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In vitro Regeneration and Over Expression of Pea DNA Helicase 45 (*PDH45)* Gene into the Local Cultivars of Chickpea (*Cicer arietinum* L.) through *Agrobacterium*-mediated Genetic Transformation

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Key words: Chickpea*, In vitro* regeneration, Genetic transformation, Salinity tolerance**,** *PDH45* gene

Abstract

In vitro regeneration studies compatible to *Agrobacterium*-mediated genetic transformation were carried out using two different types of zygotic embryo derived explants namely, decapitated embryo (DE) and decapitated embryo with single cotyledon disc (DEC) from three varieties of chickpea (*Cicer arietinum* L.) such as BARI chhola-4, -5 and -9 cultivated in Bangladesh. The best responses towards *in vitro* shoot regeneration was obtained from decapitated embryo with DEC on MS containing 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA. Healthy and effective roots from the regenerated shoots were developed on MS supplemented with 0.2 mg/l IBA. Genetic transformation was carried out with *Agrobacterium* strain LBA4404 containing the binary plasmid p*CAMBIA1301-PDH45* to integrate salt tolerant *PDH45* gene in locally grown varieties of chickpea. The transformed plantlets were successfully established in soil following adequate hardening. Integration of salt tolerant *PDH45* gene within the genomic DNA was confirmed through *GUS* histochemical assay and PCR analysis.

Introduction

Chickpea (locally known as Bengal gram, *Cicer arietinum* L.) is an important grain legume cultivated widely in more than 54 countries of the world from Asia, Africa, Europe, Australia, North and South America, but the bulk of it is produced

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and consumed in South and West Asian countries (Muehlbauer and Sarker 2017). Chickpea is a potential source of dietary protein for human nutrition and also as feed for livestock. It is particularly valued for its nutritive seeds with high protein content of 25.3 - 28.9% (Hulse 1994) for the increasing world population. In addition, as a leguminous crop, it has the unique ability to improve the soil fertility by fixing atmospheric nitrogen symbiotically and it is also considered as an important crop for the future under climate change situations. World-wide, chickpea ranks third among the pulse crops and producing about 10.1 million tons annually. It has been reported that 13.5 million hectares of land are used globally for cultivation of chickpea and its production per unit area has been increased slowly but steadily. Over 1.3 million tons of chickpea enter world markets annually to supplement the needs of countries unable to meet demand through domestic production. India, Australia and Mexico are reported to be the leading exporters of chickpea (Muehlbauer and Sarker 2017).

Chickpea is also very popular and important pulse crop in Bangladesh and ranks as third among the pulses in terms of preference. However, compared to other chickpea producing countries of the region the production and acreage of chickpea in Bangladesh is comparatively low. Therefore Bangladesh has to import a large quantity of chickpea from abroad and recent information indicates that Bangladesh is the second largest importer of chickpea (FAOSTAT 2015, Muehlbauer and Sarker 2017).

Chickpea is comprised of Desi and Kabuli types. The Desi type is characterized by relatively small angular seeds with various colouring and sometimes spotted. The Kabuli type is characterized by larger seed sizes that are smoother and generally light in colour. Desi type of chickpea is cultivated in Bangladesh. Chickpea in general is characterized by low yield potential in many countries of the world including Bangladesh. Several significant and refractory constraints are believed to be responsible for the reduced yield including biotic constraints like Ascochyta blight, Botrytis gray mould, dry root rot, collar rot, Fusarium wilt, pod borer, as well as abiotic stresses like drought, salinity and low temperature (Ahmad et al. 1988, Ghosh et al. 2013, Jha et al. 2014).

Improvement of chickpea for stress resistance through conventional breeding techniques is limited due to the lack of effective resistance in the available gene pool (Haware and McDonald 1992). Although wild species of *Cicer* have numerous desirable traits, the cross-incompatibility between the wild and cultivated varieties has deterred improvement of the crop by conventional plant breeding techniques (van Rheenen et al. 1993). Under these circumstances, there is considerable scope to exploit the modern techniques of biotechnology for chickpea improvement. Plant genetic engineering as well as genetic

transformation techniques has provided new opportunities to enhance the germplasm of crop plants by incorporating important and new gene/s of interest. Developing an efficient genetic transformation method for chickpea therefore holds promise to complement conventional breeding strategies. 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. 2004, Polowick et al. 2004, Tewari-Singh et al. 2004).

A reliable *in vitro* regeneration protocol is a prerequisite for efficient application of genetic transformation strategies. Several regeneration protocols involving somatic embryogenesis and shoot organogenesis in chickpea have been reported with varying degrees of success (Rao and Chopra 1987, Riazuddin et al. 1988, Dineshkumar et al. 1994, Jayanand et al. 2003). Considerable studies have been carried on the induction of somatic embryogenesis from mature and immature leaflets (Barna and Wakhlu 1993), mature and immature embryo axes (Sagare et al. 1993, Suhasini et al. 1994) or cell suspension cultures (Prakash et al. 1994). However, the recovery frequency of plants has been very low which has limited the genetic transformation studies.

It is worth mentioning that the total area of Bangladesh is 147, 570 km^2 where the coastal area covers about 20% of the country and over 30% of the net cultivable area. Out of 2.85 million hectares of the coastal and offshore areas about 0.83 million hectares are arable lands, which cover over 30% of the total cultivable lands of Bangladesh (Minar et al. 2013, Bhowmick et al. 2016). The cultivable areas in coastal districts are affected with varying degrees of soil salinity. Agriculture in the coastal belts of Bangladesh is mostly hampered due to the lack of salt tolerant varieties of crops. Therefore development of a salt tolerant variety is required to enhance the productivity by cultivating salt tolerant cultivars.

Recent reports unveiled that integrating salinity tolerant gene i.e. helicase as it provides duplex unwinding function in an ATP-dependent manner could solve the salinity stress tolerant problems. There is an earlier report on pea DNA helicase 45 (*PDH45*), a homolog of translation initiation factor 4A (eIF4A) to play important role in salinity stress tolerance in tobacco (Sanan-Mishra et al. 2004) and rice varieties from India and Bangladesh (Sahoo et al. 2012, Biswas et al. 2018). It was also reported that enhanced salinity tolerance gene *PgNHX1*has been achieved in a Bangladeshi rice variety Binnatoa through *Agrobacterium*-mediated transformation (Islam et al. 2009). It was also reported that overexpression of *PDH45* gene via *Agrobacterium*-mediated transformation showed salinity tolerance in the indica rice variety IR64 (Amin et al. 2011).

The present findings describe the development of an efficient transformation protocol through *Agrobacterium tumefaciens* strain containing screenable and selectable marker genes for three varieties of chickpea (BARI chhola-4, BARI chhola-5 and BARI chhola-9) growing in Bangladesh using two different explants. The transformation protocol was optimized using screenable marker gene. This method relies on the optimised co-cultivation and efficient regeneration system through multiple shoot proliferation. Present authors have carried out extensive study on various factors that can influence synchronous regeneration of multiple shoots, recovery of plants and transformation efficiency. Expression of the screenable *GUS* gene was detected by histochemical assay. Integration of *PDH45* and *hpt* genes was confirmed through PCR analysis. The protocols reported here should facilitate effective utilization of genetic transformation technology for the agronomic improvement of chickpea.

Materials and Methods

Three varieties of Desi type of chickpea (*Cicer arietinum* L.) namely, BARI chhola-4(Bch-4), BARI chhola-5 (Bch-5) and BARI chhola-9 (Bch-9) cultivated in Bangladesh were used as materials for this investigation. Seeds of these three varieties of chickpea were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur and were maintained in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka.

Two different types of zygotic embryo derived explants, namely decapitated embryo (DE) and decapitated embryo with single cotyledon disc (DEC) were used for *in vitro* regeneration as well as for *Agrobacterium*-mediated genetic transformation.

For the preparation of explants, chickpea seeds were first washed in 70% ethanol for 1 min, and then surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10 - 12 min with vigorous shaking. The seeds were then rinsed at least three times with sterilized distilled water and were kept in sterilized distilled water overnight. These surface-sterilized water soaked seeds were then cultured on a medium containing 3% (w/v) sucrose with 1.2% (w/v) agar without any growth regulators for their germination. Finally the required explants were collected from the germinating seeds. In case of DE, both root and shoot tips along with the cotyledons were removed while for DEC had a decapitated embryo, but with single cotyledon attached to it.

For initiation and development of shoot, agar solidified MS supplemented with various combinations and concentrations of BAP, Kn and NAA were used. In order to overcome shoot tip death and to improve shoot health, increased

concentration of macro-salts such as $CaCl_2$, NH_4NO_3 and KNO_3 were used in the medium (Ye et al. 2002). All media contained 3.0% sucrose with 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 the induction of roots from the base of the *in vitro* grown shoots, hormone free MS as well as MS supplemented with 0.2 mg/l IBA were used. All cultures were maintained under 16 hrs photoperiod at 25 ± 2°C. The plantlets with sufficient number of roots were transplanted to small plastic pots containing sterilized soil for their establishment.

*Agrobacterium tumefaciens*strain LBA4404 containing the binary plasmid p*CAMBIA 1301-PDH45* was used for genetic transformation (Fig. 1). This plasmid contained a scorable reporter gene *GUS* (β-glucuronidase) driven by CaMV35S promoter and selectable marker gene *hpt* gene encoding hygromycin phosphotransferase conferring hygromycin resistance as well as *PDH45* (Pea DNA helicase 45) gene conferring salinity tolerance, driven by CaMV35S promoter. Both the explants of decapitated embryo with single cotyledon disc (DEC) and decapitated embryo (DE) were utilized for transformation experiments.

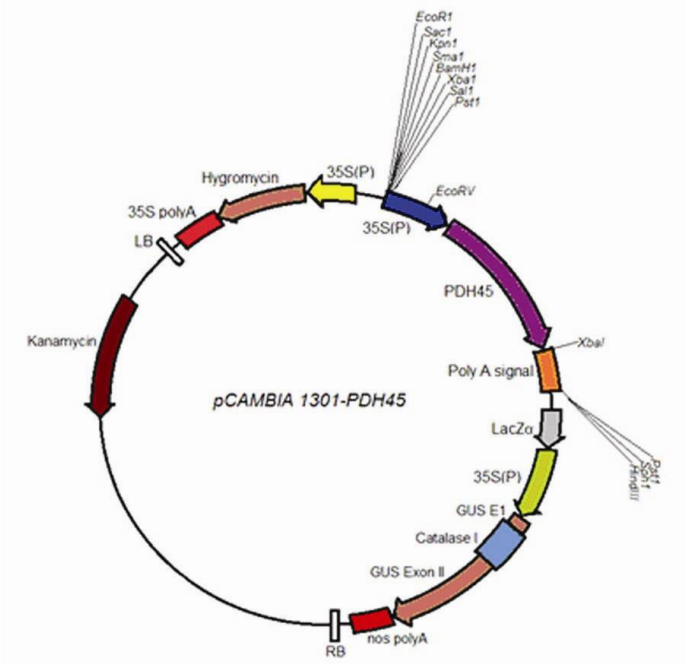


Fig. 1. Diagrammatic representation of p*CAMBIA 1301-PDH45* of *Agrobacterium tumifaciens* strain LBA4404.

The explants of DE and DEC were separated from germinating seeds (as has been mentioned earlier) with help of a scalpel and submerged immediately in *Agrobacterium* suspension. The excised explants were incubated in the *Agrobacterium* suspension in a small Petri dish for 45 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 $CaCl_2$ and KNO_3 as generally used in MS for three days in the dark at 25 ± 2°C.

Following three days of co-cultivation the explants were washed three-four times with sterilized distilled water then washed for 10 min with sterilized distilled water containing 300 mg/l ticarcillin and then finally washed with distilled water once again. After soaking on a sterile Whatman filter paper they were subcultured on the regeneration medium with 100 mg/l ticarcillin or 100 mg/l combactum to control the over growth of bacteria. After 2 weeks, the regenerated shoots were subcultured in selection medium containing 10 mg/l hygromycin and 100 mg/l ticarcillin. The infected explants were then placed in the growth room for regeneration under 16/8 hrs light/dark cycle at 25 ± 2°C.

Since *hpt* gene was present in the plasmid, putatively transformed shoots were cultured on different concentrations of hygromycin in MS for selection of transformants. To eliminate the untransformed developing shoots the explants were subcultured on fresh regeneration medium initially with 5 mg/l hygromycin. Cultures were subcultured regularly at an interval of 12 - 15 days and the selection pressure of hygromycin was gradually increased from 10 mg/l up to 15 mg/l. 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 hygromycin. It was observed that 15 mg/l hygromycin was optimum in killing the non-transformed shoots. The survival of green shoots on the optimum selection medium indicated the production of transformed shoots. 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 three days. They were then washed in 70% alcohol and scored for GUS expression.

`Genomic DNA was isolated using CTAB method (Doyle and Doyle 1990) from the transformed shoots and stable integration of *PDH45* and *hpt* genes were confirmed by PCR analysis. For the detection of the *hpt* and *PDH45* gene coding sequence, the following primers: forward-5'-CGAAGAATCTCGTGCTTTCAGC-3' and reverse- 5'-AGCATATACGCCCGGAGTCG-3', PDH45-F 5'-ATGGCGAC AACTTCTGTGG-3' and reverse PDH45-R 5'-GAGTCTAGATT ATATAAGATC

ACCAATATC-3' were used, respectively. For PCR amplification of *hpt* gene the cycling conditions were 5 min at 95°C denaturation and 30 amplification cycles using 94°C for 1 min, 50°C for 1 min (annealing) and 72°C for 1 min followed by 5 min at 72°C. For *PDH45* gene, DNA was denatured at 95°C for 5 min and then amplified in 30 cycles using 94°C for 1 min, 48°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 μl/ml).

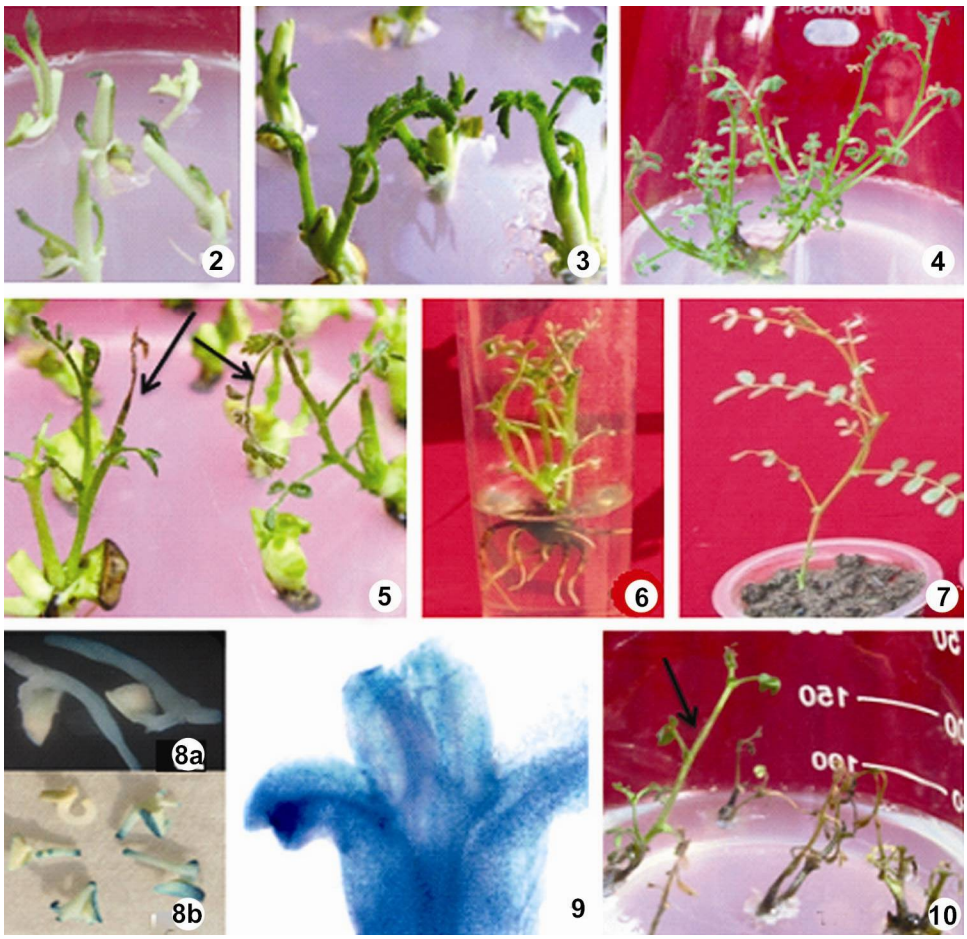
Results and Discussion

An efficient and reproducible *in vitro* regeneration system is an essential task for the development of an effective genetic transformation protocol for a particular crop of interest. In the present investigation *in vitro* regeneration studies were carried out using two different types of explants from three varieties of chickpea cultivated in Bangladesh. This *in vitro* regeneration studies were carried out following two earlier reports of Sarker et al. (2005) and Sharmin et al. (2012) with slight modification. In general pulses have been considered recalcitrant due to their passiveness to *in vitro* techniques (Mroginski and Kartha 1984). The development of desired transgenic plants in grain legumes is mainly hampered due to the lack of efficient gene delivery system (Nisbet and Webb 1990).

Responses of two different explants from the three varieties of chickpea (Bch-4, Bch-5 and Bch-9) on MS supplemented with different combinations and concentrations of BAP, NAA and Kn have been presented in Table 1. It was observed that variable number of shoots can be regenerated from both the explants of decapitated embryo with single cotyledon disc (DEC) and decapitated embryo (DE). However, the best responses towards *in vitro* shoot regeneration was obtained from decapitated embryo with single cotyledon disc (DEC) explants in all the three varieties of chickpea on MS containing the supplements of 0.5 mg/l BAP, 0.5 mg/l Kn and 0.2 mg/l NAA and all most equal responses were recorded for these three varieties for shoot regeneration. Development of healthy and green shoots with expanded leaves from decapitated embryo with single cotyledon disc (DEC) explants for Bch-5 has been presented in Figs 3 and 4.

But in most of the cases unwanted events of necrosis was found to occur on the tip of these regenerated shoots (Fig. 5) and due to these necrotic activities regenerating shoots failed to grow and survive further. To overcome this problem of shoot tip necrosis in chickpea double the concentration of $CaCl_2$ and KNO_3 were used in MS following the report of Ye et al. (2002) as found in case of lentil. Addition of twice the concentration $CaCl_2$ and KNO_3 in the regeneration

medium has been found to prevent the browning of the regenerating shoots during this study thus prevented the necrosis of young shoots.



Figs 2-10: *In vitro* plant regeneration and genetic transformation in *Cicer arietinum* var. Bch-5. 2. Initiation of shoots from decapitated embryo with single cotyledon disc (DEC) on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA with 2x $CaCl_2$ and 2x $KN0_3$. 3. Same as Fig. 2 but showing the proliferation of regenerated shoots. 4. Same as Fig. 3 showing the development of multiple shoots. 5. Necrosis of shoot tips (arrows) on MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn, 0.2 mg/l NAA without 2x $CaCl_2$ and 2x $KN0_3$. 6. Development of *in vitro* roots from regenerated shoots on MS with 0.2 mg/l IBA. 7. Plantlet transferred to soil in plastic pot. 8a. Stereomicroscopic view of *GUS* expression in the developing shoots in explants of decapitated embryo with single cotyledon disc (DEC). 8b. Histochemical localization of *GUS* activity at the cut ends of DEC explants. 9. Stable expression of *GUS* gene within the developing transformed shoot tip (× 40). 10. Putatively transformed shoot (arrow) survived on the selection medium containing 15 mg/l hygromycin. Note that the non transformed shoot became brown and failed to survive further.

Table 1. Effects of various combinations of BAP, Kn and NAA in MS on regeneration and multiplication of shoots from different explants of three varieties of chickpea (BARI chhola-4, BARI chhola-5 and BARI chhola-9).

Explants	Hormonal conc. (mg/l)			Macronutrients (mg/l)		No. of explants inoculated	% of responsive explants	Days to shoot initiation	Mean length of shoot (cm) after four weeks of culture
	BAP	Kn	NAA	$CaCl_2$	KNO_3				
					BARI chhola-4				
DEC	3.0	-	.04			40	90	10-12	4.5
DE	3.0	-	.04			40	84	15-18	4.0
DEC	1.0	0.2	-			40	95	6-12	6.5
DE	1.0	0.2	-			40	85	8-15	6.0
DEC	0.5	0.5	0.2	2x	2x	50	98	4-8	6.5
DE	0.5	0.5	0.2	2x	2x	50	88	4-10	6.5
					BARI chhola-5				
DE	3.0	-	.04			40	85	11-12	4.2
DEC	3.0	-	.04			40	80	16-20	4.0
DE	1.0	0.2	-			40	90	4-5	6.2
DEC	1.0	0.2	-			40	80	9-14	6.0
DE	0.5	0.5	0.2	2x	2x	50	94	5-9	6.0
DEC	0.5	0.5	0.2	2x	2x	50	85	5-12	6.0
					BARI chhola-9				
DEC	0.5	-	-			40	93.5	6-10	4.3
DE	0.5	-	-			40	93.5	6-10	3.0
DEC	1.5	-	-			40	86	7-12	3.0
DE	1.5	-	-			40	73	7-12	1.5
DEC	-	1.0	-			50	95	5-9	4.5
DE	-	1.0	-			50	82	4-10	3.0
DEC	-	2.0	-			50	93	7-10	3.5
DE	-	2.0	-			50	75	7-14	2.0

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

Development of roots from *in vitro* raised shoots is an essential step to obtain complete plantlets. For induction of root growth, 2 - 4 cm long regenerated healthy shoots were excised and transferred to test tubes (25 × 150 mm), each containing 10 - 15 ml of MS supplemented with various concentrations of IBA. The best responses towards the induction of roots at the base of the *in vitro* regenerated shoots were achieved on MS supplemented with 0.2 mg/l IBA (data not shown). However, development of such *in vitro* root has been presented in Fig. 6. On the other hand, in a number of previous investigations good responses for root induction was achieved when shoots were cultured on MS containing Kn (Fontana et al. 1993, Jayanand et al. 2003, Fratini and Ruitz 2003). Following induction of sufficient roots the plantlets were transferred to small plastic pots containing soil for their establishment. Plantlet transferred to soil has been presented in Fig. 7.

Experiments on *Agrobacterium*-mediated genetic transformation was conducted for the two varieties of chickpea, such as Bch-4 and Bch-5 using two DE and DEC. Transformation ability of these explants was examined through *GUS* histochemical assay (Figs 8a,b) following the methods of Jefferson (1987). Among the two explants, DE from both varieties showed higher transient *GUS* expression as confirmed by histochemical assay following their infection 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	CE	DE	CE
BARI chhola-4	30	22	11	73.33	36.66
BARI chhola-5	30	25	12	83.33	40.00

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

Stereomicroscopic view of *GUS* expression in case of decapitated embryo with single cotyledon disc explants has been presented in Fig. 8a. In most of the *GUS* positive explants produced conspicuous blue colour at their cut surfaces (Fig. 8b). In some cases the whole explants exhibited the characteristic blue colour due to expression of *GUS* gene. Stable expression of *GUS* gene in the developing shoot of BARI chhola-4 has been presented in Fig. 9.

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 to be maximum when optical

density of bacterial suspension was 1.0 at 600 nm, 45 min of incubation period and having three days of co-cultivation period (data not shown). 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 (Bch-4 an Bch-5) 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 $CaCl_2$ and KNO_3.

Since the *Agrobacterium tumefaciens* strain LBA4404 has *hpt* gene and it confers hygromycin resistance, therefore the selection of the transformants was carried out using various concentrations of hygromycin. Co-cultivated explants were cultured initially in a medium without selection pressure of hygromycin. 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 was useful in obtaining regeneration with high transformation frequency in grain legumes (McHughen et al. 1989, Sarker et al. 2000). It was also observed that when selection pressure was applied immediately after co-cultivation, the transformed explants did not show any sign of regeneration.

Gradual elimination of non-transformed shoots was done to recover transformed shoots through separating green shoots from albino and brown shoots and allowing their further growth on fresh regeneration medium containing higher concentration of hygromycin (Table 3). It was found that all the control shoots failed to survive at 15 mg/l hygromycin. Therefore, the shoots that survived in the medium containing 15 mg/l hygromycin were considered as putative transformants (Fig. 10). Stable expression of *GUS* gene was visualized through histochemical staining in the regenerating shoots (Fig. 9). Shoots those survived in hygromycin selection were cultured on MS supplemented with 0.2 mg/l IBA and 100 mg/l combactum without hygromycin for the induction of roots.

The transformation experiment indicates that, decapitated embryo explant was found to be suitable for transformation of chickpea. Shoots of BARI chhola-5

showed better survibility in selection medium when it was infected with *Agrobacterium tumefaciens*strain LBA4404 (Table 3).

Table 3. Selection of putative transformed shoots using various concentrations of selectable agent hygromycin.

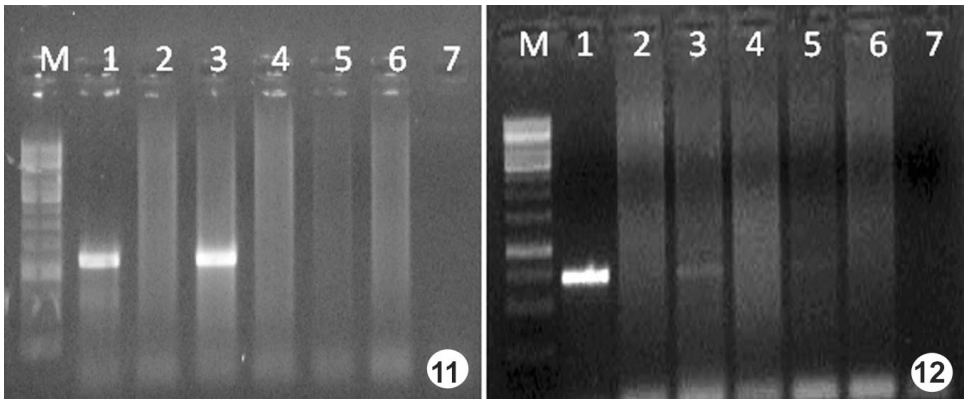
Explants	No. of shoots survived in medium with hygromycin (mg/l)			% of survived shoots
	05	10	15	
	BARI chhola-4			
DE	750	47	8	1.06
DEC	640	38	5	0.7
	BARI chhola-5			
DE	960	65	11	1.14
DEC	560	54	4	0.74

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

The integration of desired gene in the transformed plantlets was 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 *PDG45* and *hpt*genes present in *Agrobacterium* strain. Amplified DNA was analyzed through agarose gel electrophoresis. In case of *PDG45* gene a single band of 1200bp was observed in the transformed plantlet identical to the amplified DNA of bacterial strain (positive control). On the other hand in case of *hpt* gene a single band of 750 bp was found for one transformed plantlet identical to the amplified DNA of the same bacterial strain. The results indicated that the *PDG45* and *hpt* genes were inserted in the genomic DNA of transformed plantlets (Figs 10 and 11).

The *in vitro* regeneration system for locally grown chickpea varieties has been optimized and this regeneration protocol was appeared to be compatible with *Agrobacterium*-mediated genetic transformation. During this investigation it was possible to develop an efficient *Agrobacterium*-mediated genetic transformation protocol using gene/s like *PDG45* and *hpt* genes. Integration of the above genes was confirmed by *GUS*-histochemical assay as well as through PCR analysis.

Chickpea cultivars with resistance to both abiotic and biotic factors will form the backbone of desired chickpea production. Development of stress resistant varieties through genetic transformation using specific genes of interest with diverse modes of action will enhance chickpea improvement programs in the future. Earlier reports indicates that pea DNA helicase 45 gene can successfully be utilized for the introgression of salinity tolerance in a number of transgenic



Figs 11-12: 11. PCR amplification of *PDH45 g*ene in BARI chhola-5: Lane M = 1.0 kb DNA ladder; Lane 1 = DNA from positive control (Bacteria), Lanes (2, 3, 4 & 5) = DNA from putative transgenic samples, Lane 6 = DNA from negative control (plant) and Lane 7 = Water control. 12. PCR amplification of *hpt g*ene in BARI chhola-5: Lane M = 1.0 kb DNA ladder; Lane 1 = DNA from positive control (Bacteria), Lanes (2, 3, 4 & 5) = DNA from putative transgenic samples, Lane 6 = DNA from negative control (plant) and Lane 7 = Water control.

plants including tobacco and rice genotypes of Bangladesh (Sanan-Mishra et al. 2004, Biswas et al. 2018). Results of this study are the pioneering report on genetic transformation using *PDG45* gene *in* chickpea varieties cultivated in Bangladesh. Therefore, based on the findings of the present investigation, future transformation experiments may be undertaken for developing stress tolerant for the chickpea varieties cultivated in Bangladesh.

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