



## ***Agrobacterium*-mediated Genetic Transformation of Potato (*Solanum tuberosum* L.) var. Cardinal and Heera**

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### **Abstract**

Two potato varieties namely Cardinal and Heera were used in the *Agrobacterium*-mediated genetic transformation experiment to investigate the genetic transformation ability in the Biotechnology laboratory of the Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, Bangladesh during 2006 to 2007. *Agrobacterium tumefaciens* strain LBA 4404 having a binary vector pB1121 of 14 KDa containing selectable marker gene npt II (neomycine phosphotransferase II) conferring kanamycin resistance, and the CIPK antisense gene encoding calcineurin B-like protein were used. Leaf and internodes were used as explants. Expression of the transgene (GUS) was confirmed by histochemical analysis. The variety Cardinal was found more suitable for expressing best GUS response (80% GUS positive) over Heera.

**Keywords:** *Agrobacterium tumefaciens*, genetic transformation, *Solanum tuberosum* L.,  $\beta$ -glucuronidase (GUS), neomycin phosphotransferase II (nptII), CIPK

### **1. Introduction**

Potato (*Solanum tuberosum* L.) is not only an important vegetable crop but is also a substitute food crop next to rice and wheat in Bangladesh. It occupies the first position in both acreage and production among the vegetable crops grown in Bangladesh and it alone contributes about 53% of total vegetable production (Yasmin, 2002). The area and production of potato in Bangladesh has increased during the last decades, but yield per unit area has remained more or less static. In Bangladesh, the average yield of potato is 13.81 t/ha (FAO, 2003), which is very low in comparison to that of the other leading potato growing countries of the world, such as Ireland (33.33 t/ha), Netherlands (40.73 t/ha) and USA (41.15 t/ha). The yield per unit area remains low

due to the infection by fungi, viruses, viroids, bacteria and many other environmental factors which may exert a deleterious effect in yield, marketable quality, storability, germplasm conservations, distribution and international exchange. Furthermore, potato is vegetatively propagated and diverse biological constraints are encumbered with its reproductive nature.

Drought is one of the principal environmental stresses of global and eco-regional concerns (Watanabe, 2002) which is a serious threat to agriculture. Therefore, breeding for drought stress tolerance in crop plants should be given high research priority in plant biotechnology programs. To develop drought tolerant potato, conventional breeding methods were tried, but there are some limitations in conventional

breeding. Conventional breeding is time consuming, labourious and it depends on environmental conditions where breeding of a new variety takes about 8-12 years. Tissue culture techniques can play an important role in crop improvement, but this technique has contributed very little in the development of drought resistant plants. Genetic engineering is an alternative tool for crop improvement that shows a great promise in developing potato cultivars. To overcome the limitations of conventional breeding and tissue culture techniques in crop improvement, genetic transformation of crop plants has been evolved, which offers the ability to introduce single new character into a plant cultivar without altering its existing traits (Gardner, 1993).

More recently *Agrobacterium*-mediated transformation has become feasible. It is the most common method used for the genetic transformation of potato. *Agrobacterium*-mediated transformation offers several advantages over the other methods (particle bombardment, electroporation etc.), such as the possibility of transferring only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNAs fragments with minimal rearrangement (Liu *et al.*, 2002). The present study was therefore, undertaken to

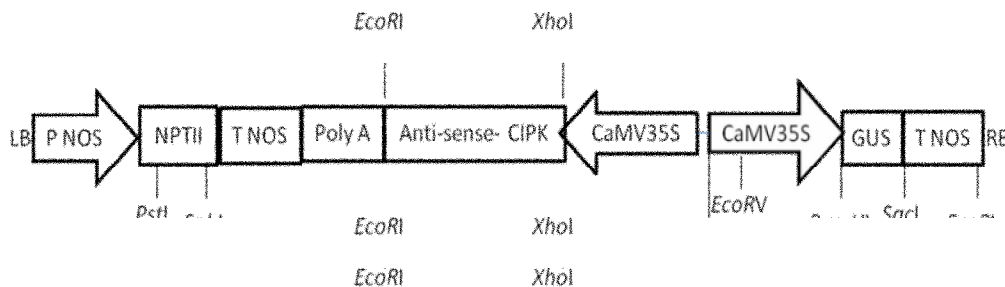
develop a reproducible and efficient protocol for the insertion of foreign genes into potato through *Agrobacterium*-mediated transformation and to identify the varieties suitable for transformation.

## 2. Materials and Methods

The present investigation was carried out in the Biotechnology Laboratory of the Department of Biotechnology, Bangladesh Agricultural University, Mymensingh, Bangladesh during 2006 to 2007.

### 2.1. Plant materials, bacterial strain and plasmid

Leaf and internodes of *in vitro* grown two potato varieties viz. Cardinal and Heera were used for this experiment. Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infection. This strain contains plasmid pBI121 of 14 KDa (binary vector) (Fig. 1). This binary vector contains the CIPK antisense gene encoding calcineurin B-like protein. CIPK works against several environmental stresses. The *uidA* gene (Jefferson *et al.*, 1986) encoding *GUS* ( $\beta$ -glucouronidase), driven by CaMV 35s promoter and, NOS terminator. This receptor gene can be used to assess the efficiency of transformation. The *nptII* gene encoding *neomycin phosphotransferase II* (*nptII*) conferring kanamycin resistance, driven by NOS promoter and NOS terminator.



**Fig. 1.** Schematic representation of the binary vector pBI121 with different restriction enzyme positions for potato genetic transformation. RB: Right border; LB: Left border, TNOS: Terminator of nopaline synthase gene; P NOS: Promoter of : nopaline synthase gene; CaMV35SW: CaMV 35S promoter of cauliflower mosaic virus; NPT II: Neomycin phosphotransferase II; Poly A: Poly A tail ; GUS:  $\beta$ -glucouronidase

### 2.2. Preparation of microplants

Sprouts of potato were first washed with tap water followed by distilled water and were then surface sterilized with 70 % (v/v) ethanol for few seconds and rinsed with sterilized distilled water. Afterwards, the sprouts were again surface sterilized by immersing in 0.1% HgCl<sub>2</sub> containing two drops of tween-20 (Polysorbate 20) per 100 ml solution for 30 seconds and then washed several times with sterilized distilled water. The sterilized sprouts were transferred aseptically to test tubes containing MS solidified medium with 3% sucrose and were incubated at 25±2 °C under (16/8 h. light/dark regime) photoperiod. Twenty five days old *in vitro* grown microplants were used as the source of explants.

### 2.3. Culture media

Two different types of culture media, namely, YMB (Yeast Mannito Broth) and LB (Luria Bertani medium broth) were used with kanamycin as antibiotic to grow the strain of genetically engineered *Agrobacterium tumefaciens*. YMB medium was used for *Agrobacterium* maintenance and LB medium was used as *Agrobacterium* working culture for transformation. MS (Murashige and Skoog, 1962) medium without growth hormones was used for Co-cultivation. Cefotaxime @ 500 mg/l was used to wash the explants after co-cultivation. MS medium with 5 mg/l NAA ( $\alpha$ -naphthalene acetic acid), 2 mg/l BAP ( $\beta$ -benzyl amino purine) and 200 mg/l cefotaxime were used for post-cultivation and callus induction. MS medium supplemented with 5 mg/l NAA, 2 mg/l BAP, 5 mg/l Kanamycin and 100 mg/l cefotaxime were used for low selection medium and for high selection medium MS medium supplemented with 5 mg/l NAA, 2 mg/l BAP, 20 mg/l Kanamycin and 100 mg/l cefotaxime were used.

### 2.4. Transformation

Leaf and internode of *in vitro* grown potato plantlets of two varieties were placed in MS medium without any growth regulators for five

days. Pre-cultured explants were floated on infection medium for five minutes. Following infection, the explants were co-cultured in co-cultivation medium. Prior to transfer of all explants to co-cultivation medium they were blotted on sterile tissue papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-cultivation media for three days. Co-cultured explants were incubated under fluorescent illumination with 16/8 hours light/dark cycle at 25±2 °C. The intensity of light was maintained at 1500 lux. After three days, the infected explants were washed twice with sterile distilled water and once with sterilized distilled water containing 500 mg/l cefotaxime.

### 2.5. Selection and regeneration of putative transformed plantlets

Following one week of post-cultivation, the explants were transferred to low selection medium containing 100 mg/l Cefotaxime. After ten days culture on low selection medium, the calli were transferred to high selection medium. The survived co-cultured materials were transferred to regeneration medium after ten days. The regeneration medium consisted of MS medium supplemented with 2 mg/l BAP and 5 mg/l NAA without any antibiotics.

### 2.6. GUS ( $\beta$ -glucuronidase) histochemical assay

Randomly selected survived co-cultured tissues were examined for GUS histochemical assay. The sample tissues were immersed in X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and incubated at 37 °C overnight. A characteristic blue color would be the expression of GUS ( $\beta$ -glucuronidase) gene in the plant tissues. Proper control for GUS histochemical assay was done with the explants having no blue color.

### 2.7. Data collection and statistical analysis

The number of calli positive response of GUS histochemical assay was recorded. The percentage of GUS positive calli were calculated

on the basis of the number of calli assayed for GUS and the total number of calli positive for GUS.

### 3. Results and Discussion

The infection and incubation were done following the methods stated in the materials and methods. After incubation and co-cultivation with *Agrobacterium*, transformation ability was checked through histochemical assay of GUS reporter gene in explants tissues. Transient GUS assay was done within 72 hours of co-cultivation with 10 randomly selected explants tissue of each type. Conspicuous GUS positive (blue color) regions were detected at the surface of the explants tissues (Fig. 2). After GUS histochemical assay, both the varieties showed positive response towards transformation. Leaf of Cardinal showed higher per cent of GUS positive (80%) tissues than internodes (60%) (Table 1)

#### 3.1. Determination of transformation ability of various explants of potato

Transformation ability of leaf and internode explants was examined. Following co-culture with *Agrobacterium*, the explants were subjected to histochemical assay to find out the expression of GUS reporter gene within the explants tissue. A very good number of both explants tissues were found to be positive in transient GUS assay (Table 1). Between the explants, leaf explants tissues showed higher percentage (80%) of positive GUS test than internodes (60%). Sultana

(2004) also reported that potato leaf explants were more effective for *Agrobacterium* infection.

#### 3.2. Selection of putative transformed callus

To select the transformed callus from various explants, all the explants were transferred to Kanamycin containing MS medium. The sensitivity of explants tested against two concentrations of Kanamycin viz. 5 and 20 mg/L.

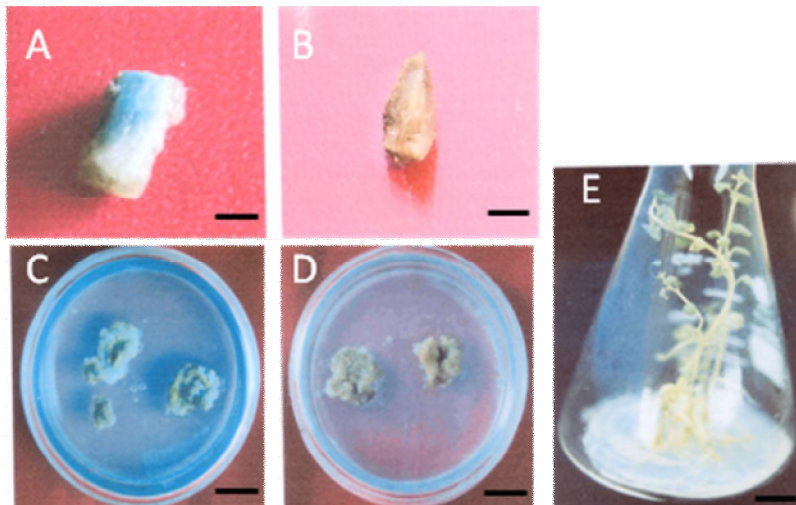
When leaf explants of cardinal cultured in different concentrations of selection media, it was found that two leaflet explants survived and regenerated in both selection media (Fig. 2). No internodal explants of Cardinal and Heera survived in selection media. Two leaflet explants of Heera also survived and regenerated in presence of kanamycin at the concentration 5 mg/l (Figure 2). However, two regenerated shoot became albino in selection medium containing kanamycin at the concentration of 20 mg/l.

#### 3.3. Histochemical GUS ( $\beta$ -glucuronidase) assay

Transformation ability was examined through histochemical assay of GUS reporter gene in explants tissue. Transient GUS assay was done with randomly selected selective transient callus at the end of selection of putative transformed callus. Conspicuous GUS positive (blue color) regions were detected at the entire cut surface on the explants.

**Table 1.** Influence of variety and explants on transformation analyzed through GUS histochemical assay

Variety	Explants	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS + ve explants
Cardinal	Leaf	40	10	8	80
	Internode	40	10	6	60
Heera	Leaf	40	10	7	70
	Internode	40	10	6	60



**Fig. 2.** Selection and regeneration of potato explants. A: GUS positive internode tissue (blue colour), B; control internode tissues, C: kanamycin resistant calli obtained from Cardinal variety, D: kanamycin resistant calli obtained from Heera variety, E: Viable shoots obtained from selection medium

#### 3.4. Transfer of putative transformed shoots in regeneration medium

Regenerated shoots that survived in kanamycin selection medium were transferred to regeneration medium (Fig. 2). The regeneration medium contained with MS medium supplemented with 3 mg/l NAA and 2 mg/l BAP were used, but no kanamycin was added because kanamycin has long term inhibitory function on plant growth (Islam *et al.*, 2005). Furthermore, the presence of kanamycin greatly hampers growth of the explants and as a result many putative transformants may have been lost (Borna *et al.*, 2010). Regeneration rate was found 2% in Cardinal leaf and 4% in Heera leaf in our study.

#### 4. Conclusions

*Agrobacterium* – mediated transformation in two potato varieties was achieved and transformation ability of leaf and internode explants was

examined. Both the varieties Cardinal and Heera showed positive response towards transformation. Leaf of Cardinal showed higher level of GUS positive (80%) tissues than internodes (60%). In our study, regeneration was found 2% in Cardinal leaf and 4% in Heera leaf. The results presented here will make an important contribution to the genetic transformation of potato var. Cardinal and Heera, two important potato cultivars in Bangladesh, demonstrating the possibilities for introduction of foreign genes for horticultural interest.

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