Establishment of an Indirect Genetic Transformation Method for Arabidopsis thaliana ecotype Bangladesh

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ABSTRACT

Arabidopsis thaliana is a small flowering plant belonging to the Brassicaceae family, which is adopted as a model plant for genetic research. *Agrobacterium tumifaciens*-mediated transformation method for *A. thaliana* ecotype Bangladesh was established. Leaf discs of *A. thaliana* were incubated with *A. tumefaciens* strain LBA4404 containing chimeric *nos. nptII. nos* and intron-*GUS* genes. Following inoculation and co-cultivation, leaf discs were cultured on selection medium containing 50 mg/l kanamycin + 50 mg/l cefotaxime + 1.5 mg/l NAA and kanamycin resistant shoots were induced from the leaf discs after two weeks. Shoot regeneration was achieved after transferring the tissues onto fresh medium of the same combination. Finally, the shoots were rooted on MS medium containing 50 mg/l kanamycin. Incorporation and expression of the transgenes were confirmed by PCR analysis. Using this protocol, transgenic *A. thaliana* plants can be obtained and indicates that genomic transformation in higher plants is possible through insertion of desired gene. Although *Agrobacterium* mediated genetic transformation is established for *A. thaliana,* this study was the conducted to transform *A. thaliana* ecotype Bangladesh.

KEYWORDS: *Agrobacterium tumefaciens*, *Arabidopsis thaliana*, brassicaceae, Bangladesh

Abbreviations: MS: Murashige and Skoog, PCR: Polymerase chain reaction

INTRODUCTION

Arabidopsis thaliana is a small flowering plant belonging to the Brassicaceae family and among several species of it, the most well known member and one of the most extensively used in basic researches is *Arabidopsis thaliana* L. Heynh, which is commonly known as “Thal cress” or “Mouse eared cress”. *A. thaliana* is native to Europe and Central Asia and now is naturalized at many places elsewhere in the world. For over 40 years *Arabidopsis thaliana* is used to understand the biology of flowering plants at the molecular level because of its small size, rapid life cycle, small simple genome, prolific seed production and the availability of numerous mutations. *A. thaliana* is extensively investigated throughout the world due to its application in a broad spectrum of studies [1, 2, 3]. Current researches on *Arabidopsis thaliana*

providing informations on phylogeny [4]; taxonomy [5], flower development [6], genomic informations [7, 8], *in vitro* regeneration [9], genetic transformation [10, 11]; seed information's [12], environmental physiology [13]. At the end of 2000, the international collaborative "*Arabidopsis* Genome Initiative" (AGI) announced that *Arabidopsis thaliana* is the first plant for which the complete genome has been sequenced, having 125 million DNA base pairs determined 25,500 genes. It is possible to transform this species indirectly by applying *Agrobacterium* to plant and recovering transformants in the progeny. Transformation approaches were done by a number of research groups [14, 15, 16, 17, 18]. *A. thaliana* is recently a known species in Bangladesh. The *A. thaliana* found in Bangladesh was identified as separate ecotype and names as *A. thaliana* ecotype Bangladesh [19]. It is needed to include this ecotype of *A. thaliana* in research material and verify with other ecotype that have been used extensively all over the world. In many cases, the lack of an efficient regeneration system is a major factor preventing the development of gene transfer technologies. The aim of the study reported here was to establish an efficient *Agrobacterium*-mediated transformation method for *A. thaliana* ecotype Bangladesh.

MATERIALS AND METHODS

Plant Material

Mature seeds from field grown plants of *Arabidopsis thaliana* ecotype Bangladesh were used as plant materials for *in vitro* plantlets formation and leaf discs if *in vitro* grown plantlets were used in transformation.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain LBA4404 (pVDH65, pTOK47) designated 1065 containing the binary vector pVDH65 and a super-virulent plasmid $_{P}$TOK47 [20] were used in this experiment. The binary vector pVDH 65 based on the pBIN19 derivative pMOG18 carried a T-DNA with the chimeric *nos. nptII. nos* and intron-*GUS* genes and was received from Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Bangladesh.

Tissue culture conditions

Arabidopsis seeds were vernalized for one week at 4°C before germination. Seeds were surface sterilized for 4 min with 0.1% $HgCl_2$ following washed with double distilled water and then transferred to agar-gelled hormone free MS medium in 9 cm petridishs to germinate. Seedlings were grown at 20°C in a 16h light (60 µE m^{-2} s^{-1}; cool white fluorescent tubes). The same growth conditions were used for tissue culture procedures. All plant media were adjusted with 0.1N NaOH to pH 5.7, solidified with 8g/l agar and autoclaved at 121°C for 20 min. Leaves were collected from 3-4 weeks old *in vitro* grown plants and used for transformation.

Effect of antibiotics concentration

Kanamycin and cefotaxime were used as antibiotic for the transformant selection. Four different concentration (25, 50, 75, 100 mg/l) of kanamycin and cefotaxime were used to test the sensitivity of leaf discs and for that leaf discs were cultured in selection medium containing four diverse concentrations of kanamycin and cefotaxime.

Transformation, selection and plant regeneration

To determine the optimum conditions for *Agrobacterium* mediated transformation, we used different culture media such as inoculation media (1:1 amount of MS liqied medium and bacterial suspension, pH 5.8), co-cultivation media (growth regulators free liquid MS, pH5.8) and selection and regeneration medium (MS +1.5 mg/l NAA + 50 mg/l kanamycin + 50 mg/l cefotaxime). Along with this tme period was also optimized for inoculation and co-cultivation with *Agrobacterium* in the co-cultivation medium. A 20-ml aliquot of bacterial culture (O.D.600=1.0) was centrifuged at 3,179g for 10 min (4°C). Following removal of the supernatant, the pellet was suspended in the same volume of inoculation medium and this suspension was used as an inoculum for infection. The explants were immersed in the bacterial inoculum for 0.5-2.0 min for inoculation; they were then cultured in 9 cm petri dishes with 20 ml of co-cultivation medium. The *Agrobacterium* infection frequency (number of GUS-positive explants/number of explants examined) was determined after co-cultivation for 1-5 days by performing a histochemical GUS assay. After co-cultivation, the explants were transferred onto selection and regeneration medium (MS +1.5 mg/l NAA + 50 mg/l kanamycin + 50 mg/l cefotaxime), blot-dried on sterile filter paper and then placed on the selection medium. After 15-21 days the explants were transferred to shoot induction medium containing the antibiotics. Induced micro-shoots were isolated, cultured on MSO medium for root induction and putative transformed rooted plants were used for *GUS* histochemical assay. Non-infected explants were also cultured onto selection media as control.

Histochemical GUS assay

The assay for β-d-glucuronidase (GUS) activity using 5-bromo-4-chloro-3-indoyl-β-d-glucuronic acid (Duchefa Biochemie) as the substrate was carried out as described by Jefferson et al. [21]. The assay culture was incubated overnight at 37°C, and the explants were subsequently soaked overnight in 95% methanol.

PCR analysis

The presence of transgenes in transformed and control (non-transformed) plants were analysed by the polymerase chain reaction (PCR). Genomic DNA was isolated from young leaves of putative transformed and non-transformed plants following the procedure of Biswas [19]. PCR analyses to detect *nptII* gene were carried out using

the PCR Screening Kit (Sigma Chemicals Ltd., USA) in the presence of following pair of primers:

Forward primer: 5/ - GTCAT CTCAC CTTGC TCCTG CC – 3/

Reverse primer: 5/ - GTCGC TTGGT CGGTC ATTTC GG – 3/.

The PCR mixtures were denatured at 94ºC for 4 min followed by 30 cycles for 1 min at 94°C, 45 sec at 58°C for annealing, 2 min at 72°C for extension and finally incubated at 72°C for 10 min. Expected PCR product size was 0.356 kb band of *nptII* gene.

RESULTS AND DISCUSSION

Effect of antibiotics

To develop a rapid and efficient *Agrobacterium*-mediated transformation method for *A. thaliana* ecotype Bangladesh, the action of different concentrations of antibiotics that are currently used as selectable markers in plant transformation procedures was assayed. Kanamycin 50 mg/l completely blocked regeneration from untransformed explants and therefore, could be used to select for transformed cells. Correspondingly, observations of *Arabidopsis* transformation experiments indicated that cefotaxime was used as an common antibiotic to kill *Agrobacterium* after co-cultivation with plant material and 50 mg/l cefotaxime noticed to be optimum. This result is in consistent with the findings of indian mulberry, *Morus indica* cv. K2 [22] and grasspea (*Lathyrus sativus* L.) [23].

Factors influencing Agrobacterium infection

To determine the optimum conditions for *Agrobacterium* infection of *A. thaliana* leaf explants, infection frequency was examined. We examined different durations of inoculation period with *Agrobacterium* inoculums and highest 72.67% of kanamycin resistance explants were observed at 1.5 min of inoculation period (Table 1). An incubation period longer than 2.5 min reduces transformation efficiency due to leaching of bacterial over growth and led to partial suppression of callus induction. Similar observation has been reported by Biswas in *A. thaliana* ecotype bangladesh [19]. The duration of the co-cultivation period with bacteria affected the infection frequency. The optimum length of the co-cultivation period was 48 h, resulting 72% of survived tissues. The blue area indicating the transient expression of the *gus* gene was initially observed after 12 h of co-cultivation; after 48 h of co-cultivation the number of GUS positive explants dramatically increased. However, when the explants were co-cultivated for 84h, we could not identified any survived tissues and, consequently, we chose 48 h as the optimum co-cultivation period for transformation. Co-cultivated explants were then transferred to solid selection medium containing MS +1.5 mg/l NAA + 50 mg/l kanamycin + 50 mg/l cefotaxime. This results are in consistent with the findings in *Arabidopsis thaliana* [14, 15], in peanut [24] and in raspberry [25]. After 14 days some tissues were noticed as kanamycin resistance and

after subculturing on the same medium microshoots were directly induced from the kanamycin resistant tissues. Findings are shown in figure 2 (A and B). Induced microshoots were isolated and then cultured on MSO medium for root induction (Fig. 2. C). The *in vitro* grown healthy plants were subcultured at least 3-4 times in MS medium with the appropriate abtibiotics to ensure that the transgenic plants were free from *Agrobacterium* and antibiotics. Putative transformed rooted plants were used for molecular detection.

Table 1. Effect of incubation period on survival percentage of explants of *A. thaliana* ecotype Bangladesh.

Explants types	Incubation period (min)	% of surviving explants ($\overline{X}$ ±SE)
Leaf	0.5	20.22±0.45
	1.0	61.56±1.05
	1.5	72.67±0.85
	2.0	42.45±0.21

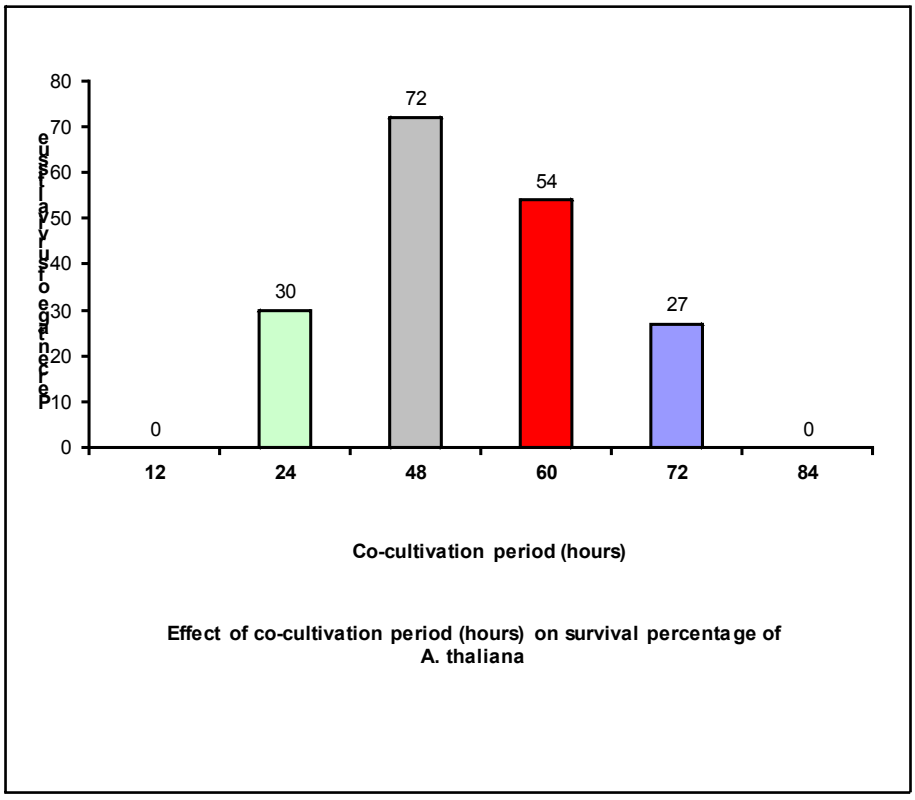
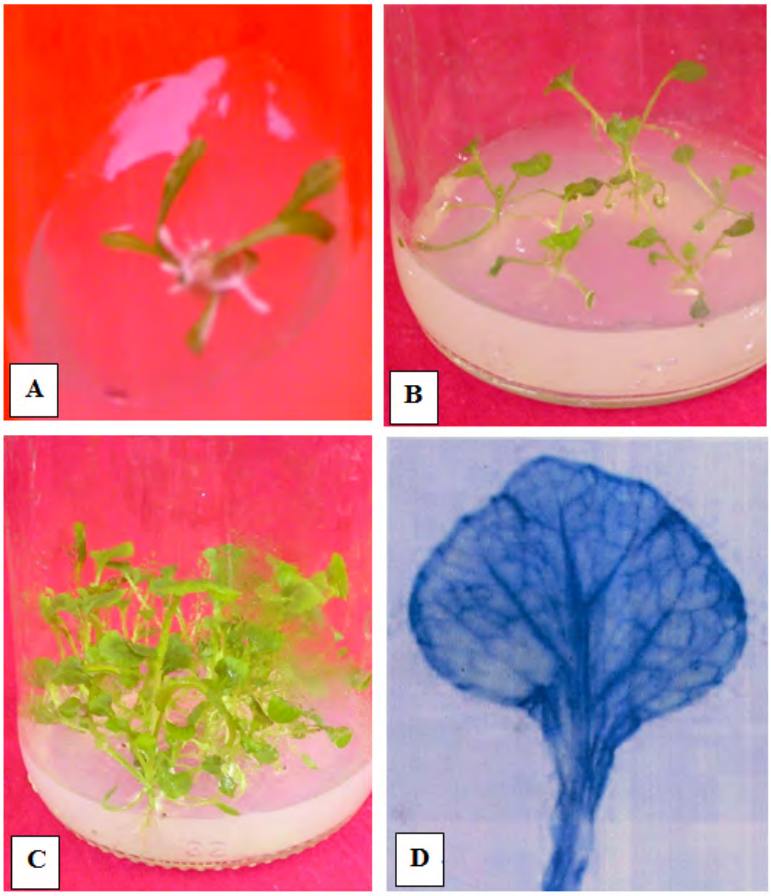


Figure 1: Effect of co-cultivation period (hours) on survival percentage of *A. thaliana* ecotype Bangladesh.

Molecular analysis of transgene

Netehouse grown putative transformed plants and control *A. thaliana* were morphologically true to type. The leaves of transformed plants were analyzed histochemically for GUS activity (Fig. 2.D). The intensity of blue GUS staining plants was also analyzed by PCR for the presence of the *hpt* gene. PCR profile of the putative transformed plants exhibited the presence 0.365 kb band of *nptII* gene as expected (Fig. 3). The same band also expressed in positive control (Lane 12) that confirmed the integration of *nptII* gene in the genome of *A. thalianla*. A similar result was also observed in *A. thaliana* [19].

In this study, leaf discs of *in vitro* grown *Arabidopsis* plantlets were transformed via *A. tumefaciens.* Therefore, it can be said that 1.5 min incubation of leaf discs with *Agrobacterium*, 48 h co-cultivation and of course selection of transformants with 50 mg/l kanamycin and cefotaxime in 1.5 mg/l NAA containing MS media are the optimal conditions for the better transfomation of *A. thaliana* ecotype Bangladesh. Also in future, real time PCR and Southern blotting would be firmly applied for the further study of transformed gene in *A. thaliana* ecotype Bangladesh.



Figures 2. (A) Single transgenic A. thaliana ecotype Bangladesh plantlet. (B) 21 days older subcultured regenerated transgenic plantlets in shoot induction medium. (C) Transgenic microshoots on MS0 medium for root induction. (D) Histochemical gus assay of the leaf from putative transformed plant.

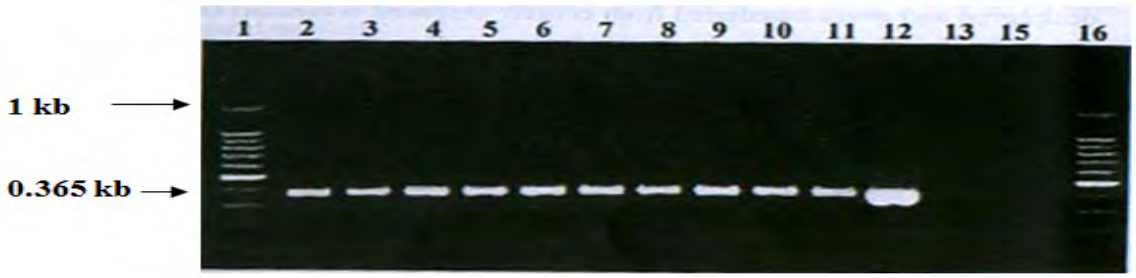


Figure 3. PCR analysis of genomic DNA of putative transformed plants of A. thaliana ecotype Bangladesh for nptII gene. Lane 1 and 16: 100 bp size marker (Promega). Lane 2-11: Transformed plants. Lane 12: Positive control. Lane 13: Water (control). Lane 15: Non transformed plants.

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