

***Agrobacterium*-mediated Genetic Transformation of Two Varieties of *Brassica juncea* (L.) Using Marker Genes**

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Abstract

Protocol for *Agrobacterium*-mediated genetic transformation using hypocotyl and cotyledonary leaf with petiole from two local varieties of *Brassica juncea* was established by optimizing various factors influencing transformation. GUS histochemical assay revealed that the cotyledonary leaf with petiole and hypocotyl explants had positive interaction with the *Agrobacterium* strain LBA4404 containing the binary plasmid pBI121 which has marker genes like, GUS and *nptII*. Maximum transformation was obtained with bacterial suspension having an optical density of 0.8 at 600 nm, 30 min of incubation and 72 hours of co-cultivation. The transient and stable integration of the marker genes were confirmed through histochemical GUS assay, as well as PCR analysis.

Introduction

The oilseed Brassica is found within *Brassica juncea*, *B. carinata*, *B. rapa* (syn. *B. campestris*) and *B. napus* collectively and are commonly called oilseed rape and mustard (Cardoza and Stewart 2004). Oilseed Brassica ranks third after soybean and palm oil in the global production (www.canolacouncil.ca). The oilseed (*Brassica* spp.) cultivation has increased tremendously from past few years and by now it is the second largest contributor to the world supply of vegetable oil (Neha and Ashutosh 2014). Mustard-seed is grown in more than 50 countries. The worldwide annual production of vegetable oil is approximately 87 million metric tons, which mainly consists of soybean, oil palm, rapeseed, and sunflower oil (Vigeolas et al. 2007). Rapeseed and mustard (*Brassica* spp.) occupy more or less the same rank among the oilseed crops of Bangladesh. They cover about 61.2%

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of the total area under oilseed and 52.6% of the total oilseed production. The annual oilseed production is 0.37 million metric tons of which rapeseed covers 62% (www.moa.gov.bd). Oilseed *Brassica* is one of the most important sources of edible vegetable oil, industrial oil, and protein-rich product of the world. Bangladesh has an incredible demand for edible oil. Among the oilseed crops grown in this country, *Brassica* occupies the first position in respect of area and production. In 2011-12, the production was 262,000 metric ton (Mt), whereas the total oilseed production was 787,000 Mt (BBS 2012). Bangladesh consumes around 1.73 million tons of oils per year of which about 1.6 million tons fully meet by import. Hence, it is important to enhance the production of *Brassica* which, if accomplished can save the country from the huge import pressure (Alam et al. 2013).

Although rapeseed and mustard are very important oil crop of Bangladesh, but these crops are affected by various biotic and abiotic stresses. The most important disease of *Brassica* in Bangladesh is leaf blight disease, caused by *Alternaria brassicae* (Ahmed 1952). The disease has been reported to cause heavy loss to the crop reducing crop yield to an extent of 30 - 40% (Rai et al. 1976 and Fakir 1980). This disease may cause 25% yield reduction at severe condition of infestation.

Conventional breeding alone was not successful enough in *Brassica* due to high degree of segregation upon cross-pollination and unavailability of suitable germplasm. Conventional breeding of *Brassica* is laborious, resource intensive and time consuming. Usually, it takes eight to ten generations to develop a new variety (Cardoza and Stewart 2006). Genetic transformation, can supplement traditional crop improvement procedures and this approach can accelerate the development of new plant varieties which is not possible through the application of breeding and tissue culture alone (Gardner 1993). A reproducible and reliable transformation system could enable us to insert genes of interest which are unavailable in present *Brassica* genotypes. *Agrobacterium*-mediated transformation has allowed the generation of viable transgenic plants from many *Brassica* species (Gupta et al. 1993 and Radke et al. 1992). The efficiency of *A. tumefaciens*-mediated transformation technique in oilseed rape is influenced by the type of cultivar, age of the donor plant and explants type (Poulsen 1996). Of various systems used for rapeseed transformation, *Agrobacterium*-mediated transformation using hypocotyl and cotyledonary leaf with petiole has been commonly employed owing to its high regeneration ability (Khan et al. 2003, Pua and Lim 2004). The transformation system of *Brassica* was low in frequency and the genotype dependent (Zhang et al. 1998). Considering the importance of *Brassica* spp. in Bangladesh and limitation in improving yield and quality, it is necessary

to improve *Brassica* varieties by incorporating gene of desired characters within the local varieties. Therefore, it is necessary to establish a suitable transformation protocol for Bangladeshi *Brassica* varieties to develop disease resistant elite genotype that would be suitable for local cultivation. Studying these facts, the present investigation was conducted with the aim to develop a protocol for *Agrobacterium*-mediated genetic transformation of local varieties of *Brassica*. Prior to that, a suitable and reproducible *in vitro* regeneration system was also developed for achieving successful transformation in *Brassica* spp.

Materials and Methods

The seeds of BARI Sarisha-11 and BARI Sarisha-16 were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. To generate explants, the seeds were treated in 0.1% (w/v) mercuric chloride for 8 - 10 mins with vigorous shaking, washed three times with autoclaved distilled water and inoculated onto the half strength MS medium with 2% sucrose for germination. These seeds were kept in the dark till the germination took place and finally transferred to 16/8 hours light/dark condition at $25 \pm 2^\circ\text{C}$ in growth room. Generally 2 - 3 days are required for germination of seeds. For initiation and development of shoot, MS supplemented with various combinations and concentrations of BAP and NAA were used. The pH of the medium was adjusted to 5.8 before autoclaving. For induction of roots from the base of the *in vitro* grown shoots, full as well as half strength of MS supplemented with or without different concentrations of IBA were used.

For genetic transformation, *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pBI121 was used. This plasmid contains a scoreable reporter gene GUS (β -glucuronidase) driven by CaMV35S promoter and NOS terminator and a selectable marker gene *nptII* fused between NOS promoter and NOS terminator encoding for the enzyme neomycin phosphotransferase conferring kanamycin resistance (Herrera-Estrella et al. 1983). The explants of hypocotyl and cotyledonary leaf with petiole were collected from 4 - 5 days old *in vitro* grown seedlings for transformation. The explants were prepared with a scalpel while submerged in the *Agrobacterium* suspension. The explants were incubated in the *Agrobacterium* suspension in a small Petri dish for 30 mins, then blotted dry and cocultured for three days in the dark on MS with 2.0 mg/l BAP and 0.2 mg/l NAA. After coculture the explants were washed in ticarcillin (300 mg/l) to control the over growth of *Agrobacterium*. They were then cultured on the regeneration medium for shoot development and maintained in the growth room.

Since *nptII* gene was present in the plasmid, putatively transformed shoots were cultured on different concentrations of kanamycin in MS for selection of transformants. To eliminate the untransformed developing shoots the explants were subcultured on fresh regeneration medium initially with 10 mg/l kanamycin. The selection pressure of kanamycin was gradually increased from 20 up to 30 mg/l. It was observed that 30 mg/l kanamycin was optimum in killing the non-transformed shoots. In the selection medium containing 30 mg/l kanamycin both albino and green shoots were observed. The survival of green shoots on the optimum selection medium indicated the production of transformed shoots. Transforming ability of the explants as well as stable expression of the GUS gene was monitored by GUS histochemical assay (Jefferson et al. 1987) by submerging them in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl α -D glucuronide) and incubating them at 37°C for 24 - 48 hours and subsequently bleached with 70% ethanol before scoring for GUS expression.

Genomic DNA was isolated from the transformed shoots and stable integration of GUS and *nptII* genes were confirmed by PCR analysis. CTAB method (Doyle and Doyle 1990) was used for DNA isolation. For the detection of the *nptII* coding sequence, DNA was subjected to PCR using the following primers: forward-5'-TAG CTT CTT GGG TAT CTT TAA ATA-3' and reverse-3'-CCA GTT ACC TTC GGA AAA AGA GTT-5'. For the GUS gene the primers were : forward 5'-CAT GAA GAT GCG GAC TTA CG-3' and reverse 3'-ATC CAC GCC GTA TTC GGC GT-5'. All primers were used at 100 pmol/ μ l. The DNA isolated from transgenic tobacco was used as the positive control. Master mixture was prepared by mixing all of the PCR components e.g. 10x buffer, dNTPs, MgCl₂ Primer - F and R, etc. except the component against which the optimization strategy was intended. In each reaction, the volume of PCR buffer was used one tenth of the total reaction volume (25 μ l). For PCR amplification of the GUS gene, DNA was denatured at 94°C for 5 min and then amplified in 30 cycles using 94°C for 1 min, 65°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C. For *nptII* gene the cycling conditions were 5 min at 94°C denaturation and 30 amplification cycles using 94°C for 1 min, 55°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C. The amplified DNA was run on 0.8% agarose gel and stained with ethidium bromide (0.05 μ l/ml).

Results and Discussion

For establishing an efficient protocol for *in vitro* plant regeneration and *Agrobacterium*-mediated genetic transformation two varieties of *Brassica juncea*,

namely, BARI Sarisha-11 and BARI Sarisha-16 were used during this investigation. From cotyledonary leaf with petiole and hypocotyl explants regeneration was obtained directly without intervention of callus and indirectly via callus formation (Figs 1 and 2). For this purpose, *in vitro* grown seedlings were developed to obtain explants for *in vitro* regeneration of shoots. Half strength of MS with 2% sucrose and 0.8% agar was used as germination media. The percentage of seed germination was recorded to be same in two varieties of *Brassica* spp. such as, BARI Sarisha-11 and BARI Sarisha-16. Explants were collected from *in vitro* grown seedlings.



Figs 1 - 9: Plant regeneration and genetic transformation in *Brassica juncea* var. BARI Sarisha-16.

1. Development of shoot buds from the callus of hypocotyl on MS supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA. 2. Initiation of shoots from cotyledonary leaf with petiole on the same medium. 3. Multiple shoots regeneration on same medium. 4. Rooting on hormone free MS. 5. Stereomicroscopic view of histochemical localization of GUS activity (blue coloured zones) at the entire cut surface of hypocotyl explants ($\times 100$). 6. Selection of transformed shoots in presence of 30 mg/l kanamycin (note that the non transformed shoots became albino). 7. Putative transformed shoots showing blue colour on GUS histochemical assay. 8. Microscopic view of the blue coloured leaf cells of the putative transformed shoots. 9. Transformed plant survived on soil.

Different concentrations of BAP and NAA were used in MS to determine the optimum media composition for initiation and development of multiple shoots from explants. The effect of BAP without auxin was found to be very poor towards shoot regeneration in all varieties. In case of BARI Sarisha-11 and BARI Sarisha-16, MS supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA was found to be the most suitable for callus induction and multiple shoot regeneration among all the media combinations used (Fig. 3). Earlier Mollika et al. (2011) reported that in case of these two varieties 2.0 mg/l BAP, 0.2 mg/l NAA and 0.5 mg/l Kn were effective in developing multiple shoots. George and Rao (1980) observed maximum regeneration from cotyledon explant in *B. juncea* with BAP and NAA rather than BAP alone. Hachey et al. (1991) had also reported efficient regeneration in *B. campestris* with BAP in combination with NAA.

Among two explants, hypocotyl was most responsive in terms of percentage of shoot regeneration as well as the number of shoots per explants in all the varieties. In case of BARI Sarisha-11, the percentage of responsive explant producing shoots was 84% and in case of BARI Sarisha-16 it was 87%. The number of shoots per explant was 3 - 5 in BARI Sarisha-11 and 3 - 6 shoots in BARI Sarisha-16 (Table 1). Hypocotyl as a suitable explant for regeneration of shoots was observed by Tang et al. (2003) and Khan et al. (2010). Next to hypocotyl, cotyledonary leaf with petiole showed second highest response regarding percentage of shoot development. In case of BARI Sarisha-11, the percentage of responsive explant producing shoots was 81.30 and in BARI Sarisha-16 it was 82.66. Shoot primordia originated from the cut end of petiole of cotyledonary leaf. So, the presence of petiole was found critical for shoot regeneration when cotyledon were used as explant. This finding also supported the view that considerable variation in shoot regeneration from cotyledon explants was observed both between and within *Brassica* species (Tang et al. 2003).

For root induction and development, MS without plant growth regulator was found to be best for BARI Sarisha-11 and BARI Sarisha-16. The highest percentage of root producing shoots (55) was recorded in BARI-11 and 50 shoots produced root in BARI Sarisha-16 (Fig. 4).

Transformation ability of explants was detected using GUS histochemical assay. In all the varieties hypocotyl explants were found to show the best transformation ability, followed by cotyledonary leaf with petiole. The transformation ability of *B. juncea* var. BARI Sarisha-11 and BARI Sarisha-16 with *Agrobacterium* strain LBA4404 was examined. About 85.55% GUS positive explants were observed following transformation with the above gene construct (Fig. 5). Results of these observations are presented in Table 2. The various

factors that influence successful transformation such as, bacterial strain, incubation period, density of bacterial suspension and co-cultivation period, etc. were optimized. During optimization of regulatory factors it was found that maximum transformation was obtained with bacterial suspension having an optical density of 0.8 at 600 nm. Moreover, 30 minutes of incubation followed by 72 hours of co-cultivation were most effective towards transformation. Various methods used for *Brassica* transformation and the factors affecting transformation efficiencies have been reviewed by Poulsen (1996). In the present study, regeneration from transformed explants has also been observed after 3 - 4 weeks following inoculation.

Table 1. Effects of different concentrations and combinations of BAP and NAA on MS medium for multiple shoot regeneration from hypocotyls and cotyledonary leaf with petiole of BARI Sarisha-11 and 16.

Explants	BAP+NAA mg/l	No. of explants inoculated		Days to shoot initiation		% of shoot regeneration		Mean no. of shoots/explants	
		B-11	B-16	B-11	B-16	B-11	B-16	B-11	B-16
Hypocotyl	2.0 + 0.2	60	60	20 - 25	18 - 25	84.00	87.00	5.10	5.17
	2.0 + 0.5	60	60	22 - 25	18 - 30	66.66	65.08	4.00	4.50
	2.0 + 1.0	60	60	25 - 30	18 - 32	56.66	54.00	3.33	2.07
Cotyledonary leaf with petiole	2.0 + 0.2	60	60	15 - 20	15 - 18	81.30	82.66	4.00	4.06
	2.0 + 0.5	60	60	15 - 16	18 - 20	67.00	74.00	7.10	3.90
	2.0 + 1.0	60	60	12 - 16	16 - 18	70.00	64.33	2.16	2.16

B-11 (BARI Sarisha-11 and B-16 (BARI Sarisha-16).

Table 2. Responses of various explants of BARI Sarisha-11 and BARI Sarisha-16 towards GUS histochemical assay following co-cultivation.

Variety	Explant source	No. of explants assayed for GUS expression	No. of GUS +ve explants	% of GUS +ve explants
B-11	Hypocotyl	120	100	84.00
	Cotyledonary leaf with petiole	120	80	66.00
B-16	Hypocotyl	120	105	87.00
	Cotyledonary leaf with petiole	120	94	79.00

Agrobacterium strain (LBA4404/pBI121) used in this investigation has *nptII* gene within its T-DNA and this gene confers kanamycin resistance to the transformed cells. For the selection of transformed tissue kanamycin was applied to the regeneration medium at a concentration higher than that of the natural

tolerance of the respective explant tissue. To determine the level of kanamycin for selection of explants, different concentrations of kanamycin were tested. Following the report of Krishnamurty et al. (2000) and Tewari-Singh et al. (2004) selection pressure with different concentrations of kanamycin was not applied immediately after co-cultivation. It was also observed that when selection pressure was applied immediately after co-cultivation, the transformed explants did not show any sign of regeneration.

The purple shoots developed along with green or albino shoots after transferring them onto the selection media containing kanamycin. The purple shoots developed from both control and infected explant did not show blue colour when screened for GUS activity. Mukhopadhyay et al. (1992) observed that emerging green buds turned white or purple on selection medium within seven days of culture or after first sub-culture in *B. campestris*.

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 (Table 3). It was found that all the control shoots failed to survive at 30 mg/l kanamycin within 15 days. Therefore, the shoots that survived in the medium containing 30 mg/l kanamycin were considered as putative transformants (Fig. 6). Stable expression of GUS gene was visualized through histochemical staining in the regenerating shoots (Figs 7 and 8).

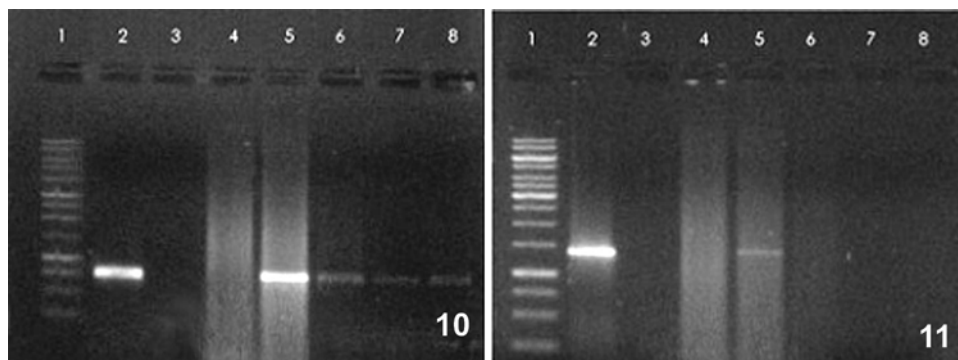
Table 3. Effect of kanamycin on shoots during selection.

Varieties	No. of infected explants	No. of regenerated shoots	No. of shoots survived in medium with kanamycin (mg/l)			
			10	20	25	30
B-11	500	1100	500	330	30	10
B-16	500	1130	563	352	25	12

After sufficient development of roots from the selected shoots, the plantlets were successfully transplanted to soil (Fig. 9). But the survival rate of these plantlets was low compared to the controls. The low survival rate of the transformed plantlets in the soil is most likely due to the lack of adequate root development or the proper acclimatization of the plantlets.

The transgenic nature of the transformed plantlets were confirmed through the application of specific molecular techniques like PCR analysis. The DNA isolated from both transformed and non-transformed shoots was subjected to PCR for the amplification of GUS and *nptII* genes present in *Agrobacterium*

strain. Amplified DNA was analyzed through agarose gel electrophoresis. In case of GUS gene a single band of 700 bp was observed in each of the four transformed plantlets identical to the amplified DNA of bacterial strain (positive control). On the other hand in case of *nptII* gene a single band of 800 bp was found for one transformed plantlets identical to the amplified DNA of the same bacterial strain. The results indicated that the GUS and *nptII* genes were inserted in the genomic DNA of transformed plantlets (Figs. 10 and 11).



Figs 10-11: PCR amplification of GUS gene and *nptII* gene of *Brassica juncea* var. BARI Sarisha-16. 10. PCR amplification of GUS gene: Lane 1-1kb ladder, lane 2-positive control, lane 3-water control, lane 4-negative control, lanes 5-8 transformed shoots. 11. PCR amplification of *nptII* gene: Lane 1-1kb ladder, lane 2-positive control, lane 3-water control, lane 4-negative control, lanes 5-transformed shoots.

From the foregoing discussion it may be concluded that through this investigation it has been possible to develop an efficient *Agrobacterium*-mediated genetic transformation protocol using marker gene/s like GUS and *nptII* gene. Integration of the above genes was confirmed by GUS- histochemical assay as well as through PCR analysis. Therefore, based on the findings of the present investigation the future transformation experiments may be undertaken for developing fungus resistant Bangladeshi *Brassica* varieties.

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