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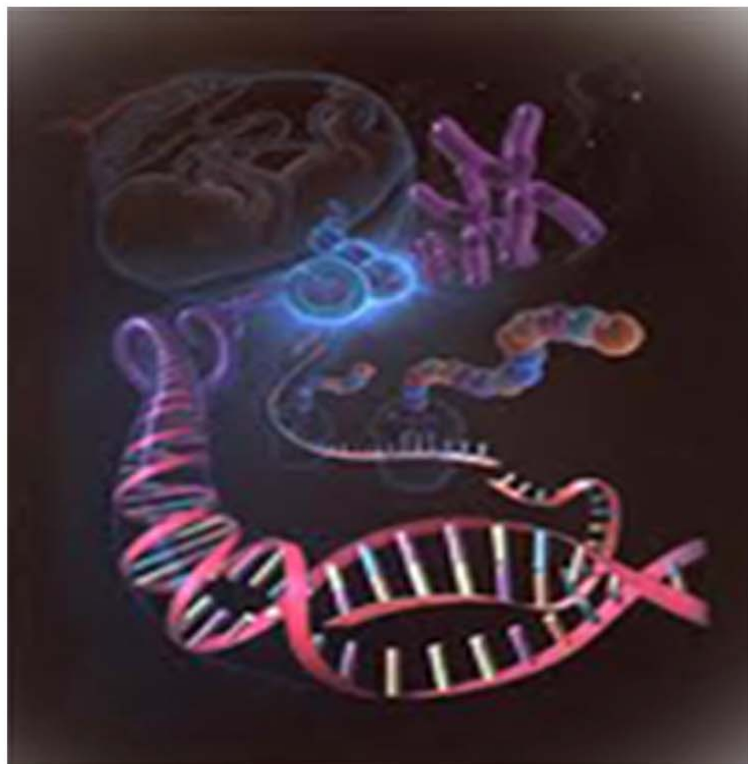

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Research Paper

AN EFFICIENT AND IMPROVED METHOD OF *IN PLANTA* TRANSFORMATION IN *ARACHIS HYPOGAEA.* L

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Arachis hypogaea (Peanut) has long been the focus of conventional breeding efforts because of its importance as a source of high quality oil and proteins. Genetically modifying peanut plants by molecular techniques has been an important tool for introduction of agronomical useful genes responsible for improvement of economically important traits. In the present study, an improvised *in planta Agrobacterium tumefaciens* mediated genetic transformation was carried out in *Arachis hypogaea* L.cv. TMV-2. *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA 1301 was used for transformation of embryo axes with one of the cotyledon intact. The putative transformed embryos were germinated on MS basal medium supplemented with 40 mg/L hygromycin along with 250 mg/L cefataxime thus allowing direct selection of plants. After germination on MS medium, the putative transgenic plants were moved to sterile soilrite, maintained in greenhouse. The transgenic plants were validated by GUS histochemical assay and integration of transgene into peanut genome was confirmed by genomic DNA PCR. The optimized protocol could be applicable for other plant species for high transformation efficiency.

Keywords: *In Planta* transformation, GUS, *Arachis hypogaea*, Hygromycin phosphotransferase

INTRODUCTION

Genetically modified plants have incredible potential to revolutionize agriculture. The underlying principle of genetic modification in plants is to improve nutritional value, providing resistance to stress, various diseases and herbicides, enabling production of significant chemicals that finds application as nutraceuticals, pharmaceuticals and production of plant based edible vaccines and antibodies (Untergasser *et al.,* 2012). There are numerous methods and techniques that are used to transfer foreign genes into cells that include electroporation of protoplasts, particle bombardment and *Agrobacterium*-mediated transformation. In plant biotechnology, the latter

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is an extensively used customary method for inserting foreign genes into plant genome for obtaining transgenic plants. This method has been successfully used to genetically transform various agronomical important monocot and eudicot species. The process of *Agrobacterium*-mediated gene transfer is governed by various factors that include genotype of the host plant, explants used for transformation, Agrobacterial strain and temperature of co-cultivation. The reported standard tissue culture and *in planta* transformation techniques like floral dip or seedling infiltration, apical meristem transformation and the pistil dip methods have been currently used for plant transformation (Mehrotra and Goyal, 2012).

Arachis hypogaea (peanut) is important oil yielding legume plant and is also a source of resveratrol, a polyphenol having anti-inflammatory and antioxidant properties. Although conservative methods of breeding has led to the improvement of peanut, modification of groundnut plants using genetic engineering techniques has been a very important tool for introduction of genes responsible for improvement of economically important traits such as disease resistance, high seed quality and to enhance oil yield. A successful protocol for groundnut transformation work was carried out earlier by microprojectile bombardment of the embryogenic calli (Livingstone and Birch, 1999), though this procedure was reliable it was found to be laborious and in many cases prone to microbial contamination and has low transformation efficiency. *Agrobacterium*-mediated gene transfer is the most widely applied system in peanut gene manipulation experiments.

A major limitation in the peanut transformation is the tissue culture based transformation protocol which is time-consuming, recalcitrant to regeneration and genotype-dependent. To overcome the recalcitrance nature, minimizing the role of tissue culture in the transformation procedure is considered as advantageous. Thus *in planta* transformation method is a promising technique over other methods and has been optimized in rice (Supartana *et al.*, 2005) , buckwheat (Kojioma *et al.,* 2000) , Peanut (Rohini and Sankara Rao, 2000), mulberry (Ping *et al.,* 2003) Cotton (Keshamma *et al.,* 2008), etc.

Earlier reports on peanut transformation have shown the generation of transgenic peanut plants using various explants. Eapen and George (1994) had co cultivated *Agrobacterium tumefaciens* LBA4404 harboring pBI121 plasmid using leaf as explants and generated transgenic peanut plants. *Arachis hypogaea* .L, cv JV24 transgenic plants was generated by transforming *Agrobacterium tumefaciens* harboring binary vector p35SGUSINT using cotyledonary node explant (Anuradha *et al.,* 2006). Highest regeneration and transformation efficiency was reported by Sharma and Anjaiah (2000) using cotyledon explants. Marker free transgenic peanut was reported by Bhatnagar *et al.* (2010). Zygotic embryos were transformed with the plasmid pCAMBIA1301 via particle bombardment method (Yang *et al.,* 2001). An improved biolistic method for efficient recovery and analysis of transgenes using embryogenic tissues has been developed (Chu *et al.,* 2013). In this study we examined the applicability of improvised *in planta* transformation of peanut for direct selection of transformants.

MATERIALS AND METHODS

Plant Material: The groundnut seeds (*var* TMV -

2) were procured from Department of Crop Physiology, university of agricultural Sciences, GKVK, Bangalore. The seeds were soaked for 48 h in water; one of the cotyleydon was removed retaining the other with the intact embryo (explant). The explants were surface sterilized with 70% ethanol for 5 min followed by 10 min wash in 2% sodium hypochlorite. The explants were then washed with 0.1% mercuric chloride for 2 min and thoroughly washed with sterile water to remove traces of mercuric chloride. After surface sterilization, the embryo axes with one of the cotyledons intact were inoculated in hormone free basal MS medium for two days for the initiation of plemule and radicle primordia. The seeds were germinated at 25^0C under standard fluorescent tubes with a flux rate 35 µmol s^{-1} m^{-2} and exposed to 16 h photoperiod (day length). After two days, the embryo axes pricked with sterile needle were cocultivated with *Agrobacterium tumefaciens* LBA4404 harboring plant expression promoter::reporter construct pCAMBIA1301.

Transformation – Inoculation and Coculture

In planta genetic transformation of embryo axes with one of the intact cotyledons was inoculated for 2 days in hormone free basal MS (Murashige and Skoog, 1962) medium and was used as explants. *Agrobacterium tumefaciens* harboring the plasmid pCAMBIA 1301 was streaked on LB agar plate and incubated at 28^0C for 48 h. Later the *Agrobacterium* was scraped and suspended in MS liquid medium supplemented with 100 µM acetosyringone. The explant was suspended in the Agrobacterial culture for 20 min and later cocultivated. After two days of cocultivation on MS solid medium supplemented with 100 µm acetosyringone, the explant tissue were washed thoroughly with sterile double distilled water followed by final wash with cefotaxime (250 mg/L) for 3 h and transferred to MS medium with hygromycin (40 mg/L) and cefotaxime (250 mg/L) supplemented with BAP (2 mg/L) and NAA (0.5 mg/L).

Establishment of Transgenic Plants

After 10-15 days in hygromycin selection half strength MS medium, the putative transformed plantlets were transferred to sterile soil rite for acclimatization for 20 days. Later the transgenic plants were moved to soil and maintained in greenhouse under controlled temperature.

PCR Analysis to Confirm the Insertion of Transgene

To confirm the insertion of the transgene, genomic DNA was isolated by CTAB method (Doyle and Doyle, 1987) from the leaves of the putative peanut transgenic plants. PCR was carried out with genomic DNA as the template using GUS specific primers, the FP (5-GAATGGTGATTACCGAGAA -3') and the RP-(5 - TAATGCGAGGTACGGTAGGA -3'). The PCR was carried out under the following conditions: Denaturation at 94°C for 1 min, Annealing: 55°C for 1 min followed by extension/elongation at 72°C for 1 min and the PCR was carried out for 25 cycles, final extension at 72°C for 5 min. The amplified product (550 bp) was assayed on agarose electrophoresis (1.5% agarose gel) and image was captured in Gel documentation system.

GUS Assay

GUS activity assays was carried out to confirm the transgenic plants. Different tissues of the transgenic peanut plants were collected, treated with 80% acetone for 15 min in ice to remove

chlorophyll and washed in GUS buffer. The tissues was then incubated with 5- bromo, 4-chloro, 3- indolyl β D-glucoronide (X-Gluc) dissolved at a concentration of 1 mg mL^{-1} in 50 mM potassium phosphate buffer (pH 7.0) containing 0.05% Triton X-100 and 5 mM each potassium ferricyanide and potassium ferrocyanide (Jefferson *et al.*, 1987) for 12 h at 37°C in dark.

RESULTS AND DISCUSSION

In the present study optimization of *Agrobacterium* mediated genetic transformation was carried out by *in planta* transformation method in *Arachis hypogaea var* TMV-2. *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pCAMBIA 1301 having the 35S CAMV promoter driving the expression of *β*-glucurodinase (GUS gene), and Hygromycin phosphotransferase as the antibiotic resistance gene marker (hptII); driven by CAMV 35S promoter was used for transformation. The promoter::reporter (35SCAMV::GUS) plasmid with nos terminator and hygromycin resistant gene sequences was transformed to *Agrobacterium tumefaciens* strain LBA4404 by Freeze thaw method. After transformation, plasmid DNA preparation was carried out using alkali lysis method followed by restriction enzyme digestion with Xho1 to authenticate the plasmid transformation.

For carrying out *in planta* transformation the embryo axes with one of the cotyledon were cocultivated in Agrobacterial cells harboring pCAMBIA1301 suspended in MS medium supplemented with 100 µM acetosyringone for 24 h and washed thoroughly with sterile double distilled water followed by final wash with cefataxime (250 mg/L) for 3 h. The cocultivated explants were then inoculated onto MS medium with hygromycin (10-50 mg/L) and cefotaxime (250 mg/L) supplemented with BAP (2 mg/L) and NAA (0.5 mg/L).

To check the sensitivity of hygromycin, transformed embryo axes were inoculated on MS medium with various concentration of hygromycin (10-50 mg/L), cefotaxime (250 mg/L), BAP (2 mg/L) and NAA (0.5 mg/L). A high regeneration and development of plantlets was seen with low concentration of hygromycin (10-20 mg/L). As the concentration increased, the plantlets developed in 40 mg/L hygromycin were decreased and at 50 mg/L hygromycin was lethal for development without any regeneration (data not shown). The plantlets developed in low concentration of hygromycin with high incidence of germination were found to be more of false positive transgenic 'escapes' and low frequency of germinated plantlets on 40 mg/L were all positive transgenics (Figure 1A, B, C, D) as indicated by the PCR analysis of genomic DNA using GUS gene specific primers which showed expected 550 bp amplified product. However, there was little effect on transformed plants on their germination capacity when NAA and BAP were not added to MS medium.

The histochemical GUS expression assay was carried out in these plants using root and shoot bits from wild type and pCAMBIA1301 transformed groundnut plants. The tissues of wild type and transformed plants were treated with 90% acetone in ice, the tissues were then washed with GUS buffer and incubated followed by incubating the tissues in 5- bromo, 4-chloro, 3-indolyl (D –glucoronide (X-Gluc -GUS substrate) at 37°C in dark and after 12 h of incubation the transformed root and shoot segments had turned blue and showed positive for GUS assay (Figure

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Figure 1A: Peanut embryo axis with one of the cotyledon intact was used as the explant for *in planta* transformation. B: The putative transgenic plant harboring GUS gene maintained on MS medium supplemented with BAP (2 mg/L) and NAA (0.5 mg/L) and hygromycin (40 mg/L). C: The putative germinated transgenic plants were transferred from MS medium and moved to soilrite for acclimatization. D: After acclimatization, the transgenic plants being maintained in green house

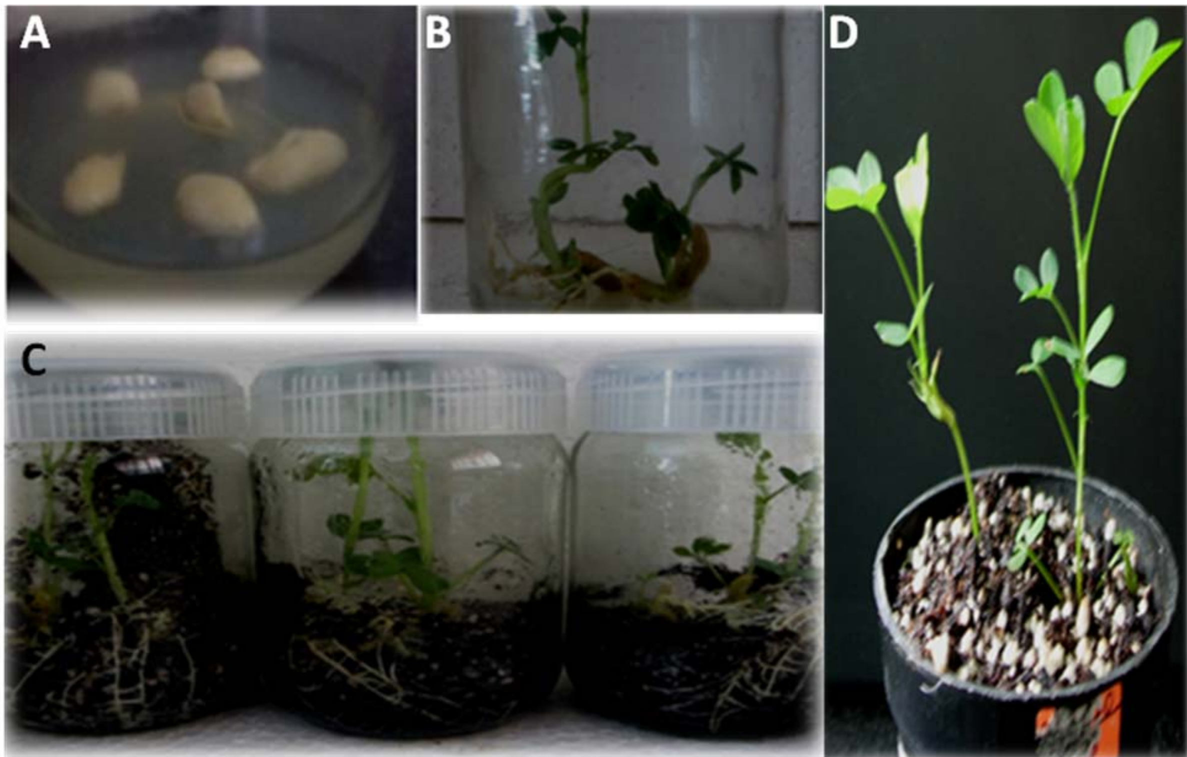


Figure 2: Genomic DNA was isolated from the leaves of wild type and putative transgenic plants of *Arachis hypogaea.* PCR was carried out using GUS specific primers.The presence of 550 bp amplicon in transgenic plant. Line number 1 and 4 confirms the presence of transgene in the transgenic plants

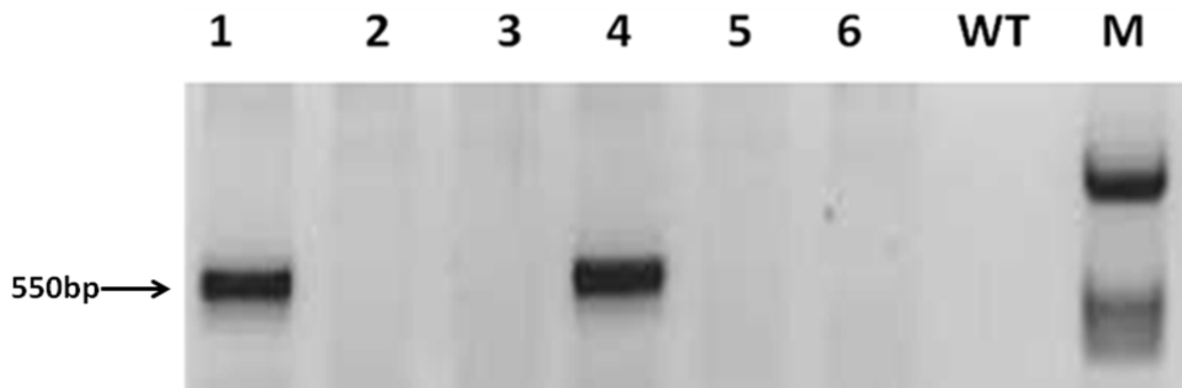
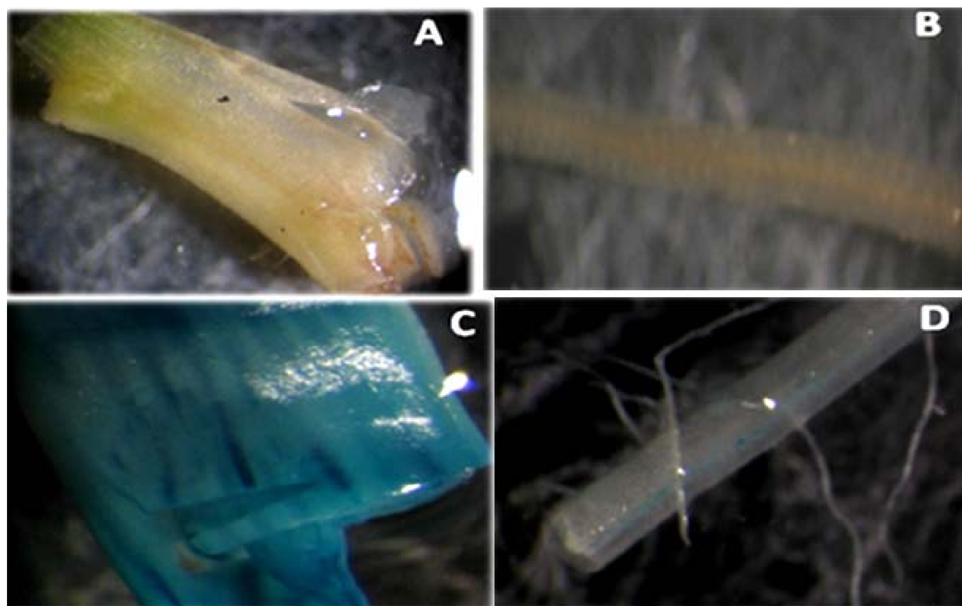


Figure 3: Wild type shoot segment (A) and root segment (B) showing negative for GUS assay. GUS expression in pCAMBIA 1301 transformed peanut shoot (C) and root (D)



3, A-D). Out of approximately 80 seeds taken for transformation at 10-20 mg/L hygromycin as selection pressure, thirty plants proved to be transgenic which was confirmed by GUS assay and genomic DNA PCR (Figure 2) indicating low concentration of hygromycin do not eliminate the nontransformed tissues completely, whereas approximately 48% plants were positive for GUS and genomic PCR at 40 mg/L hygromycin.

CONCLUSION

The present study describes an efficient and reproducible protocol for *Agrobacterium tumefaciens* mediated genetic transformation in *Arachis hypogaea* using embryo axes with one of the cotyledon. Efficient peanut transformation using *Agrobacterium tumefaciens* will depend on several physical and chemical factors like genotype, explants, bacterial strains, selection pressure, etc. (Egnin *et al.,* 1998). The hygromycin based *in planta* selection regime optimized here on cotyledon with one of the embryonal axes resulted in recovery of large number of transgenic plants within a short period, with higher efficiency of genetic transformation in peanut. This method could also very well be applied for other economically important seed crops for which efficient transformation protocol is not available.

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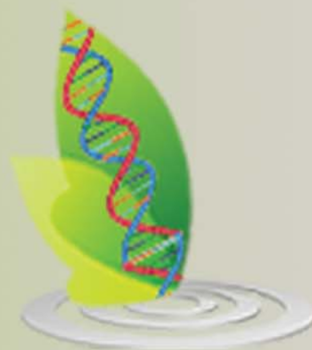

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