

***Agrobacterium*-mediated Genetic Transformation of Local Cultivars of Chickpea (*Cicer arietinum* L.)**

Ripa Akter Sharmin¹, Jasmin Akter, R.H. Sarker* and M.I. Hoque

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Agrobacterium-mediated genetic transformation protocol was established for two chickpea (*Cicer arietinum* L.) varieties, namely Barichhola-4 (Bch-4) and Barichhola-5 (Bch-5). Transformation ability of different explants such as decapitated embryo with single cotyledon disc (DEC), decapitated embryo (DE) and slice embryo decapitated at shoot end with single cotyledon disc (SEC) were tested using *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid pBI121, containing the GUS and *nptII* genes. Maximum transformation ability was exhibited by explants of decapitated embryo (DE) from Barichhola-5 (Bch-5). The optimum regeneration from the transformed tissue was achieved on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA along with double the amount of CaCl₂ and KNO₃. Selection of the transformed shoots was carried out by gradually increasing the concentration of kanamycin to 150 mg/l. Stable expression of the GUS gene was detected in various parts of the transformed shoots through GUS histochemical assay. Stable integration of *nptII* gene within the genomic DNA from these transformed shoots was confirmed through PCR analysis.

Introduction

Chickpea (*Cicer arietinum* L.) is considered as an important pulse crop in the Indian subcontinent as far as the production and area under cultivation are concerned. Chickpea is valued for its nutritive seeds with high protein content of 25.3-28.9% (Hulse 1991). Along with proteins it also provides a good proportion of carbohydrates, fats and minerals. In Bangladesh chickpea is ranked as the third most important pulse crop and it is considered as an important source of dietary protein both for human and animal. In addition, as a leguminous crop, it has the unique ability to improve the soil fertility by fixing atmospheric nitrogen symbiotically.

*Author for correspondence: <rhsarker200@yahoo.co.uk>. ¹Department of Botany, Jagannath University, Dhaka-1100, Bangladesh.

A number of biotic and abiotic stresses severely affect the yield potential of this crop all over the world. Among the biotic stresses the disease caused by various fungi mostly affecting the productivity of this important grain legume. The main fungal diseases of chickpea are Botrytis gray mold caused by *Botrytis cinerea*, Fusarium wilt caused by *Fusarium oxysporum* and Ascochyta blight caused by *Ascochyta rabiei*. Moreover, the damage caused by a number of insect pests is also one of the main constraints which limit the production of chickpea. Pod-borer (*Helicoverpa armigera* and *Heliothis* sp.) has been reported to cause almost 80% of total damage of this crop in Bangladesh (Ahmed et al. 1982). Therefore, there is an urgent need to develop disease and pest resistant cultivars of chickpea and to increase the productivity in order to meet the growing need for this crop in Bangladesh.

Improvement in chickpea through conventional breeding is limited due to the lack of adequate and satisfactory levels of genetic variability within cultivated chickpea germplasm. Interspecific hybridization in chickpea has been largely unsuccessful (Ahmad et al. 1988) and the wild species did not respond well to introgression of genes through conventional breeding techniques for yield improvement (Van Rheenen et al. 1993).

Under these circumstances techniques of genetic transformation provide an unique opportunity in improving the agronomic performance of this grain legume crop. *Agrobacterium*-mediated transformation technique has been utilized in the past to generate variability in a number of leguminous crops including soybean (Hinchee et al. 1988), pea (Schroeder et al. 1993) and subterranean clover (Khan et al. 1994).

A few reports are also available on genetic transformation of chickpea (Fontana et al. 1993, Kar et al. 1996, Khishnamurthy et al. 2000, Sarmah et al. 2003, Polowick et al. 2004 and Tewari-Singh et al. 2004). These reports clearly demonstrated that *Agrobacterium*-mediated genetic transformation can be exploited in transforming various types of chickpea explants.

Following the above reports in the present investigation *Agrobacterium*-mediated transformation was carried out using various explants from two varieties of chickpea (Barichhola-4 and Barichhola-5) growing in Bangladesh. Here, a simple protocol for multiple shoot regeneration from different explants following *Agrobacterium*-mediated gene transfer has been reported. Expression of the GUS gene was detected by histochemical assay and integration of *nptII* gene was confirmed through PCR analysis.

Materials and Methods

Two varieties of chickpea (*Cicer arietinum* L.), namely Barichhola-4 (Bch-4) and Barichhola-5 (Bch-5) were used. Seeds of these two varieties of chickpea were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur.

A variety of embryo explants, namely decapitated embryo with single cotyledon disc (DEC), decapitated embryo (DE) and slice embryo decapitated at shoot end with single cotyledon disc (SEC) were used. These explants were collected from surface sterilized and overnight soaked seeds following the protocol described by Sarker et al. 2000. For shoot initiation and development MS supplemented with various combinations and concentrations of BAP, Kn and NAA were used. When required the MS macrosalts such as CaCl₂ and KNO₃ were used in higher concentrations than those are used in MS. All media had 3% sucrose and 0.8% agar with pH 5.8, adjusted before autoclaving. *In vitro* regenerated shoots were subcultured regularly to fresh medium at an interval of 12 - 15 days for further multiplication of shoots. For root induction MS supplemented with various concentrations of IBA was used. Cultures were maintained under 16hr photoperiod at 25 ± 2°C. The plantlets with sufficient number of roots were transplanted to small pots containing sterilized soil for their establishment.

Agrobacterium tumefaciens strain LBA4404 harbouring the binary plasmid pBI121 conferring β-glucuronidase (GUS) and *nptII* gene was used for transformation.

The excised explants were incubated in the *Agrobacterium* suspension for 30 min. The explants were then soaked on a sterilized Whatman filter paper to remove the bacterial suspension and co-cultured on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA along with double the amount of CaCl₂ and KNO₃ as generally used in MS for three days in the dark. Following co-culture the explants were washed several times with sterile distilled water. The infected explants were finally washed with 300 mg/l ticarcillin (Duchefa, Netherlands). After soaking on a sterile Whatman filter paper they were subcultured on the regeneration medium with 50 mg/l combactum (Pfizer, Germany) and 50 mg/l ticarcillin to control the over growth of bacteria. The infected explants were then placed in the growth room for regeneration under 16/8 hrs light/dark cycle at 25 ± 2°C.

To select transformed tissues, the regenerating explants were subcultured on a fresh regeneration medium with 50 mg/l kanamycin after three weeks of initial culture. The selected antibiotic was increased with each subculture at 14 days intervals up to 150 mg/l kanamycin. During each subculture the dead and deep

brown tissues were discarded and green shoots and shoot buds were sub-cultured to fresh medium containing the next higher concentration of kanamycin. Transformation ability of explants 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 two-three days. They were then washed in 70% alcohol and scored for GUS expression.

The integration of the *nptII* gene in the chickpea was analysed by PCR. Genomic DNA was isolated from both transformed and nontransformed chickpea shoots. 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 and conditions: forward 5'-TAG CTT CTT GGG TAT CTT TAA ATA-3' and reverse 5'-CCA GTT ACC TTC GGA AAA AGA GTT-3'. 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 0.80% agarose gel and stained with ethidium bromide (0.05 μ l/ml).

Results and Discussion

Development of an *in vitro* regeneration system is an essential task before embarking upon a research project on genetic transformation of any particular crop. In the present investigation *in vitro* regeneration experiments were carried out using different explants following the report of Sarker et al. (2005) with slight modification. Results of *in vitro* regeneration from various explants of two varieties of chickpea using MS supplemented with different combinations and concentrations of BAP, NAA and Kn are presented in Table 1. It was observed that healthy and green shoots with expanded leaves were regenerated from decapitated embryo with single cotyledon disc (DEC) from both the varieties on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA along with double the amount of CaCl₂ and KNO₃ used in MS. It was observed that variable number of shoots can be regenerated from different explants on the medium containing the hormonal supplements of with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA without the supplements of additional CaCl₂ and KNO₃ (Table 1). The second highest regeneration responses were obtained from DE explants. But in most of the cases necrosis was found to occur on the tip of these regenerated shoots and such shoots failed to grow and survive further. To overcome this problem of shoot tip necrosis in chickpea double the concentration of CaCl₂ and KNO₃ used in MS was added in the regeneration medium following the report of Ye et al. (2002) as they could overcome shoot tip death in lentil.

Table 1. Effect of various combinations of BAP, Kn, and NAA in MS on regeneration and development of multiple shoots from various explants in two varieties of chickpea (Barichhola-4 and -5)

Explants	Hormonal conc. (mg/l)					No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean length of shoot (cm) after four weeks
	BAP	Kn	NAA	CaCl ₂	KNO ₃				
Barichhola-4									
DEC	3.0	-	.04			50	90	10-12	4.5
DE	3.0	-	.04			50	82	15-18	4.0
DEC	-	1.0	0.1			60	95	6-12	6.0
DE	-	1.0	0.1			60	85	8-15	5.8
DEC	0.5	0.5	0.2	2×	2×	50	98	4-8	6.3
DE	0.5	0.5	0.2	2×	2×	50	90	4-10	6.1
Barichhola-5									
DEC	3.0	-	.04			50	84	11-12	5.0
DE	3.0	-	.04			50	80	16-20	4.0
DEC	-	1.0	0.1			60	83	8-10	6.2
DE	-	1.0	0.1			60	80	9-15	6.0
DEC	0.5	0.5	0.2	2×	2×	50	93	5-9	6.5
DE	0.5	0.5	0.2	2×	2×	50	88	5-12	6.4

DEC = Decapitated embryo with single cotyledon disc, DE = Decapitated embryo.

Best root induction at the base of the *in vitro* regenerated shoots was achieved on MS supplemented with 0.2 mg/l IBA. However, previous workers (Fontana et al. 1993, Jayanand et al. 2003, Fratini and Ruitz 2003) obtained good root induction when shoots were regenerated on MS containing Kn.

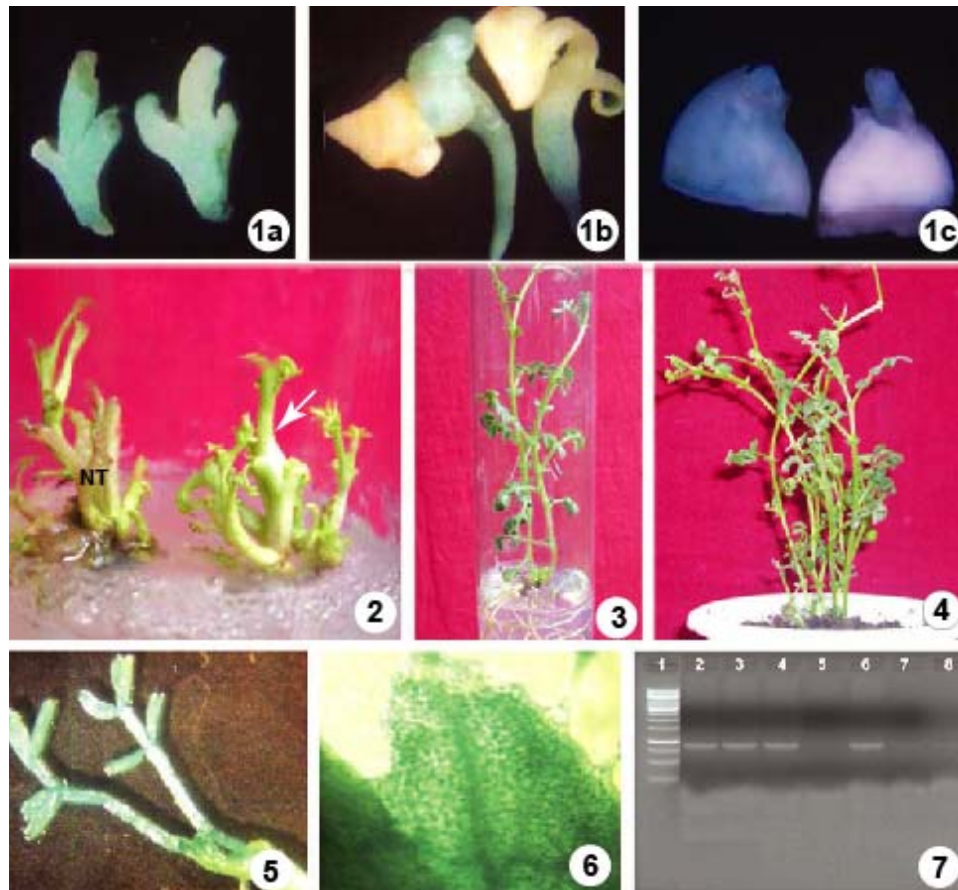
The second part of the present investigation was confined to the development of an *Agrobacterium*-mediated genetic transformation in two varieties of chickpea using three types of explants, namely decapitated embryo (DE), decapitated embryo with single cotyledon disc (DEC) and slice embryo decapitated at shoot end with single cotyledon disc (SEC). Transformation ability of these various explants was studied through histochemical GUS assay following the methods of Jefferson (1987). Among the different explants, decapitated embryo (DE) from both varieties of chickpea (Bch-4 and Bch-5) showed high transient GUS expression as confirmed by GUS histochemical assay following their infection with strain LBA4404 of *Agrobacterium tumefaciens* (Table 2). In most of the GUS positive explants conspicuous blue color was observed at the cut surface of these explants. In some of the cases the whole explants exhibited the characteristic blue colour due to expression of GUS gene (Fig. 1a-c).

Several factors influencing *Agrobacterium*-mediated genetic transformation, namely optical density of bacterial suspension, incubation period, and co-cultivation period were optimized during present study. Transformation efficiency of explants, in both varieties was found maximum when optical density of bacterial suspension was 1.0 at 600 nm, 30 min of incubation period and three days of co-cultivation period. In chickpea Krishnamurthy et al. (2000) incubated mature embryo explants for 20 min and then co-cultivated for 3 days during transformation, while Tewari-Singh et al. (2004) employed the same co-cultivation periods but they incubated explants in bacterial suspension for 1 - 2 hrs. In the present study it was observed that a longer infection and co-cultivation period enhanced over growth of bacteria in culture medium, thereby hampering proper growth of infected explants.

Following co-cultivation the explants were transferred to regeneration medium in obtaining regeneration of shoots through organogenesis. The best responses of shoot regeneration was observed from co-cultivated decapitated embryo (DE) from both the varieties on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA along with double the amount of CaCl₂ and KNO₃ used in MS.

Since the *Agrobacterium tumefaciens* strain LBA4404 has *nptII* gene and it confers kanamycin resistance, therefore the selection of the transformants was carried out using various concentrations of kanamycin. In the present study, co-cultivated explants were cultured initially in a medium without selection

pressure of kanamycin. Following initiation of regeneration about 2.0 cm long shoots were transferred to the selection media. In chickpea, Tewari-Singh et al. (2004) applied selection pressure after culturing the explants in selection free regeneration medium for 3 weeks. It was noticed that a preculture period and a delayed selection using kanamycin was useful in obtaining regeneration with high transformation frequency (McHughen et al. 1989, Sarker et al. 2000).



Figs 1 - 7: Transformation of chickpea using GUS and *nptII* gene. 1. Histochemical localization of GUS activity at the cut ends of DE explant (a), SEC (b) and DEC (c) of Bch-5 variety. 2. Putatively transformed shoot (arrow) of Bch-5 survived on the selection medium containing 150 mg/l kanamycin . Note that the non transformed shoot (NT) became brown. 3. Fully developed roots at the base of regenerated transformed shoots of Bch-5. 4. Putatively transformed plantlets (T₀) transferred to soil. 5. A part of a shoot from a transformed plantlet showing stable expression of GUS gene in case of Bch-5. 6. Magnified view of young leaf from transformed plantlets (Bch-5) showing the presence of blue colour following GUS assay (x 8) 7. PCR amplification of transformed shoots for *nptII* gene (lane-1: 1 kb ladder; lanes 2, 3, 4 and 6: transformed plantlets showing the amplification of *nptII* gene; lane -7: non-transformed plantlet; lane-8: water control).

In the present study, the initial selection pressure was 50 mg/l kanamycin and it was increased gradually in subsequent subcultures. It was observed that all control shoots died in the selection medium having 150 mg/l kanamycin. To screen the transformed shoot more effectively, higher concentrations of selective agents were applied. Krishnamurthy et al. (2000) applied 100 mg/l and Tewari-Singh et al. (2004) applied 250 mg/l of kanamycin, respectively as a final selection pressure. But in the present investigation, it was found that selection medium containing 150 mg/l kanamycin was sufficient to select transformed shoots (Fig. 2).

Shoots those survived kanamycin selection were cultured on MS supplemented with 0.2 mg/l IBA without kanamycin to induce roots (Fig. 3). After the development of sufficient roots, the plantlets were transplanted to soil (Fig. 4). Histochemical localization of GUS expression was also carried out with the fully developed plantlets growing in soil. Conspicuous blue color was observed in leaves of such plantlets that indicates the stable integration of GUS gene (Figs 5 and 6).

The transformation experiment indicates that, decapitated embryo explant was found to be the most suitable for transformation of chickpea. Shoots of Barichhola-5 showed better survivability in selection medium when it was infected with *Agrobacterium tumefaciens* strain LBA4404 (Table 2).

Table 2. Response of different explants from two chickpea varieties towards transformation with *Agrobacterium* strain analyzed by transient GUS histochemical assay.

Varieties	No. of explants assayed for GUS expression	No. of GUS +ve explants			% of GUS +ve explants		
		DE	DEC	SEC	DE	DEC	SEC
Barichhola-4	30	22	11	20	73.33	36.66	66.66
Barichhola-5	30	25	12	22	83.33	40	73.33

DEC = Decapitated embryo with single cotyledon disc, DE = Decapitated embryo, SEC = slice embryo decapitated at shoot end with single cotyledon disc.

The transgenic nature of the shoots was confirmed by PCR amplification of the *nptII* gene present within the genomic DNA of 5 randomly selected putatively transformed plantlets (Fig. 7). Specific primers were used for this purpose as mentioned in the Materials and Methods. Such observation primarily indicates the presence of transgenes within the DNA of the host plant shoots.

Agrobacterium-mediated genetic transformation protocol developed through the present investigation may be used to incorporate the useful candidate genes conferring resistance against disease, insect and pest in the chickpea varieties grown in Bangladesh.

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