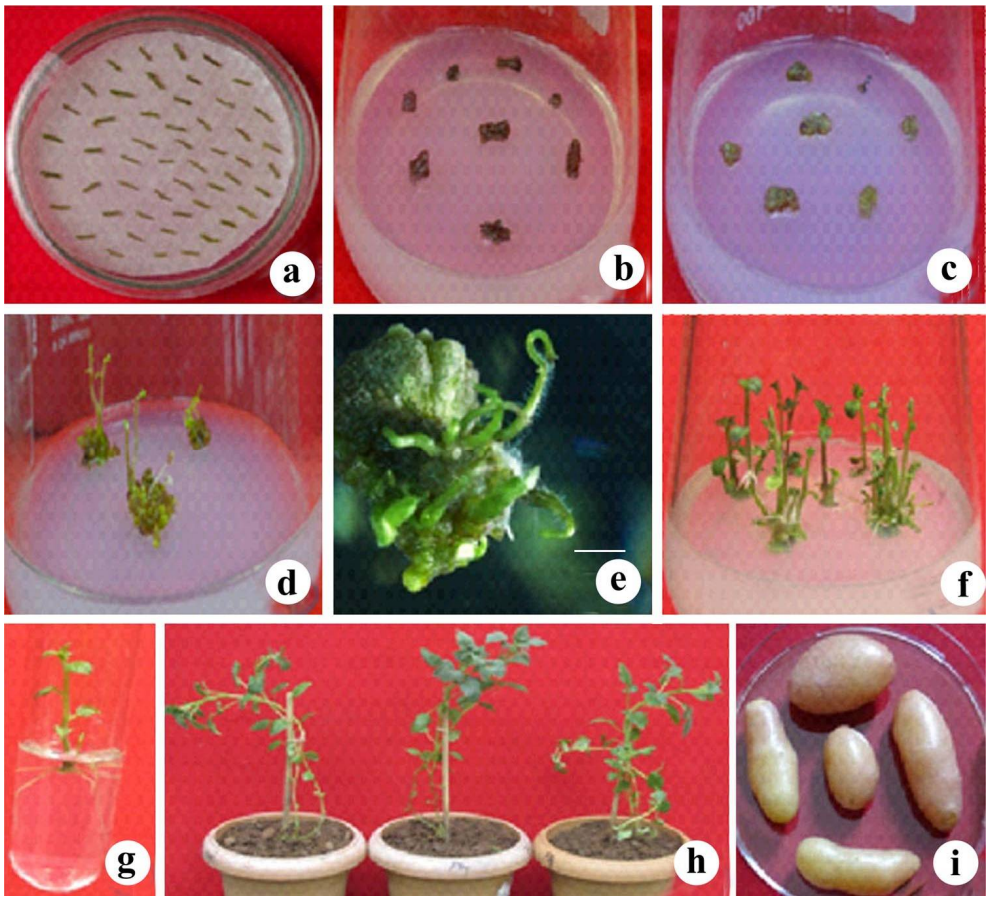

Fig. 2. Development of transformed plantlets and tubers from nodal segment explants infected with *Agrobacterium* strain LBA4404 containing plasmid pBI121. a. Co-culture of nodal segments of Asterix. b. Non-transformed explants (negative control) turned albino and turned deep brown in colour. c. Callus formation from brown nodal segments (infected with *Agrobacterium* strain LBA4404 containing plasmid pBI121) after 5-6 weeks. d. Initiation of shoots from callus of nodal segments. e. Stereomicroscopic view of initiation of shoots from callus derived from nodal segments after 98 days of infection. f. Multiple shoots formation. g. Fully developed roots from regenerated shoots of Asterix on MS. h. Regenerated plantlets established in pots containing soil. i. Minitubers developed from putatively transformed plants.

The transgenic nature of the shoots was confirmed by PCR amplification of the *GUS* and *nptII* genes present within the genomic DNA (Figs 4 - 5) of 15 randomly selected transformed individuals. Specific primers were used for this purpose as detailed in the Materials and Methods section. Amplified DNA was analyzed using agarose gel electrophoresis. From the gel it was observed that the single band formed in each of the 15 transformed plantlets were identical to the amplified DNA of positive control. This result indicated that both the GUS and *nptII* genes were inserted in the genomic DNA of 15 transformed plantlets and they showed prominent positive band. The band size was 750 and 720 bp for GUS and for *nptII* gen, respectively.

Fig. 3. Histochemical localization of GUS activity of different parts of fully developed transgenic plants of Asterix. a. GUS expression of transgenic plantlets with a control one. b. same as Fig. 3a but in case of leaf lets. c. GUS expression in germinated microtubers and d. microtuber discs of a T_0 plant showing conspicuous blue colour. Note that the control materials in all Figs. did not produce characteristic blue colour of GUS expression

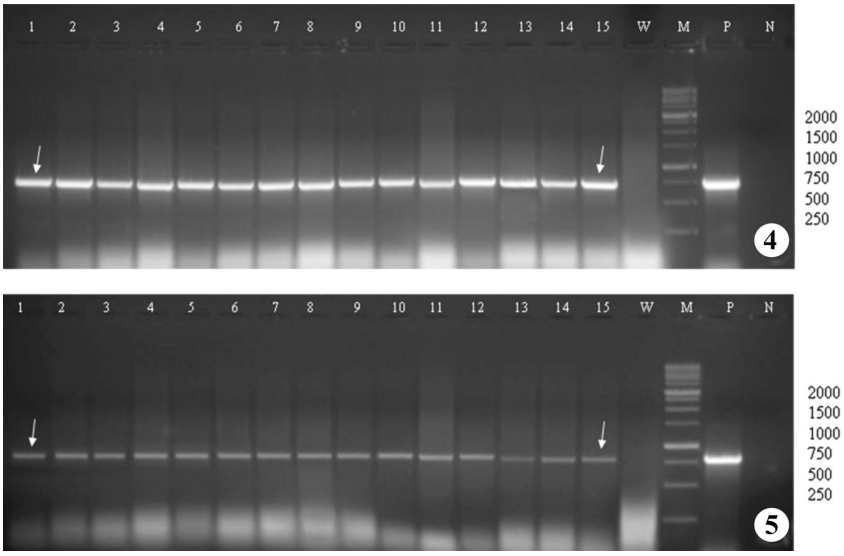

Fig. 4 - 5: 4. Molecular confirmation of the putative transformants of Asterix. PCR amplification of GUS and *nptII* genes (lanes 1-15 genomic DNA of transformed plants; lane W- wild; lane M-1kb ladder; lane P-plasmid DNA of pBI121 as positive control; lane N- negative control). 5. Same as 4 but with *nptII* gene.

The protocol of *Agrobacterium*-mediated genetic transformation developed here can be used for the production of transgenic potato plants for any specific purpose. Here the protocol was primarily developed using screenable marker gene like GUS and selectable marker gene like *nptII*. Using this protocol, in future agronomically important gene/s namely, salinity and drought tolerance can be incorporated to the potato varieties grown in Bangladesh. Incorporation of the above-mentioned abiotic stress resistant genes could facilitate potato to acclimatize in diverse agro-ecological zones, thus impeding food-shortage in less fertile or water deficit lands.

Acknowledgement

The first author (SRM) is grateful to Jagannath University, Dhaka for granting her study leave and the Ministry of Science and Technology, Govt. of the People's Republic of Bangladesh for offering Bangabandhu Science & Technology Fellowship. She is also thankful to Prof. Dr. Mohammad Nurul Islam, Dr. Rita Sarah Borna, Dr. Tahmina Islam and Mrs. Sabina Yesmin (Ph.D. Fellow), Department of Botany, University of Dhaka for their valuable advice and encouragement during the research work.

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(Manuscript received on 14 May, 2020; revised on 30 May, 2020)