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Establishment of an efficient *Agrobacterium*-mediated Genetic Transformation protocol for Potato (*Solanum tuberosum* L.) var. Diamant

Sanjida Rahman Mollika[1], Tahmina Islam, M. I. Hoque and R. H. Sarker*

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Key words: Genetic transformation, *Agrobacterium*, Neomycin phosphotransferase II (NPTII), Potato, Diamant

Abstract

The experiment aimed to standardize the transformation capability of leaf disc, internode, and nodal segment explants from the Diamant variety of potato (*Solanum tuberosum* L.). Nodal segments, internodes and leaf disc explants were infected with *Agrobacterium tumefaciens* strain LBA4404, which harbors the binary vector pBI121 containing the *GUS* reporter gene and the neomycin phosphotransferase *(NPTII)* kanamycin resistance gene. Optimal transformation responses were observed in nodal segments with a bacterial suspension exhibiting an optical density of 0.6 at 600 nm in the Diamant variety. Furthermore, a 30 min incubation followed by 72 hrs of co-cultivation was identified as the most successful method for transformation, as indicated by the transient GUS histochemical assay. Transformed shoots were selected utilizing MS medium 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. The stable integration of *GUS* and *NPTII* genes was validated using PCR analysis utilizing genomic DNA extracted from transformed plants.

Introduction

Potato (*Solanum tuberosum* L.) is an important crop plant of Solanaceae, ranks third among edible and first among non-cereal crops in terms of consumption. It ranks as the fifth most important food crop globally, following wheat, maize, rice, and sugarcane (Dangol et al. 2018). Nevertheless, the potato is susceptible to several biotic and abiotic variables, and numerous strategies, including conventional breeding methods, have been employed in the past to mitigate these challenges. Traditional procedures are generally

*Author for correspondence: <rhsarker2000@yahoo.co.uk>. [1]Department of Botany, Jagannath University, Dhaka-1100, Bangladesh.

regarded as complicated and need a relatively extended duration to yield a variety with the required traits (Grafius and Douches 2008). Potato breeding efforts to enhance specific features using wild species have yielded limited success (Carputo and Barone 2005). Researchers and breeders face difficulties in modifying the genome of potato effectively through conventional breeding methods due to the tetraploid (2n = 4x = 48) genome of the cultivated potato which contains four allelic copies of genes (Consortium 2011). Conventional breeding for gene stacking in polyploid crops is a complex, laborious, and protracted endeavor (Weeks 2017). To mitigate these constraints, plant genetic engineering utilizing advantageous genes from diverse organisms has yielded encouraging outcomes in enhancing the yield and quality of potato cultivars (Urwin et al. 2001, Chue et al. 2004). The development of an effective *in vitro* regeneration strategy is certainly essential for successful plant genetic transformation (Soto et al. 2007). Numerous *in vitro* regeneration procedures for potato have been developed to facilitate genetic transformation (Borna et al. 2010, Chakravarty and Wang-Pruski 2010, Shin et al. 2011, Mollika et al. 2024). Reports are available concerning the impact of genotype, explant type, and culture conditions on the efficacy of *in vitro* regeneration in potato cultivars (Sarker and Mustafa 2002). Recently, Borna et al. (2010), Chakravarty and Wang-Pruski (2010), Shin et al. (2011) and Mollika et al. (2020) demonstrated the successful transformation of potato genotypes. The efficiency of transformation techniques is significantly dependent on genotype; thus, optimization must be required for each specific potato genotype/varieties (Vinterhalter et al. 2008). Diamant variety of potato is popularly cultivated in Bangladesh and there are several studies are being conducted for the improvement of this variety. It is understood that an efficient technique is essential for the desired improvement of this potato variety through genetic transformation. Therefore, the aim of this study was to establish a regeneration and transformation protocol for the Diamant variety of potato cultivated in Bangladesh, utilizing *Agrobacterium tumefaciens* containing *GUS* and *NPTII* genes.

Materials and Methods

The tubers of the Diamant variety of potato (*Solanum tuberosum* L.) utilized in this study were obtained from the Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur. Tubers were stored at 4°C for 7 days to induce dormancy break. Following a duration of 7 days, the tubers were placed in a dark chamber for 15 days at a temperature of 25 ± 2°C to facilitate sprout development. Sprouts (2.0 cm) were subsequently utilized as main explants for the initiation of *in vitro* cultures. The sprouts were initially washed three times with distilled water and subsequently surface sterilized with 0.1% (w/v) $HgCl_2$ for 7 to 8 min under a laminar flow cabinet. The surface-sterilized sprouts were grown on MS medium to generate shoots. Nodal segments, internodes and leaf explants were harvested from *in vitro* cultured shoots. MS medium containing diverse combinations of BAP, IAA and GA_3 was employed for *in vitro* regeneration. The medium's pH was calibrated to 5.8 prior to autoclaving. Cultures were sustained in a

growth chamber with a photoperiod of 16 hrs at 25 ± 1°C and a light intensity of 2500-3000 lux. To induce root formation, regenerated shoots were severed and placed in MS medium devoid of plant growth regulators (PGR). Glycerol stocks of *Agrobacterium tumefaciens* LBA4404 harboring the binary plasmid pBI121 were utilized to generate bacterial plates (YEP plates) through streaking. This plasmid has a reporter gene, *GUS* (β-glucuronidase), regulated by the CaMV35S promoter and NOS terminator and a selectable marker gene, *NPTII*, which is integrated between the NOS promoter and terminator, expressing the enzyme neomycin phosphotransferase. The plates were incubated at 28°C for 48 hrs in an incubator. Bacteria were sub-cultured onto a new YEP plate. The plate was incubated at 28°C for 48 hrs in an incubator. Bacterial cells were suspended from the plates in 25 ml of bacterial suspension medium (liquid MS with 9% (w/v) sucrose) using a vortex mixer (FISONS Whirl Mixer). The optical density (OD) of this suspension was measured at 600 nm using a spectrophotometer (Shimadzu, Japan). A bacterial culture with an optical density of 0.6 to 0.8 was selected for transformation. This bacterial solution was produced 30 min prior to inoculation. 200 μmol of acetosyringone was included into the suspension before to infection. The explants were immersed in the bacterial suspension and incubated for 20, 30 and 40 min. The explants were subsequently immersed in filter papers briefly to eliminate surplus bacterial suspension before transferring all explants to co-cultivation media (MS enriched with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA_3 and 100 μmol acetosyringone). All explants were cultured on co-culture medium for 2, 3 and 4 days at 25 ± 2°C in darkness. Co-cultured explants were rinsed with distilled water three to four times until no opaque solution remained. Subsequently, they were rinsed for 15 min with distilled water containing 300 mg/l of carbenicillin. The explants were subsequently immersed in sterile filter paper and transferred to a selection medium (MS with BAP, IAA and GA_3) containing 200 mg/l kanamycin and 300 mg/l carbenicillin. The sub-culture of infected explants was conducted consistently every 20 to 22 days on the selection medium. Following a duration of 70 to 75 days, the green calli were isolated and sub-cultured on MS medium augmented with BAP, IAA and GA_3. Following 100 to 105 days of inoculation, the small multiple shoots and shoot buds were transferred to the same medium for shoot elongation. The elongated shoots were removed and placed in root induction medium (PGR-free MS). Subsequent to root production, the plantlets were transferred to small plastic pots filled with dirt. Twenty randomly selected co-cultured explants from each treatment were analyzed using the GUS histochemical technique to assess transformation efficiency. Co-cultured explants were submerged in an X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) solution and incubated at 37°C for 24 to 48 hrs. The manifestation of a characteristic blue hue would indicate the expression of the *GUS* gene in the plant tissue. The GUS assay was conducted using explants from a normal plant for the control treatment. Leaves, shoots, roots, germinated microtubers, and microtuber discs were evaluated from randomly chosen plants. Explants and plant segments were immersed in GUS buffer solution for 24 to 48 hrs at 37°C. Following 24 to 48 hrs of

treatment, explants were subjected to bleaching with 70% ethanol to eliminate chlorophyll prior to assessing GUS expression. Genomic DNA was extracted from the transformed potato plants and the stable integration of the GUS and *NPTII* genes was verified using PCR analysis. The CTAB method (Doyle and Doyle 1990) was employed for DNA extraction. The primers for the *GUS* gene, 5´-CATGAAGATGCGGACTTACG-3´ (forward) and 3´-ATCCACGCCGTATTCGGCGT-5´ (reverse), were utilized at a concentration of 100 pmol/µl. To detect the *NPTII* gene, DNA underwent PCR with forward and reverse primers consisting of 5´-TAGCTTCTTGGGTATCTTTAAAATA-3´ and 3´CCAGTTACCTTCGGAAAAAGAGTT-5´, respectively. 0.2 µl of *Taq* DNA polymerase, 2.5 µl of 10 × buffer and 1.0 µl of dNTPs were utilized for the PCR process. In each reaction, the volume of PCR buffer was one-tenth of the total reaction volume, which was 25 µl. The first denaturation of the *GUS* gene PCR was conducted at 95°C for 5.0 min, followed by 30 cycles of denaturation at 95°C for 1.0 min each. The annealing temperature was set at 56°C for 30 sec, followed by elongation at 72°C for 1.0 min, specifically for the *GUS* gene. A concluding step of 10 min at 72°C was incorporated following the final cycle to ensure the complete extension of all amplified fragments. Upon completion of the cycling exercise, the response was maintained at 4°C. The cycling conditions for the *NPTII* gene consisted of an initial denaturation at 95°C for 5.0 min, followed by 30 amplification cycles including denaturation at 95°C for 1.0 min, annealing at 55°C for 1.0 min and extension at 72°C for 1.0 min, concluding with a final extension at 72°C for 10.0 min. The amplified DNA was subjected to electrophoresis on a 1.0% (w/v) agarose gel and subsequently stained with ethidium bromide at a concentration of 0.08 µl/ml.

Results and Discussion

A series of experiments was conducted to establish an appropriate protocol for *Agrobacterium*-mediated genetic transformation of Diamant. The transformation efficiency was significantly affected by various factors, including the optical density (OD) of the *Agrobacterium* suspension, the duration of the incubation period, and the length of co-cultivation. The optimization of these parameters was conducted through the monitoring of the histochemical assay of the *GUS* reporter gene in the explant tissue. The expression of the *GUS* gene correlates with the development of a distinct blue color in the cell or tissue. Prominent GUS positive (blue colored) regions were detected at the cut surface and in the meristematic part of the nodal segment explants. Control explants that were non-infected were also analyzed to compare the expression of *GUS* genes throughout this study. One of the most important influencing factors for transformation efficiency is optical density of *Agrobacterium* suspension. Optical density was assessed at a wavelength of 600 nm. In these experiments optical densities of 0.6, 0.7 and 0.8 were used. Optical density 0.6 resulted in the highest percentage of GUS positive nodal explants in Diamant, at 90%. The incubation period is a critical parameter, during which the explants are placed in bacterial suspension for a specified duration. To achieve this,

various incubation periods of 15, 30 and 45 min were utilized with a bacterial suspension maintained at a constant optical density of 0.6. From the result it was evident that, maximum percentage (95%) 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. Sarker et al. (2009) found that an incubation period of 40 min was optimal for the varieties Cardinal and Atlas in a separate set of experiments. The co-cultivation period is a significant factor affecting transformation efficiency. The transformed explants were co-cultivated for durations of 2, 3 and 4 days under a constant incubation period. Prolonged co-cultivation periods exceeding four days resulted in significant bacterial proliferation on the co-culture medium, rendering it unsuitable for the growth and survival of co-cultured explants. The co-cultivation period of 3 days was identified as the most suitable duration for transformation experiments conducted under optimal conditions.

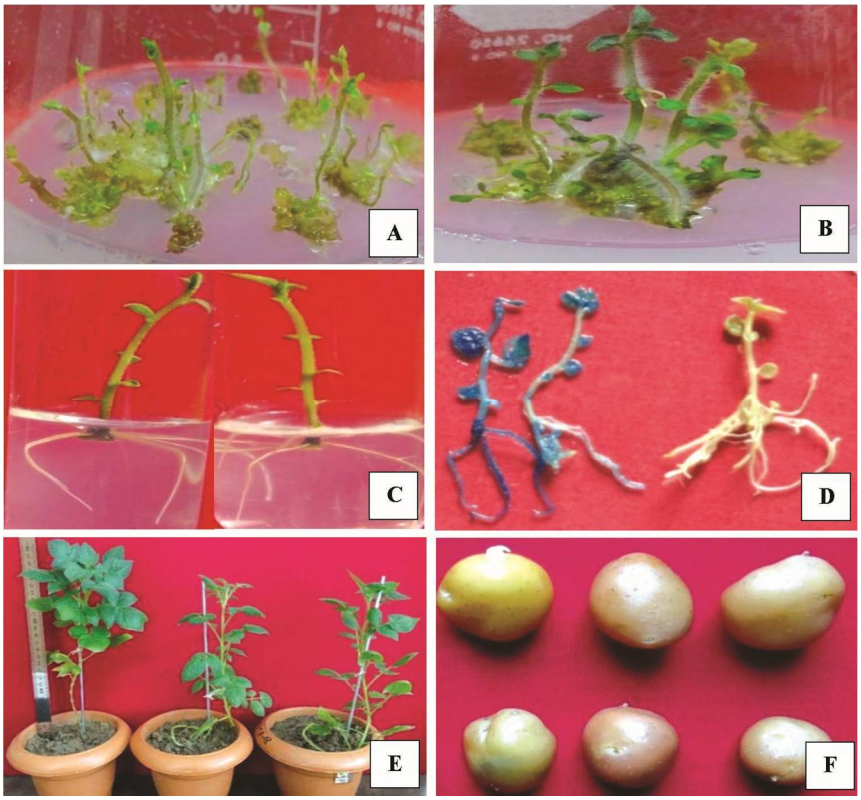

Fig. 1 (A-F). Regeneration and transplantation of transgenic plantlets infected with LBA4404 (pBI121): (A) Initiation of shoots from callus of nodal segments on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA and 0.5 mg/l GA_3, 300 mg/l carbenicillin and 200 mg/l kanamycin, (B) Multiple shoots formation on same medium, (C) Fully developed roots of Diamant on MS, (D) GUS expression of transgenic plantlets with a control one, (E) Transplantation of regenerated plantlets in pots containing soil and (F) Minitubers formation from putatively transformed plants.

The maximum transformation efficiency in Diamant, Cardinal and Granola was observed with a bacterial suspension exhibiting an optical density of approximately 1.0 at 600 nm, an incubation period of 60 min, followed by 72 hrs of co-cultivation (Borna et al. 2010). Antibiotic selection pressure was applied immediately following co-cultivation for the selection of transformed tissues. The selection of transformed shoots was conducted using kanamycin, as the kanamycin resistance *NPTII* gene was incorporated in the plasmid of the 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. Control explants were inoculated in selection medium devoid of infection. Direct selection facilitates the regeneration of exclusively transformed shoots after co-cultivation. It was noted that the application of kanamycin immediately following co-cultivation resulted in non-transformed explants exhibiting albino or deep brown coloration. The transformed explants exhibit a green or light brown coloration. The non-transformed albino explants were eliminated.

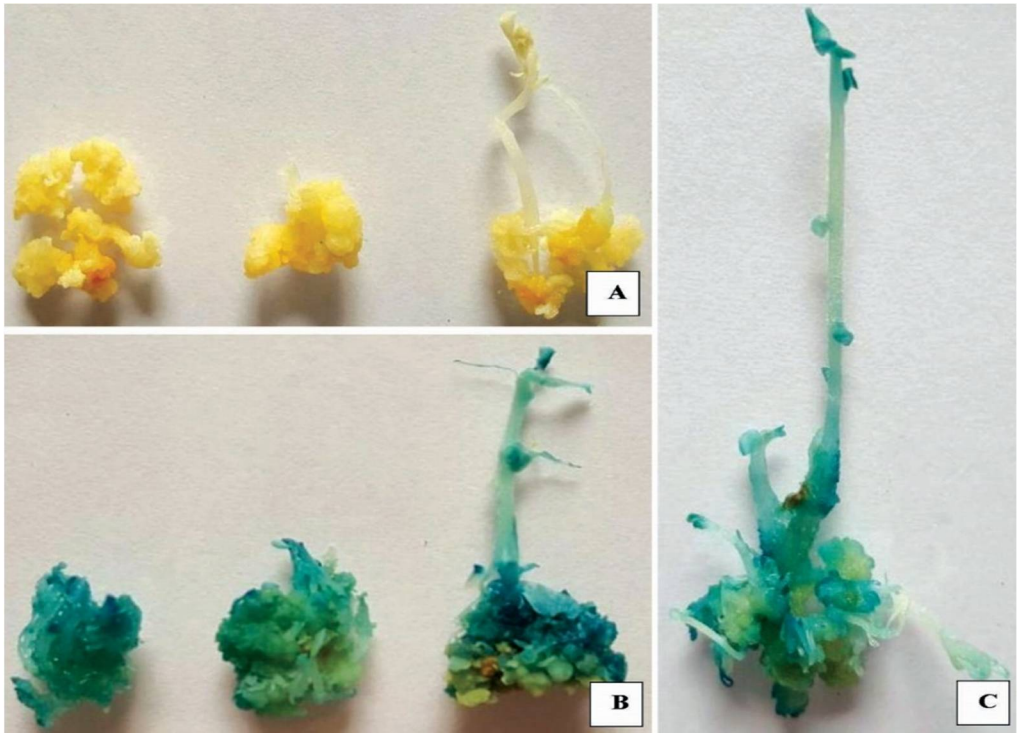

Fig. 2 (A-C). Transgenic plantlets development from callus of nodal segment explants infected with *Agrobacterium* strain LBA4404 containing plasmid pBI121: (A) Different stages of shoot initiation from control callus did not show any blue colour, (B) Photomicrographs of different stages of shoot initiation from callus showing expression of GUS (blue colour) and (C) Photomicrographs of elongated shoots from callus of nodal segments, showing expression of GUS.

The green and brown explants were sub-cultured regularly after every 20-22 days. After 70 to 75 days, the brown explants began to green and developed green callus. Green callus regeneration and shoot proliferation were observed after 100-105 days of infection on MS medium supplemented with 4.0 mg/l BAP, 1.0 mg/l IAA, 0.5 mg/l GA3, 300 mg/l carbenicillin and 200 mg/l kanamycin (Figs 1A-B). The transformed shoots (2-3 cm in length) survived on the selection medium were separated and transferred to MS containing 100 mg/l carbenicillin. Induction of roots required approximately 8 to 10 days (Fig. 1C). Some plantlets were immersed in GUS histochemical stain. The stable expression of the *GUS* gene was observed in the transformed regenerating shoots (Fig. 1D). Transformed plantlets were transplanted into soil, where they demonstrated survival after appropriate acclimatization (Fig. 1E).

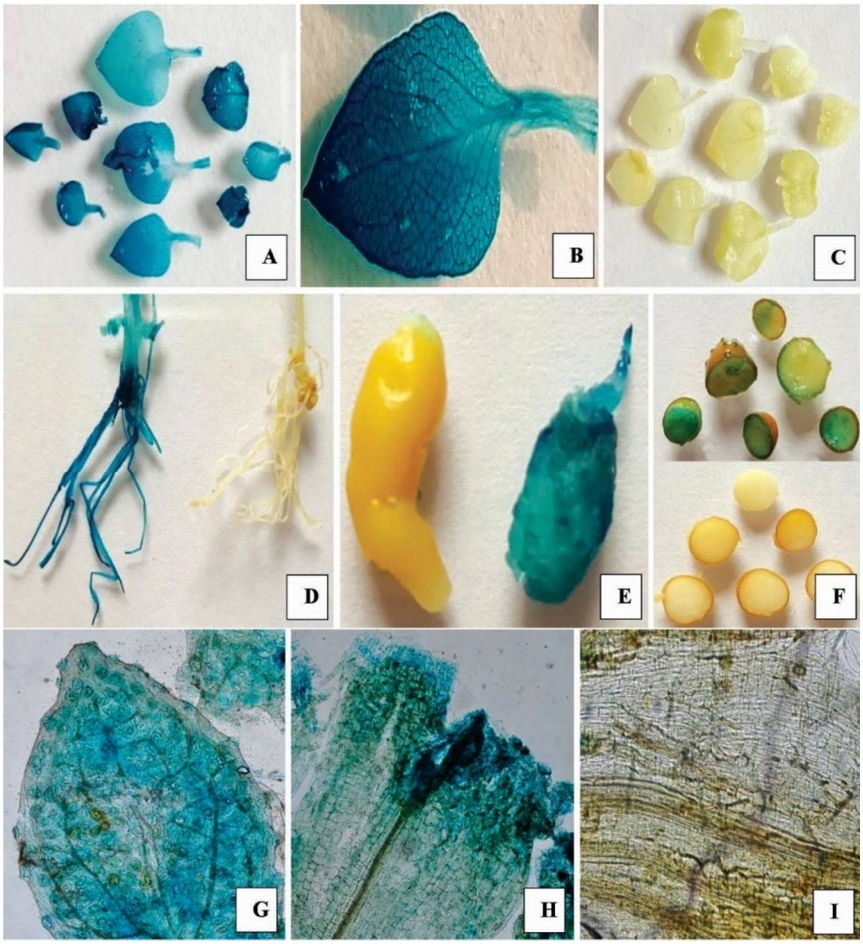

Fig. 3 (A-I). GUS expression of different parts of fully developed transgenic plants of Diamant with control: (A-C) Leaves, (D) Roots, (E) Germinated microtubers, (F) Microtuber discs and (G-I) Sterio microscopic view of leaf and stem tissue showing the expression of GUS.

The plants were kept in a growth room, and after 90-95 days, several minitubers (Fig. 1F) were harvested from them. The various stages of callus development and the formation of shoots from this callus were analyzed using GUS histochemical staining. The control callus did not exhibit any blue coloration (Fig. 2A), whereas the transformed callus displayed a significant blue color (Figs 2B-C). The leaf, roots, germinated microtuber and microtuber discs from the transformed shoots demonstrated significant *GUS* gene expression; however, no blue coloration was observed in the non-transformed (control) leaf, microtuber and microtuber discs (Figs 3A-F). The stereomicroscopic examination of transgenic leaf and stem revealed a blue coloration (Figs 3G-H), whereas the control stem exhibited no blue coloration (Fig. 3I).

This finding aligns with Sarker and Mustafa (2002), who demonstrated the expression of the *GUS* gene in the leaf tissues of transformed shoots through histological GUS assays. Mollika et al. (2020) reported the expression of the *GUS* gene in the leaf, microtuber, and microtuber discs of transformed shoots in the Asterix variety of potato. The transgenic nature of the shoots was confirmed through PCR amplification of the *GUS* and *NPTII* genes in the genomic DNA of 18 randomly selected transformed individuals (Figs. 4A-B).

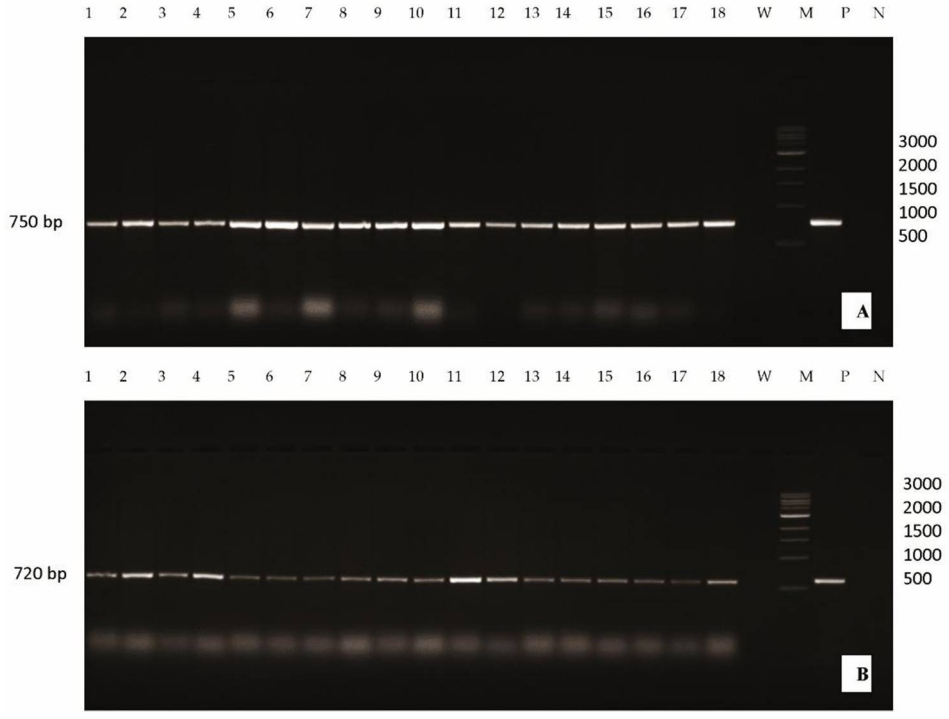

Fig. 4 (A-B). Molecular confirmation of the putative transformants of var. Diamant: PCR amplification of *GUS* (A) and *nptII* (B) gene (lanes 1-18 genomic DNA of transformed plants; lane W- wild; lane M-1kb ladder; lane P-plasmid DNA of pBI121 as positive control and lane N- negative control).

Specific primers were employed for this purpose, as outlined in the Materials and Methods section. Amplified DNA underwent analysis through agarose gel electrophoresis. The gel analysis revealed that the single band present in each of the 15 transformed plantlets was identical to the amplified DNA of the positive control. The results demonstrated that both the *GUS* and *NPTII* genes were integrated into the genomic DNA of 18 transformed plantlets, which exhibited distinct positive bands. The band sizes were 750 bp for GUS and 720 bp for the *NPTII* gene, respectively. The *Agrobacterium*-mediated genetic transformation protocol established in this study is applicable for generating transgenic potato plants for various specific objectives. The protocol was primarily developed utilizing screenable marker genes such as *GUS* and selectable marker genes like *NPTII*. This protocol can facilitate the incorporation of agronomically significant genes, specifically those related to salinity and drought tolerance, into potato varieties cultivated in Bangladesh. The integration of the abiotic stress-resistant genes may enable potatoes to adapt to various agro-ecological zones, thereby mitigating food shortages in less fertile or water-scarce regions.

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