Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **44** Issue S-**05 Year 2023** Page **2430:2434***

Agrobacterium Mediated Genetic Transformation Of Ground Nut (Arachis Hypogaea L.) Cultivar Icgv-15311 Embryo Axis Explants For Defensin Gene Against Fungal Resistance

Swapna Mittapalli[1], Thriveni Dumpeti[1] , Gade Pavan Kumar[1], Sadhu Suman Kalyan[2], Potta Jyothi[3], Rajender Vadluri[1*]

[1]Department of Biotechnology, Chaitanya (Deemed to be University), Kishanpura, Hanumakonda – 506009.*
[2]Department of Microbiology, Kakatiya University, Hanumakonda-506009.
[3]Department of Microbiology, Chaitanya (Deemed to be University), Kishanpura, Hanumakonda – 506009.

***Corresponding Author:** Rajender Vadluri*
**Department of Biotechnology, Chaitanya (Deemed to be University), Kishanpura, Hanumakonda – 506009.*

Article History	*Abstract*
Received: 22/10/2023 Revised: 22/12/2023 Accepted: 25/12/2023	Genetic transformation of Ground nut (*Arachis hypogaea* L.) was attempted using plasmid vector pCAMBIA '3365' Present with in Agrobacterium tumifaciens strain LBA4404 with CaMV35sp (Cauliflower mosaic virus 35s RNA Promoter). A total of 323 Embryo axis, Half cut embryo, Cotyledonary nodes were employed in independent co cultivation events, of which 13 putative transgenic plants were established in *Invitro* conditions. The transgenic shoots obtained were initially confirmed for the introduced reporter gene by GUS histochemical assay and later by the PCR analysis. The transformed shoots were checked for the transcription of the gene by RT-PCR analysis. Southern blot analysis was carried out to confirm the stable integration of the foreign DNA.
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Introduction:

Ground nut (Arachis hypogaea.L.) belongs to the family Leguminosae, sub family papilionidae, tribe Aeschnomenae, sub tribe stylosanthinae, genus Arachis and species hypogaea (Isleib et al 1994).The genus name Arachis derived from a –rachis (Greek meaning without spine) in reference to the absence of erect branches. The species name hypogaea term derived from hypo-ge (Greek, meaning below earth) that grows downward in to the earth so that the pod develops underground. It is also known as peanuts, earth nut, monkey nuts, manila nut, panda, unpredictable legume and energy capsule (Sheshadri, 1962). The cultivated ground nut is an ancient crop of the new world, which is originated in South America (southern Bolivia) where it was cultivated as early as 1000 BC. Dissemination of the crop to Africa Asia, Europe and the pacific islands occurred presumably in the 16th and 17th centuries with the discovery voyages of the Spanish, Portuguese, British and Dutch(krapovickas,1969,1973: Gregorferal 1980; Hammons 1982;Isleib et al 1994) Ground nut was introduced in to India by the Magellan expedition around 1519 (subbaRao1909). Ground nut varieties ICGV 15311, ICGV15287, ICGV13074 seeds from explants Embryo axis, Half cut embryo axis, Leaf and Cotyledonary node. various concentrations and combinations of different plant growth regulators are used for multiple shooting and rooting to obtain a complete ground nut plantlets on MS (Murashige and Skoog) medium supplemented with different concentrations of auxins (IAA,NAA,IBA and2,4-D) and

cytokinins (Kin and BAP). Especially for legumes like Tephrosia purpurea like medicinal plants are showing good results at this combinations (Rajender et al 2016), More shoots are obtained from embryo axis on a medium containing BAP (2.0mg/L) and NAA (0.5mg/L) and more roots are obtained from embryo axis supplemented with 2.0mg/L IBA on MS medium.

Materials & Methods:

Agrobacterium mediated genetic transformation of shoot Explants

For genetic transformation of shoot explants of ICGV15311 cultivar, *Agrobacterium* strain LBA 4404 harbouring p CAMBIA plasmid vector carrying 3365 gene under the control of CaMV 35Sp cauliflower mosaic virus 35S RNA promoter and NOS –poly A Nopaline synthase terminator was used in the study.
In this study, we used ICGV15311 cultivar of Ground nut (Arachis hypogaea.,L.) explants embryo axis ,cotyledonary nodes and half cut embryo's were growned in MS medium with BAP(2mg/l) and NAA(0.5mg/l) for shoot proliferation. For selection of transformants 25µl of Kanamycin and 50µl of Cefatoxime were used. Agrobacterium tumifaciens carrying gene 3365 construct was grown in LB medium containing 250µl of Kanamycin and 40µl Acetosyringone grown for 48hr at 28ºc with 200 rpm shaking. A pre culture of the shoot explants was done for 2 days on MS medium.On the day of transformation 24hr grown culture of chimeric Agrobacterium tumifaciens was pelleted at a speed of 6000rpm for 15 min used cold centrifuge then suspended and diluted to OD 600 nm of 0.5 in liquid MS medium supplemented with25µl of Acetosyringone. This bacterial suspension was used for infecting the explants. For better infection performed sonication or pricking with sterile needle then soaked the explants in liquid MS medium for 30 min and then explants were blot dried on to sterile filter paper and transferred on to petridishes containing MS shoot induction medium(SIM). For the preparation of shoot induction medium MS medium supplemented with BAP of 250µl and NAA of 250µl along with the glutamic acid of 250mg, cysteine 100 mg. After completion of sterilization medium was cooled and added Acetosyringone 25µl and poured the medium in to Petri plates). Petri dishes were sealed with parafilm and cocultivated for 2 days at 25±2ºc in the dark.
The co-cultivated explants were rinsed with sterile distilled water to remove bacterial contamination then blot dried and some were transferred to GUS solution for Histochemical assay in order to get the transformation frequency and the remaining were transferred to selection medium for shoot induction. For this MS medium supplemented with BAP 500µl and 500µl NAA along with 25µl of kanamycin and 50µl of cefatoxim50µl.Sub culturing on to fresh medium was carried out every fortnight. Once the Agrobacterium contamination was brought under control, Cefatoxime was eliminated from the media. Shoots separated and transferred to rooting medium with IBA(2mg/l) containing 50mg/l Kanamycin, rooted plantlets were transferred to pots containing sterile soil, sand and vermi compost(1:1:1) and maintained in glass house.

Transgene integration and PCR analysis

The presence of transformants was checked initially by GUS histochemical assay by the reporter gene and later by the PCR analysis using gene 3365 specific primers. Total DNA from transgenic plants was isolated by c-TAB method. DNA from transformed and non transformed (control) plants was extracted by c-TAB method and used for PCR analysis (Doyle and Doyle, 1990). For the amplification of the desired DNA fragments PCR analysis was done with appropriate gene specific primers (5′-CTCGTCCGTCCTGTAGAAACCC-3′), (5′-TCATAGAGATAACCTTCACCC-3′) and conditions. A reaction volume of 20µl solution was prepared in sterile 0.2ml thin wall PCR tubes with 25µM each of forward and reverse primers, 50-100 mg of genomic DNA or 5µg/µl plasmid DNA (or) bacterial colony (colony PCR) or PCR product or 2-5µl of first strand c DNA as a template,100µM of each d NTP, 1 unit of Taq DNA polymerase,1X concentration of Taq DNA polymerase buffer (10Mm Tris-Hcl, pH 9.0,1.5Mm $Mgcl_2$,50 Mm KCl, 0.01% (Gelatin) and milliQ water to make up the total volume. Each PCR aliquor was mixed and PCR reaction was carried out in Eppendorf Master Cycler. The amplified products were run on 1% Agarose gel with a DNA marker. As a positive control the corresponding plasmid DNA was used as a Template. The negative control and plant control were set with no DNA and untransformed plant DNA respectively. The amplified products were mixed with 1X loading dye. Electrophoresed using horizontal Electrophoresis unit on 1% Agarose gel in 1X TAE buffer (0.5µg/ml Ethidium bromide). The amplified products were visualized and documented in gel documentation unit.

Results:

Genetic transformation & Regeneration of Transgenic plants

A high frequency shoot regeneration system employing Embryo axis explants of Ground nut *(Arachis hypogaea*.L.) ICGV 15311 Cultivar was obtained using MS medium supplemented with 2mg/l BAP and 0.5m/l NAA. This optimized protocol was further used for the genetic transformation of Ground nut.
For genetic transformation, embryo axis explants were co-cultivated with *Agrobacterium tumifaciens* containing expression cassette of gene 3365 in p CAMBIA vector following the protocol with Shoot induction medium supplemented with BAP 500µl and NAA 500µl, througout the regeneration process. The selection of transformants was done by 25µl of kanamycin and 50µl of Cefatoxime. A total of 566 explants were employed in five independent co-cultivation events, of which 323 explants survived up on two sub culturing on selection medium containing 50mg/l kanamycin. Survival percentage of transformed explants shoots and plantlets of Ground nut are detailed in Table. 1

Table1: Percentage survival of transformed explants, shoots and plantlets of Ground nut.

Experiment No.	No. Of explants infected	No. Of Plants responded	No. Of putative Transformants (After PCR)	Transformation efficiency (%)
1.	120	72	04	3.33
2.	115	64	02	1.73
3.	117	71	03	2.56
4.	109	62	02	1.83
5.	105	54	02	1.90
Total	566	323	13	11.35

$$\text{Transformation efficiency } (\%) = \frac{Number\ of\ Transgenic\ plants}{Number\ of\ explants\ infected} \text{ X } 100$$

$$\text{Average Transformation efficiency } (\%) = \frac{11.5}{5} = 2.27\%$$

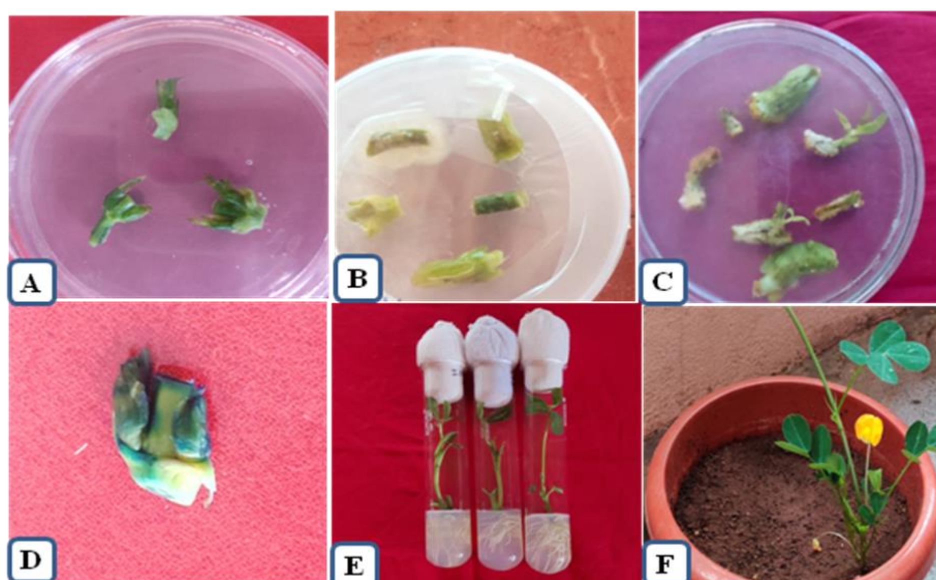
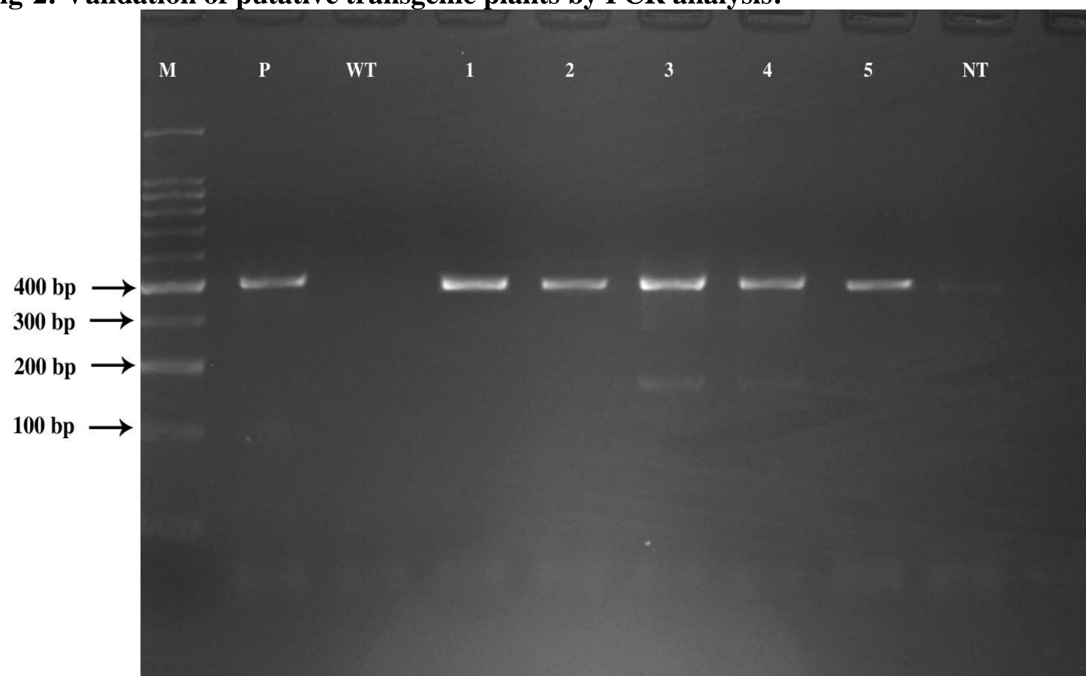


Fig-1: Agrobacterium mediated genetic transformation of emryo axis explants with LBA 4404 strain with gene 3365 construct.

A-Shoot bud induction from explants. **B-** Agro infection of the explants, **C-** Shoot elongation after four weeks on SIM, **D-** Transiant gus expression in putative transgenic shoot, **E**- Putative transgenic plant on RIM, **F-**Acclematization of trnsgenic plant.

Fig-2: Validation of putative transgenic plants by PCR analysis:



M: Marker DNA ladder (100 bp ladder, Genei)
P: Positive control (Plasmid DNA)
WT: Wild type (Control plant DNA)
Lane 1-5: Putative transgenic plants in T0 generation
NT: No template (Negative control)

Conclusion:

The *Agrobacterium* mediated genetic transformation protocol has been optimized by studying various factors influencing the transformation efficiency using Embryo axis explants. *Agrobacterium*-mediated genetic transformation efficiency was significantly increased by several factors such as pricking, bacterial cell density, and infection duration, pre-culture of explants, co-cultivation duration, acetosyringone and L-cysteine concentrations. The transgenic Ground nut (T0) plants were confirmed by Histochemical gus assay by stable integration of Gus A gene. Transgenic Ground nut (T0) plants were generated with Defensin Gene 3365 and stable integration of transgene was confirmed using PCR analysis.

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