

***Agrobacterium*- mediated Genetic Transformation of oilseed *Brassica juncea*(L.)**

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Abstract

Brassica is an important oilseed crop in Bangladesh. The available *Brassica* varieties cultivated in Bangladesh are susceptible to many diseases and pests specially, with fungal diseases. In this context, an alternative to trait improvement especially disease resistant variety would be evolved by applying biotechnological approaches like genetic transformation. So, the present investigation was carried out for the development of *Agrobacterium*-mediated genetic transformation system for oilseed *Brassica* grown in Bangladesh. Prior to the transformation an efficient *in vitro* regeneration protocol was developed for two varieties of *Brassica juncea* namely, BARI Sarisha-11 and BARI Sharisha-16. The best shoot regeneration was obtained from hypocotyl explants using MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA. MS medium hormone was found to be effective in inducing roots. Three gene construct (LBA4404/pBI121, LBA4404/ pCAMBIA2300polyA AFPFP, EHA105/pGII-pSOUP-VST-N-CHITIN) of *Agrobacterium* were used for transformation experiments. Transformation frequency regarding the recovery of shoots for BARI Sarisha-11 and BAR Sarisha-16 using gene construct I was 0.84% and 0.96% respectively, while that was 0.63% and 0.78% respectively in case of construct III. The stable integration of the putatively transformed shoots developed by using EHA105/pGII-pSOUP-VST-N-CHITIN (containing *bar* and *chitinase* gene) was confirmed through PCR analysis.

Keywords: Oilseed Brassica; In vitro Regeneration; Genetic Transformation Bangladesh.

1. Introduction

Brassica is one of the major crop genera belongs to the family Brassicaceae (syn. Cruciferae). Economically important crop plants of this genus are rapeseed (*Brassica campestris* L. and *B. napus* L.), mustard (*B. juncea* L., Czern and Coss.), turnip (*B. rapa*), cabbage and broccoli (*B. oleracea*) [1]. Oilseed *Brassica* is one of the most important sources of edible vegetable oil, industrial oil and protein-rich product of the world. Species of *Brassica* contain 40-45% oil and 20-25% protein. It is a good source of protein with well-balanced amino acid and mineral. It is one of the best cooking oil particularly for heart patient because it has an high levels of omega-3 (6-11%) and are a common, cheap, mass-produced source of plant-based (therefore, vegetarian) omega-3 fatty acids (linolic and alpha linolic acid respectively).

Mustard, *Brassica juncea* L., rapeseed, *Brassica campestris* L. (syn *Brassica rapa*) are important oil yielding crops in Bangladesh. Acute shortage of edible oil has been prevailing in Bangladesh during last several decades. Bangladesh is ranking number one mustard seed importing country of the world [2].The productivity and quality of these crops are affected by various biotic and abiotic stresses. Rapeseed and

mustard suffer from 14 diseases in Bangladesh. Among these diseases leaf blight, downy mildew and the parasitic plant are important. The most important disease of *Brassica* in Bangladesh is leaf blight disease, caused by *Alternaria brassicae* [3]. This disease causes leaf blight, pod blight and seed abnormalities [4]. Crop loss between 30-100% due to this disease has been reported [5]. Downy mildew caused by *Peronospora brassicae* is predominant at early growing stage. Farmers store their seeds in traditional methods, where heavy infestation of different fungi occurs. The most seed borne pathogens are *A. brassicae*, *A. brassicola*, *A. raphani*, *Botrytis cinerea*, *Fusarium avenaceum* and *Phoma lingam* [6]. The most important pest of this crop is aphid (*Lipaphis erysimi*).

Although a number of new varieties have been released from different research institutes of Bangladesh, none has come up to the expectation of breeders, because the released varieties are not resistant to above diseases and pests. Conventional breeding programmes alone were not successful enough in *Brassica* due to high degree of segregation upon cross pollination and unavailability of suitable germplasm. Conventional breeding of *Brassica* is labor and resource intensive and time consuming; it takes several generations to develop a new variety.

In this context, an alternative to trait improvement especially disease resistant variety would be evolved by applying biotechnological approaches like genetic transformation. Genetic transformation offers a powerful tool for introducing desired genes into *Brassica* species. Significant progress has been achieved in developing transformation technology for many species of *Brassica* [7], [8].

Considering the importance of *Brassica* spp. in Bangladesh and limitation in improving yield and quality through conventional breeding techniques, it is necessary to improve *Brassica* varieties by incorporating gene of desired character within the local varieties. Therefore, it is necessary to establish a suitable transformation protocol for Bangladeshi *Brassica* varieties to develop biotic stress resistant lines of *Brassica* that would be suitable for local cultivation. Thus, the present investigation was conducted with an aim in developing a protocol for *Agrobacterium*- mediated genetic transformation of local varieties of *Brassica*. It may be mentioned here that for a successful genetic transformation protocol a suitable and reproducible regeneration system is required. Considering the requirement of a reproducible *in vitro* regeneration attempts were also made to develop a regeneration protocol for locally grown *Brassica*.

2. Materials and Methods

Two varieties of *Brassica juncea* namely, BARI sarisha-11 and BARI sharisha-16 cultivated in Bangladesh were used as plant materials for this research. Seeds of both the varieties of *Brassica juncea* (L.) were collected from Oil Seed Division of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. The surface sterilized seeds were inoculated on half strength of MS with 2% sucrose and 0.8% agar for germination and seedling development. Hypocotyl explants were excised from *in vitro* grown seedlings. Isolated explants were cultured on MS medium [9] containing BAP, NAA, IAA and Kn (mg/l) singly or in combinations for regeneration. *In vitro* regenerated shoots were sub-cultured regularly to fresh medium at an interval of 12 - 15 days for further multiplication. Elongated shoots were separated and cultured on rooting medium for root formation. About 2 - 3 cm long shoots were separated and cultured on rooting medium containing full and half strengths of MS without hormonal supplement or with different concentrations of IBA. The plantlets with sufficient root system were then transplanted to small plastic pots containing sterilized soil.

Three genetically engineered *Agrobacterium*, LBA4404/pBI121 [10] (Gene Construct I) containing Gus and nptII gene, *Agrobacterium tumefaciens* LBA4404 contains plasmid pCAMBIA2300 polyA-AFPFP (Gene Construct II) [10] harbouring AFP (anti-fungal protein gene) and nptII gene, EHA105/pSOUP-pGII-VST-N-CHITIN (Gene Construct III) [11],[12] harbouring bar and chitinase gene were used for Transformation experiments. The overnight grown *Agrobacterium* culture was centrifuged to make the *Agrobacterium* suspension. This *Agrobacterium* suspension was used for infection of explants. Prior to this "Optical Density" (OD) of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). In obtaining sufficient infection, cut explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium. Following infection and incubation, the explants were soaked in sterile Whatman filter papers for 2 - 3 min to remove the excess bacterial suspension.

All the explants were maintained in co-culture medium for 2 - 4 days in dark condition at $25 \pm 2^\circ\text{C}$. Following co-culture, the explants were washed with distilled water for three to four times until no opaque suspension was seen, then washed for 15 min with distilled water containing 300 mg/l ticarcillin (Duchefa, The Netherlands). Then explants were dried with a sterile Whatman filter paper and transferred to shoot regeneration medium with 100 mg/l ticarcillin. After 15- 18 days, the regenerated shoots were subcultured in selection medium (containing kanamycin/phosphinothricin). The survival of green shoots on the optimum selection medium indicated the production of transformed shoots.

Genomic DNA was isolated from the transformed *Brassica* shoots and stable integration of *bar* gene was examined by PCR (ependorf Master cycler gradient) analysis. For this purpose DNA was isolated from transformed and non- transformed shoots using the CTAB method (Doyle and Doyle 1990). The primers that used for amplification were Bar Fwd: 5'-AAC TTC CGT AAC GAG CCG CA -3' and Bar Rev: 3'-GAT TTC GGT GAC GGG CAG GA -5'. The plasmid isolated from *Agrobacterium* strain EHA105/pGII-pSOUP-VST-N-CHITIN was used as positive control. The PCR analysis and electrophoresis of the PCR products was followed by the protocol described by Das et al 2012. For PCR amplification of the *bar* gene, DNA was denatured at 94°C for 5 min and then amplified in 30 cycles using 94°C for 1 min, 59°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C . The amplified DNA was run on 0.80% agarose gel and stained with ethidium bromide (0.05 $\mu\text{l/ml}$). Electrophoresis was accomplished at 40 volts.

3. Results and Discussion

Two varieties of *Brassica juncea* namely, BARI Sarisha-11 & BARI Sarisha-16, were used for *Agrobacterium* mediated genetic transformation. A number of experiments were carried out in the past [13] with identical materials to determine the suitable media for regeneration of *Brassica* spp. According to them *Brassica juncea* namely, BARI Sarisha-11 & BARI Sarisha-16 was found to be most responsive varieties towards regeneration as well as in transformation using marker gene [14]. That's why these two varieties were used for transformation experiments. Prior to transformation experiments regeneration of BARI Sarisha-11 & BARI Sarisha-16 were conducted to develop control plants using the best suitable media MS with 2 mg/l BAP and 0.2 mg/l NAA. In vitro regeneration and transformation experiments were conducted using cotyledonary leaf with petiole and hypocotyl explants of BARI Sarisha -11 and BARI Sarisha -16. In case of BARI Sarisha-11 and BARI Sarisha-16, the above mentioned media showed best response for direct and indirect (through callus from hypocotyl explants) multiple shoot regeneration. Initiation of shoots was found to occur within 15-18 days of inoculation of explants. Percentage of shoot

regeneration as well as the number of shoots per explants was maximum (5-6) on this medium composition (Figure 1 a-c.). In this case, 85.00% explants of BARI Sarisha-11 and 88.33% (Table-1) explants of BARI Sarisha-16 exhibited shoot regeneration capability. This finding is also supported by previous authors [13], [14]. Profuse root induction in *Brassica juncea* (BARI-11 and and BARI-16) was achieved on MS medium without any hormonal supplements. Rooting from excised regenerated shoot of BARI-11 and BARI -16 are presented in Figure 1(d, e). After successful root formation plantlets were transferred into small plastic pots for acclimatization.

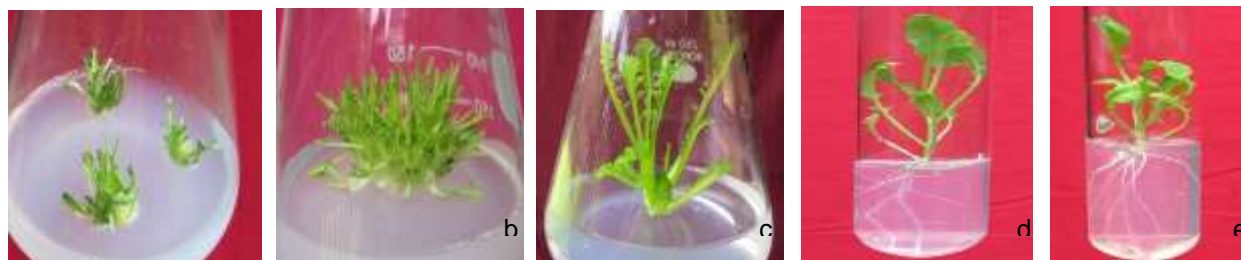


Figure 1(a-e). Different stages of in vitro regeneration of *B. juncea*. a) Initiation of multiple shoots of BARI Sarisha-11 from cotyledonary leaf with petiole explants. b) Newly formed multiple shoots from hypocotyl of BARI Sarisha-16, c) Elongated single shoot of BARI Sarisha-16, d) Rooting of *in vitro* shoots of BARI Sarisha-11 on MS without hormonal supplement, e) Root formation of BARI Sarisha-16.

Table 1. Responses of hypocotyl explants towards the shoot regeneration in MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA for two varieties of *B. juncea*.

variety	Responsive explants (%)	Days to shoot initiation	Mean no. of shoots/ explant
BARI-11	85.00	18-21	5.01
BARI-16	88.33	16-20	6.00

Series of experiments were carried out to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation using BARI Sarisha-11 and BARI Sarisha-16. Several factors influencing the *Agrobacterium*-mediated genetic transformation procedure *viz.* optical density of bacterial suspension, incubation period and co-cultivation period [15] were optimized. During optimization of regulatory factors it was found that maximum transformation was obtained with bacterial suspension having an OD of 1.0 with 30 minutes of incubation and 3 days of co-cultivation period in case of *Agrobacterium* gene construct I (LBA4404- pBI121) for transformation of cotyledonary leaf with petiole and hypocotyl of two varieties of *Brassica juncea*. However, for gene construct II (LBA4404/pCAMBIA2300polyA AFPPF) bacterial suspension having an OD of 1.0 with 35 minutes of incubation and 3 days of co-cultivation period was found to be optimum. In case of *Agrobacterium* construct III (pGII-pSOUP-VST -N- CHITIN) OD 1.0 with 15 minutes of incubation and 3 days of co-cultivation period (Table 2) was found to be optimum for transformation of cotyledonary leaf with petiole and hypocotyl explants.

Table 2. Influence of different co-cultivation period on transformation of cotyledonary leaf with petiole and hypocotyl explants analyzed based on the rate of survival for Gene construct III (EHA105/pGII-pSOUP-VST-N-CHITIN).

Varieties	Co-cultivation Period (in days)	No. of survived explants	Survival (%)
BARI	2	29	58
Sarisha-11	3	38	76
	4	27	54
BARI	2	30	60
Sarisha-16	3	40	80
	4	25	50

In this part of investigation, *Agrobacterium* strain LBA4404 (Gene construct I) was used to test its compatibility with the explants of *Brassica* varieties. Following *Agrobacterium* infection and co-cultivation for 72 hours transformation ability was monitored by transient histo-chemical assay (Fig.2a-e) of the GUS reporter gene in the explant.

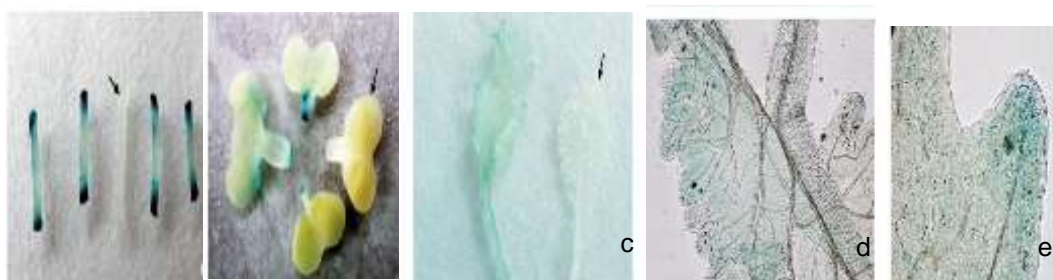


Figure 2 (a-e). GUS-histochemical assay of the explants and shoots transformed with gene construct I. a) GUS gene positive hypocotyl explants of BARI Sarisha-16, b) GUS positive cotyledonary leaf with petiole explants of BARI Sarisha-11, c) Gus-histochemical assay of leaf of BARI Sarisha-16 isolated from the putatively transformed shoots. Control shoots showed no blue colour (arrow). d & e) Sterio Microscopic(Olympus, Japan, x 40) view of leaf cells of the putatively transformed shoots showing the expression of GUS gene.

The *Agrobacterium* gene construct I and gene construct II used in these experiments contains npt II gene. This gene confers kanamycin resistance of the transformed cells. Selection pressure starting with lower concentrations of kanamycin was applied after 2 weeks following initiation of regeneration. It was observed that the purple shoots were developed along with green or albino shoots (Figure 3.) after transferring them onto selection media containing kanamycin. It was observed that, all control shoots died in the selection medium in presence of 50 mg/l kanamycin. For this purpose kanamycin concentration was gradually increased from 25-50 mg/l during the selection of transformed *Brassica juncea* [14] shoots. To recover transformed shoots, gradual elimination of non-transformed shoots and shoot buds was done through separating green shoots from albino shoots and allowing their further growth on fresh regeneration medium containing higher concentration of kanamycin. The shoots that survived on the medium containing 50 mg/l kanamycin were considered as putative transformants. In case of

Agrobacterium gene construct I (LBA4404- pBI121) the number of survived shoots in the final selection pressure was extremely low BARI Sarisha-11(0.84%) and BARI Sarisha-16 (0.96%). In case of gene construct II (LBA4404/pCAMBIA2300polyA AFPFP), no shoots were found to survive in the final selection medium containing 50 mg/l kanamycin.

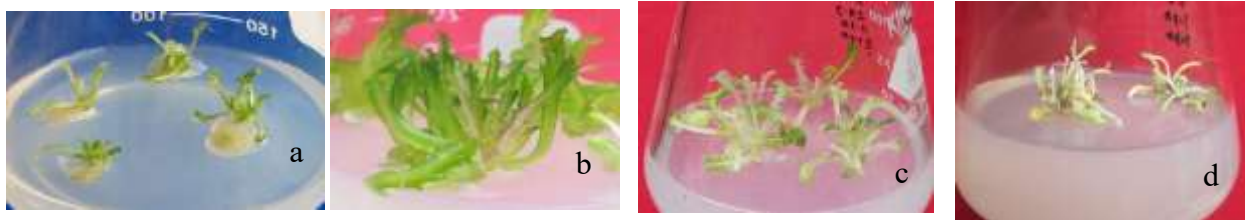


Figure 3. a) Initiation of shoots after co-cultivation, b-d) selection of transformed shoots with selectable agents.

Gene construct III EHA105/pGII-pSOUP-VST-N-CHITIN harbouring bar gene as selectable marker that conferring phosphinothricin resistance and chitinase as antifungal gene (the gene of interest). So, selection of transformed plants was done using phosphinothricin(PPT) Co-cultivated explants were cultured initially in a medium without selective agents. After 2 weeks following initiation of regeneration, the regenerated shoots were sub-cultured in the same media but with selectable agents. It was observed that, all control shoots died in the selection medium in presence of 2.5 mg/l phosphinothricin. For this purpose phosphinothricin(PPT) concentration was gradually increased from 1.5-2.5 mg/l (Figure 4) during the selection of transformed *Bassica juncea* var. BARI-11 and BARI-16 shoots. Therefore, the shoots that survived on the medium containing 2.5 mg/l PPT or any higher PPT for more than two weeks were considered as transformed. The number of survived shoots of the final selection pressure was extremely low for BARI-11 and BARI-16, 0.63% and 0.78% respectively (Table 3). Similar findings also reported in lentil [16].

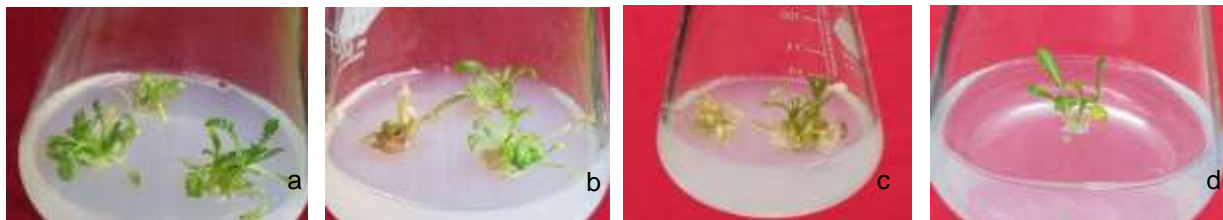


Figure 4 (a-d). Genetic Transformation of *B. juncea* using gene construct III. a) Multiplication of shoots after co- cultivation. b & c) Selection of shoots of BARI Sarisha-11 and BARI Sarisha- 16 on the selection medium containing 2.5 mg/l PPT and 3.0 mg/l PPT showing that non transformed shoots became brown. d) Putatively transformed shoots of BARI- 16 that survived on the selection medium containing 3.0 mg/l PPT.

Table 3. Effect of phosphinotricin on regenerated shoots during selection infected with Gene Construct III (EHA105/pGII-pSOUP-VST-N-CHITIN).

Varieties	No. of infected explants	No. of regenerated shoots after transformation	No. of shoots survived in medium with Phosphinotricin (mg/l)				% of survived shoots
			1.5	2	2.5	3	
BARI-11	504	1268	625	87	11	9	0.63
BARI-16	506	1400	685	91	19	11	0.78

In the present investigation the transgenic nature of the transformed plantlets were confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR), PCR analysis was conducted to confirm the integration of fungal diseases resistant gene in transformed *B. juncea* varieties i.e. BARI-11 and BARI-16 (Figure 5) that was developed using *Agrobacterium* gene construct III EHA105/pGII-pSOUP-VST-N-CHITIN. The DNA isolated from both of transformed and non-transformed shoots was subjected to PCR for the amplification of *bar* and *chitinase* gene present in *Agrobacterium* gene construct III. Amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band (500 bp) formed in the transformed plantlets of BARI-16 were identical to the amplified DNA of bacterial gene construct III (positive control). This result indicated that the *bar* gene was inserted in the genomic DNA of three transformed plantlets. This investigation confers the integration of *bar* gene into the transformed plants.

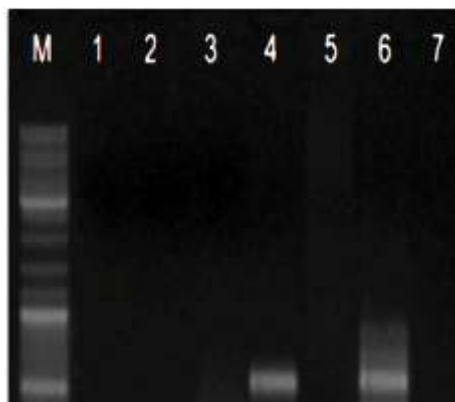


Figure 5. PCR amplification of *bar* gene: Lane M- 1Kb DNA ladder, lane 1-3 - non transformed shoots of BARI Sarisha- 16, lane 4 - transformed shoots of BARI- 16, lane 5- negative control, lane 6 - positive control, lane 7 - water control.

4. Conclusion

Based on the results it may be concluded that in the present investigation it has been possible to develop a suitable *in vitro* regeneration system for *Brassica* spp. The protocol of *Agrobacterium*-mediated genetic transformation developed through the present investigation is applicable for the transformation of locally grown *Brassica* varieties using antifungal genes as well as other agronomically important genes.

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