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RESEARCH ARTICLE

Agrobacterium-mediated transformation in chickpea (*Cicer arietinum* L.) with an insecticidal protein gene: optimisation of different factors

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Abstract *Agrobacterium*-mediated transformation in chickpea was developed using strain LBA4404 carrying *npt*II, *uidA* and *cryIAc* genes and transformants selected on Murashige and Skoog's basal medium supplemented with benzyladenine, kinetin and kanamycin. Integration of transgenes was demonstrated using polymerase chain reaction and Southern blot hybridization of T_0 plants. The expression of CryIAc delta endotoxin and GUS enzyme was shown by enzyme linked immunosorbent assay and histochemical assay respectively. The transgenic plants (T_0) showed more tolerance to infection by *Helicoverpa armigera* compared to control plants. Various factors such as explant source, cultivar type, different preculture treatment period of explants, co-cultivation period, acetosyringone supplementation, *Agrobacterium* harboring different plasmids, vacuum infiltration and sonication treatment were tested to study the influence on transformation frequency. The results indicated that use of epicotyl as explant, cultivar ICCC37, *Agrobacterium* harboring plasmid pHS102 as vector, preculture of explant for 48 h, co-cultivation period of 2 days at 25°C and vacuum infiltration for 15 min produced the best transformation results. Sonication treatment of explants with *Agrobacteria* for 80 s was found to increase the frequency of transformation.

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Keywords *Agrobacterium tumefaciens* · Chickpea · *Cicer arietinum* · *cryIAc* gene · Genetic transformation

Abbreviations

BA	Benzyladenine
ELISA	Enzyme Linked Immunosorbent Assay
IBA	Indole butyric acid
Kan	Kanamycin
Kn	Kinetin
MS	Murashige and Skoog's medium
PCR	Polymerase chain reaction
SR	Shoot regeneration medium

Introduction

Agrobacterium-mediated transformation has been used successfully in grain legumes for over a decade. Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes of the tropics and subtropics and is a rich source of dietary proteins. Advances in biotechnology of grain legumes may lead to introduction of novel traits through genetic transformation into chickpea. Although a few reports on *Agrobacterium*-mediated transformation are available in chickpea (Fontana et al. 1993; Kar et al. 1996; Altinkut et al. 1997; Krishnamurthy et al. 2000) the frequency has been low ranging from 0.4 to 4%. Here, we report optimization of conditions for efficient delivery of *Agrobacterium* T-DNA, harboring *cryIAc* gene, along with selectable marker *npt*II and reporter gene *uidA* into chickpea. Complexity of *Agrobacterium* species and labor intensive procedure of cell-culture and difficulties associated with shoot regeneration in some plants still need to be improved for improving transformation frequency. Among factors, Sonication-Assisted *Agrobacterium*-mediated

Transformation (SAAT) (Joersbo and Brunstedt 1992; Trick and Finer 1998; Santarem et al. 1998) and vacuum-infiltration (Charity et al. 2002; Park et al. 2005; Paz et al. 2006) methods have been reported to enhance the efficiency of *Agrobacterium*-mediated transformation of plant species. Hence in the present study, we have tested sonication and vacuum-infiltration of explants to study their influence on transgenic efficiency. Several parameters that influence the *Agrobacterium*-mediated delivery of T-DNA into chickpea like explant type, genotype, preculture period, co-cultivation time, acetosyringone treatment and bacterial cell density were also investigated. Stable transgenic chickpea plants expressing cryIAc protein were established and characterized for protection against pod borer insect—*Helicoverpa armigera*.

Materials and methods

Plant materials and culture initiation

Seeds of chickpea (*Cicer arietinum* L.) cvs. Chafa and PG12 (MPKV, Rahuri), ICCC37 and ICCC32 (ICRISAT, Patencheru, AP) were used for the experiments. Cultivar-ICCC37 was used for all experiments, except one experiment where different cultivars were compared. Seeds were sterilized with 70% ethanol for 30 s followed by 0.1% mercuric chloride (w/v) for 5 min. The sterilized seeds were rinsed 5 times with sterile water and inoculated on Murashige and Skoog's (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.8% agar and incubated in 50 μmol/m^2/s light with 16/8- h photoperiod. For studying the influence of different explants on transformation, mature embryonal explants, stem and epicotyl explants were used. Mature zygotic embryonic axes were dissected out from overnight soaked, sterilized seeds, their meristematic regions excised and embryonic axes used as explants for experiments. Other explants such as epicotyls and stem explants were excised form seven day old axenically grown plants cultured on MS medium solidified with 0.8% agar. Epicotyl explants were used for all experiments, except the one where different explants were compared.

Agrobacterium strain and plasmids

To study the influence of different *Agrobacterium* strains, disarmed *Agrobacterium tumefaciens* strain LBA4404 harboring three plasmids pHS101, pHS102 (Kamble et al. 2003) and pBI121 (Clonetech, USA) were used for transformation experiments. All the three plasmids used were binary vectors containing selectable marker gene *npt*II and reporter gene *uidA* driven by CaMV35S promoter. In addition to this, pHS101 and pHS102 also possess *cryIAc* gene from *Bacillus thuringiensis* for insect resistance. The plasmid pHS101, in addition, contains a waxy locus from maize under the control of tandem 35S promoter.

Agrobacterium tumefaciens strain LBA4404 carrying pHS101, pHS102 and pBI121 were used for studying the influence of different plasmids on transformation. For all other experiments *Agrobacterium* with pBI121 was used. Glycerol stock of each *Agrobacterium* culture was thawed and then streaked onto solid YEP medium (An et al. 1988) with kanamycin 50 μg/mL and rifampicin 50 μg/mL. A single bacterial colony of each strain was inoculated into 2 mL of liquid YEP medium with appropriate antibiotics and grown overnight at 28°C on a rotary shaker at 180 rpm, until an optical density of 0.5 at 600 nm (OD_{600}) was reached. 20 μL of each bacterial suspension was added to 20 mL of their respective medium and grown overnight. These overnight grown cultures at a density of 5×10^8 cells per mL (OD_{600}=1) were used for transformation experiments.

Co-cultivation of explants with *Agrobacterium*

Epicotyls from 7 day old seedlings of chickpea precultured on shoot regeneration (SR) medium {MS+benzyladenine [BA] (0.5 mg/L)+kinetin [Kn] (0.1 mg/L)} for 48 h were wounded with a sterile needle and co-cultivated with overnight grown bacterial culture with infection time of 20 min. Preconditioned explants were incubated with *Agrobacterium tumefaciens* suspension of OD_{600} between 0.8 to 1.0. *Agrobacterium* cultures were pre-induced with 100 μM of acetosyringone, half an hour before use. Co-cultivated explants were blotted dry on sterile filter paper sheets to remove the excess bacteria and placed horizontally on shoot regeneration medium. Co-cultivation was carried out for 2 days by incubating the cultures at 16 h/8 h light/dark photoperiod at 25±2°C.

Selection and maintenance of transformants

Explants co-cultivated for 2 days were transferred to selection medium. The composition of selection medium was same as shoot regeneration medium, but additionally containing cefotaxime (500 mg/L) and 50 mg/L kanamycin (Kan). After 15 days, explants were transferred onto fresh selection medium of the same composition but with Kan increased to75 mg/L and cefotaxime concentration reduced to 250 mg/L. In subsequent subcultures, cefotaxime was completely omitted from the selection medium, but Kan maintained at 75 mg/L concentration. Cultures were maintained by transferring them to fresh medium at regular intervals at 25–30 days. Maintenance of cultures with 75 mg/L Kan was done to eliminate the possibility of escapes. Each subculture involved the elimination of

explants which turned brown and selection of only healthy green shoots, which were subsequently maintained for 5–6 passages on selection medium. Putatively transformed shoots were transferred to rooting medium {MS+indole butyric acid [IBA] (0.5 mg/L)+1% sucrose+0.8% agar} and plantlets were hardened for transferring to greenhouse. Hardened plantlets were further subjected to molecular analysis for confirmation of transformation.

Histochemical assay

Expression of β-d-Glucuronidase (*gus*/*uidA*) gene in chickpea transformants was assayed as described by Stomp (1992) with 5-bromo-4-chloro-3-indolyl-β–D-glucuronide (X-Gluc) as a substrate (Jefferson 1987). To analyze the transient expression, explants cultured for 48 h on selection medium with respective co-cultivation conditions were subjected to GUS assay. The explants were processed for histochemical localization by incubating sliced explants in a mixture of potassium ferricyanide (50 mM), potassium ferrocyanide (50 mM), 5-bromo-4 -chloro-3-indolyl-β-D-glucuronide (0.3%), sodium phosphate buffer (0.2 M, pH 7.0) and triton X-100 at 37°C overnight. The tissues were treated with 70% ethanol for a few hours before observation.

PCR amplification and southern blot analysis

Genomic DNA was isolated from 50 randomly selected putatively transformed plants each obtained by *Agrobacterium* mediated transformation with pBI121, pHS101 and pHS102 plasmids using the method of Dellaporta et al. (1983). The DNA pellet was dissolved in TE buffer and concentration of the DNA was monitored spectrophotometrically. PCR amplification was carried out with gene specific primers for *npt*II, *cryIAc* and *uidA/gus* genes using genomic DNA from fifty 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 1.4 kb fragment. For amplification of *npt*II gene, primers used were 5′ GAG GCT ATT CGG CTA TGA CTG 3′ (*npt*II F) and 5′ ATC GGG AGC GGC GAT ACC GTA 3′ (*npt*II R), which amplified a 0.7 kb fragment. For *cryIAc* amplification, primers used were 5′ ATG GAT AAC AAT CCG AAC ATC AAA GA 3′ (*cryIAc* F) and 5′ TTA TTA GCC CTA GTT GGT TTG TAC A 3′ (*cryIAc* R), which amplified a 2 kb fragment.

Genomic DNA was extracted from randomly selected T_0 plants (using pHS102 plasmid) using the protocol as described earlier (Dellaporta et al. 1983). 10 μg of DNA was digested with *Hind*III and DNA fragments separated on 0.8% agarose gel. The separated DNA fragments were blotted onto positively charged nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, Sweden). Probes were labeled using Dig-DNA labeling kit. Pre-hybridization, hybridization, washing and detection were carried out using chemiluminescent detection system as per kit manufacturer's protocols (Roche Biochemicals, Germany).

Immunological assay

Enzyme linked immunosorbent assay (ELISA) was performed using Desigen Quan T–ELISA-96 well plate kit for quantitative estimation of CryIAc protein (Desigen, Jalna, Maharashtra). Sample preparation was done by macerating 5 mg of leaf tissue in 500 μL of sample extraction buffer as per kit protocol. Samples were chilled and spun at 7800*g* for 15 min and supernatant pipetted out for loading. For the estimation of CryIAc, the 96 well titre plate was coated with 150 μL per well (1: 1000) of goat anti-CryIAc antibodies. Plate was then loaded with 100 μL samples and buffer was used in control wells. Plate was incubated at 37°C for 1.5 h followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1: 1000 with 250 μL per well for 45 min at 37°C. Plate was then washed with wash buffer twice and 250 μL of freshly prepared substrate (p-nitrophenyl phosphate, 1 mg/mL) was added per well. Plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a microplate reader (Biotek Instuments, Inc.).

Insect bioassay

Entomocidal activity of the toxin CryIAc expressed in the tissues of chickpea transformants was assayed through insect feeding bioassay. Detached leaf feeding tests were done using the third instar larvae of insect—*Helicoverpa armigera*. Larvae of *H. armigera* were initially reared in laboratory at 27±1°C on young castor leaves. About 500 mg of fresh leaves from transgenic and control plants were kept in small glass beakers with moist filter paper disc. Five larvae were released in each beaker. Beakers were sealed with moist cloth to prevent desiccation of leaves and kept in the insect rearing room at 27±1°C, 16 h photoperiod and 70% relative humidity. Feeding was allowed for four days with one change of fresh leaves on alternate days. Data were taken on larval weight, survival and mortality. Each treatment was done with three replicates and repeated twice and data analyzed using ANOVA.

Factors influencing *Agrobacterium*-mediated transformation

For studying the influence of different factors on *Agrobacterium*—mediated gene transfer, epicotyl explants of

chickpea cv. ICCC37 were treated with *Agrobacterium tumefaciens* harboring pBI121 except for specific experiments. Both transient expression (GUS) assay and stable transformation based on number of shoots growing on Kan (75 mg/L) at the end of second passage (60 days) of incubation were assayed. To study the influence of explants on transformation, different explants such as embryonic axis, epicotyl and stem explants of cv. ICCC37 were used. Effect of different periods of preculture treatment such as 24 h, 48 h, 72 h and 96 h before *Agrobacterium* treatment on transient GUS assay using epicotyls of chickpea was determined. To study the influence of *Agrobacterium* infection period on transformation, epicotyl explants were wounded with a sterile needle and infected with an overnight grown bacterial culture of *Agrobacterium* harboring pBI121 preinduced with acetosyringone (100 μM) and incubated in bacterial medium for 5, 10, 15, 20, 25 and 30 min. Co-cultivation of epicotyl explants was carried out on shoot regeneration medium for 1, 2, 3 and 4 days. To study the influence of acetosyringone, various concentrations of acetosyringone 50, 100, 200 and 300 μM were added to the bacterial culture medium half an hour prior to infection of the explants.

To study the effect of vacuum infiltration on transformation, vacuum-infiltration was carried out by using precultured (48 h on SR medium) epicotyls of chickpea. On the day of treatment, explants were transferred to sterile 1.5 mL microcentrifuge tubes containing 500 μL of liquid SR medium. When all explants were prepared, SR medium was removed using a micro-pipette and replaced with 500 μL of *Agrobacterium tumefaciens* (pBI121). In the case of controls, SR medium remained as such without replacement with bacterial culture medium. Explants kept in open-capped 1.5 mL microfuge tubes were vacuum infiltrated in the bacterial suspension (24 in Hg) for different periods such as 5, 10, 15 and 20 min. After treatment, explants were washed by pouring 200 mL of liquid SR medium. They were then blotted dry on sterile paper towels and transferred to SR medium for 2 days prior to transferring to selection medium.

To study the influence of sonication on transformation, epicotyl explants were placed in 1.5 mL microcentrifuge tubes containing 0.5 mL of *Agrobacterium* suspension (pBI121). Explants were gently resuspended and placed in a float at the center of an ultrasonic bath (Model No. TEC 40, Roop Telesonic Ultrasonix, Mumbai, India) and working frequency was 33 KHz. Epicotyls were sonicated for 0, 20, 40, 60, 80, 100 and 120 s using *Agrobacterium* strain pBI121 at 1 OD_{600nm} and transient expression levels and stable transformation frequency recorded.

Statistical analysis

All experiments were carried out using completely randomized design (CRD). Values reported are mean of three replicates and each replicate consisted of 50 explants. All data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) was calculated to find out significance among means of the treatments (Gomez and Gomez 1984), using IRRISTAT software (IRRI 2003). In all tables and figures (presented in the "Results" section), means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$).

Results

Regeneration of putative transgenic plants from epicotyl explants of chickpea

Epicotyl explants of cv. ICCC37 treated with *Agrobacterium* strain with pHS102 and pHS101 were grown on selection medium with 50 mg/L Kan. After 15 days of culture, explants with green shoot primordia were subcultured in the selection medium with Kan (75 mg/L) for second round of selection. Final selection of transformed shoots was carried out by two more subcultures in fresh selection medium with 75 mg/L Kan. These shoots elongated in the same culture medium and could be rooted upon transfer to rooting medium. The negative control did not show any regeneration of shoots in kanamycin supplemented medium, while in a medium without kanamycin, all control explants produced shoots. After 15 days of shoot formation, some of the putative chickpea shoots were excised from each culture and tested for GUS activity and they showed blue color, indicating that the putative transformed plants are transgenic, since both the vectors (pHS101, pHS102) used in these studies contained *uidA* gene, while the leaves and shoots from negative control did not show any blue color.

Over 70% of shoots regenerated in presence of kanamycin were rooted on the MS medium after pulsing with indole butyric acid (10 mM) for 30 s (Fig. 1a–f).

Molecular analysis of T_0 transgenic chickpea plants

The genomic DNA isolated from fifty primary transformants (T_0) transformed with pHS102, along with a control plant was used for PCR analysis using *npt*II, *uidA* and *cryIAc* primers for amplification of DNA fragments of 0.7 kb, 1.4 kb and 2.0 kb respectively. Results revealed that all transgenic chickpea plants tested were positive for all the three genes. Control (non transformed) plants failed to show any amplified fragments of the expected size with gene specific primers (Fig. 2a–c).

Southern hybridization analysis performed to confirm the stable integration of these genes in the chromosome of kanamycin resistant chickpea plants obtained through independent transformation events was shown in this study using *cryIAc* gene probe (Fig. 3), *npt*II and *uidA* probes

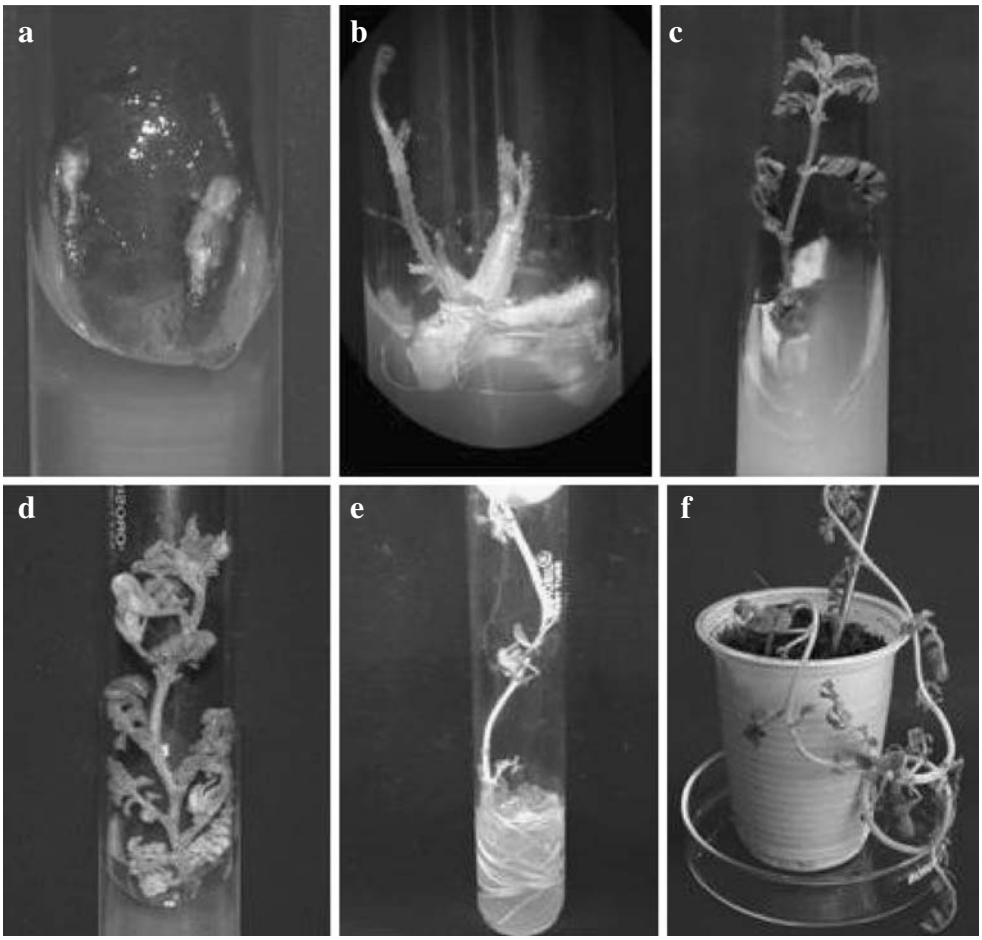

Fig. 1 **a–f** Development of transgenic chickpea plants via *Agrobacterium*-mediated transformation (LBA 4404 harboring pHS102) using epicotyls **a** Non transformed explant on SR medium+Kan 50 mgl^{-1}. **b** Initiation of shoot buds from epicotyls cultured on selection medium {SR medium+Kan (50 mgl^{-1})}. **c** Well developed shoots with leaves on SR+Kan 50 mgl^{-1}. **d** In vitro flowering in shoots. **e** Putatively transformed rooted plant. **f** Putatively transformed plants transferred in paper cup 3 weeks after transfer

(data not shown). Southern hybridization of T_0 with homologous probe for *cryIAc* from plasmid pHS102 showed integration of *cryIAc* gene in these plants, while non-transformed plants did not show any band hybridizing with the probe (Fig. 3). Southern blot analysis clearly demonstrated the integration of the transgene into the genome of chickpea.

Expression of *cryIAc* in transgenic plants

Expression of *cryIAc* in transgenic chickpea plants was performed by ELISA. Selected 5 plants were subjected to immunological assay. The amount of CryIAc protein amongst T_0 chickpea plants varied from 4 to 21 ng/mg of total soluble protein. Results indicated that three T_0 plants showed high levels of endotoxin expression ranging from 15–21 ng/mg of soluble protein (Fig. 4). Remaining plants exhibited moderate levels of CryIAc expression. Quantitative ELISA indicated efficient expression of CryIAc in transgenic chickpea plants.

Insecticidal activity

All the confirmed positive transgenic plants were subjected to feeding by larvae of 3rd instar of the insect *H. armigera*. Mortality and loss in weight/retardation in growth were recorded for assessing the effect of the protein on the larvae. Experiments were conducted along with a non-transformed chickpea plant for comparison. Highest mortality found in transgenic chickpea plants was 76%. Total five independent confirmed transgenic plants selected after testing for resistance for *H. armigera* manifested a range of response, which might be due to the differences in the expression of levels of the *cry* gene (Table 1). Leaves from non-transformed plants were damaged completely within 24 h after releasing the larvae. The larvae fed on leaves of transgenic chickpea plants showed severe stunted growth when compared to larvae fed on control leaf. These results indicated the expression of the transferred gene and its effectiveness in controlling larvae of *H. armigera* (Fig. 5).

Factors influencing *Agrobacterium*-mediated transformation

When three different explants of chickpea namely embryonic axes, epicotyls and stem explants were tested for transformation, epicotyls showed the highest % of stable transformation compared to embryonic axes and stem explants (Table 2). When different cultivars such as ICCC37,

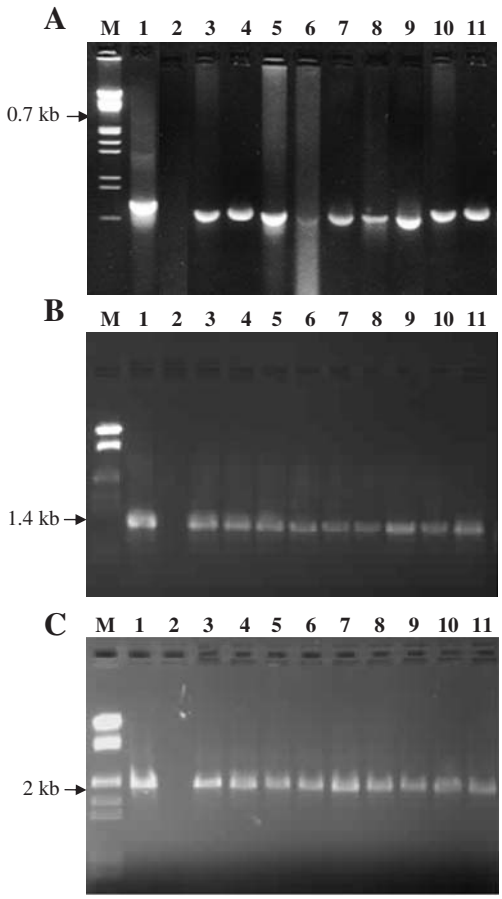

Fig. 2 Detection of transgenes in genetically transformed plants of chickpea. PCR amplification was carried out by using the genomic DNA of control (lane 2), transformants (lanes 3–11) and plasmid DNA (lane 1) as positive control, with gene specific primers for *npt*II (A), *uidA* (B), *cryIAc* (C), {M- λ marker *Hind*III/*Eco*RI double digest}

ICCC32, Chafa and PG-12 were screened for their susceptibility for *Agrobacterium*-mediated transformation, cvs. ICCC37 and PG-12 produced the highest % GUS expression, followed by cvs. Chafa and ICCC32 (Table 3).

Preculturing of explants in shoot regeneration medium had a positive effect on transformation efficiency. Explants

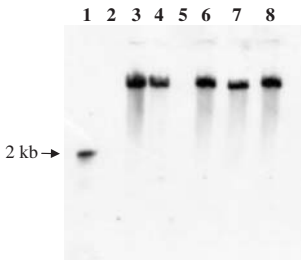

Fig. 3 Detection of *cryIAc* gene in genetically transformed plants of chickpea by Southern blot analysis. Genomic DNA of control plant (lane 2 and 5); transformants (lanes 3,4,6,7,8) digested with *Hind*III and hybridized with *cryIAc* probe and compared with PCR product of *cryIAc* gene (lane 1) as positive control

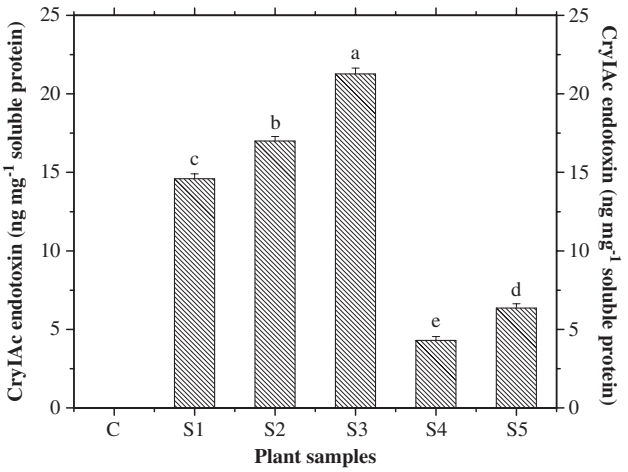

Fig. 4 Quantitative estimation of expression level of CryIAc protein in different transgenic chickpea plants (S1, S2, S3, S4, S5—five plant samples confirmed by Southern analysis were analyzed by immunological assay)

preconditioned for 48 h before co-cultivation produced the highest % of transient expression and stable transformation using epicotyls. Preculture treatment lower or higher than 48 h resulted in a decrease in % GUS response as well as percentage of stable transformation (Fig. 6a). We evaluated the effect of different bacterial infection periods on transformation efficiency of chickpea. Twenty minutes of incubation of epicotyl explants of chickpea with *Agrobacterium tumefaciens* culture resulted in highest % GUS expression and % stable transformation (Fig. 6b), followed by 15 min incubation. Duration of co-cultivation was also an important factor for improving the efficiency of transformation. Highest % of GUS expression and % stable transformation was obtained at 2 days of co-cultivation using epicotyls (Fig. 6c). Extending the co-cultivation up to 2 days increased the transient transformation frequency and subsequently, further increase in co-culture time decreased the transformation frequency resulting in bacterial overgrowth (Fig. 6c).

The influence of addition of acetosyringone at various concentrations (0–300 μM) into bacterial culture medium half an hour prior to infection of explants was analyzed for transient expression efficiency and also for regeneration. Acetosyringone at all concentrations increased the efficiency of T-DNA delivery in terms of the number of explants displaying expression of *uidA* gene compared to control (Fig. 6d). Highest % GUS expression using epicotyls of chickpea was obtained by supplementation with 100 μM acetosyringone. Acetosyringone at higher and lower concentrations showed a decline in % of GUS expression. The regeneration frequency of epicotyls declined with increase in concentration of acetosyringone (Fig. 6d).

A serial dilution was used in inoculation to test the effect of bacterial cell density on transformation frequency in

Table 1 Insect bioassay with *Helicoverpa armigera* third instar larvae on leaf feeding assay on transgenic T_0 plants of chickpea

Plant no.	Mean initial wt.(mg)±SE	Mean final wt. (mg)±SE	% survival±SE	Wt. Loss of control (%)±SE
Control	7.14±0.06[a]	55.0±0.57[a]	100±0.0[a]	Nil
Sample 1	7.11±0.069[a]	20.82±0.59[d]	33.3±3.33[cd]	62.26±1.22[c]
Sample 2	6.82±0.066[a]	18.47±0.22[e]	40.0±5.7[c]	66.37±0.55[b]
Sample 3	6.87±0.008[a]	13.6±0.48[f]	23.33±3.31[d]	75.34±0.62[a]
Sample 4	6.92±0.03[a]	29.1±0.55[b]	50.0±2.25[b]	46.7±0.65[e]
Sample 5	7.23±0.20[a]	23.86±0.10[c]	36.6±3.33[cd]	56.41±0.42[d]

Mean of 3 replicates, with each replicate consisting of 5 insects

Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

chickpea. The concentration of *Agrobacterium* density in the co-cultivation suspension with chickpea explants revealed that optimal density of bacterial culture was an OD of 1.0 at 600 nm, which produced maximum GUS expression. Increasing or decreasing the density caused a substantial decrease in the number of GUS expressing spots (Fig. 7a). OD values of bacterial culture at 1.2, 1.4 and 0.8 produced 55–70% GUS expression.

Vacuum-infiltration was tested as a means to increase gene transfer efficiency by improving penetration of *Agrobacterium* into the cell layers beneath the epidermis of plant tissue. Results showed that 15 min of vacuum treatment of epicotyls in the presence of *Agrobacterium* produced the highest percentage of GUS positive explants and stable transformation. Explants treated for periods longer or lesser than 15 min decreased the % GUS expression and % stable transformation (Fig. 7b). In all tissues tested, the sonication treatment significantly enhanced the levels of transient expression. When epicotyl explants were treated with *Agrobacterium* without sonication, percentage explants displaying GUS expression was relatively lower than the sonication treated explants. Although tissues responded to a wide range of treatment durations (Fig. 7c), the tissue was often damaged by longer sonication treatments and the regeneration response was reduced. Results showed that sonication treatment at 80 s to pre-conditioned epicotyl explants resulted in maximum level of GUS expression (Fig. 7c). The regeneration % declined with increase in period of sonication treatment.

Agrobacterium tumefaciens harboring three different plasmids namely pBI121, pHS102 and pHS101 when tested for co-cultivation of epicotyl explants of chickpea

Fig. 5 Insect bioassay of chickpea transformants using *Helicoverpa armigera*. **a** Larvae showing normal growth on control samples; **b** Treatment showing larvae fed on transformants; **c** Larval mortality on transgenic shoots; **d** Early pupation of larvae inflicted by endotoxin stress

Table 2 Influence of different chickpea explants on transformation

Explants	% GUS expression±SE	% Stable transformation±SE
Embryonic axes	78.07±0.50[a]	15.08±0.08[b]
Epicotyls	74.96±1.93[a]	15.92±0.057[a]
Stems	21.97±0.72[b]	8.77±0.17[c]

Mean of 3 replicates, with each replicate consisting of 50 explants

Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

cv. ICCC37, showed a variation in *uidA* expression. *Agrobacterium* with pBI121 and pHS102 produced higher % of GUS expression compared to pHS101, while pHS102 produced the highest frequency of stable transformation (24%) compared to pBI121 and pHS101 (Table 4).

Discussion

Although a few crop legumes have been tried for the production of transgenic plants using different methods of plant genetic transformation, most of the methods have reflected limitations for efficient production of transformed plants. There is an urge to improve *Agrobacterium tumefaciens* mediated grain legume transformation to circumvent these limitations and for development of stable transgenic plants. Efficiency of *Agrobacterium*-mediated transformation and delivery of T-DNA into plant cells is influenced by several physico-chemical and physiological conditions. Present study focuses on the optimization of mentioned conditions in chickpea transformation with special reference to introduction of *cryIAc* gene. Transient expression of *uid*A (GUS) marker gene to assess and optimize *Agrobacterium*-mediated delivery was done 48 h after experiment and % stable transformation scored at the end of 40 days.

On the basis of maximum GUS positive response, epicotyls and embryonic axes were found to be the explants of choice for transformation of chickpea. Most of the earlier reports on *Agrobacterium*- mediated transformation in chickpea have used embryonic axes as the explants of choice (Fontana et al. 1993; Krishnamurthy et al. 2000; Polowick et al. 2004; Sarmah et al. 2004; Tewari-Singh et al. 2004). Leaf and stem explants have also been used (Srinivasan et al. 1991). In the present study, use of epicotyl as explant of choice for *Agrobacterium*-mediated transformation in chickpea has shown that it is an efficient and novel transformation system. *A. tumefaciens* differs in its ability to infect different species and different genotypes of plants. Generally, the specificity of genotype is related to the cell physiological conditions, which include cell physiological reaction after wounding, concentration of cell internal hormone and structure of cell wall. It was likely that for these reasons chickpea cv. ICCC37 displayed better response compared to other genotypes, endowing higher transformation efficiency. Similarly, genotypic influence on transformation efficiency in chickpea has been demonstrated by previous workers (Kar et al. 1996; Krishnamurthy et al. 2000; Senthil et al. 2004; Tewari-Singh et al. 2004). Preconditioning of epicotyl explants on SR medium played an important role in increasing the transformation frequency, with 48 h of preculture giving the best transformation frequency.

In the present study using chickpea, a co-cultivation period for 2 days produced the highest transformation frequency, while co-cultivation for 1, 3 and 4 days decreased the transformation frequency. *Agrobacterium* living in the wound of plant for a minimum period only can transfer its T-DNA for integration. Therefore, too short a co-cultivation period is not favorable for transformation. However, too long a co-cultivation period result in over-growth of *Agrobacterium* and therefore it is harmful to the plant cells. Role of co-cultivation period correlating with transformation frequencies have been reported by earlier workers (Fontana et al. 1993; Krishnamurthy et al. 2000; Sarmah et al. 2004; Tewari-Singh et al. 2004). Previous reports that 48 h of co-cultivation is optimal period for chickpea transformation (Srinivasan et al. 1991; Sanyal et al. 2003, 2005) is in agreement with the present study. In the present study, 20 min of infection with *Agrobacterium* was found to be best for transformation experiments using epicotyls. Long-term explant suspension beyond 20 min in the liquid infection medium inhibited the growth of chickpea explants. Infection time influencing the frequency of transformation has been already demonstrated in soybean (Liu et al. 2004; Ko and Korban 2004). Infection periods of 5–10 min for rice and 5–30 min for soybean transformation have also been used (Ke et al. 2001).

Acetosyringone, a plant phenolic compound naturally secreted by wounded plant cells is known to act as an inducer of virulence (*vir*) genes of *Agrobacterium* (Stachel

Table 3 Influence of different cultivars of chickpea on *Agrobacterium*-mediated transformation using epicotyls

Cultivar	% GUS expression±SE	% Stable transformation±SE
ICCC32	28.80±0.14[c]	7.8±0.41[d]
Chafa	45.59±0.04[b]	9.03±0.04[c]
PG-12	70.83±0.67[a]	14.89±0.05[b]
ICCC37	72.30±1.27[a]	16.11±0.08[a]

Mean of 3 replicates, with each replicate consisting of 50 explants

Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

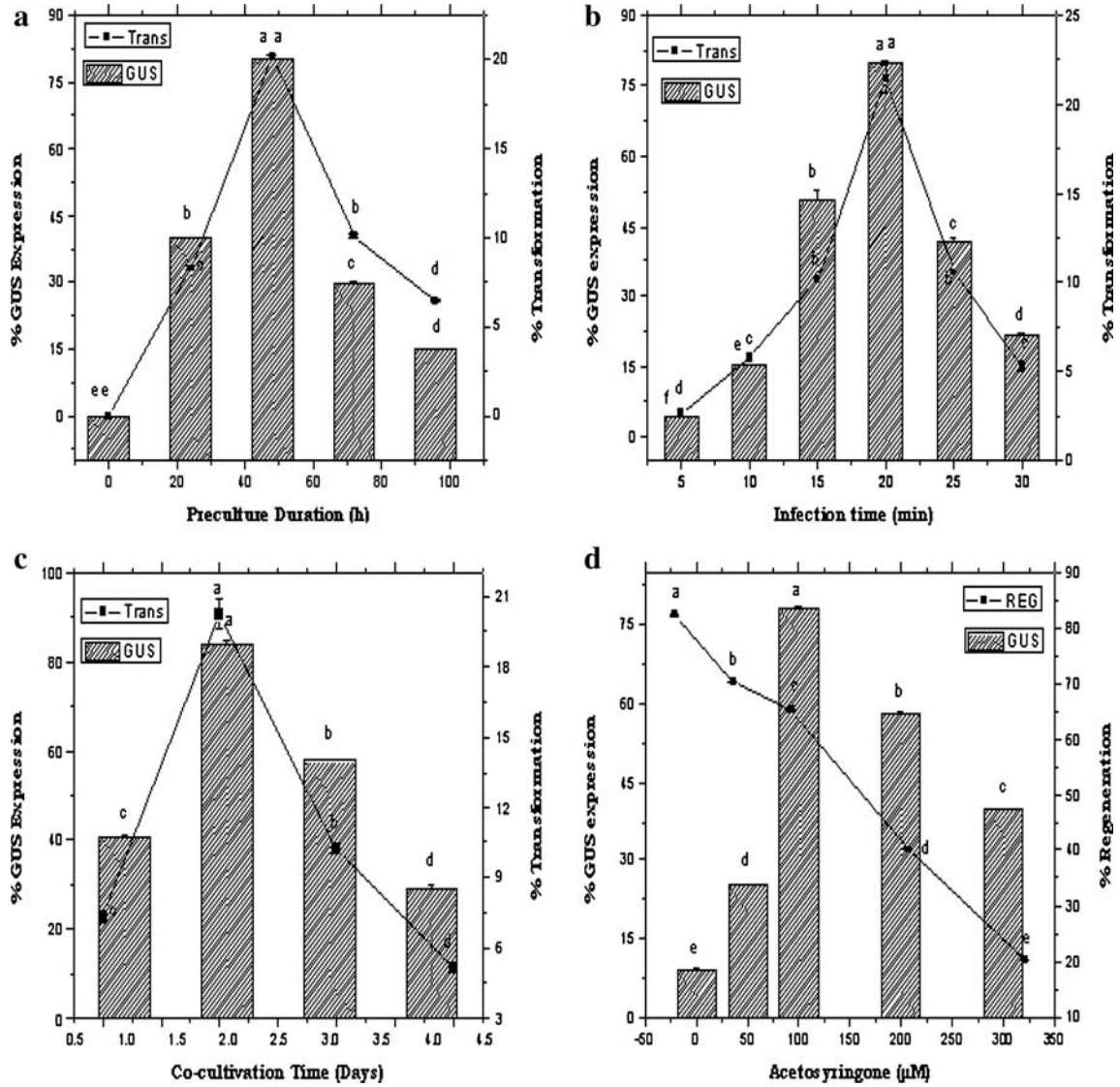

Fig. 6 Response of different parameters on the transformation efficiency using chickpea cv ICCC37 as evident of GUS expression and in vitro regeneration*: **a** influence of preculture duration on transformation; **b** effect of *Agrobacterium* infection period on transformation; **c** effect of co-cultivation time on chickpea transformation; **d** effect of different concentrations of acetosyringone on expression of GUS and shoot regeneration. *Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

et al. 1985). Acetosyringone (100 µM) was utilized based on preliminary studies of transient X-Gluc staining at the end of co-cultivation. In the presence of acetosyringone, there was extensive blue coloration in the explants, while in the absence of acetosyringone, X-Gluc staining was comparatively less in chickpea explants. The present observation that addition of acetosyringone improved the transient expression efficiency in chickpea is in agreement with the earlier reports (Sanyal et al. 2003, 2005; Polowick et al. 2004). However, acetosyringone at all concentrations studied resulted in a decline in regeneration in chickpea.

For optimization of transformation efficiency, different *Agrobacterium* concentrations were tested in the present study using chickpea. In the present study, no bacterial overgrowth was observed up to the bacterial cell density equivalent to $OD_{600}=1$. Optimizing the bacterial density for effective transformation and recovery of transformants has been considered as an important factor (Paz et al. 2005; Yu et al. 2002; Sanyal et al. 2005; Ko and Korban 2004).

Use of vacuum-infiltration technique for improving transformation efficiency was advantageous in chickpea explants and the highest % of GUS expression and stable transformation was obtained at 15 min. Effect of vacuum-infiltration of bacterial suspension on transient expression had been demonstrated earlier (Jaiwal et al. 2001; Charity et al. 2002).

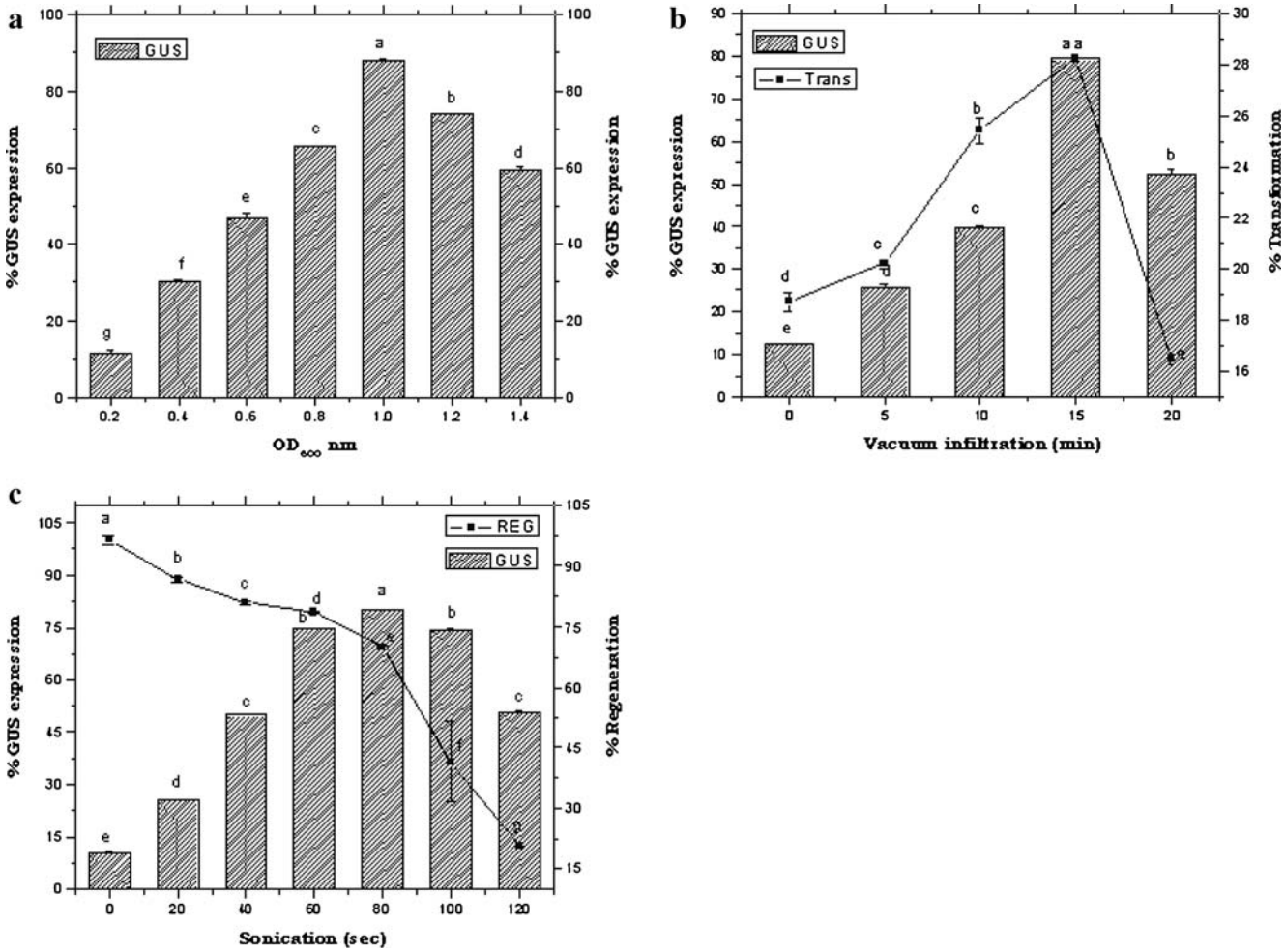

Fig. 7 Response of various parameters on the transformation efficiency using chickpea cv ICCC37 as evident of GUS expression and in vitro regeneration*: **a** influence of *Agrobacterium* density on % GUS expression; **b** influence of vacuum-infiltration period on transformation efficiency; **c** effect of sonication treatment period on % GUS expression and shoot regeneration. *Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

The present study explores the use of sonication for enhancing transformation efficiency in chickpea. Increased transformation rates of chickpea explants using sonication (Sanyal et al. 2005) is supported by the present observations that explants of chickpea subjected to sonication showed higher GUS expression than non-sonicated explants. When sonication treatments longer than 80 s were used, regeneration response of explants reduced to a great extent. To achieve efficient transformation, the intensity of sonication treatment should be carefully monitored to control micro-wounding and cell disruption (Santarem et al. 1998).

Table 4 Influence of plasmids on transformation using epicotyl explants of chickpea

Plasmids	% GUS expression±SE	% Stable transformation±SE
pBI121	55.47±1.61[a]	13.24±0.63[b]
pHS102	57.80±0.60[a]	24.31±0.97[a]
pHS101	41.10±0.45[b]	8.24±0.12[c]

Mean of 3 replicates, with each replicate consisting of 50 explants

Means followed by same letter do not differ significantly at 0.01 probability ($p<0.01$)

The present study has shown that different recombinant plasmids used influenced the efficiency of transformation in chickpea. The construction of pHS101 and pHS102 plasmids was exactly similar, except that in pHS101, a waxy locus leader peptide was fused with the transgene, which targets the synthesized protein to chloroplasts. In earlier studies on particle gun bombardment mediated gene transfer in *V. aconitifolia* (Kamble et al. 2003) the influence of plasmid constructs on the transformation frequency was reported. The present results on chickpea that pHS102 produced the highest stable transformation frequency is in agreement with the report on *V. aconitifolia* transformation (Kamble et al. 2003).

Agrobacterium-mediated transformation has been used successfully in grain legumes for over a decade (Christou 1997). Efficient transformation system for pea was developed based on direct shoot regeneration and meristem proliferation from *Agrobacterium*- treated seedling explants (Davies et al. 1993). To date, a few reports are available on the production of transgenic chickpea plants using *Agrobacterium tumefaciens*-mediated transformation (Fontana et al. 1993; Kar et al. 1996; Krishnamurthy et al. 2000; Polowick et al. 2004; Senthil et al. 2004; Tewari-Singh et al. 2004; Sanyal et al. 2005). In chickpea, earlier work using *Agrobacterium* - mediated gene transfer showed a transformation frequency rate of 0.5–3% (Polowick et al. 2004; Senthil et al. 2004), while the best transformation frequency in the present study was 14%. In the earlier reports on transformation in chickpea, embryonic axes were used (See Sonia et al. 2003). However, in the present work, epicotyl explant served as a good source of *de novo* regenerating cells and use of this explant resulted in successful *Agrobacterium*—mediated transformation in chickpea. Stable transformation frequency was determined on the basis of % Kan resistant shoots produced on kanamycin (75 mg/L) containing medium. In the present study, epicotyls of chickpea showed high regeneration potential and transgenic plants could be recovered using *Agrobacterium* as a vector. PCR and Southern hybridization analysis proved the integration of transgenes into chickpea genome. Southern blot analysis (using DNA isolated from plants transformed with pHS102) with *Hind*III digested DNA suggested that, all five transgenic plants, showed positive signal with *cryIAc* probe, indicating the integration of *cryIAc* gene into the genome of chickpea.

The results of bioassay study with transgenic chickpea plants revealed significant reduction in the survival of *Helicoverpa armigera* fed on transgenic chickpea tissues compared to nontransformed chickpea control samples. The study on quantification of CryIAc indicated that transformed plants contained good amount of endotoxin than control chickpea plants, which indicate the presence of the *cryIAc* gene (Sanyal et al. 2005). Bioassay studies proved the expression pattern in tissues of five transgenic plants, which revealed the CryIAc activity.

In conclusion, the present studies have developed an efficient method for the production of transgenic plants for chickpea using *Agrobacterium* as vector. A number of factors which are important in the consistent production of transgenic chickpea plants including explant type, acetosyringone concentrations, *Agrobacterium* concentrations and co-cultivation conditions were evaluated. The present results demonstrated the feasibility and effectiveness of *Agrobacterium tumefaciens* strain LBA4404 harboring plasmids with *npt*II, *uidA* and *cryIAc* genes under the optimized conditions of co-cultivation for chickpea transformation. Although we got a few T_0 seeds, T_1 plants could not be generated due to lack of germination of the seeds and hence the inheritance of transgenes could not be done. In conclusion, high frequency of *Agrobacterium*-mediated transformation and development of transgenic chickpea plants using epicotyl explants, expressing *cryIAc* gene, against *H. armigera* has been documented in the present study. As the transformation efficiencies continue to improve for recalcitrant plant species including grain legumes such as chickpea, development of promising transgenic plants for major agronomic traits are expected in the next few years.

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