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Agrobacterium-mediated Genetic Transformation of two Peanut *(Arachis hypogaea* L.) Varieties using Salinity Tolerant PDH45 Gene

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Key words: Peanut, genetic transformation, pea DNA helicase (*PDH45*), salinity tolerance

Abstract

An efficient *Agrobacterium*-mediated genetic transformation protocol has been developed to integrate salinity tolerant pea DNA helicase (*PDH45*) gene in two peanut varieties, Dhaka-1 and BARI Chinabadam-8. Decapitated half-embryo explants from these two varieties of peanut were also used to develop a transformation compatible *in vitro* regeneration system. MS medium supplemented with 5.0 mg/l BAP and 0.5 mg/l Kn showed maximum responses towards shoot induction in both the varieties. Half-strength of MS medium supplemented with 0.2 mg/l IBA showed better responses for root induction in case of these varieties. Genetic transformation experiments were performed using *Agrobacterium* strain LBA4404 containing pCAMBIA1301-*PDH45* conferring *gus r*eporter gene, hygromycin resistance *hpt* gene, and salinity tolerant gene *pdh45*. The transformation variables were optimized, OD_{600} 1.4 of the bacterial suspension with 45 minutes of incubation period, and three days of co-culture showed the best performance towards transformation. The transformed shoots were selected using 30.0 mg/l hygromycin. The overall transformation frequency was found to be very low, where 0.10% shoots of Dhaka-1 and 0.07% shoots of BARI Chinabadam-8 survived through required selection pressure. The stable integration of the transgene in the transformed shoots was confirmed through PCR analysis.

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is one of the most important food legume crops. It is a good source of protein (23%), edible oil (43%), as well as, vitamin E, niacin, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine, potassium, etc. (Bandyopadhyay and Manivel 2001). In Bangladesh, it is cultivated primarily for human

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consumption and as fodder, either as a rabi or as a kharif crop, and cultivated on about 99,000 acres. The annual production is about 74,000 metric tons (BBS 2021-2022). The cultivated peanut varieties of Bangladesh are characterized by their low yield potential. Biotic stress, like diseases, and abiotic stresses, like climatic factors, drought, salinity, water logging, etc. are highly responsible for peanut production and quality degradation. The studied peanut varieties Dhaka-1 and BARI Chinabadam-8 show significant susceptibility towards salt stress and their seed germination is completely inhibited at NaCl concentration higher than 200 mM (Haider et al. 2020).

To meet the increasing demand of crop production and yield stability the importance to establish stress or salinity tolerant peanut variety is very high. *Agrobacterium*-mediated genetic transformation system can be used to transfer desirable gene(s) e.g. salinity tolerance gene. Several attempts have been made to develop transgenic peanut plants through *Agrobacterium*-mediated genetic transformation using marker gene/s (Sarker et al. 2000, Sarker and Nahar 2003). In addition to the selectable marker and reporter genes, genes for fungus, insect and virus resistance have been introduced into peanut (Brar et al. 1994, Li et al. 1997, Magbanua et al. 2000, Singsit et al. 1997, Yang et al. 1998, Rohini and Rao 2001, Tiwari et al. 2011). Several salinity tolerance genes have been identified and transferred in plants to enhance salinity tolerance, e.g. *SOS1*, *AhLea-3, PDH45, etc.* (Shi et al. 2000, Qiao et al. 2021 and Nath et al. 2015). The *PDH45* is a DEAD-box helicase protein, which was originally isolated from *Pisum sativum* (Pham et al. 2000). This gene can induce stress tolerance, in both monocot and dicot plants (Nath et al. 2015, Amin et al. 2012). Studies showed that PDH45 can increase salinity tolerance of rice without affecting its yield (Sahoo et al. 2012). There are also reports that showed overexpression of *PDH45* gene in peanut can induce high tolerance against drought stress (Manjulatha et al. 2014). Since this gene has an account of conferring salinity tolerance in many plant species and drought tolerance in the k-134 variety of groundnuts, this gene could be a potential transgene to confer salinity tolerance in local peanut varieties of Bangladesh.

The objective of the present investigation was to develop salinity-tolerant peanut varieties using *Agrobacterium*-mediated genetic transformation. First, to achieve this goal, an *in vitro* regeneration system was tested using the decapitated half-embryo explant of Dhaka-1 and BARI Chinabadam-8 varieties. Then, utilizing that regeneration system, an efficient *Agrobacterium*-mediated genetic transformation protocol was developed to integrate salinity tolerant gene (PDH45) into the same varieties.

Materials and Methods

Two varieties of peanut were used in the present investigation, Dhaka-1 and BARI Chinabadam-8. As explant, decapitated half-embryo was used for *in vitro* regeneration. as well as for the genetic transformation. The explants were collected from surface sterilized and overnight soaked seeds following the protocol described by Sarker et al. 2000. For shoot development MS medium supplemented with various combinations of

hormones, BAP (6- Benzyl aminopurine) and Kn (Kinetin) were used. For root induction of the *in vitro* grown shoots, half strength of MS medium supplemented with IAA (Indole-3 acetic acid), IBA (Indole-3 butyric acid) or NAA (α- naphthalene acetic acid) were used.

The genetically engineered *Agrobacterium tumefaciens* strain LBA4404 containing pCAMBIA1301 plasmid was used for genetic transformation experiments (Fig. 1). This vector contained (1) the *uid*A gene encoding GUS (β-glucuronidase), (2) the *hpt* gene encoding hygromycin phosphotransferase conferring hygromycin resistance, and (3) the *PDH45* gene conferring salinity tolerance.

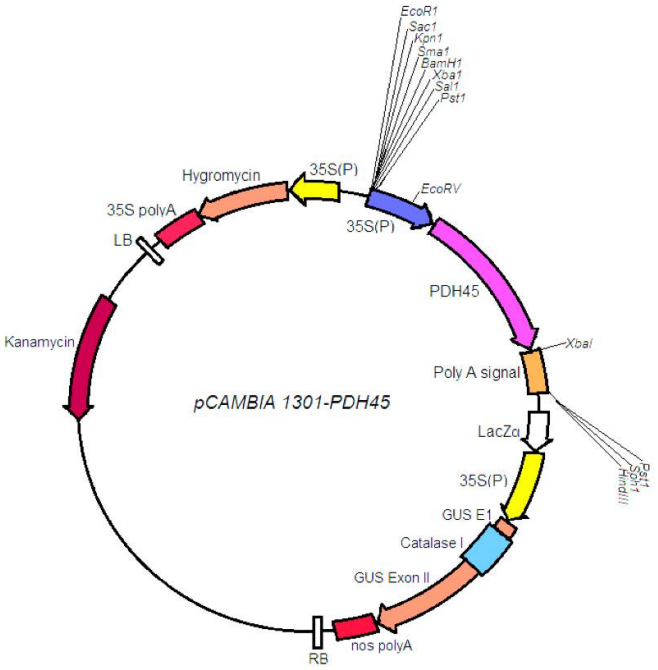

Fig. 1. Diagrammatic representation of the plasmid pCAMBIA1301-*PDH45* of *Agrobacterium* strain LBA4404.

The preparation of *Agrobacterium* suspension and transformation experiments were conducted following the procedures described by Sarker et al. (2003). The explants were co-cultured on MS medium for three days in the dark chamber, and then transferred to regeneration medium containing 100 mg/l ticarcillin. Following each transformation experiment, randomly selected co-cultured tissues were examined for GUS-histochemical assay and non-transformed explants were used as control. Hygromycin was used as selectable agent in this experiment. The hygromycin concentration in the selection medium was increased gradually (15, 20 and 30 mg/l hygromycin) in subsequent subcultures. In each subculture the dead necrotic shoots were discarded and only the healthy green shoots were transferred to the culture media.

The integration of the *hpt* gene in the putative transformed shoots were analyzed by PCR method. Genomic DNA was isolated from both transformed and non-transformed peanut shoots. Here, CTAB method (Doyle and Doyle 1990) was used for DNA isolation. DNA was subjected to PCR using the following primers and conditions: forward HPT-F 5′-CGAAGAATCTCGTGCTTTCAGC-3′ and reverse HPT-R 5′-AGCATATACGCCCG GAGTCG-3′. DNA was denatured at 94℃ for 5 min and then amplified in 30 cycles using 94℃ for 1 min, 56.4℃ for 1 min (annealing) and 72℃ for 1 min followed by 5 min at 72℃. 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 a prerequisite for developing a transformation protocol for a particular plant species (Gardner 1993). During the present study the regeneration experiment was conducted by using decapitated half-embryo explant. Direct regeneration was observed in case of this explant. Regeneration using half-embryo decapitated at shoot end with single cotyledon disc has been reported for Pea (Schroeder et al. 1993), Chickpea (Tewari-Singh et al. 2004), etc. Here, decapitated half embryo explants were used for direct shoot regeneration using MS medium supplemented with two different cytokinins, namely, BAP and kinetin. And the highest number of multiple shoots from decapitated half embryo explants was achieved when explants were cultured on MS medium supplemented with 5.0 mg/l BAP and 0.5 mg/l Kn in case of both Dhaka-1 and BARI Chinabadam-8 variety (Table 1, Fig. 2a-d). Similar results were also observed by Sarkar and Nahar (2003) in case of leaflet explants of DM-1, Jhinga Badam varieties. Although MS medium supplemented with 5 mg/l BAP also showed considerable number of explants initiation and multiple shoots in this experiment, but the efficiency of regeneration is comparatively low than the application of the hormonal combination of 5 mg/l BAP and 0.5 mg/l Kn.

For root induction, regenerated shoots were cultured on half strength of MS medium supplemented with 0.2 mg/l of IBA, IAA, or NAA. It was observed that although roots were induced in all the three combinations of auxins, however, 0.2 mg/l of IBA was found most effective for root induction in regenerated shoots of Dhaka-1 and BARI Chinabadam-8 varieties (Table 2, Fig. 2e). Similar result was observed by Nguyen and Le Tran (2012), they found 0.3 mg/l IBA to be best for rooting in peanut.

After establishing the *in vitro* regeneration protocol, both the peanut varieties were used for genetic transformation experiments. For this purpose, a strain of genetically engineered *Agrobacterium,* LBA4404/pCAMBIA1301-*PDH45* was used. Transformation experiments were undertaken using decapitated half-embryo explants. *Agrobacterium*-mediated genetic transformation is influenced by several factors, such as optical density (OD) of *Agrobacterium* suspension, duration of incubation period, duration of co-cultivation period, etc. These parameters were optimized during this study by

monitoring the transient expression of the GUS gene (Fig. 2f-g). Following GUS histochemical assay, it was confirmed that both the varieties showed positive responses towards transformation. It was evident that maximum transformation was obtained with bacterial suspension having an OD_{600} 1.4, with 45 minutes of incubation period. Also, three days of co-cultivation periods were found to be most effective towards transformation (Fig. 3a-b). Sarker et al. (2000) applied 60 mins of incubation period for transformation of peanut variety DM-1 and co-cultured for 72 hours.

Table 1. Response of decapitated half embryo explants of Dhaka-1 and BARI Chinabadam-8 towards shoot regeneration on different combination of BAP and Kn in MS medium.

Variety	Hormonal concentration (mg/l)		Number of Explants inoculated	% of responsive explant	Days to shoot initiation	Mean no. of shoots/explant after 45 days
	BAP	Kn				
Dhaka-1	1.0	-	40	72.5	7-9	1.60
	2.5	-	40	75.0	5-7	2.50
	5.0	-	40	87.5	5-6	3.80
	7.0	-	40	87.5	6-8	3.00
	2.5	0.25	40	75.0	8-10	3.13
	2.5	0.50	40	70.0	7-9	2.30
	5.0	0.25	40	62.5	8-10	2.25
	5.0	0.50	40	87.5	7-8	4.00
BARI Chinabadam-8	1.0	-	40	72.5	7-9	1.87
	2.5	-	40	72.5	5-8	3.00
	5.0	-	40	87.5	6-7	4.00
	7.0	-	40	75.0	6-8	2.80
	2.5	0.25	40	87.5	7-9	3.41
	2.5	0.50	40	75.0	8-10	2.41
	5.0	0.25	40	70.0	8-9	2.11
	5.0	0.50	40	95.0	6-7	4.12

Agrobacterium strain used in this investigation contains *hpt* gene which confers hygromycin resistance to the transformed cells. So, hygromycin was used as selectable agent in this experiment (Fig. 2h). To determine the optimum level of hygromycin in medium for selection of explants, different concentrations of hygromycin were tested on control shoots. All control shoots died in the selection medium in presence of 30 mg/l hygromycin (data not shown). However, Tiwari and Tuli (2012) found that 40 mg/l hygromycin concentration prevented complete shoot regeneration of non-transgenic explants. In the present investigation, the selection pressure was increased gradually (15, 20 and 30 mg/l hygromycin) in subsequent subcultures.

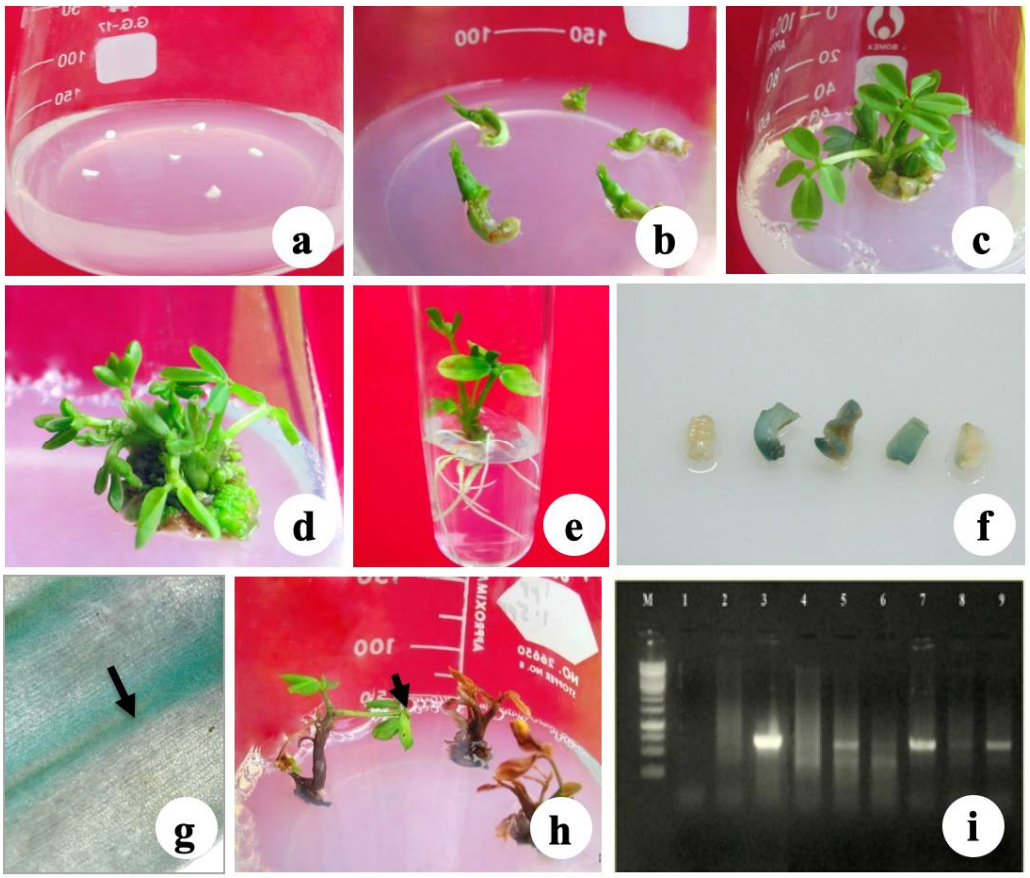

Fig. 2 (a-i): *In vitro* regeneration and genetic transformation of peanut varieties Dhaka-1 and BARI Chinabadam-8. **a.** Decapitated half-embryo explants of Dhaka-1 on MS medium supplemented with 5.0 mg/l BAP. **b.** Same as figure a, but showing shoot initiation from explants. **c.** Multiple shoot regeneration from decapitated half-embryo explants of Dhaka-1 on MS medium supplemented with 5.0 mg/l BAP + 0.5 mg/l Kn. **d.** same as figure c for BARI Chinabadam-8 variety. **e.** Root formation of Dhaka-1 on half strength MS medium containing 0.2 mg/l IBA. **f.** Histochemical localization of GUS activity of decapitated half embryo explants infected with LBA4404/pCAMBIA1301-PDH45 strain of *Agrobacterium* in case of Dhaka-1 with control. **g.** A part of transformed macerated half-embryo explant of BARI Chinabadam-8 variety showing the presence of GUS positive blue color. **h.** Selection of putative transformed shoots (arrow) of BARI Chinabadam-8 on medium containing 30 mg/l hygromycin. **i.** PCR amplification of transformed shoots for hpt gene: Lane M = 1kb ladder, lane 1 = water control, lane 2 = negative control, lane 3 = positive control, lanes 4, 6 and 8 = non-transformed shoots, lane 5, 7 and 9 = transformed shoots.

But the number of survived shoots in the final selection pressure was extremely low for Dhaka-1 (0.07%) and for BARI Chinabadam-8 (0.10%). Table 3 shows the effect of hygromycin on selection of infected shoots of Dhaka-1 and BARI Chinabadam-8 from decapitated half embryo explants with strain LBA4404/ pCAMBIA1301-*PDH45*.

Table 2. Effect of different auxins on root formation from regenerated shoots of Dhaka-1 and BARI Chinabadam-8 variety.

Variety	Growth regulators	Conc. of growth regulators (mg/l)	N0. of shoots inoculated for rooting	% of shoots forming roots	Days to root formation	Mean no. of roots/ shoot
Dhaka-1	IAA	0.2	20	65	10-15	5.5
	IBA	0.2	20	75	12-20	7.0
	NAA	0.2	20	50	16-22	3.0
BARI Chinabadam-8	IAA	0.2	20	60	10-13	6.5
	IBA	0.2	20	80	11-18	8.0
	NAA	0.2	20	45	15-20	3.5

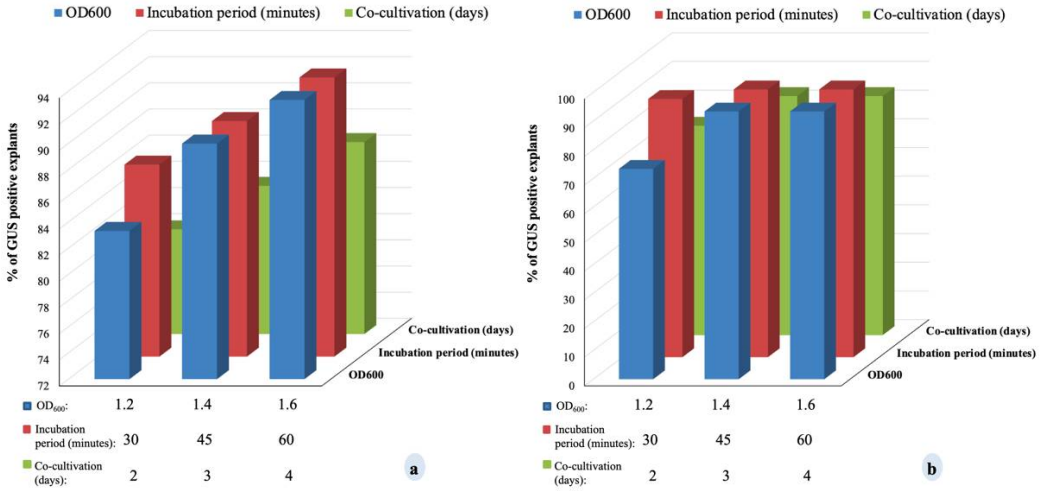

Fig. 3. Influence of different OD_{600}, incubation period, and co-cultivation period on transformation of decapitated half embryo explants analyzed by transient GUS histochemical assay for Dhaka-1 (a) and BARI Chinabadam-8 variety (b).

Table 3. Effect of hygromycin on selection of infected shoots of Dhaka-1 and BARI Chinabadam-8 from decapitated half embryo explants with strain LBA4404/ pCAMBIA1301-PDH45.

Varieties	No. of infected explants	No. of regenerated shoots after transformation	No. of shoots survived in culture with hygromycin (mg/l)				% of survived shoots
			10	15	20	30	
Dhaka-1	1090	2725	894	108	34	1	0.07
BARI Chinabadam-8	1169	2922	907	134	37	3	0.10

To confirm the transgenic nature of the transformed shoots polymerase chain reaction (PCR) was carried out. The DNA isolated from both transformed and non-transformed shoots were subjected to PCR for the amplification of *hpt* gene. Then

amplified DNA was analyzed through agarose gel electrophoresis. From the gel it was observed that the single band (750 bp) formed in each of the three transformed shoots were identical to the amplified DNA of bacterial strain (positive control) (Fig. 2i). This result indicated that the *hpt* gene was inserted in the genomic DNA of three transformed plantlets. As the *hpt* and *PDH45* (salinity tolerant gene) genes are situated in the same T-DNA of the *Agrobacterium* strain, so, it could be assumed that the *PDH45* gene has also been inserted into these shoots.

An efficient *in vitro* regeneration system was established in this investigation using decapitated half-embryo explant, which can be used to develop transgenic peanut plants. An efficient *Agrobacterium*-mediated genetic transformation protocol has also been developed using the same explant with the strain LBA4404 containing pCAMBIA1301-*PDH45* conferring salinity tolerant gene *PDH45*. Previous data showed that the *PDH45* can potentially induce high salinity tolerance in plants without affecting its yield (Sanan-Mishra et al. 2005). Utilization of this potential game changing trait could play a vital role in improving agriculture, and socio-economic conditions of marginal peanut farmers of our country.

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