ENHANCEMENT OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION METHOD FOR THE PRODUCTION OF HEME-PROTEIN (FERRITIN PROTEIN) RICH POTATO

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ABSTRACT

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The experiment was conducted at plant breeding and gene engineering laboratory, Department of Botany, Rajshahi University, Rajshahi during 2008-09 to establish an *Agrobacterium tumefaciens* mediated transformation procedure for *Solanum tuberosum.* For increasing the iron storage in the potato, explants (*in vitro* and *ex vitro* leaf and internode) were incubated with *Agrobacterium tumefaciens* strain LBA4404 bearing Ti based binary pCAMBIA2301 plasmid containing selectable marker neomycin phosphotransferase (*npt*II) and *ferritin* gene. Following co-cultivation explants were cultured on selective medium containing 50 mg/l Kanamycin + 50 mg/l Cefotaxime. Kanamycin resistant shoots were induced from explants after 5-6 weeks. Shoot regeneration was achieved after transferring the tissue on to fresh medium of same combination and the shoots were rooted on MS medium containing 50 mg/l kanamycin. Incorporation and expression of the transgenes were confirmed by PCR and RT-PCR analysis. Transgenic potato could be developed with *ferritin* gene using this protocol.

Keywords: Agrobacterium, Heme protein, Ferritin gene, Transformation method

INTRODUCTION

Potato *(Solanum tuberosum* L.) is an herbaceous tuber-bearing annual plant belongs to the Solanaceae family. It is one of the most agronomically and economically important plants and it is the most productive, common and multiuse horticultural vegetable crop. Potato is one of the first crop plants in which the transgenic plants were successfully regenerated (An *et al., 19*86, Shanin and Simpson 1986). Successful genetic transformation has been reported through *Agrobacterium tumefaciens* in many plants species with ferritin gene such as Rice (Khalekuzzaman *et al.*, 2003), Wheat (Drakakaki *et al.*, 2000), Tobacco (Goto *et al.*, 1998) and Lettuce (Goto *et al.*, 2000).

IDA (iron deficiency anemia), the most common nutritional disorder in the world, impairs immunity and reduces the physical and mental capacities of people of all ages. Anemia in pregnancy is an important cause of maternal mortality, increasing the risk of hemorrhage and sepsis during childbirth. An inadequate dietary iron intake is the main cause of IDA. Ferritin is an iron storage protein which stores 4500 iron atoms in its central cavity in nontoxic (Korcz and Twardoski 1992). Iron stored in ferritin is completely bioavailable (Goto *et al.,* 2001) and is released when need arises for metabolic functions (Briat 1996). There are reports of increasing iron content by ovarexpressing ferritine gene in transgenic rice (Goto *et al.,* 1999; Vasconcelos *et al.,* 2003) and wheat (Drakakaki *et al*., 2000)

Potato is an important food and cash crop ranks third after rice and wheat. Most of the potato varieties produce negligible amount of different vitamins, proteins and bio-available iron in the tubers consumed by humans. Heme iron, which is relatively well absorbed by the human intestine, is found primarily in foods containing blood and muscle. Transgenic potato for different purposes was already developed such as human serum albumin production (Sijmons *et al.,* 1990), Cyclodextrin producing potato (Oakes *et al.,* 1991), amylose free starch containing potato (Kuipers *et al.,* 1994). The present study was undertaken to develop of transgenic potato plants with *ferritin* construct with *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

The experiment was conducted at plant breeding and gene engineering laboratory, Department of Botany, Rajshahi University, Rajshahi during 2008-09.

Plant material: Following two different types of explants of potato cv. Cardinal were used.

(i) Third leaf and the internodes of in-between 3rd and 4th leaves of 28-days old field grown plants.(ii) Leaves and internodes (4th, 3rd and 2nd leaves) and to corresponding internodes of 21 days old in vitro grown plants.

Agrobacterium strain and plasmid: *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al.,* 1982) contains binary plasmid pCAMBIA-2301 was used. cDNA sequence of *ferritin* gene was inserted into LBA4404 Ti plasmid under the control of 35S promoter and Nos terminator to develop pCAMBIA-2301 plasmid. The recombinant vector pCAMBIA-2301 was transferred from *Escherichia coli* DH5α into *A. tumefaciens* LBA4404 by triparentalmating (Holsters *et al*., 1978) and was received from Professor M. Monzur Hossain, Plant Breeding and Gene Engineering Lab., Dept. of Botany, University of Rajshahi, Bangladesh.

Tissue culture condition: In *ex vitro* condition, young leaves and internodes of field grown potato plants were collected and surface sterilized with 0.1% $Hgcl_2$ for 3 minutes, washed thrice with distilled water. In *in vitro* condition, explants from aseptically grown 21 days old seedlings were excised and cultured onto MS basal medium. All plant media were adjusted with 1N NaOH to pH 5.7, solidified with 6gm/l agar and autoclaved at 121^0 for 20 min.

Transformation of explants: Explants were inoculated with *Agrobacterium tumefaciens* strain LBA4404 containing pCAMBIA-2301 plasmid having *ferritin* gene in liquid MS medium with 50 mg/l kanamycin for 30-90 sec. The density of *Agrobacterium* inoculums of 0.50-2.00 at 600 nm and co-cultivation for 24-48 h on agar gelled MSo medium. After, co-cultivation the explants were transferred and placed on selection and regeneration medium (MS+1.0 mg/lNAA+0.5 mg/l BA+50 mg/l kanamycin+50 mg/l cefotaxime). After 5-6 weeks shoots began to regenerate in selection medium from the cut surface of the explants and transferred on to same fresh medium for shoot induction. Kanamycin resistant shoots were transferred to MS basal medium supplemented with 50 mg/l kanamycin for shooting.

PCR and RT-PCR analysis: To detect the transgene in transformed and control (Non-transformed) plants were analysed by the polymerase chain reaction (PCR) and its transcripts by reverse transcriptase polymerase chain reaction (RT-PCR). Genomic DNA of potato was extract from young leaves following the protocol of Edwards et al, (1991). PCR analysis to detect the presence of the *ferritin* gene were carried out using the PCR Screening Kit (Sigma Chemicals Ltd., USA) in the presence of following pair of primers forward primer (5´GTTGCTCTCAAGGGACTTGC3´) and reverse primer (5´CACACACCGTGACCCTTTC 3). Amplification condition was 94 °C for 4 min followed by 35 cycles of 94 °C 1 min, 55 °C 1 min, 72 °C 1.30 min and 72 °C 10 min for final extension. Expected PCR product size was about 365 bps. *Ferritin* transcripts analysed were carried out to confirm the transgenic status of plants showing a positive reaction in RT-PCR analysis. Total RNA was isolated from the transgenic plants according to the manufacture's instructions (Quagene, UK). First strand cDNA synthesis with poly-T proimers and subsequent cDNA amplification with the target gene specific primers were done with RT-PCR kits (Applied Biosystem, UK) according to suppliers manual. Amplified cDNA were on 1.8 % agarose gel, stained with ethidium bromide and documented.

RESULTS AND DISCUSSION

Standardization of plant regeneration from potato explant: Though the main objective of this study is to transformed potato with *ferritin* gene, but before doing this, plant regeneration media composition was standardized for potato explants. In *in vitro* condition, explants were excised and cultured onto MS basal medium supplemented with different concentration of NAA (0.5, 0.1 and 1.0 mg/l) and BA (0.05, 0.1, 0.5 and 1.0 mg/l) and incubated in a growth chamber. This experiment reveals that 1.0 mg/l NAA and 0.5 mg/l BA in MS medium was the most effective formulation where the highest percentage (70%) of the explants was induced to regenerate shoots. Similar result has already been reported in peanut *Arachis hypogaea* (Anuradha *et al,* 2006) and lettuce (*Lactuca sativa*) (Ahmed *et al*, 2007).

Effect of Kanamycin Concentration: The sensitivity of potato (leaf and internode) explants to kanamycin was assayed by culturing the explants without co-cultivation with *A. tumefaciens* on selection medium contained different concentrations kanamycin (0, 25, 50, 75, 100 mg/l). The explant growth was completely inhibited at the level of kanamycin concentration of 50 mg/l. So, 50 mg/l kanamycin was chosen for the selection of kanamycin resistant tissues in subsequent transformation experiments. Similar result have been reported in Indian mulberry (Bhatnagar and Khurana, 2003), grasspea (Barik *et al.*, 2005) and lettuce (Ahmed *et al.*, 2007).

Effect of acetosyringone concentration: In the present study, different concentrations (0, 10, 15, 20, 25 and 30 mg/l) of acetosyringone were added into the co-cultivation medium. The highest percentage of kenamycin resistant explants reseeming new growth was obtained at the 25 mg/l concentration of acetosyringone.

Transformation of potato explants using optimum parameters: Explants were inoculated with a *Agrobacterium tumefaciens* strain LBA4404 ferritin gene for different infection period (30-90s). Bacterial inoculum density was measured and the highest percentages of kanamycin resistant of *in-vitro* and *ex-vitro* leaves were respectively, 2.99% and 2.00% and internodes were respectively, 1.9% and 1.85% observed at 1.50 OD 600nm. The post infection process, an incubation period 60 s (Table 1) and 24 h of co-cultivation period was optimized (Table 2) for subsequent transformation experiments. After co-cultivation explants were transferred onto the selection and regeneration medium containing 50 mg/l kanamycin and 50mg/l cefotaxime. Similar result was reported in Arabidopsis (Abel and Theologis, 1994), in peanut (Barik *et al.*, 2005), in raspberry (Faria *et al.*, 1997), in sweet orange (Bhatnagar and Khurana, 2003) and in lettuce (Ahmed *et al.*, 2007). After 5 weeks, shoots began to regenerate from explants. The experiment was repeated three times. The highest nos. of transgenic plants were recovered from *in vitro* derived leaf explant from experiment no-2, that showed the highest transformation

efficiency (35%) than *ex vitro* leaf (21.67%) among three experiments. On the other hand, *in vitro* internodes showed better transformation efficiency (16.67%) than *ex vitro* internodes (10%). Kanamycin resistance shoots rooted on MS medium containing 50 mg/l kanamycin.Similar result was reported in pear (Mourgues *et al.*, 1997) and sweet orange (Changhe *et al.*, 2002). Results are shown in figure 3 (A, B, C, D). The putative rooted plants were assumed as to line.

Molecular analysis of transgenic plants: PCR analysis of transgenic plants revealed that 21 out of 27 plants were positive for the *ferritin gene*. PCR profile of the putatively transformed transgenic plants exhibited the presence of expected 365 bps exons of *ferritn* gene (Figure1). Among 21 *ferritin* positive plants, randomly selected 18 plants. To ensure the presence of transgene in the transgenic plants, *ferritin*-PCR positive plants were further analyzed by RT-PCR. RT-PCR profile of the putative transformed plants exhibited the presence of 365 bps ferritin cDNA as expected (Figure 2). All the PCR positive plants did not show the same positive results for RT-PCR. Only 12 out of 18 were RT-PCR positive.

In summary, this finding suggests that the potato with enhanced iron content developed by genetic transformation may contribute to solve the iron deficiency nutritional problem of the population that consumes potato as a major food.

Table 1. Effects of infection period on survival explants of potato. (Explants were selected by growing them onto regeneration medium containing 50 mg/l kanamycin, 50 mg/l cefotaxime, 1.0 mg/l NAA and 0.5 mg/l BA)

Explant source	Explants type	Incubation period (sec)	No. of explants inoculated	No. of explants survived	Surviving percentage of explants	Mean (X±SE)
Ex vitro	Leaf	30 60 90	20	5 11 5	25 55 25	35+±0.46
	Internode	30 60 90	20	4 6 -	20 30 -	25±0.25
	Mean			**6.2**		
In vitro	Leaf	30 60 90	20	9 **15** 10	45 **75** 50	56.67±0.28
	Internode	30 60 90	20	6 11 -	30 55 -	42.50±0.91
	Mean			**10.20**		

Table 2. Effect of co-cultivation period on development of kanamycin resistance tissue with *Agrobacterium tumefaciens* LBA4404 containing *Ferritin* construct

Explant source	Explant type	Co-cultivation period (hour)	No. of explant. co-cultured	No. of explants survived	Percentage of surviving explants
Ex vitro	Leaf	24 48	20	11 8	55 40
	Internode	24 48		4 5	20 25
	Mean			**7**	
In vitro	Leaf	24 48	20	**13** 7	**65** 35
	Internode	24 48		7 6	35 30
	Mean			**8.25**	

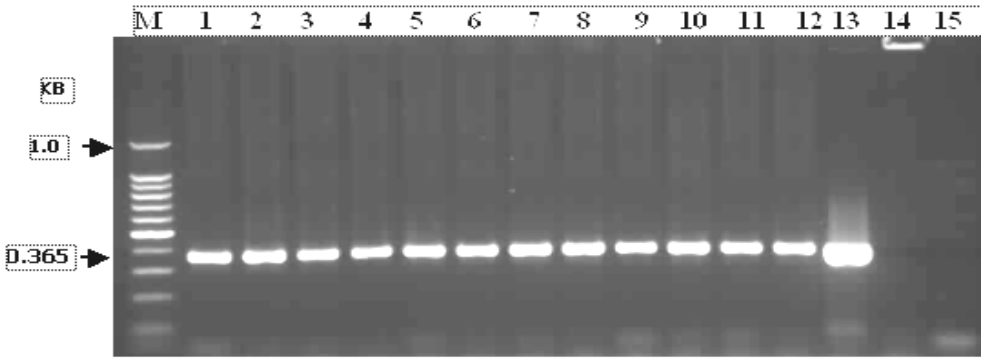

Figure 1. PCR analysis of genomic DNA of transgenic potato (cv. Cardinal) plants to detect the presence of ferritin (Soybean) gene (CLVT8). The genomic DNA of the one month old in vitro grown putative transgenic plants. The PCR products were separated on a 1.8 % agarose gel and stained with ethidium bromide. *M*: 100 bp size marker (Promega). Lanes *1 -12*: plants regenerated from infected leaves showing expected 365 bp band. Lane *13*: plasmid control. Lane *14*: control plant regenerated from uninfected leaf culture. Lane *15*: water (control).

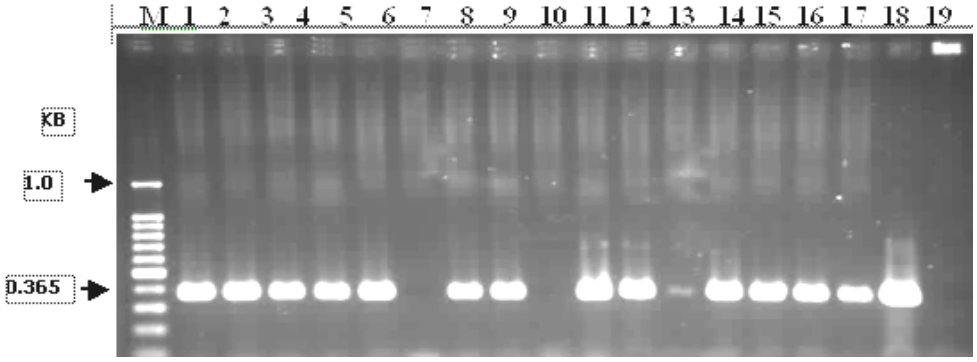

Figure 2. RT-PCR analysis of total RNA of transgenic potato plants to detect the transcript of *ferritin* (Soybean) gene (CLVT8). *M*: 100 bp size marker (Promega). Lanes *1 -17*: To plants showing expected 365 bp ferittin cDNA band; lane *18*: RNA of to plant used as template without RT-reaction and lane *19*: control plant regenerated without infection.

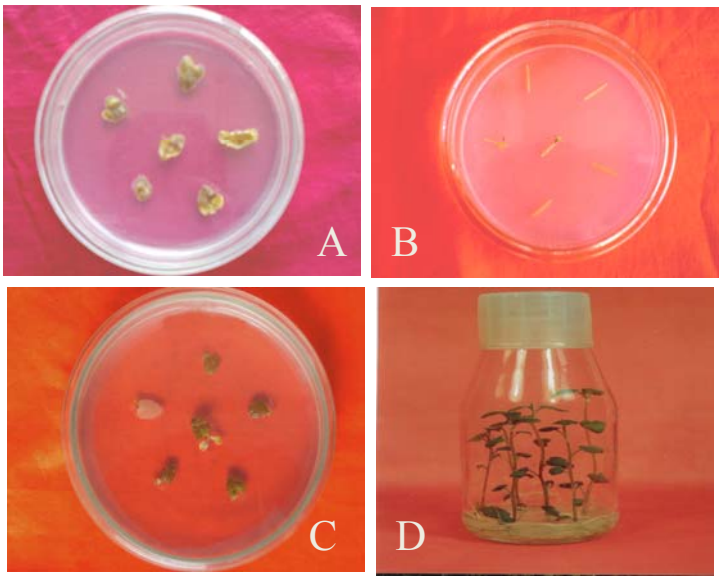

Figure 3. Selection and plant regeneration of potato with *ferritin* gene. A-B Infected leaves and internodes with pCAMBIA2301 vector containing *ferritin* gene under the control of 35S promoter and Nos terminator. (C) Regenerated plantlets in the regeneration medium. (D) Multiplication of transgenic plants.

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