

Agrobacterium tumefaciens-mediated Transformation of Chickpea (*Cicer arietinum* L.) using Mature Embryonic Axes and Cotyledonary Nodes

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The mature embryo axes and cotyledonary nodes of chickpea (*Cicer arietinum* L.) were evaluated for *Agrobacterium*-mediated transformation and the production of stable transgenic plants expressing the reporter gene GUS was documented. The major limitation in delivering the T-DNA in these explants of grain legume has been the low frequency and inconsistency. With manipulation of co-cultivation conditions and preparation of excised explants with exposed regenerative cells in L2 and L3 layers followed by the stringent screening on selection pressure of 100-150 mg l⁻¹, kanamycin exhibited significantly higher transformation frequency as indicated in the transient GUS expression. Amongst various strains of *Agrobacterium*, the strain, LBA 4404 was found to be most suitable for maximal transfer of T-DNA and minimum induction of hypersensitive response into the excised explants of chickpea. Kanamycin resistant chickpea shoots selected after 3-4 cycles of kanamycin screening were grafted onto 8-day-old seedlings. Mature plants recovered thereafter showed integration of T-DNA in PCR and Southern analysis and expression of GUS gene in histochemical assay. Screening of putative transformants of chickpea harbouring modified *gfp* gene (pCambia 1303) at an early stage was possible on the basis of green fluorescence but the transformants failed to grow further. The quantitative evaluation of GUS activity in primary transformants showed expression levels ranging from 580-1950 p mol methyl umbelliferone (MU) per mg protein per min. Out of 22 primary transformants, only six showed setting of F1 seeds while others failed to produce mature seeds.

Keywords: chickpea, *Agrobacterium tumefaciens*, β -glucuronidase, transformants, *Cicer arietinum* L., southern hybridization

Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume, as a major source of dietary protein in Indian and Mediterranean subcontinent for human and live stock consumption. Despite its nutritional significance, grain productivity of the crop has been declining primarily due to fungal diseases (*Fusarium* and *Ascochyta* spp.) and field infestation of *Heliothis*, a pod-borer insect (Singh *et al.*, 1994). Considerable efforts have been made for the introduction of agronomically useful traits into chickpea and other grain legumes through conventional breeding and hybridization. However, progress has been restricted due to non-availability of useful traits in the existing

germplasm including their wild relatives and barriers for hybridization and sexual incompatibility amongst grain legumes (Ahmad *et al.*, 1988; Kumar & Davey, 1991). Therefore, introduction of specific genes of diverse origin into chickpea through biotechnological tools is a promising alternative that precisely depends on high frequency simple procedures of transfer of foreign DNA into regenerative cells and tissues. Dicotyledonous grain legumes, although susceptible to *Agrobacterium* infection, are highly recalcitrant crop plants for *in vitro* regeneration and genetic manipulations (Parrott *et al.*, 1992; Babaoglu *et al.*, 2000). For successful transformation in chickpea, a simple procedure of *in vitro* regeneration compatible to DNA-mediated system is the pre-requisite. Particle bombardment has been used for mature embryoaxes and cotyledonary nodes to obtain fertile transgenic plants of soybean and other legumes, but both require screening of shoots in subsequent generations due to development of chimeric situation (McCabe *et al.*, 1988; Christou *et al.*, 1988). To overcome the problem of low frequency, inconsistency and chimeras as consequence of particle bombardment, this paper has

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Abbreviations: KIN-Kinetin; BAP-6-Benzylaminopurine; IBA-Indole-3-butyric acid; MEA-Mature embryonic axes; CN-Cotyledonary node; GUS and *uidA*- β -glucuronidase; *npt* II-Neomycin phosphotransferase II; *hpt*-Hygromycin phosphotransferase; *gfp*-Green fluorescent protein; PCR-Polymerase chain reaction; MU-4 -Methyl umbelliferone

optimised *Agrobacterium*-mediated transformation in chickpea using embryoaxes and cotyledonary nodes (CNs).

Materials and Methods

Plant Material and Tissue Culture Conditions

All the experiments were conducted with mature authentic seeds of *Cicer arietinum* L. (variety C-235, BG-256, Pusa 362 and Pusa 372) obtained from Indian Agricultural Research Institute, New Delhi. The seeds were surface sterilized by sequentially treating with mercuric chloride (0.1%, w/v) for 3 min, sodium hypochlorite (1.5%, v/v) for 10 min followed by repeated washing in sterile distilled water. The mature zygotic embryonic axes (MEA) were dissected from overnight soaked seeds and their meristematic regions were excised. Remaining explants were obtained from axenically grown plants on culture medium consisted of Murashige & Skoog (MS) salts (1962), B5 vitamins, sucrose (3%, w/v) and agar (0.8%, w/v) along with appropriate growth regulators. The medium (pH 5.7) was autoclaved at 1.09 kg cm² for 20 min. Cultures were incubated in the culture room maintained at 24±1°C under cool white light of intensity 60 µmol m⁻²s⁻¹ for 16 hrs photoperiod. The preparation of CN began after 21 days of growth with complete removal of the regenerated shoots and pre-existing axillary meristematic cells while apical shoot tips were collected from 7-day-old seedlings. The regenerated shoots were rooted in MS-medium containing IBA (0.5 mg l⁻¹) and agar (0.5%, w/v). The rooted plantlets were acclimatized for 15 days in culture room before transferring to glasshouse in pots containing a mixture of soilrite, sand and soil. The transformed Ro shoots selected after screening on the antibiotic-supplemented medium were grafted on 8-day-old stock for rapid and consistent development of transgenic plants.

Agrobacterium Strains and Plasmids

Agrobacterium tumefaciens strains used for transformation were LBA 4404 (Hoekema *et al.*, 1983) and GV 2260 (Deblaere *et al.*, 1985) harbouring either pBI 121 (Jefferson, 1987), p35S GUS-INT (Vancanneyt *et al.*, 1990) or pCambia 1303 (Roberts *et al.*, 1998) plant binary vectors. The plasmid DNA was prepared from bacterial cultures grown at 28°C in YEB medium containing appropriate antibiotics (Rifampicin, 20; Kanamycin, 50 and Streptomycin, 50 mg l⁻¹) depending on the type of plasmid. The binary plasmid pBI 121 has a *uid A* gene for β -glucuronidase

(GUS) and plant selectable marker *npt II* for kanamycin resistance while p35S GUS-INT has same set of genes (*npt II* and *uid A*) except *uid A*, which has an intron at STLS1 position that makes the gene non-expressible in prokaryotic cells. The plasmid pCambia 1303 has a *uid A* gene along with modified green fluorescent protein (*gfp*) and hygromycin-resistant (*hpt*) gene driven by CaMV35S promoter for plant selection (Fig. 1).

Co-cultivation Procedure

Transformed *A. tumefaciens* strains harbouring a binary vector, grown overnight on a shaker at 28°C in 25 ml of YEB medium with appropriate antibiotics were harvested and the pellet was resuspended in 50 ml of MS medium fortified with BAP and 100 µM acetosyringone to yield an OD_{600nm} between 0.8 to 1.0. Batches of 30 excised MEA or 15 prepared CN of chickpea, incubated in *A. tumefaciens* suspension for 30 min, were sonicated at 43 kHz for 30-60 sec in water bath sonicator and then subjected to vacuum for 30 sec. The explants were blotted dry on sterile filter paper and co-cultivated on agar plates containing regeneration medium and 100 µM acetosyringone for 48 hrs under normal growth conditions. After co-cultivation, the explants were washed with sterile medium containing Cefotaxime (500 mg l⁻¹), blotted dry and cultured in 250 ml glass jars containing shoot regeneration medium (MS medium; BAP, 0.5-2.0 mg l⁻¹; sucrose, 3% w/v; agar, 0.8% w/v) supplemented with Cefotaxime (500 mg l⁻¹) and either

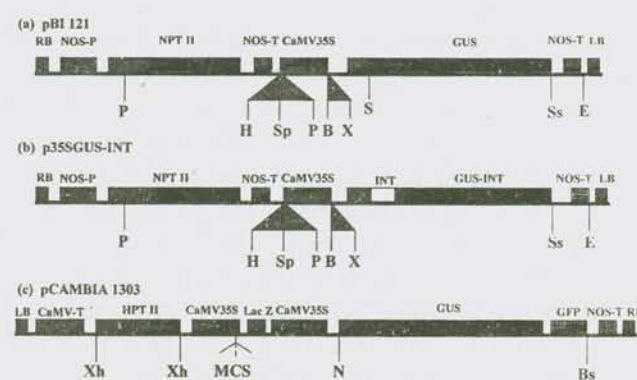


Fig. 1—T-DNA region of different binary vectors: a) pBI 121; b) p35SGUS-INT; and, c) pCambia 1303. LB, Left border; RB, Right border; *NPT II*, Neomycin phosphotransferase gene for kanamycin resistance; *HPT II*, Hygromycin phosphotransferase gene for hygromycin resistance; *GUS-INT*, β -glucuronidase gene with intron; *CaMV35S* promoter, terminator and *mgfp*, modified green fluorescent protein; MCS, Multiple cloning site; B, *Bam*HI; Bs, *Bst* E II; E, *Eco*R I; H, *Hind* III; N, *Nco* I; P, *Pst* I; S, *Sna*B I; Ss, *Sst* I; X, *Xba* I; Xh, *Xho* I.

Kanamycin (100 mg l^{-1}) or Hygromycin (20 mg l^{-1}) depending upon the nature of binary vector. After two cycles of selection on antibiotic-supplemented medium, healthy individual green shoots were separated and subcultured on fresh selection medium containing Kanamycin but without Cefotaxime to retain the selection pressure for subsequent two cycles of 15 days each. The surviving shoots were rooted in presence of antibiotics and developed for further analysis.

Gus Assays

The histochemical and fluorometric quantitative assay for GUS activity in different explants of chickpea was performed (Jefferson, 1987) with some modifications. The explants were processed for histochemical localization and preparation of cell free extracts for quantitative estimation of GUS activity by fluorometric assay (Shrivastava *et al.*, 2001). The specific GUS activity is expressed as pmol MU produced per mg protein per min. Histological sections of $10 \mu\text{m}$ thickness of both the explants were prepared with rotary microtome (Steeves & Sussex, 1989). Light microscopic observations including *gfp* visualizations were performed with Leica stereo zoom microscope equipped with long range UV source (320 nm) and filter for monitoring *gfp*.

Polymerase Chain Reaction (PCR) and Southern Hybridization

The genomic DNA from non-transformed and putative transformed chickpea plants was isolated by homogenizing leaf tissues ($100\text{--}200 \text{ mg}$) in liquid nitrogen (Shrivastava *et al.*, 2001). PCR amplification of GUS and *npt II* genes was performed in Perkin Elmer Gene Amp 9700 system using the set of specific primers and 35 cycles, each comprising of 1.5 min denaturation at 92°C , annealing at 58°C for 1 min followed by extension at 68°C for 2 min. The set of primers for *uid A* and *npt II* were, forward 5'-TTT AAC TAT GCC GGG ATC CAT CGC-3' and reverse 5'-CCA CTC GAG CAT CTC TTC AGC GTA -3' and forward 5'-TAT TCG GCT ATG ACT TGG CC-3' & reverse 5'-GCC AAC GCT ATG TCC TGA TA-3' amplifying a 530-bp and 700-bp amplicon of GUS and *npt II*, respectively. PCR was performed in $25 \mu\text{l}$ reaction mixture containing $1\times$ DNA polymerase buffer, $100 \mu\text{M}$ dNTP's, 25 ng of each primer, 2 mM MgSO_4 , 200 ng template DNA and 1 unit of Deep Vent polymerase (New England Biolabs, USA). The amplified DNA fragments were separated on 1.2% agarose gel and visualized on UV transilluminator.

Southern blot hybridization was performed (Sambrook *et al.*, 1989). About 8 to $10 \mu\text{g}$ of DNA from each plant was digested overnight with *Hind III*, separated on agarose gel (0.8% , w/v) and blotted onto Zeta Probe nylon membrane (BioRad Labs, USA). A 2.06-Kb *BamHI* and *EcoRI* fragment containing the GUS gene and about 1.8-kb *Pst I* fragment containing the *npt II* gene excised from pBI 121 were radiolabelled with $\alpha^{32}\text{P}$ -dCTP and used as probe for hybridization at 68°C for 12-14 hrs. The membrane blots were washed under the stringent conditions and exposed to X-ray films for autoradiography at -70°C .

Microprojectile Bombardment

Microprojectile mediated delivery of DNA was performed either with indigenously developed pneumatic particle bombardment device based on the acceleration of DNA coated gold or tungsten microcarriers by pulse of compressed nitrogen under partial vacuum or in BioRad Biolistic PDS-1000 He system. About 6.0 mg of sterilized gold ($1.0 \mu\text{m}$) or tungsten ($1.1 \mu\text{m}$) particles suspended in $50 \mu\text{l}$ of 50% glycerol were mixed while vortexing with $12 \mu\text{l}$ plasmid DNA ($12 \mu\text{g}$), $50 \mu\text{l}$ CaCl_2 (2.5 M), and $20 \mu\text{l}$ spermidine (5.0 M). The vortexed particle-DNA suspension was allowed to settle for 10 min, centrifuged and supernatant was completely discarded. The pellet ($6.0 \mu\text{l}$ consisting about $0.7 \mu\text{g}$ microcarriers), washed with 70% ethanol and resuspended in $40 \mu\text{l}$ absolute alcohol, was used for each bombardment. The target explants were placed at a distance of 11-13 cm from screen and bombardment was either at 900, 1100 or 1350 psi pressure of helium depending on the nature of the explants under 26-28 mm Hg vacuum. The bombarded explants were incubated under normal growth conditions and after 48 hrs used for histochemical GUS assay to monitor transient expression of reporter gene, whereas for stable integration the bombarded explants were subjected to selection on antibiotic supplemented medium as described earlier.

Results

The excised mature embryonic axes of chickpea on an average induced 3-5 and 6-9 shoots per explant when cultured on MS-medium supplemented with KIN, 1.0 mg l^{-1} or BAP, 2.0 mg l^{-1} respectively. Whereas CN explants, prepared after 20-21 days of culture on MS medium supplemented with BAP, 1.0 mg l^{-1} and IBA, 0.05 mg l^{-1} resulted into 8-12 shoots

per explant. Addition of 4.0 μ M L-glutamine or L-arginine either singly or in combination, into the BAP supplemented medium improved the overall health and efficiency of survival of shoots on transferring to glass house. Incubation of shoot apex region on MS medium fortified with BAP, 1.0 mg l⁻¹ and IBA, 0.02 mg l⁻¹ revealed 6-10 shoots per explant after 15 days of culture. Histological examinations of regenerating embryo axes and CNs revealed adventitious nature of shoot induction from the non-meristematic region of the excised surface of the explant while proliferation of pre-existing meristem in shoot apex on cytokinin medium resulted into induction of large number of shoots. The individual shoots after 15 days of growth were rooted with 80-86% efficiency, irrespective of their genotype and origin from the explants, in MS medium supplemented with IBA, 0.5 mg l⁻¹, 1/20th of nitrate and sucrose (1%, w/v). The hardening of *in vitro* grown plantlets of chickpea with primary roots was performed for 20 days in growth chamber in pots containing soilrite before transferring to glass house (Fig. 2).

Prior to transformation, an effective threshold concentration of antibiotics for selection of transformants was determined by culturing the explants on regeneration medium supplemented with different levels of Kanamycin (0-200 mg l⁻¹) and Hygromycin (0-100 mg l⁻¹) respectively. Kanamycin (50 mg l⁻¹) completely suppressed shoot development in all the different explants. Hence, in the present study following *Agrobacterium* co-cultivation, explants were screened either on 100 mg l⁻¹ Kanamycin or 50 mg l⁻¹ Hygromycin supplemented medium along with 500 mg l⁻¹ of Cefotaxime instead of 50 mg l⁻¹ Kanamycin, as reported earlier (Fontana *et al.*, 1993; Kar *et al.*, 1996). There was no influence of Cefotaxime, even up to 1000 mg l⁻¹ on the induction and development of shoot regeneration in chickpea.

All the three constructs harbouring GUS reporter gene were evaluated for efficiency of expression in chickpea. The plasmid DNA was coated onto gold and tungsten microcarriers and the extent of transient expression of GUS in bombarded explants was monitored through histochemical assay. It was consistently observed that level of expression as index of number of GUS foci per explant was higher with gold particles as compared to tungsten with all the three constructs (Fig. 3, Table 1). Maximum expression of GUS was noticed with p35S GUS-INT

followed by pBI 121 and pCAMBIA 1303. Results of the experiments conducted with different strains of *Agrobacterium* (LBA 4404, EHA 105 and GV 2260) harbouring p35S GUS-INT plasmid has shown maximum number of blue spots of GUS expression in transient assay with EHA 105 strain followed by LBA 4404 and minimum numbers with GV 2260 strains. However, explants co-cultivated with EHA 105 strain also showed maximum necrosis and significant suppression of multiple shoot induction, therefore, in rest of the studies strain LBA 4404 was used. Amongst the various explants CNs reflected maximum response compared to shoot tip, embryo axes and leaf explants. Microscopic observations of the bombarded explants (intact and sections) revealed that gold particles penetrated deeper into cell layers whereas majority of the tungsten particles and their agglomerates did not pass beyond the epidermal or deeper into the explants excepting in leaves. The gold particles in all the explants of chickpea, induced smaller blue spots while tungsten particles were accompanied with large diffused spots around the transfected cells (Fig. 3).

The embryo axes devoid of apical and root meristems were co-cultivated with *A. tumefaciens* strain LBA 4404 harbouring pBI 121, p35S GUS-INT or pCAMBIA 1303 constructs under optimized conditions. Twelve kanamycin resistant Ro shoots were recovered from 1350 explants reflecting an average frequency between 0.05-0.2%. The frequency of transformation was improved to 0.6-0.8% using the excised CNs for co-cultivation with *A. tumefaciens*. Various parts of these putative transformed Ro plantlets like shoots, leaves and roots were analyzed for GUS expression and molecular characterization. Histochemical assays of these plants showed GUS activity localized in the stem, leaves and uniformly in the aerial parts in few plantlets (Fig. 3). The GUS activity was not detectable in roots. The quantitative estimation of GUS activity in 12 randomly selected Ro plantlets in fluorometric assay reflected differential levels of expression amongst the transformants ranging from 580 to 1950 p mol MU per mg protein per min. Two to three-fold higher expression of GUS activity was observed in plants harbouring *uidA* gene with intron (GUS-INT) as compared to plants with *uidA* gene without intron. With pCAMBIA 1303, authors were able to easily screen out putative transformed chickpea plantlets at a very early stage of development on the basis of green fluorescence at 320 nm and could recover several

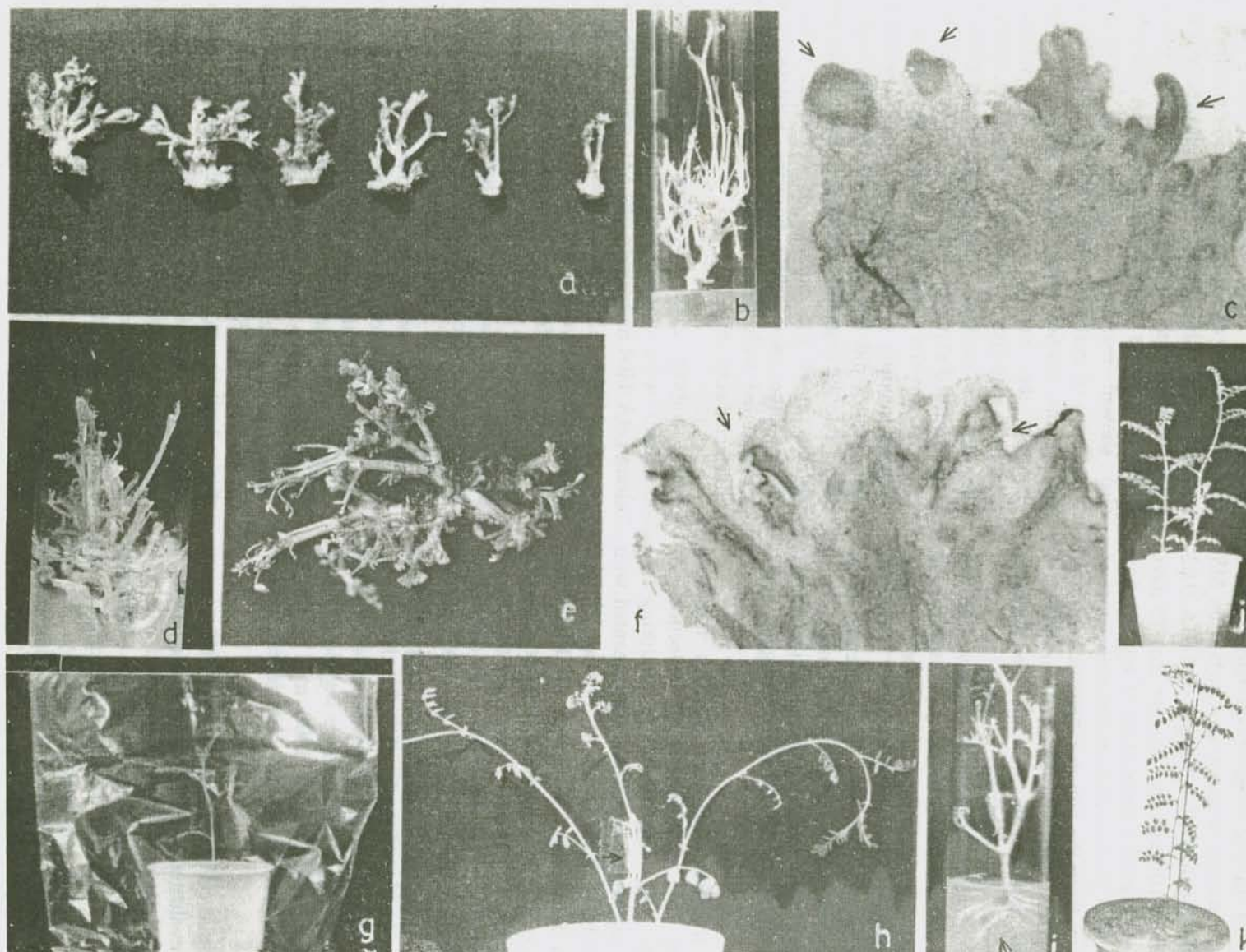


Fig. 2—*In vitro* regeneration in chickpea through direct organogenesis from excised mature embryonic axes and cotyledonary nodes: a) Different stages of shoot development from MEA; b) Fully-developed multiple shoots; c) Longitudinal section of MEA showing development of shoot primordia; d) Multiple shoots from cotyledonary node; e) Developed multiple shoots from cotyledonary node; f) Longitudinal section showing development of multiple shoot primordia; g) Hardening of individual shoot in contained condition; h) Micrografting on stock; i) Development of roots in individual shoot; j) Hardened plants in soilrite pots; and, k) Plantlets in earthen pots transferred to glasshouse.

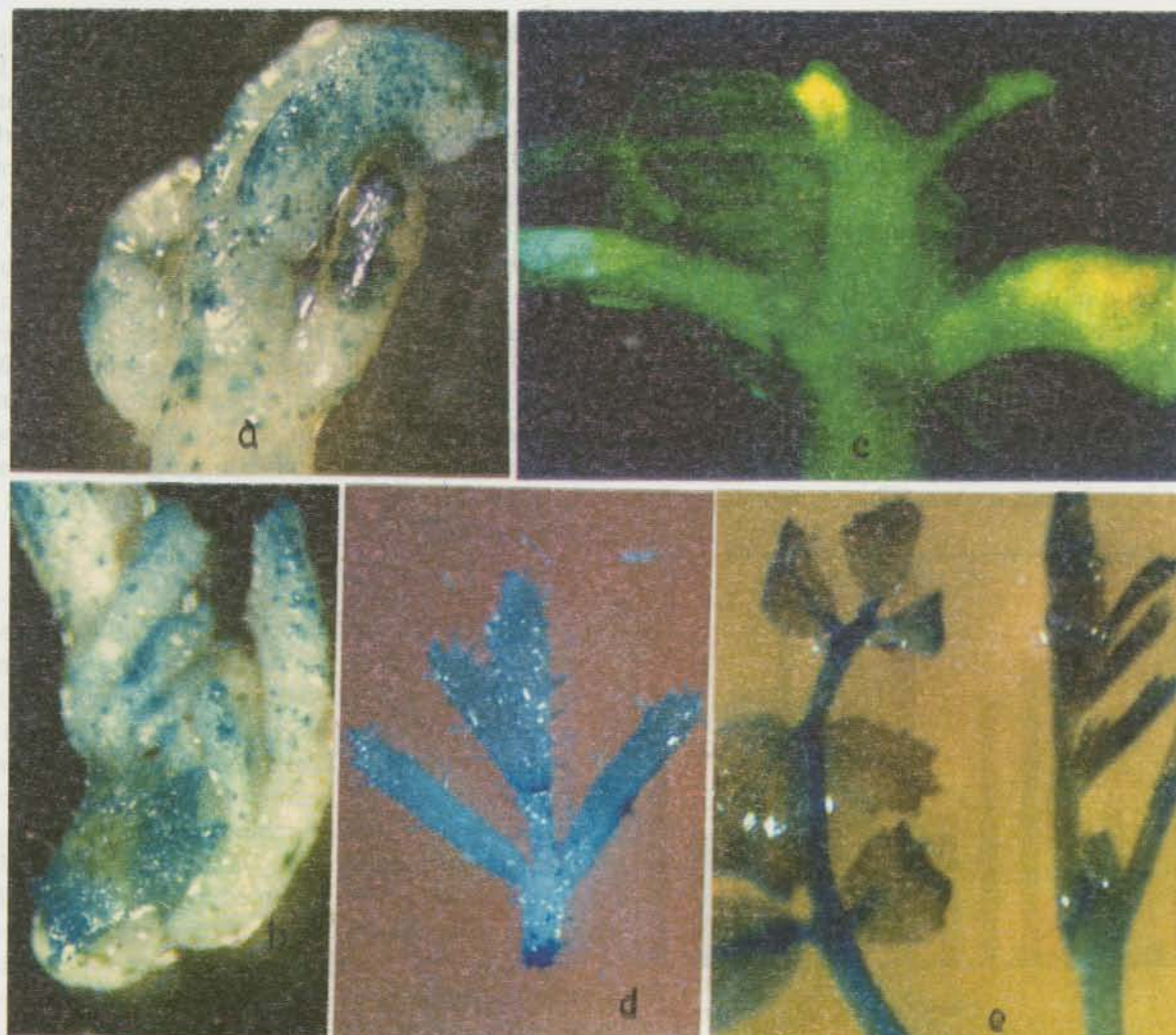


Fig. 3—Histochemical assay of GUS expression in chickpea explants bombarded with gold particles coated with p35SGUS-INT DNA: a) Shoot tip; b) Cotyledonary node; c) Expression of GFP in leaves of putative transformants with pCAMBIA 1303 as seen under UV; and, d) Stable expression of GUS in leaves of transgenic plants transformed with p35SGUS-INT via *Agrobacterium*.

Table 1—Comparative transient GUS expression in different explants of chickpea after bombardment with gold or tungsten microcarriers coated with plasmid DNA

Plasmid Constructs	Microcarriers	Per cent response of explants							
		Leaf		Embry oaxes		Shoot tips		Cotyledonary node	
		1	2	1	2	1	2	1	2
pBI 121	Gold	40±3	21±5	51±3	12±2	61±2	29±5	68±3	23±5
p35SGUSINT	Tungsten	26±3	7±5	27±3	10±2	32±2	17±5	39±3	16±5
	Gold	58±3	19±5	65±3	17±2	62±3	57±5	79±3	39±5
pCAMBIA 1303	Tungsten	37±3	10±5	36±3	8±2	30±3	21±5	56±3	24±5
	Gold	33±3	12±5	43±3	13±2	52±3	32±5	42±3	22±5
	Tungsten	19±3	10±5	27±3	11±2	28±3	13±5	30±3	17±5

1) Average number of responding explants; 2) Average number of GUS foci per responding explant

small plantlets after 2 cycles of selection. However, authors failed to grow Ro putative transformants despite best efforts. The plantlets showing GFP fluorescence also exhibited expression of GUS activity ranging between 1250 to 1676 ± 10 pmol MU per mg protein per min (Fig. 4).

PCR analysis of total genomic DNA isolated from randomly selected seven Ro plants showed amplification of the expected *npt II* and GUS internal fragment of about 700 bp and 530 bp with specific primers in all the plants (Fig. 5). The Southern hybridization of their genomic DNA digested with *Hind III* and *EcoRI* showed strong signals of hybridization in three plants with GUS and *npt II* specific homologous probes. Whereas remaining four

Ro plants did not show intense hybridization signals (Fig. 5), probably due to low amount of total DNA available in these plants for Southern blotting. Considering the significant difficulty in developing high frequency of rooting in transformed Ro plantlets, the transformed shoots were grafted on 8 days old seedling of chickpea and allowed to grow for 20 days before transferring to glass house. A total of 22 plants were transferred into the glass house and only 6 flowered to deliver either 1-2 seeds per plant. Nine F1 seeds were obtained and only three germinated. While other six F1 seeds failed to germinate on kanamycin supplemented medium. The further molecular analysis of F1 population is in progress to reveal the performance of transgene in the R1 and R2 generations.

Discussion

Regeneration in legumes is accomplished mostly by two procedures: i) Shoot proliferation from the region adjacent to pre-existing meristems and; ii) The occurrence of unorganized callus phase giving rise to shoots or embryos (Davey *et al*, 1994). The frequency and consistency of second type of regeneration is extremely low and variable amongst various grain legumes including chickpea (Parrott *et al*, 1992; Davey *et al*, 1994). The present study has modified and utilized the first type of regeneration system in chickpea to establish *Agrobacterium*-mediated transformation using the explants having pre-existing meristematic tissues, which are considered inefficient and recalcitrant to *Agrobacterium* (Matthyse & Gurlitz 1982; McClean *et al*, 1991; McCabe *et al*,

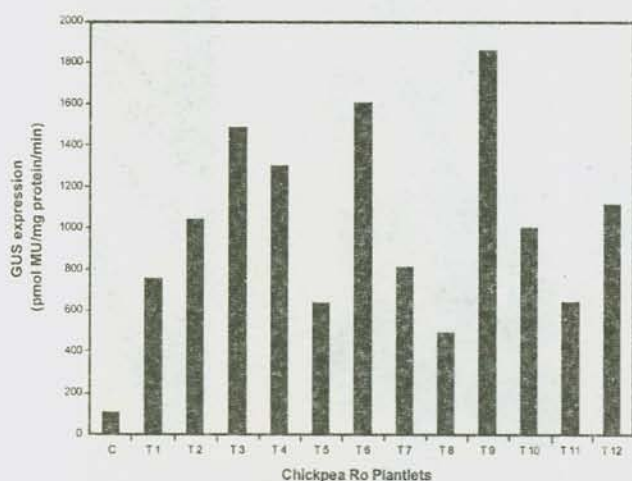


Fig. 4—Quantitative assessment of GUS expression in various primary transformed (Ro) plantlets of chickpea.

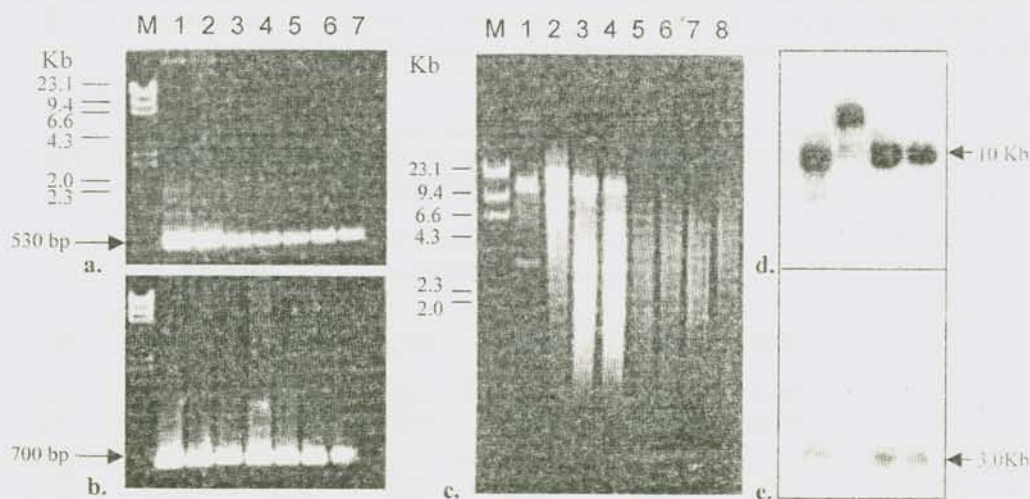


Fig. 5—Molecular analysis of primary transgenic (Ro) chickpea plantlets: PCR amplification of-a) GUS; b) NPT II and their amplicons of 530 and 700 bp respectively; c) Genomic DNA digested with *Hind III* and resolved on 0.8% agarose gel; d) Southern hybridization with 1.8 Kb *Pst I* fragment of pBI 121 as NPT II probe; and, e) 2.06 Kb *Bam HI* and *EcoRI* fragment of pBI 121 as GUS probe.

1988). To facilitate effective interaction of agrobacterial cells with deep seated regenerative tissues in explants like CNs and embryonic axes, the cells of L1 and part of L2 layers were precisely removed to expose the regenerative cells of the meristematic zone (Steeves & Sussex, 1989) having pluripotent stem cells for induction of organogenesis on appropriate cytokinin-supplemented medium (Kartha *et al.*, 1981; Parrott *et al.*, 1992). Removal of L1 and L2 layers has obviously exposed the inaccessible deep-seated organization of potentially regenerative cells to *Agrobacterium*, beside their immediate contact with the shoot induction medium. Perhaps owing to this major limitation, particle bombardment has always been preferred for delivering DNA in grain legumes and other recalcitrant plant species (McCabe *et al.*, 1988; Potrykus, 1991; Hinchey *et al.*, 1994).

The results with particle bombardment of different explants of chickpea have shown significant levels of transient expression of reporter gene GUS, comparable to earlier reports in soybean (Christou *et al.*, 1988; McCabe *et al.*, 1988), pea (Schroeder *et al.*, 1995), and cereals (Wang *et al.*, 1988). The GUS foci induced by gold microcarriers was sharp, well-defined and deep-seated whereas tungsten particles has shown foci of large, irregular shape and mostly confined onto superficial cell layers. Similarly gold particles have induced relatively higher number of GUS spots per explant than tungsten particles. This may be attributed to gold particles being nearly spherical and mostly individual while tungsten particles have an irregular surface and form agglomerates of varying sizes. Owing to these properties, penetration of tungsten particles is relatively restricted in the explants and reflected larger GUS foci of irregular shape over the surface of explants (Hunold *et al.*, 1994).

Results of *Agrobacterium* strains evaluated for cocultivation and agroinoculation of T-DNA has shown that strain EHA 105 reflected maximum transient expression of GUS expression associated with significant necrosis and suppression of organogenesis. This may be attributed to the extra copy of *vir G* gene in strain EHA 105 that eventually induces relatively higher hypersensitive response in the excised tissues of chickpea together with increased release of phenolic compounds which suppresses *in vitro* regeneration (Hood *et al.*, 1993; Meurer *et al.*, 1998).

Molecular characterization of the primary transformants of chickpea showed incorporation of

single copy of GUS and *npt II* gene. These plantlets, however, showed different levels of GUS expression driven by CaMV 35S promoter in leaves and shoots of transformed plants. Expression of cauliflower mosaic virus 35S promoter is often referred as constitutive in all plant tissues, but its expression is subjected to several regulatory elements and may reflect differential expression even in different tissues of the same plant (Benfey & Chua, 1990). The differential expression of GUS in Ro population may be attributed to incorporation of T-DNA at different locations in the individual plant, to the surrounding flanking sequences and copy number. Interestingly, quantitative levels of GUS expression were 2 to 3-folds higher in plants harbouring the *uid A* gene with intron similar to earlier reports (Vancanneyt *et al.*, 1990; Dillen *et al.*, 1997). It was possible to screen out putative transformants of chickpea on the basis of GFP fluorescence and plantlets also showed co-expression of GUS activity. However, these plants could not be grown further perhaps due to *in vivo* accumulation of GFP protein that suppressed further growth and development of plantlets. Toxicity of the GFP protein expressing from native *gfp* gene has been documented in transformed *Arabidopsis thaliana* and tobacco plantlets (Haseloff & Amos, 1995; Rouwendal *et al.*, 1997) and suggested utilization of completely modified *gfp* gene to eliminate the *in vivo* toxicity of the native GFP protein in plants.

With modifications in the preparation of explants, co-cultivation procedures and screening on higher threshold of antibiotics right from beginning, authors have achieved *A. tumefaciens*-mediated transformation in chickpea with relatively higher frequencies (Fontana *et al.*, 1993; Krishnamurthy *et al.*, 2000). This offers a simple and better alternative for delivery of transgene in grain legumes with higher frequency, consistency and low copy number than the biolistic gun (Jaiwal *et al.*, 2001; Hinchey *et al.*, 1994). However, further optimization, is required for recovery of high frequency of fertile stable transgenic plants.

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