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In vitro regeneration and genetic transformation of diverse genotypes of chickpea (*Cicer arietinum* L.)

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Abstract

Lack of an efficient *in vitro* regeneration protocol is a limiting factor for application of genetic engineering approaches for improvement of chickpea. Although regeneration and genetic transformation system has been optimized in few desi genotypes, there is an urgent need to optimize the regeneration and transformation system in kabuli types as well. Four genotypes of chickpeas (two kabuli and two desi) were screened for shoot induction and multiplication. Genotype L550, which produced highly embryogenic, rapidly growing good-quality shoots capable of regenerating at a high frequency, was selected for transformation experiments. Using a binary vector (pBinAr), frequency of GUS expression was studied. Bombardment of embryonic axes with gold particles coated with pBinAr at a distance of 9 cm, pressure of 1300psi, and vacuum of 27mm Hg passing through 100µm mesh produced higher transformation frequency. The stable GUS-expressing embryonic axes were multiplied during selection on MS medium containing 50 mg/l kanamycin, incubated at 16/8hr light/dark. Several transformed plants with very strong GUS expression were recovered using the particle gun mediated method. Transformed shoots were confirmed through polymerase chain reaction and Dot blot analysis. These results demonstrated that *Cicer arietinum* is amenable to particle gun mediated genetic transformation using a binary vector.

Key words: Chickpea, *Cicer arietinum, Kabuli*, organogenesis, particle gun bombardment.

Introduction

Chickpea is the most important grain legume in India with an area of 8.75 million ha, total production of 8.25 million tons and productivity of 945kg/ha. India is the largest producer of chickpea in the world sharing 78.29 and 76.51 percent of the total area (11.55 m ha) and production (10.46 mt), respectively [1]. It is also called poor man's meat because of its high protein content (~20% in the husked grains). However, its production has been almost static in the India over past few decades. One of the main reasons for low yield and lack of stability is susceptibility of chickpea to various biotic and abiotic stresses. Conventional method of chickpea improvement has not been very successful in developing varieties resistance to biotic and abiotic stresses. In recent years, tools of genetic engineering and biotechnology are suggested to address these issues. However, high efficiency of regeneration is pre-requisite for application of genetic engineering in chickpea improvement. Direct shoot organogenesis and establishment of plantlets from different explants of chickpea was reported earlier [2, 3]. Plantlets were also developed through direct somatic embryogenesis and through callus from different explants of chickpea [4]. Few successes are available in desi type of chickpea for regeneration and transformation [5]. However, the success achieved in *kabuli* chickpea is very limited [6] and the recovery of transgenic plants in most of the studies has been very low, limiting their application in the routine genetic transformation studies. Therefore, development of an efficient genetic transformation system is prerequisite for generating successful insect resistant transgenic chickpea. Earlier, embryonic axes and cotyledonary nodes were used for genetic transformation by previous workers [7, 8]. However, the frequency of transformation was not sufficient enough for effective applications. In this study, transformation of chickpea embryonic axes with

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uidA gene constructs (binary vector) followed by kanamycin selection to obtain transformed plants expressing the *uid A* gene is contemplated.

Materials and methods

Shoot induction and plant regeneration

Seeds of two chickpea cultivars (L550, JGK-1) and two desi cultivars DCP 92-3 and C235 were washed thoroughly in Tween 20 (Polyoxyethylene sorbiton monolaurate, MERCK) for 10-15 minutes. The seeds were then rinsed under running tap water and surface sterilized with 10% sodium hypochlorite for 5 minutes followed by repeated washing (5-6 times) with sterile double distilled water. The seeds were soaked in sterile water for 16-18 h. The seed coat was removed and the embryonic axes were used as explant for *in vitro* shoot induction and regeneration. The explants were cultured on the Murashige and Skoog (MS) medium supplemented with different concentrations of NAA, IBA, IAA, BAP and Kinetin. Sucrose (4% w/v) was added to the media as carbon source. The pH of the media was adjusted to 5.8 and solidified with 0.8% agar before autoclaving at 15 psi (pound per square inches) pressure for 15 minutes. All the cultures were maintained at a temperature of 25±1°C under a 16/8 h (light/dark) photoperiod provided by cool white, fluorescent light (3000 lux). Regular subculturing was done at an interval of 15 days. Explants were inoculated for 15 days in medium supplemented with 2mg/l BAP and 0.1mg/l NAA for multiple shoot induction. Explants were further transferred in medium containing 2mg/l BAP + 0.1mg/l NAA+ 1mg/lGA_3 for another 15-20 days. The shoots at least 3 cm long were placed in the rooting medium. After formation of well developed roots, plantlets (4-5 cm long) were taken out from culture tubes, washed thoroughly with tap water to remove the medium and transferred to pre-sterilized potted soil and sand mixture (1:1).

Particle gun mediated genetic transformation

Plasmid preparation

Large-scale plasmid DNA of pBinAr was extracted, purified, and presence of plasmid was confirmed by using restriction analysis. Embryonic axes from overnight soaked seeds were used as an explant. Transformation experiments were carried out using a PDS-1000/He gene gun [9] with gold particles coated with plasmid DNA [10]. Mixed together, 10µl gold particles (60 mg/ml), 20µl plasmid DNA, 25µl $CaCl_2$ solution (2.5 M), and 20µl spermidine solution (0.1M free base) were left to settle for 10 min on ice. The

Table 1. Effect of different genotypes on direct shoot bud induction and multiple shoot formation

Cultivars	Embryonic axes			Embryonic axes slices			Apical shoot meristem			Cotyledonary node		
	Frequency (%) ±SE	Efficiency (mean no. of shoots/explant) ±SE	Range	Frequency (%)±SE	Efficiency (mean no. of shoots/explant) ±SE	Range	Frequency (%)±SE	Efficiency (mean no. of shoots/explant) ±SE	Range	Frequency (%)±SE	Efficiency (mean no. of shoots/explant) ±SE	Range
L550	77.85±0.30	28.89±0.10	26-32	72.50±0.26	19.55±0.08	18-22	68.77±0.37	12.5±0.07	11-15	56.04±0.33	2.08±0.05	2-4
JGK-1	62.02±0.25	4.4±0.02	3-8	60.3±0.19	4.89±0.02	3-6	34.44±0.34	3.6±0.03	3-5	43.92±0.31	0.73±0.01	1-2
DCP92-3	77.89±0.3	15.0±0.06	14-18	78.62±0.29	9.68±0.03	8-12	73.46±0.25	6.86±0.09	6-8	40.79±0.23	1.8±0.01	1-3
C235	77.60±0.19	7.2±0.01	6-8	64.58±0.41	4.68±0.08	4-6	65.37±0.37	5.45±0.03	5-7	45.75±0.49	0.44±0.01	1-2

mixture was centrifuged at 14,000 rpm for 2 min on a microcentrifuge and the supernatant was removed. The pellet was washed twice with ethanol, without resuspending the pellet. Ethanol was removed and the pellet was dissolved in 10µl sterile distilled water by rigorous vortexing.

Bombardment treatment

Ten to 15 explants were used per target plate and bombarded with DNA and gold mixture (10µl per shot) at a distance of 6 cm and 9 cms through a 100µm steel mesh with a helium pulse at 1100 and 1300 psi pressure [11] using *pBinAr* plasmid (CaMV35S promoter-*uidA*). The explants were later transferred to MS medium for 48 h and incubated in dark.

Histochemical assay and selection

The bombarded explants were assayed for transient GUS, β-*glucuronidase*. They were dipped in GUS stain under vacuum for 10 min followed by incubation at 37°C for 48 h. The stain was removed and explants were resuspended in 70% ethanol. The blue spots in each explant were counted under stereomicroscope. Frequency of GUS expression was calculated as the number of GUS expressing explant to the total number of explants stained and expressed as percentage. The explants which were found GUS positive were selected and transferred to MS medium containing 50mg/l Kanamycin. These explants were left on selection medium for 2 weeks and regular subculturing was done after every 15 days.

Regeneration of transgenic plants

Surviving explants on selection medium after 6 weeks were transferred to regeneration medium MS with 2 mg/l BAP + 0.2 mg/lNAA+1.0mg/l GA_3 supplemented with 50mg/l Kanamycin for proliferation. The regenerating shoots were subcultured at 2-week intervals. Leaves of putative transgenic plants were cut into small disc-shaped pieces under aseptic conditions. These leaf discs were dipped in GUS stain under vacuum for 10 min, followed by incubation at 37°C for 48 h [12]. The GUS spots in these leaf discs were also counted under stereomicroscope.

Statistical analysis

The regenerative potentials of genotypes and explants were tested on MS and B5 media supplemented with varying concentrations of auxin and cytokinin in factorial experiment laid down in Completely Randomized Design (CRD). Data were subjected to analysis of variance to test the level of significance among various treatments *viz*., Genotype (G), Hormone (H) and Explant (E) (Table 2). These analyses were further used to estimate significant interaction effects for Genotype x Explant and Genotype x Hormones at 0.5% level of significance. Besides, frequency and efficiency of regeneration were estimated using standard statistical procedures.

Construct used

Agrobacterium strain EHA105 harbouring binary vector pBinAr was used for transformation work. The binary vector pBinAr contains a GUS gene and a neomycin phosphinothricin gene, both under the control of cauliflower mosaic virus (CaMV) 35S constitutive promoter. The T-DNA of pBinAr (13.0 Kb) contains one *EcoR*I site located near the left border region. The *uidA* gene is not expressed in bacteria due to

Table 2. Analysis of variance for direct regeneration using different concentrations of growth hormones in chickpea

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Replication	0.026	4	0.006	0.633	0.639
Genotype	7687.316	3	2562.439	252856.534	0.000
Explant	6906.544	3	2302.181	227174.840	0.000
GR	6150.000	5	1230.000	121374.046	0.000
Genotype * Explant	3740.329	9	415.592	41009.833	0.000
Genotype * GR	3016.813	15	201.121	19846.222	0.000
Explant * GR	3010.012	15	200.667	19801.482	0.000
Genotype * Explant * GR	2454.536	45	54.545	5382.419	0.000
Error	3.851	380	0.010		
Total	50338.341	480			
Corrected Total	32969.427	479			

R Squared = 1.000 (Adjusted R Squared = 1.000)

deletion of the bacterial ribosomal site [13].

PCR Amplification

Genomic DNA was isolated using CTAB method from putatively transformed plants, each obtained by bombardment with pBinAr plasmid. The DNA pellet was dissolved in TE buffer and concentration of the DNA was monitored spectrophotometrically. PCR amplification was carried out with *uidA* (*gus*) gene using genomic DNA from putative transgenic plants, control plants and plasmid DNA as templates. For amplification of *uidA* gene, primers used were 5' GGT GGG AAA GCG CGT TAC AAG 3' (*gus* F) and 5'GTT TAC GCG TTG CTT CCG CCA 3' (*gus* R), and these amplified a 0.2 kb fragment.

Dot blot analysis

Dot blot was done to confirm the presence and integration of *gus* gene in the genome of chickpea. The plant genomic DNA was isolated using CTAB method from leaves of transformed plants as described above. Genomic DNA was denatured in a boiling water bath for 10 minutes and then quickly chilled on ice. The DNA was spotted on nylon membrane (Hi-bond).The DNA were fixed on the membrane by incubating at 130^{o}C for 30 minutes.DIG labelling kit was used (Fermentas). Labelling of probe was done according to the instruction of manufacturer through PCR method. Pre-hybridization of blot was also carried out as instructed by manufacturer of DIG Kit. The hybridization was carried out for overnight at 42^{o}C. After hybridization membrane was washed for 10 minutes in 2X SSC +0.1% SDS solution at room temperature followed by stringent washing with 0.1X SSC+0.1%SDS for 20 minutes at 65^{o}C. The excess liquid was removed from the membrane by placing it on filter paper. After washing, the membrane was incubated in blocking solution for 30 minutes at room temperature with moderate shaking. After blocking in solution the membrane was incubated in detection buffer for colour development using BCIP and Streptavidin-AP- conjugate for 30 minutes at RT in dark. The blue purple precipitate becomes visible after 15-30 minutes of incubation.

Results and discussion

Experiments were undertaken to establish direct organogenesis protocol amenable to genetic transformation through particle bombardment mediated method. The regenerative potentials of genotypes and explant were tested on MS salts and B_5 vitamins supplemented with varying concentrations of auxin and cytokinin. The explants on inoculation to appropriate media gave rise to induction of shoot buds within a week (Fig. 1). Genotypic differences were visible as early as 6-7 days of explant culture, and could be observed through shoot induction response.

Significant differences were also observed among different cultivars for both frequency (no. of explants responded/total no. of explants inoculated) as well as efficiency (mean number of shoots/explants) for four different explants. However, the three genotypes L550, DCP 92-3 and C235 were at par, in case of embryonic axes, with a frequency(%) of 77.85±0.30, 77.89±0.30 and 77.60±0.19 but resulted significant differences in efficiency(shoots/explants) with 26-32 shoots/explant (L550), 14-18 shoots/explant (DCP 92-3), 6-8 shoots/explant (C235) and 3-8 shoots/explant (JGK-1) (Table 1). Genotype DCP 92-3 showed a maximum frequency of 78.62±0.29 in case of embryonic axes slices. Genotype L550 again responded better in case of cotyledonary node with a frequency of 56.04±0.33. However, genotype L550 exhibited maximum efficiency in case of all the four explants *viz.*, embryonic axes (28.89±0.10), embryonic axes slices (19.55±0.08), apical shoot meristem (12.5±0.07) and cotyledonary node (2.08±0.05) followed by DCP 92-3 for embryonic axes (15.0±0.06), embryonic axes slices (9.68±0.03), apical shoot meristem (6.86±0.09) and cotyledonary node (1.8±0.01) (Table 1). When all the cultivars were compared irrespective of the explants used then genotype L550 (68.77±0.31) and DCP 92-3 (67.69±0.27) were at par followed by C235 (63.32±0.35) and JGK-1(50.17±0.27). However, maximum efficiency was again obtained for L550 (15.78±0.07) followed by DCP 92-3 (8.35±005), C235 (4.44±0.02) and JGK-1 (3.40±0.02) (Fig. 1).

It was possible to obtain regeneration levels (>65%) for cultivars L550 and DCP92-3. In contrast, JGK-1 yielded least regeneration frequency (50.17) and did not regenerate on shoot induction medium (Fig. 1). Such variation in the ability to regenerate adventitious shoots from different chickpea cultivars have been reported previously [14]. Genotype oriented responses has also been observed in *V. radiata*, *V. mungo* and *Lens culanaris* [15]; pigeonpea [16]; mungbean [17]; chickpea [18]. Genotypic differences with regard to response to *in vitro* could be due to different genetic background of genotypes being used under present investigation [19]. It was observed that difficulty in regeneration of cultivars when cultured *in vitro* was also linked with the production of phenolics

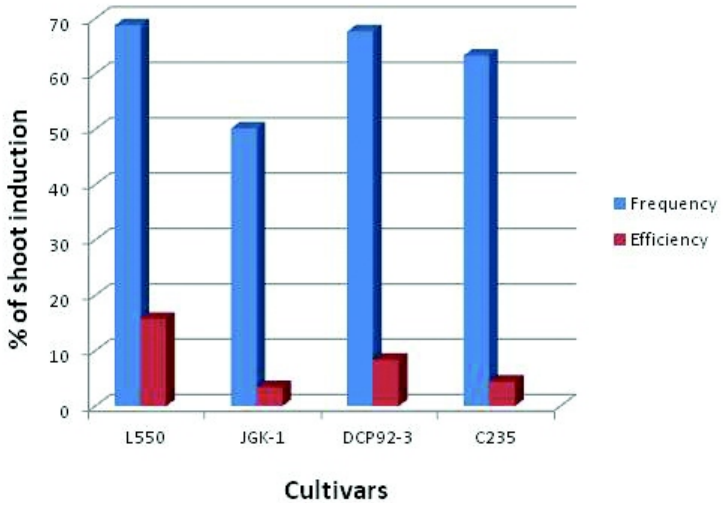

Fig. 1. Response of different cultivars to direct shoot regeneration (%)

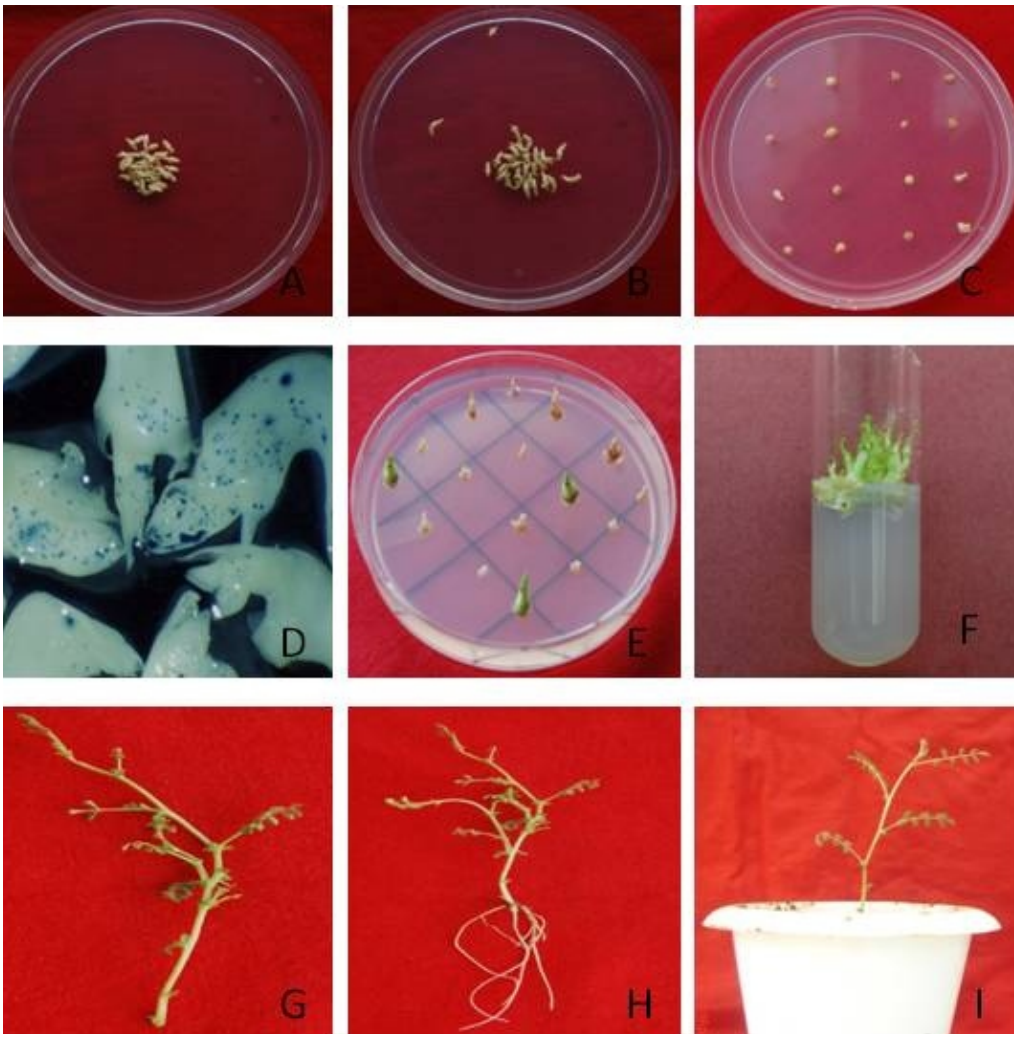

Fig. 2. Microprojectile bombardment mediated transformation in chickpea : A-Explant used for bombardment; B-Explant after bombardment; C-Explant transferred to kanamycin selection; D-GUS assay of bombardment explant; E-Transformed shoots regeneating under selection pressure; F-A separated shoot containing a green pesumptive transformed shoots; G-Elongated shoot; H-Elongated shoot ; I-Hardened *in vitro* shoots

in the media. The highest levels of regeneration 68.79% was obtained from L550 followed by DCP 92-3 (67.69%). This study reports regeneration rate from embryonic axes as high as 77.85% for L550, 77.89% for DCP92-3, 77.60% for C235 and 62.02% for JGK-1 (Table 1), as compared to 40-50% observed for a local cultivar. For cotyledonary node explants, only cultivar L550 responded with a frequency of 56.04% having 1-3 shoots/explant. Similar results were also obtained with genotype B-108 [19].

Analysis of variance showed significant differences among various treatments *viz*., Genotype (G), Hormone (H) and Explant (E). These analyses further showed significant interaction effects for Genotype x Explant and Genotype x Hormones at 0.5% level of significance (Table 2). This regeneration protocol was utilized for optimizing transformation parameters in chickpea. Earlier reports using *Agrobacterium*-mediated gene transfer in chickpea showed a transformation frequency of 0.5-3% [20]. Transformation frequency using particle gun bombardment was also low [21] or not recorded [22]. In many of the transformation experiments, epicotyl was used as the choice of the explants. After bombardment, embryonic axes explants were transferred to kanamycin (50mg/l) enriched medium and *de novo* shoot bud initiation was observed within 15-20 days (Fig. 2E). The well developed shoot buds were further subcultured on MS media supplemented with 2.0mg/l BAP+0.1mg/l NAA +50mg/l kanamycin + 40gm/l sucrose. All the transformed shoots were rooted on medium containing 1mg/l IAA (Fig. 2H). There are also reports of successful rooting in chickpea on MS solid medium containing 2.5 mM IBA and 2.5 mM NAA [23]. High frequency of rooting (80% and 93.3%) were also recorded with media $^1/_4$th MS + 10.4 mM NAA + 2% sucrose and $^1/_4$th MS + 10.7 mM NAA + 1% sucrose, respectively [2]. Besides, direct rooting with different PGR combinations [24] or in PGR free media [25] were reported though with lower frequency in chickpea after prolonged incubation periods. The transformed shoots with fully developed roots were transferred to a green house and grown at 25±1°C with a 16-h photoperiod and light intensity of 400 m mol m^{-2} s^{-1}, where these were acclimatized to progressive declining of relative humidity on soil and sand mixture (1:1), these plants flowered and produced seeds (Fig. 2I).

Effect of helium (He) pressure

Experiments were conducted to optimize different parameters determining transformation frequency. Only embryonic axes were used for direct gene transfer studies using PDS-1000/He bombardment device. The rationale was to enhance the chances of obtaining transformation for both the *gus* and *nptII* genes. DNA coated microparticles must have sufficient velocity to penetrate the cells besides, it should also cause the minimum amount of damage. The He pressure used under experiment was 1100 and 1300psi and there is a clear difference in increase of the number of explants surviving selection pressure in microbombarded embryo axes of chickpea with 1300psi compared to 1100psi bombardment pressure. At rupture disk of 1100psi, a total of 4% transformed shoots were obtained which increased 10% by increasing disk pressure to 1300psi (Table 3).

Effect of DNA concentration

The preparation of the DNA coated microparticles is an important step in particle bombardment and can effect the efficiency of transformation. Apparently, the ratio of DNA particle was very important as number of shoots surviving selection pressure as well as *gus* expression was generally reduced when the precipitation mixture contained higher amounts of DNA. A concentration of 1mg/5ml volume of DNA was found to be optimum resulting in 7% transformation frequency compared to higher DNA concentration (2mg plasmid DNA/5ml volume) which resulted in 3% transformation frequency (Table 3). These results are in agreement with other studies which have examined the effect of particle and plasmid DNA ratio [26]. It was observed that high amounts of DNA tend to form large agglomerates of particles, thereby causing extensive cell damage and reduced foreign gene expression. In chickpea, plasmid DNA at a concentration of 12mg/ml of tungsten particles when accelerated with an inflow of helium gas at 1300psi pressure through a distance of 9 cms in a chamber maintained at a negative pressure of 71.12cm of mercury, resulted in optimal transient expression of the b-*glucuronidase* gene in chickpea embryonic axes.

Effect of target cell distance

To optimize the length of the pathway through which DNA coated gold particles traversed, the plant tissues were placed at two different distances (6 and 9 cms) from the plastic assembly holder. Target cell distance of 9 cm showed a better response and yielded 11% transformation frequency as compared to 5% for target cell distance of 6 cm (Table 3). Similar results were also obtained in pea with 9 cm distance indicating the

Table 3. Effect of different parameters on transformation frequency (%)

Parameters		No. of explants bombarded	Transformation frequency (%)			
			L550	JGK-1	DCP92-3	C235
Rupture disc pressure (psi)	1100	100	3	0	1	0
	1300	100	5	2	2	1
Ratio of DNA: gold particles	1μg/5μl	100	4	1	1	1
	2μg/5μl	100	3	0	0	0
Target distance (cms)	6	100	2	1	1	1
	9	100	4	3	3	1

importance of distance between the target tissue and the stopper plate which may influence the number of cells transformed [27]. In cowpea, maximum *GUS* gene expression occurred when the cultures were initiated one day before the bombardment, adjusting the distance between stopping screen and the target tissue to 9 cm and using the 1100psi rupture disk [28]. It is therefore, sensitivity of target tissue of a plant species is more important in deciding the target cell distance during the process of standardization.

PCR analysis

The integration and presence of gene was confirmed by PCR analysis using *uidA* gus gene primers. PCR analysis results showed that all gus-positive plants produced amplified fragments of 207bp with gus (*uidA*) gene specific primers (Fig. 3), while control plants showed no amplification. These PCR +ve plants were subjected to Dot Blot analysis for further confirmation of true transformants. The advantage of performing dot blot as compared to Southern Blot is that it is simple in operation, quick in identification and avoids the inconvenience of DNA transfer. About 5μg of DNA either isolated from kit or manually was spotted on nylon membrane. The relevant plasmids of 2-10 ng were used as positive controls and DNA from untransformed plants were used as negative control. The membrane spotted with DNA was hybridized with DIG labelled probe of 207 bp digested fragment of *gus*. The labelled probe hybridized only with complementary sequence of *gus* gene present in the spotted DNAs and gave coloured reaction. No purple colour was observed in negative control while very dark and distinct colour was developed in positive control. The PCR positive plants were subjected to Dot Blot analysis and all the plants were found positive in Dot blot assay which further confirmed results obtained through PCR analysis (Fig. 4). The other non-transformed plants failed to give positive signals. Those may be the false transformants (escapes) or they contained the low copy number of gene below dot blot detectable level. Dot blot detection is only sufficient for confirming the presence of foreign gene sequences in transformed plants. Hence, a simple, rapid, high frequency transformation system in chickpea by particle gun bombardment has been demonstrated in the present study. Compared to low transformation frequency in earlier studies using *Agrobacterium*-

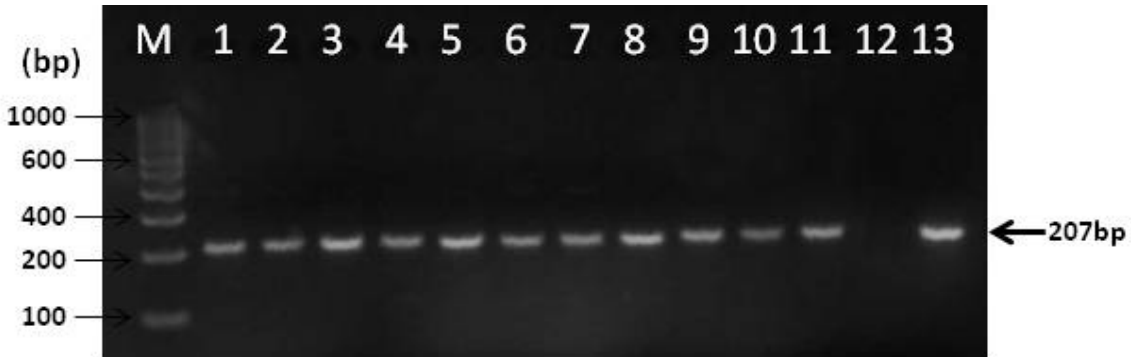

Fig. 3. PCR amplification using *gus* gene specific primers : Lane M : 100 bp ladde, 1-11: tansformed shoots; 12 : non-transformed shoot; 13 : positive control (plasmid DNA)

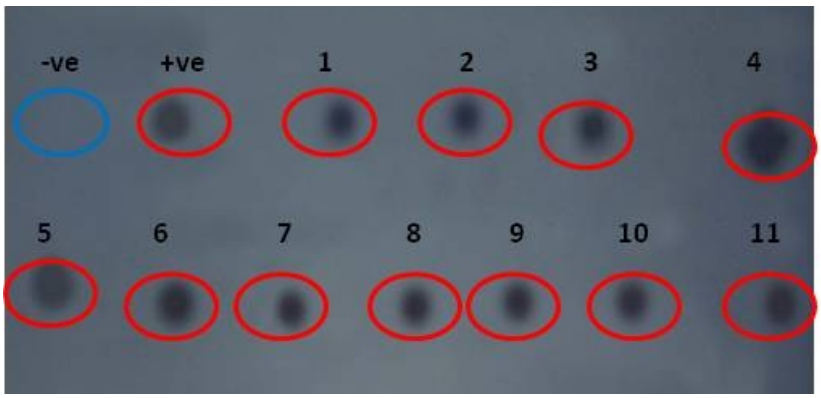

Fig. 4. Dot blot analysis using biotin labeled probes : –ve: negative contol (non-tansformed plant), +ve: control (plasmid DNA), 1-11: PCR positive plants

mediated transformation of chickpea [7], the present study reports a high frequency transformation in *Cicer arietinum* using particle gun bombardment of embryonic axes explants. The development of high frequency plant regeneration and transformation protocol, using a *de novo* regenerable source such as embryonic axes in combination with selected suitable genotype, plasmid type and optimum conditions of particle gun bombardment as employed in the present study will be helpful in genetic modification of chickpea using genes of economic importance.

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