Analysis of factors influencing successful *Agrobacterium*-mediated genetic transformation in two Bangladeshi peanut (*Arachis hypogaea* L.) varieties BINA Chinabadam-2 and BINA Chinabadam-6 using embryonic leaflet explant

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by
Iftekhar Mahmud Chowdhury
ID 10336001

Department of Mathematics and Natural Sciences
Biotechnology Programme
BRAC University
Dhaka, Bangladesh

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DECLARATION

I, hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Analysis of factors influencing successful *Agrobacterium*-mediated genetic transformation in two Bangladeshi peanut varieties (*Arachis hypogaea* L.) BINA Chinabadam 2 and BINA Chinabadam 6 using embryonic leaflet explant"** submitted by the undersigned has been carried out under the supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree of diploma.

(Iftekhar Mahmud Chowdhury)
Candidate

Certified

(Dr. Aparna Islam)
Supervisor

Associate Professor
Biotechnology Programme
Department of Mathematics and Natural Sciences
BRAC University, Dhaka.

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I dedicate this work to my parents hoping that there are more of such works in near future with their blessings.

March, 2014 **Iftekhar Mahmud Chowdhury**

ABBREVIATIONS

The following abbreviations have been used throughout the text.

A.	*Agrobacterium*
BAP	6-Benzylaminopurine
BINA	Bangladesh Institute of Nuclear Agriculture
GUS	ß-glucuronidase
HCl	Hydrocholric Acid
MS	Murashige and Skoog medium (1962)
NaOH	Sodium hydroxide
nptII	Neomycin phosphotransferase II
OD	Optical Density
T-DNA	Transfer DNA
Ti	Tumor inducing
Vir	Virulence region
YEP	Yeast Extract Peptone

Abstract

Peanut (*Arachis hypogaea* L.) is an important crop both for its nutritional value and for its oil content in the seeds. But this economically important crop is susceptible to both biotic and abiotic factors that hamper its growth both in quality and quantity. Due to change in agricultural environment and also due to lack of resistance towards diseases the production is compromised. For this reason, genetic engineering is a possible tool to enhance the quality of the crop by introducing novel trait in it. To achieve that it a prerequisite to established a transformation protocol prior to any transformation event. Establishment of an efficient *Agrobacterium*-mediated transformation protocol requires optimization of several factors that affects the transfer of T-DNA. In the present study, these were optimized for two peanut varieties, namely, BINA Chinabadam 2, and BINA Chinabadam 6 using embryonic leaflet as a source of explant. In this study, *Agrobacterium tumifaciens* strain LBA4404 carrying pBI121 having *GUS* and *nptII* marker genes was used. T-DNA transfer was monitored through transient transformation assay utilizing *GUS* gene expression. Bacterial suspension having density (OD_{600}) more than 1.0 with at least 1 hour of incubation period and minimum of 2 days of co-cultivation period found to be efficient for leaflet explants in both the varieties. Following transfer of T-DNA, presence of transgene within the infected tissue was observed by GUS histochemical transient assay.

CONTENTS

Introduction

The peanut, or groundnut (*Arachis hypogaea L.*), is a species in the legume or "bean" family (Fabaceae). It is suggested that peanut was probably first cultivated in the valleys of Paraguay. This is are known by many other local names, such as, earthnuts, groundnuts, goober peas, monkey nuts, pygmy nuts and pignuts. In spite of its name and appearance, the peanut is not a nut, but a legume.

Peanut is an annual herbaceous plant growing 30 to 50 cm tall. The leaves are opposite, pinnate with four leaflets; each leaflet is 1 to 7 cm long and 1 to 3 cm across. Peanut flower is a self-pollinating flower and grows low on the plant. Most flowers bloom for one day and then wilt. The flowers are a typical pea flower in shape, yellow with reddish veining. The pollination occurs before the flowers open, cleistogamous pollination, because of which genetic variation doesn't occur in peanut. The specific name*, hypogaea*means "under the earth" after pollination, the flower stalk elongates, causing it to bend until the ovary touches the ground. Continued stalk growth then pushes the ovary underground where the mature fruit develops into a legume shell, which is the peanut. Shells are 3 to 7 cm long, containing 1 to 4 seeds (McGraw *et al.*, 1979).

Peanut is cultivated in 24 million ha area all over the world for the purpose of extracting edible oil and also to be used as a source of healthy food. (Janila *et al*., 2013). Most of the world peanut production takes place in Asia followed by the production in Africa and North America. China leads in production of peanuts, having a share of about 41.5% of overall world production, followed by India (18.2%) and the United States of America (6.8%). In Europe, the leading producer is Greece, approximately 2000 tons of peanuts per year (Fletcher *et al.*, 2002).

In Bangladesh peanuts are cultivated in Noakhali, Faridpur, Kishoreganj, Patuakhaliu, Rangpur and Dhaka districts in approximately 35000 hectors of lands and about 40,000 metric tons of peanuts produced annually (BINA, 2012). Compared to the world production of peanuts the average peanut production in Bangladesh is very low. Commercially seeds are being sold to farmers in the salt region of Noakhali and other districts for cultivation of peanut varieties.

Peanuts contain 25 to 32% protein (on average of 25% digestible protein) and 42 - 52% oil. Peanuts are naturally cholesterol-free. Moreover, peanuts contain over 30 essential nutrients and phytonutrients. Even they have a more anti-oxidants than grapes, green tea, tomatoes, spinach, broccoli and carrots (Fletcher *et al.*, 2002). Peanuts are good sources of vitamin E, niacin, folate, protein and manganese. Peanuts also provide resveratrol, the phenolic antioxidant also found in red grapes and red wine. In Bangladesh peanut is very much popular as leisure time snacks. Research published in the Journal of Neurology, Neurosurgery and Psychiatry indicates regular consumption of niacin-rich foods like peanuts provides protection against Alzheimer's disease and age-related mental decline.

There are several factors that limits the productivity of peanut crop that results in great economic losses annually both by the degradation in quality and quantity. Initially conventional breeding was used to enhance several traits such as seed yield and drought tolerance in peanuts. However, many important agronomic traits have yet to be improved. These traits may include resistance to insect pests and diseases caused by nematodes, viruses such as Tomato spotted wilt virus (TSWV) (Source: (http://www.cropsci.ncsu.edu/PDF_Files/ag-638factsheet.pdf), dated: 03 March 2014) and peanut stripe viruses, bacteria, leaf spot fungi and to fungi such as *Aspergillus flavus* and *A. parasiticus* which produce carcinogenic aflatoxin (Higgins and Dietzgen, 2000).

It is assumed that there is a single hybridization event between two diploid species of *Arachis duranensis* and *A. ipaensis* and the resulted species is the present day peanut (Higgins and Dietzgen, 2000). Due to the narrow genetic base and self-pollination the resulted peanuts have very minimum genetic diversity, which results into the vulnerability of diseases both microbial and fungal (Anuradha *et al*., 2006; Iqbal *et al*., 2012). In many countries and also in Bangladesh different types of fungal diseases infect peanut which includes early and late leaf spots caused by *Cercospora arachidicola* and *Cercosporidium personatum,* respectively as well as leaf rust caused by *Puccinisa rachides* are responsible for the reduction of production of peanuts (Talukder, 1997).It has been reported that wilting caused by *Fusarium oxysporum* to be highly destructive to the plant (Iqbal *et al.*, 2012). The assault of such disease is controlled mostly by the use of harmful chemicals that are toxic to the nature. Apart from the biotic factors, abiotic factors can be tackled by the introduction of novel gene, as climactic change will bring about more extreme weather. These traits include tolerance to salt, water stress etc. Nutritional qualities

like uniform fruit maturity; enhanced nutritional quality by modification of the amino acid and lipid composition can enhance the production and nutritional value of peanut (Higgins and Dietzgen, 2000).

Genetic engineering has opened new opportunities to modify crops, and provided new solutions to satisfy specific needs for creation of genetic variability and selection of desired traits. Genetic transformation techniques provides a faster and alternative to conventional breeding, only when a optimized protocol is stablished (Sticklen, 1994). In addition to conventional breeding approaches, genetic engineering is being widely used to introduce desirable agronomic traits to produce crops that are high in quality (Mansur, 1995).

The low success of production of transgenic grain legumes is the poor regeneration and lack of proper transfer of desired gene into the crop. Many factors influence transformation efficiency Monitoring transient expression within the explants enables optimization of procedures to target specific cells that are easily regenerated for assaying gene transfer efficiencies. Apart from explant, efficiency of plant transformation also depends on the genotype, technique employed and regeneration system (Gelvin *et al.,* 2000). The successful production of transgenic plants relies on several factors: i) means of stably introducing foreign DNA into the genome of a plant cell, ii) method of plant regeneration directly or indirectly from transformed cell, iii) and method by which transgenic plants can be identified among population of untransformed individuals (Somers *et al.*, 2003).

Two commonly used technique used to transfer genes into plant cells are via *Agrobacterium tumejaciens* or direct gene transfer using microprojectile bombardment (Chandra and Pental, 2003). *A. tumejaciens* is a soil bacterium, which transfers specific DNA molecules into cells of wounded host plants, causing crown gall disease. *A. tumefaciens* carries a tumor inducing (Ti) plasmid which contains a region called the T-DNA that is transferred into a plant cell where it becomes integrated as part of the plant's genetic material (Chilton, 1983). The gall-inducing genes within this T-DNA are replaced with genes of agricultural interest. When *A. tumejaciens* transfers these genes to plant cells at the wound site that are capable of regeneration, stably transformed plants carrying the new trait can be obtained.

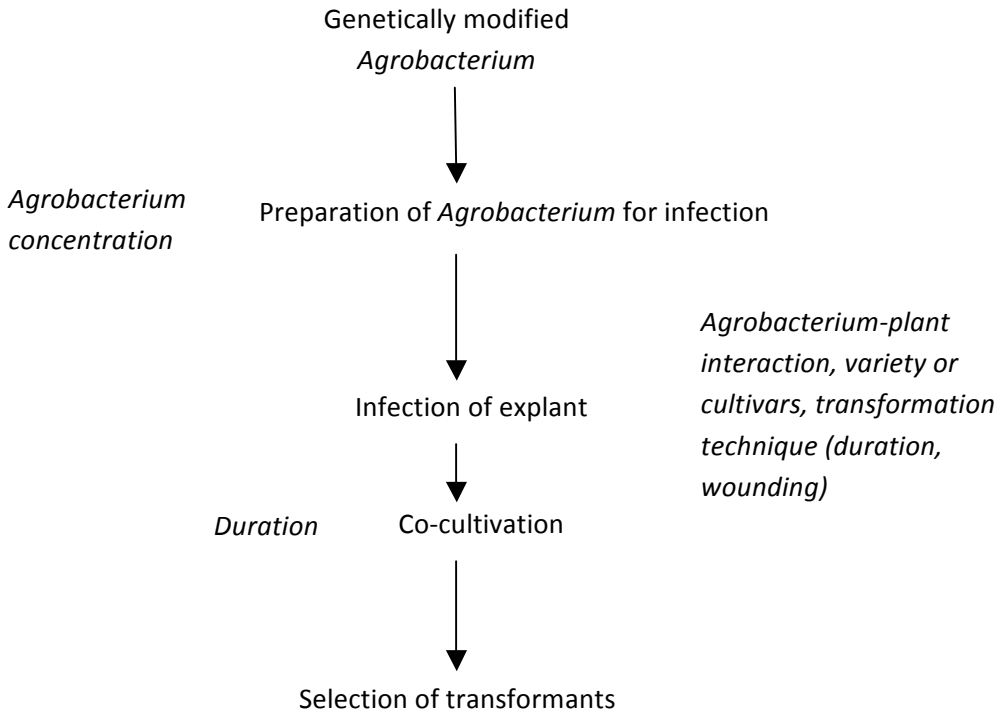

Figure 1. Steps of *Agrobacterium*-mediated transformation with influencing factors at respective steps.

Agrobacterium-mediated transformation technique is hugely affected by several parameters, which are shown in Fig. 1. According to Christou (1997) experiments conducted with other legumes (*Vigna* species, *Lens culinaris* and *Phaseolus vulgaris*) suggested that transfer of foreign gene in peanut is not an easy task. Problems associated with *Agrobacterium* host specificity and low frequencies need to be tackled. For this reason efficient transformation protocol needs to be established for consistent outcome (Sarker *et al.*, 2003).

Successful genetic transformation of plants requires a tissue culture system to regenerate whole fertile plants from single cells as well as a method to deliver the gene(s) of interest to those cells that have the capacity to regenerate. By either organogenesis or embryogenesis peanut plants have been regenerated from explants such as mature and immature embryonic cuts (Baker, 1995; Brar, 1994; Hazra, 1989; Atreya, 1984), cotyledons (Ozias-Akins 1989; Atreya, 1984, Tiwari and Tuli, 2011), leaves (Livingstone and Birch 1995; Baker and Wetzstein 1992, Sharma and

Anjaiah, 2000; Anuradha, *et al.*, 2006; Tiwari and Tuli, 2011) using cotyledonary nodes (Anuradha, *et al.*, 2006; Iqbal, *et al.*, 2012) as explants. Inefficient shoot regeneration was observed in explant when *Agrobacterium*- mediated gene transfer was used. To overcome the problems associated with inconsistencies of *in vitro* shoot regeneration responses non-tissue culture technique using peanut embryo has also been explored (Rohini and Rao, 2000). From these reports it is seen that choice of explants and choice of genetic transfer method plays an important role for successful transformation.

Sarker and Islam (1999) commenced with Bangladeshi peanut variety DM-1 using epicotyl, hypocotyl and leaflet as source of explant for the *Agrobacterium*-mediated transformation. They reported to establish a working protocol using leaflet as a source of explant. In 2000 another work has been published by them using Tridana variety peanut using leaflet as an explant. It showed that leaflet is a good source of explant for *Agrobacterium*-mediated transformation of peanut (Sarker, *et al.*, 2000).

Once a suitable explant has been identified the next task is to analyze the factors involving the gene transfer mechanism. There have been several reports stating various factors involved in several species. Such factors involve the effect of age of explant, co-cultivation duration, infection duration; effect of plant growth regulators present in the co-cultivation media, bacterial density was optimized for several species. Seventy two hours was considered as an optimum co-cultivation for transformation for tomato using cotyledon explants (Sharma *et al.*, 2009). For peanuts when leaflet was used as a source of explant (Sarker *et al.*, 2000) optimum co-cultivation duration of Tridana variety was found to be also 72 hours. Same duration was also observed in cotyledonary nodes in another study (Anuradha *et al.*, 2006; Iqbal *et al.*, 2012). When cotyledons of peanut were used as explant co-cultivation duration of two weeks was required before transforming it into kanamycin stress (Sharma and Anjaiah, 2000).

In 2010, a report was seen where the effect of bacterial density (OD at 600nm), co-cultivation duration and infection period was optimized in tomato varieties of Bangladesh and India (Islam *et al.*, 2010). Similar research was seen where factors were analyzed for only Indian variety of tomato (Sharma *et al.*, 2009). They measured the bacterial density by measuring optical density using a spectrophotometer. Evidence was found that the optical density at 600nm to determine

bacterial density is very critical. In a study using wheat it was seen that optimum density was found to be from OD 0.6 to 1.3 any higher than this resulted into bacterial overgrowth and thus causing death of the explants (Pérez-Piñeiro *et al.*, 2012).

For the intended improvement of peanuts using genetic modification it is required to develop a transformation protocol using a suitable explant and identifying the transfer DNA or selection of the transformants is also important. For this purpose, transient GUS assay is performed to identify the presence of the transferred foreign gene into the leaflet after infection by *Agrobacterium*. Transient gene expression systems offer several advantages over stable transformation for studying gene expression and regulation, including being independent of regeneration from transformed cells. The factors which as bacterial density, co-cultivation duration, infection period which can affect the transformation efficiency, are studied for the two peanut varieties (BINA Chinabadam2 and BINA Chinabadam6) using leaflet as explant and then transformation is verified by transient GUS histochemical assay.

Objective of the present study

- To analysis various factors effecting transformation efficiency to establish an *Agrobacterium*- mediated transformation protocol.
- Establishment of transformation protocol in two peanut varieties of Bangladesh.

Materials and Methods

2.1 Materials

2.1.1 Plant materials:

Seeds of two varieties of peanut (*Arachis hypogaea* L.) were used in this study. They are BINA Chinabadam 2 and BINA Chinabadam 6. All the seeds are collected from Bangladesh Institute of Nuclear Agriculture (BINA). Important characteristics of both the varieties are described below:

2.1.1.1 BINA Chinabadam 2

To create a more enhanced and stable variety of chinabadam, Dhaka-1 (Maijchor) seeds were introduced to gamma rays and a stable structural changed mutant variety was produced, which was named Mutant – 6 (Mut-6). In 1994 this mutant seed was again introduced to gamma ray to produce another three mutant seed which were M_6 /20/42-M (2), M_6/20/44-3 and M_6/20/62-4. The mother seed for these three mutants were Dhaka-1 variety and it was seen that these three had huge productivity compared to their mother seed. Thus, they were named BINA Chinabadam 1, BINA Chinabadam 2 and BINA Chinabadam 3 respectively. In year 2000, Bangladesh Seed Board gave the permission for the seeds to be commercially sold to the farmers all over the country (BINA, 2012).

When compared with BINA Chinabadam 1 (BINA1), BINA Chinabadam 2 (BINA 2) is comparatively shorter to that of BINA1. Compared to the mother seed there is a 24% increase in fruit and 28% increase in the size of the seeds. It is seen that BINA 2 seeds are vulnerable to leaf curl, leaf spot and other leaf diseases. Life span for the seeds in winter season is 150-160 days where in summer season is 125-135 days. The total production of BINA 2 variety in Bangladesh in 2012 was 3.19 ton per hector km. and total production of such variety in only winter season is 2.9 ton per hector km. Which showed that majority of the production takes place in winter season. Percentage of fats and protein present in the seeds of BINA 2 are 50% and 28% respectively, making it a good source of fat and protein (BINA, 2012).

2.1.1.2 BINA Chinabadam 6

For the production of this variety, locally cultivated Dhaka- 1varieties seed undergoes permanent genetic transformation by the treatment of 250 gray gamma ray radiations and produces a mutant variety, which is named Mutant – 3. Next a new mutant variety is obtained from the population of Mutant–3 and it was named Mutant – 6. Again this mutant variety undergoes 250 gray gamma radiations and a mutant is obtained named M_6/25/64-82. In 2011, National seed board commercially launches this seed to the farmers in the salt rich area of Bagerhaat, Potuakhali and Noakhali (BINA, 2012).

Percentage of protein present in this variety of seed is 28.68% and the percentage of collection of the seeds is 48.51%. When compared with other BINA Chinabadam varieties it is seen that BINA Chinabadam 6 variety's plant is short and straight in structure. Life span of the seeds is 140-150 days and the maximum production is 2.9 ton per hector and 1.17 ton per hector during winter season (BINA, 2012).

2.1.2 Agrobacterium strain and vector plasmid:

Agrobacterium is well known for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for genetic engineering. Genetically modified *Agrobacterium tumifaciens* strain LBA4404 was used for infection in the transformation experiments (Figure. 1). It contains plasmid pBI121 of 14KDa (binary vector). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

1. The *nptII* gene (Herrera, 1983) encoding neomycin phosphotransferase II presenting kanamycin resistance, propelled by NOS promoter and NOS terminator.
2. The *udiA* gene (Jefferson, 1986) encoding GUS (ß- glucuronidase), propelled by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation through the GUS histochemical assay.

The *Agrobacterium* also contains plasmid pAL4404; which is disarmed Ti plasmid (132KDa) containing virulence genes.

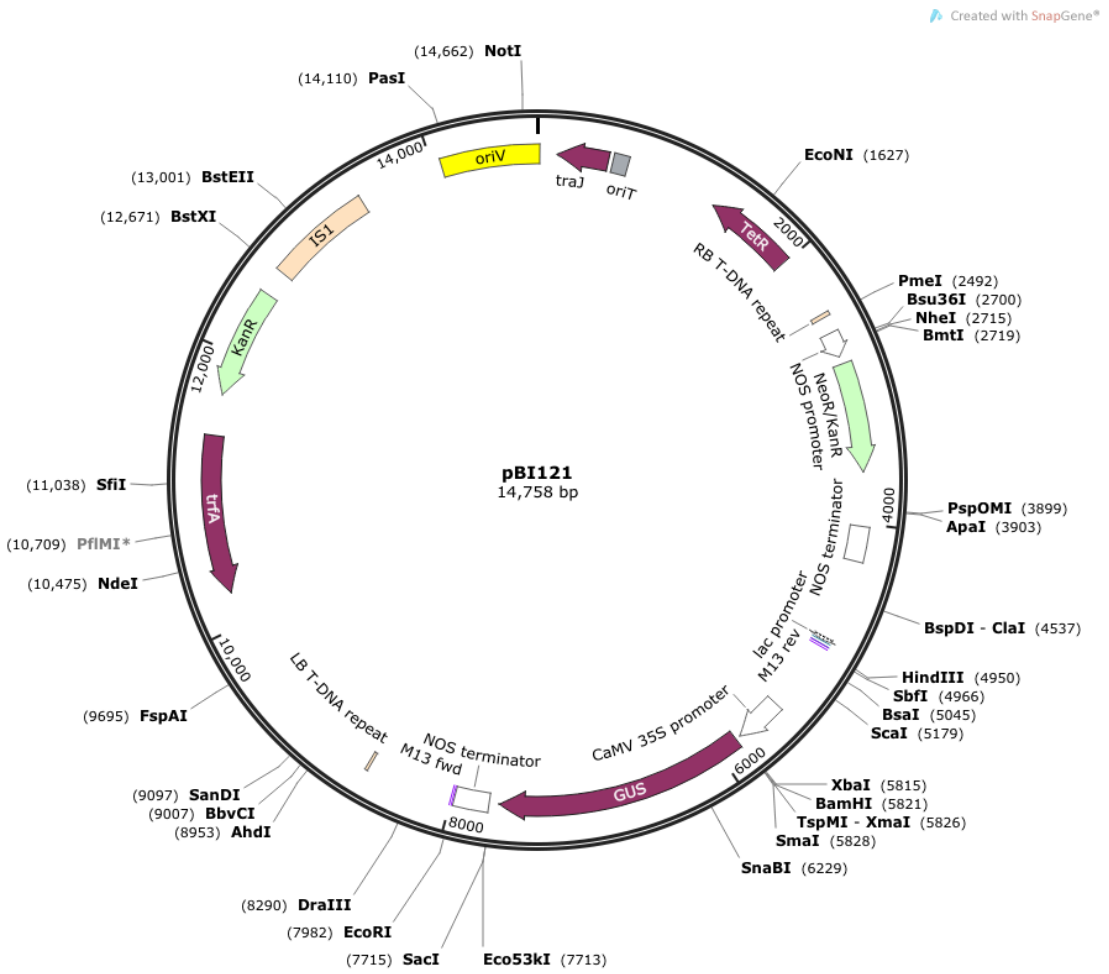

Figure 2. Genetic map of plasmid pBI121.

(Source: http://www.snapgene.com/resources/plasmid_files/plant_vectors/pBI121/)

2.1.3 Different culture media

Different culture media were used in this investigation for various purposes which are as follows:

2.1.3.1 Agrobacterium culture media:

YEP media is used as the growth media for *Agrobacterium tumefaciens* both in plates and in liquid media. The organism is sub-cultured at regular interval from the plates and grown in the liquid culture every week. The composition of YEP media is 10g peptone, 5g NaCl, 10g yeast extract and 15g agar for production of 1liter of the media.The media is then made up to 1 liter by adding required amount of distilled water and then autoclaved for 30mins. Genetically transformed *Agrobacterium tumifaciens* strain LB4404 was maintained in the liquid media with appropriate antibiotic concentration.

Table 1. YEP media composition.

Component	**Amount (1 liter)**
Yeast Extract	10 g
Peptone	10g
Sodium Chloride	5g
Agar	13g (for solid media)
Distilled water	Till 1 liter

2.1.3.2 Co-cultivation media:

Co-cultivation media is used for both the explant and the *Agrobacterium* strain. Following the infection the explants are transferred in the co-cultivation media and kept in dark chamber. The co-cultivation media contains Murashige and Skoog (1962) medium with 2mg/l BAP and 1mg/l Kinetin. BAP and Kinetin is used for the growth of the plant cell. Mixing several components, which are separately prepared, makes the Murashige and Skoog media.

The stock solutions are prepared and then mixed in a conical flask according to the following composition. The total amount is made up to 1liter by addition of distilled water. The pH is then adjusted to 5.8 using 1N NaOH or HCl accordingly. When preparing solid medium, agar was added in 0.6% (w/v) ratio. The composition of the stock solution and MS media is represented in Table 2 and 3.

2.1.3.3 Regeneration medium:

When using embryonic leaflet as a source of explant then regeneration media is prepared using 2mg/l BAP and 0.5mg/l Kinetin along with 200mg/l cefotaxime to kill the *Agrobacterium*.

Table 2. Stock solution composition of MS medium.

Component	**Amount**
Macro nutrients (10x)	mg/l
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.2H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
Minor salts (100x)	mg/l
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron EDTA solution (100x)	mg/l
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organics (100x)	/mg/l
Nicotinic acid	0.5
PyridoxinH	
ThaiminHC	
Glycine	2.0

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Table 3. MS medium composition.

Component (Stock concentration)	Amount (for 100ml)
Macro nutrients (10x)	10ml
Minor salts (100x)	1ml
Iron EDTA solution (100x)	1ml
Organics (100x)	1ml
Myo-inositol	0.01g
Sucrose	3g

2.1.4 Materials for GUS histochemical assay:

The composition of component's for the GUS assay is given below:

Table 4. Composition of GUS histochemical assays.

Components	Final Concentration
Sodium Phosphate buffer pH=7	50mM
EDTA pH=8	50mM
$K_3Fe(CN)_6$	0.5mN
$K_4Fe(CN)_6$	0.5mM
Triton X-100	0.1%
X- gluc (5-bromo-4-chloro-3-indolyl ß-D glucoronide) dissolved in DMSO	1mM

2.1.5 Cotton soaked media:

Cotton was placed inside conical flasks and then soaked in water so that ample amount of water is present to support the seed germination. The flasks were sealed with aluminum foil and parafilm, and then autoclaved.

2.2 Methods:

2.2.1 Media sterilization:

Different stock solutions and MS media were prepared, and placed in conical flask and then autoclaved for 15mins at 120psi.

2.2.2 Seed sterilization:

Seeds of the two varieties were first surface sterilized. The seeds were taken in a conical flask and sterilized by washing in autoclaved distilled water for 2 minutes. Following a further wash of the seeds in 70% ethanol. They were again washed in autoclaved distilled water for three times with 1-minute interval. After the third wash the seeds are completely emerged in mercuric chloride for 18mins and placed in an orbital shaker at 180 rotations per minute. Mercury chloride is poured off carefully and the seeds were then washed with autoclaved distilled water until all the bubbles disappear. It is usually three to four times wash.

2.2.3 Seed germination and explant collection:

The seeds were placed giving equally in the cotton soaked media so that there is no over population in the conical flask. After doing so the flasks were kept in dark chamber for approximately 10-13days and were checked regularly within this time-frame to check the formation of embryonic leaflet. Sprouted seeds were then taken in a Petri dish and split open to check the presence of embryonic leaflet. The two sets of four leaflets are cut from the stem and kept separately in another Petri dish.

2.2.4 Agrobacaterial culture growth:

Before the night of the experiment fresh colony of *Agrobacterium tumefaciens* strain LBA4404 was inoculated into YEP media containing the antibiotic kanamycin (200mg/l) for selection and was kept overnight to increase the growth of the culture. The bacterial density was measured

using spectrophotometer (PG Instruments) at 600nm wavelength. The liquid culture is kept on ice to stop the any further growth of the bacteria.

2.2.5 Transformation of explants:

Freshly cut leaflets from the seeds that are kept separately in another Petri dish is taken. Each leaflet is cut from both the sides and made vertical cuts on the surface of the leaflet to increase cut ends. This cut ends are the site for the *Agrobacterium* to enter the explant. After creating the cut ends the explants are dipped into liquid *Agrobacterium* culture in a Petri dish. The Petri dish was hand shaken and the explants are retrieved into the co-cultivation media after every 30mins. Thus, creating an infection period of 30, 60, 90 and 120 mins. The steps were repeated for both the BINA Chinabadam verities.

The infected explants are allowed to cultivate in the co-cultivation media for 1, 2 and 3 days before placing them on regeneration media containing 200mg/l cefotaxime, which kills all the bacteria. Roughly 25% of the explants are used for the transient expression using GUS histochemical assay where the rest are kept to regenerate in the regeneration media.

2.2.6 GUS histochemical assay

For each batch of explants following each transformation experiment, randomly selected co-cultivation explants were selected for GUS histochemical assay. For this experiment co-cultured explants were fixed in fixation solution for 3-5 mins then explants were washed three times in 50mM phosphate buffer and immersed in X-gluc solution and incubated at 37˚ C overnight. A characteristic blue colour would be the expression of GUS gene in the plant tissue. After X-gluc treatment explants were transferred to 70% ethanol for degreening. Following degreening explants were observed under stereomicroscope (Olympus, Japan).

Results

The aim of the present study was to establish a suitable protocol for *Agrobacterium*-mediated transformation of two peanut varieties cultivated in Bangladesh. For this purpose a genetically modified *Agrobacterium* strain was used to transform the embryonic leaflet of the two varieties, namely, BINA Chinabadam 2 and BINA Chinabadam 6, and different factors responsible for transformation was examined.

3.1 Influence of optical density (OD) of Agrobacterium suspension on transformation:

The relationship between optical density of *Agrobacterium* suspension at 600 nm (OD_{600}) and transformation efficiency of embryonic leaflet explant was investigated. Bacterial suspension with optical density of 0.7, 1.4, and 1.6 were used in these experiments. For both the test varieties in most of the cases it is seen that with the increase of optical density of the *Agrobacterium* suspension transformation efficiency was found to increase. It was found that maximum percentage of transformation was observed at OD_{600} of 1.4 for both BINA Chinabadam 2 and BINA Chinabadam 6 maximum transformation of 66.67% and 100% GUS positive explants, respectively was found at this OD_{600} (Fig. 3). The minimum transformation efficiency of GUS positive explants at 0.7, 1.4 and 1.6 OD were 50%, 66.7% and 40%, respectively. It is seen that as the increases from 0.7 to 1.4, doubling the bacterial density resulted into the maximum GUS positive explants for both the varieties whereas further increasing the bacterial density resulted decrease of GUS positive explants (Table 5-6). So, it is seen that bacterial density of (OD_{600}) 1.4 gives maximum GUS positive explants for both the varieties.

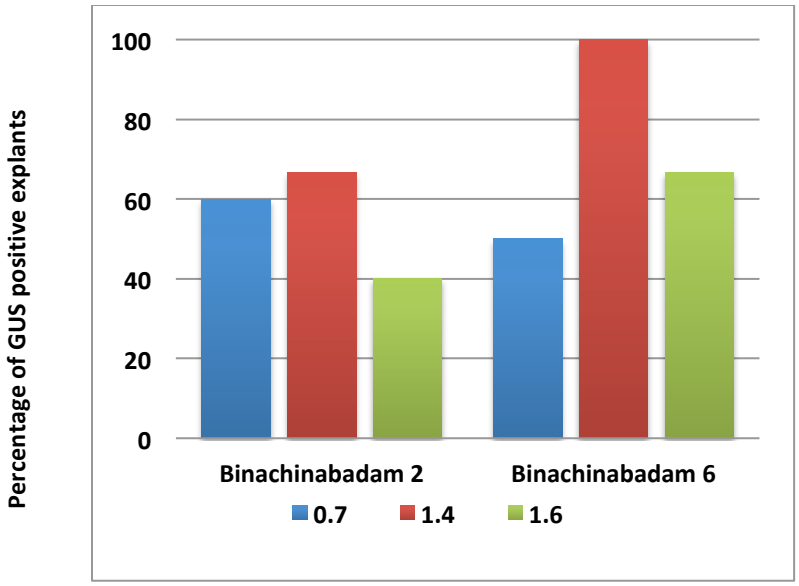

Figure 3. Influence of Optical Density (OD_{600}) of *Agrobacterium* suspension on transformation efficiency analyzed by transient GUS histochemical assay for the two peanut varieties.

3.2 Influence of incubation period on transformation of embryonic leaflet explants:

Infection/ incubation time is an important factor for transformation efficiency. In this study effect of different infection period on transformation efficiency was analyzed. The infection durations were 30, 60, 90 and 120 mins. For each of the varieties it was seen that infection period plays a diverged role but the general trend was transformation efficiency increased from 30mins incubation period to 60mins incubation period (Fig. 4). For BINA Chinabadam 2 maximum GUS positive explant was observed at 60 mins of infection period and further increase of infection duration resulted in the decrease of transformation efficiency. However for BINA Chinabadam 6 the number of GUS positive explants increased with the increasing of infection period and reached 100% at 120 mins (Table 5-6).

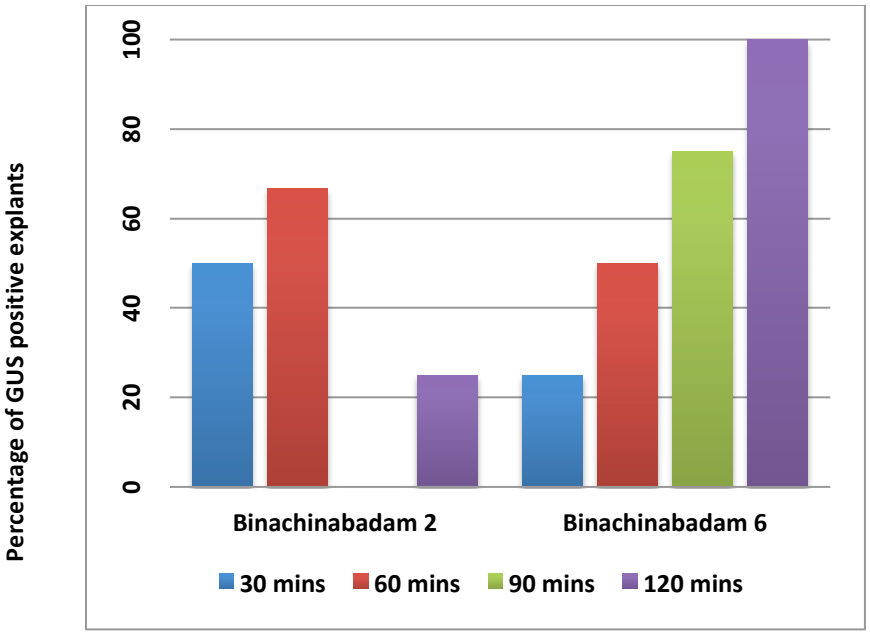

Figure 4. Influence of infection duration on transformation efficiency analyzed by transient GUS histochemical assay in twopeanut varieties.

3.3 Influence of co-cultivation on transformation of embryonic leaflet explant:

Duration of co-cultivation was found to influence the transformation efficiency. This analysis was done with bacterial density (OD_{600}) of 1.4 and infection period of 60mins and 120mins for both the peanut varieties (Table 5-6). It is seen that for BINA Chinabadam 2 as the co-cultivation period increases the percentage of GUS positive explants decreases which maintain the low level in further increase of time. Whereas for BINA Chinabadam 6 at 1 day of co-cultivation there is no transformation but improves in further co-culture. It is seen that with the increase of co-cultivation period from 2 to 3days the percentage of GUS positive explant also increases (Fig. 5).

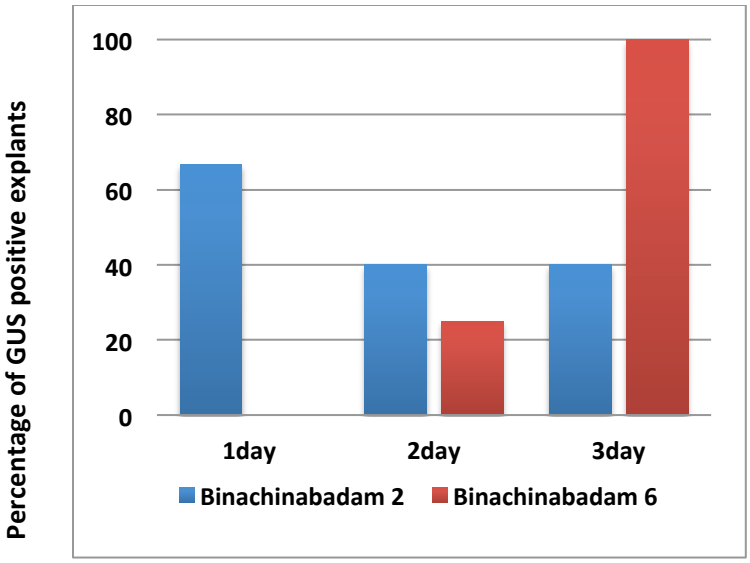

Figure 5. Influence of co-cultivation period on transformation efficiency analyzed by transient GUS histochemical assayin two peanut varieties.

3.4 Overall response of two peanut varieties towards transformation:

The transformed explants were confirmed for the transfer of the T-DNA by GUS histochemical assay by observing the blue coloration. The result represented the transient assay of the explants and the coloring was seen at the cut ends of the leaves and as well as within the tissue underneath the epidermis. Where BINA Chinabadam 6 showed 100% GUS positive explant and BINA Chinabadam 2 showed 66.7% GUS positive explant (Figs. 6-7). Thus, both the varieties were found to be compatible towards the *Agrobacterium* strain LBA4404 (Fig.8).

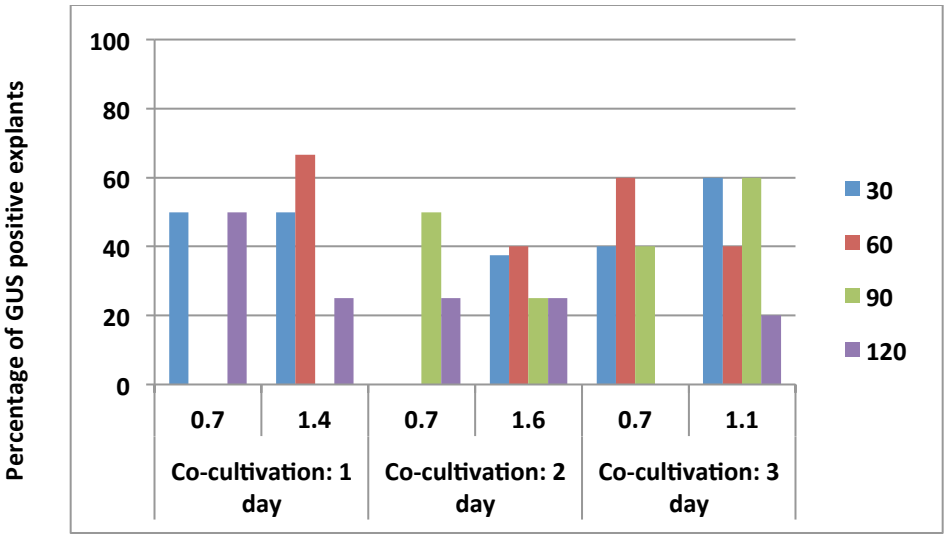

Figure 6. Comparison of all the factors influencing transformation efficiency of BINA Chinabadam 2.

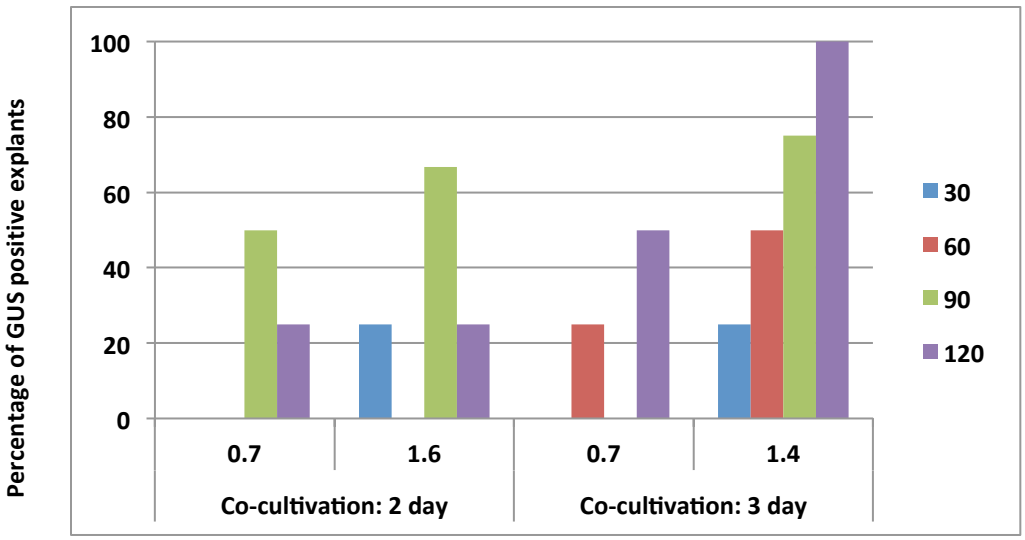

Figure 7. Comparison of all the factors influencing transformation efficiency of BINA Chinabadam 6.

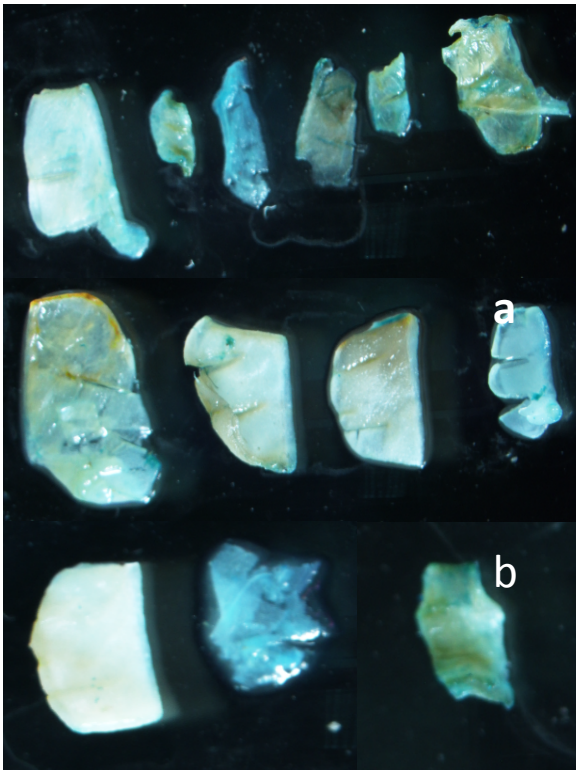

Figure 8. Blue coloration observed under stereomicroscope (Olympus, Japan) due to GUS histochemical assay.

(a) Different BINA Chinabadam 2 embryonic leaflet
(b) Different BINA Chinabadam embryonic 6 leaflet.
(c) Comparison between control, BINA Chinabadam 2 leaflet and BINA Chinabadam 6 leaflet.

From table 5 and 6 it is seen that maximum transformation efficiency is achieved for BINA Chinabadam 2 when the co-cultivation duration is 1day, infection period of 60mins and bacterial density of (OD_{600}) of 1.4. However for BINA Chinabadam 6 it is seen that maximum transformation efficiency of 100% is achieved when the co-cultivation period is 3days, infection

duration is further increased to 120mins, but with the same bacterial density of (OD_{600}) of 1.4. Thus, from this study it can be concluded that the optimized *Agrobacterium*- mediated transformation condition of peanut using embryonic leaflet as a source of explant for both the variety is bacterial suspension of greater than 1, infection period of minimum 60mins and co-cultivation period of minimum 1day. Though the study is just using GUS histochemical assay, there is no permanent evidence of transfer of gene into the plant tissue so further molecular analysis is required to give any concrete evidence on permanent transformation.

Table 5. Effect of different factors on transformation of BINA Chinabadam 6 leaflet.

Co-cultivation duration (days)	Optical density (OD_{600})	Infection time (mins)	No. of explants used in GUS assay	Percentage of GUS positive explants
2	0.7	30	4	0
		60	4	0
		90	4	50
		120	4	25
	1.6	30	4	25
		60	4	0
		90	3	66.67
		120	4	25
3	0.7	30	4	0
		60	4	25
		90	4	0
		120	3	50
	1.4	30	4	25
		60	8	50
		90	4	75
		120	2	100

Table 6. Effect of different factors on transformation of BINA Chinabadam 2 leaflet.

Co-cultivation duration (days)	Optical density (OD_{600})	Infection time (mins)	No. of explants used in GUS assay	Percentage of GUS positive explants
1	0.7	30	2	50
		60	2	0
		90	2	0
		120	2	50
	1.4	30	2	50
		60	3	66.67
		90	4	0
		120	4	25
2	0.7	30	4	0
		60	4	0
		90	4	50
		120	4	25
	1.6	30	8	37.5
		60	5	40
		90	4	25
		120	4	25
3	0.7	30	5	40
		60	5	60
		90	5	40
		120	5	0
	1.1	30	5	60
		60	5	40
		90	5	60
		120	5	20

Discussion

The aim of the present study was to establish an efficient protocol of *Agrobacterium*- mediated transformation for two peanut varieties of Bangladesh using embryonic leaflet as a source of explant. For this purpose different factors responsible for the transformation were monitored. Such factors include bacterial density, infection duration and co-cultivation period. In Bangladesh peanut variety of DM-1 and Tridana was previously used to establish *in vitro* regeneration protocol using several explants. However only leaflet explants has been reported to be the most compatible (Sarkar and Islam 1999, 2000).

Bacterial density was measured using a spectrophotometer (OD at 600nm). This procedure of measuring bacterial density was seen in many researches (Islam *et al.*, 2010; Sharma *et al.*, 2009; Pérez-Piñeiro*et al.*, 2012). From our study it was seen that as the bacterial density (OD_{600}) was increased from 0.7 to 1.4 the percentage of GUS histochemical assay also increased. However further increase in the optical density resulted in decrease of transformation efficiency. Similar results were reported in tomato by Islam (2007)

As it was reported that co-cultivation is genotype dependent (Zhang *et al.*, 1997) thus effect of co-cultivation depends on the species. From our study we found that for BINA Chinabadam 6 with the increase of co-cultivation period percentage of GUS positive explant increases, while for BINA Chinabadam 2 transformation efficiency decreases. For Jingha Badam and DM-1 varieties of peanut using leaflet as explants it was reported that 72 hours of co-cultivation gave the optimum result (Sarker *et al.*, 2000). Same duration was reported when cotyledonary nodes were used as explant in peanut (Anuradha *et al.*, 2006; Iqbal *et al.*, 2012) and cotyledon explant for tomato (Sharma *et al.*, 2009). Sharma and Anjaiah (2000) used cotyledons of peanut as explant and mentioned co-cultivation duration of two weeks for optimum transformation.

As it is seen for BINA Chinabadam 2, increase in infection duration resulted in increase in GUS expression till 60mins.However further 30mins increase resulted in 0% GUS positive explant and then again increasing to 25% at 120mins. On the other hand, an increasing pattern was

observed for BINA Chinabadam 6. Their increase in infection duration resulted in an increase in GUS positive explants. All explants found positively transformed at 120mins, without any sign of bacterial overgrowth even after 3days of co-cultivation. It was seen that for gherkin increase of infection time to more than 35 min caused browning of the target tissue and did not allow it to flourish. (De Clercq *et al.*, 2002; Kumria *et al.*, 2001). For a study using chickpea seedling it was reported to have varied infection time while carrying *Agrobacterium*- mediated transformation (Akbulut *et al.*, 2008). The infection durations were 2hour, 8hour, 16hour and 24hour and was concluded that 16hour and 24hour were optimum. They also pointed out that the longer the infection period, higher is the rate of transformation but this also resulted in bacterial overgrowth, which ultimately reduces viability of seedlings. According to Sarker *et al.,* 2000 60min infection time was found to be optimum for DM-1 peanut variety using leaflet explant, our present study came to the agreement for BINA Chinabadam 2 variety of peanut.

In the present investigation, the transgenic nature was confirmed through GUS histochemical assay only. However, for stable integration of transformation into plant tissue specific molecular techniques like Polymerase Chain Reaction (PCR) and Southern blot hybridization are also necessary. This study establishes the protocol of transformation of peanut using a popular explant, leaflet. Following this protocol in future attempts can be taken to develop transgenic peanut, which will be better tolerant to various biotic and abiotic constrains and also improve in nutritional quality.

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