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Optimization of regeneration and *Agrobacterium*-mediated transformation of immature cotyledons of chickpea (*Cicer arietinum* L.)

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Abstract Immature cotyledons collected at different time intervals from four genotypes of chickpea (C 235, BG 256, P 362 and P 372) were cultured adaxially on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine, thidiazuron, kinetin, zeatin and dimethylallylaminopurine (2-iP), either alone or in combination with indole-3-acetic acid (IAA) or α-napthoxyacetic acid (α-NOA) for dedifferentiation and regeneration of adventitious shoots. Morphogenesis was achieved with explants cultured adaxially on MS medium with 13.68 μM zeatin, 24.6 μM 2-iP, 0.29 μM IAA and 0.27 μM α-NOA. Explants prepared from pods of 21 days after pollination, responded favourably to plant growth regulator treatment in shoot differentiation. Histological studies of the regenerating explants, revealed the initiation of meristematic activity in the sub-epidermal region during the onset of morphogenesis, which can be correlated with elevated activity of cytokinin oxidase-dehydrogenase, for cytokinin metabolism. The regenerated shoots were efficiently rooted in MS medium supplemented with 2.46 μM indole-3-butyric acid and acclimatized under culture room and glasshouse conditions for normal plant development leading to 76–80 % survival of the rooted plantlets. The immature cotyledon explants were used for *Agrobacterium*-mediated transformation with critical manipulation of cultural conditions like age of explant, O.D. of *Agrobacterium* suspension, concentration of acetosyringone, duration of sonication and co-cultivation for successful genetic transformation and expression of the reporter gene *uid*A (GUS). Integration of transgene was confirmed by molecular analysis. Transformation frequency up to 2.08 % was achieved in chickpea, suggesting the feasibility of using immature cotyledon explants for *Agrobacterium*-mediated transformation.

Abbreviations
BA	6-Benzyladenine
CKX	Cytokinin oxidase-dehydrogenase
DAP	Days after pollination
IAA	Indole-3-acetic acid
2-iP	Dimethylallylaminopurine
MS	Murashige and Skoog
α-NOA	α-Naphthoxyacetic acid
PGR	Plant growth regulator
MU	4-Methylumbelliferone

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Introduction

Chickpea (*Cicer arietinum* L.) is an important crop and major source of dietary protein for human and livestock consumption in tropical and sub-tropical countries. Several large-seeded grain legumes, particularly chickpea, pigeonpea, and *Phaseolus* spp., are classified as marginal orphan legumes, owing to their recalcitrant nature to in vitro regeneration and genetic transformation (Varshney et al. 2009). The grain productivity of this legume has been stagnating for the last few decades primarily because of the

limited success in the genetic improvement by conventional breeding methods, owing to a narrow genetic base and sexual incompatibility for inter-specific hybridization and restricted success of biotechnological approaches (Somers et al. 2003; Popelka et al. 2004). However, recent developments in tissue culture technology seem to provide opportunities for widening the genetic base and incorporation of desirable traits into these important grain legumes including chickpea (Kumar and Davey 1991; Parrott et al. 1992, 1995; Christou 1997). The recalcitrant nature of the grain legume chickpea to tissue culture could be attributed to the inadequate proportions of competent regenerative cells, extensive DNA methylation, the presence of high phenolic and flavonoid contents (Mok and Mok 2001; Somers et al. 2003; Popelka et al. 2004). Protocols for regeneration in grain legumes through direct organogenesis from pre-existing meristems, leaflet callus and immature explants have been demonstrated with limited success (Parrott et al. 1992; Davey et al. 1994). Somatic embryogenesis and shoot regeneration via direct organogenesis has been reported in chickpea from leaflets and embryo axis (Barna and Wakhlu 1993; Dinesh Kumar et al. 1994; Suhasini et al. 1994; Pathak and Hamzah 2008; Mehrotra et al. 2011a). Direct somatic embryogenesis has been reported from immature cotyledons in two cultivars of chickpea cultured on MS medium (Murashige and Skoog 1962) supplemented with indole-3-acetic acid (IAA) or α-napthoxyacetic acid (α-NOA) and high conversion of somatic embryos into plantlets in the presence of zeatin (Hita et al. 1997; Ghanti et al. 2010). There are several reports of efficient genetic transformation using *Agrobacterium tumefaciens* (Tewari-Singh et al. 2004; Senthil et al. 2004) as well as by sonication assisted *Agrobacterium*-mediated transformation method (Pathak and Hamzah 2008). This communication reports an efficient and genotype-independent in vitro regeneration protocol from immature cotyledons of chickpea, where a large proportion of cells are provoked for dedifferentiation and morphogenesis on MS medium supplemented with zeatin and dimethylallylaminopurine (2-iP). We have successfully demonstrated *Agrobacterium*-mediated transformation of chickpea with the optimization of critical parameters for co-cultivation of immature cotyledons for high-frequency genetic transformation and regeneration of transgenic plants.

Materials and methods

Plant material and culture conditions

Seeds of chickpea genotypes (C 235, BG 256, P 362 and P 372) used in this study were obtained from the Crop Research Centre, Gobind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India. The plants were grown to the pod-filling stage under field conditions. The green pods were collected every 7 days after pollination (DAP) until the maturation of pods (45 days). Pods were surface disinfested by rinsing for 1 min with 70 % (v/v) ethanol, followed by 1.0 % (v/v) sodium hypochlorite for 15 min and rinsed thoroughly four to five times with sterile water. Pods were dissected aseptically and the immature developing seeds ranging from 2 to 8 mm were obtained. The immature seeds were surface disinfested with 0.5 % (v/v) sodium hypochlorite and rinsed thoroughly with sterile water. After removal of green seed coat, the immature cotyledons and embryo axis were carefully dissected and separated. The excised cotyledons were cultured on MS medium containing vitamins of B5 medium (Gamborg et al. 1968), supplemented with different cytokinins either alone or in combination with 0.8 % (w/v) agar (Cat # A1296 Sigma Chemical Co., USA) and 3 % (w/v) sucrose (HiMedia Labs, Mumbai, India). The medium was adjusted to pH 5.8 before autoclaving at 1.09 MPa for 20 min. All cultures were incubated in culture room maintained at 24 ± 1 °C under cool-white fluorescent light of 60 μmol m^{-2} s^{-1} intensity and 16 h photoperiod unless otherwise mentioned.

Cultural conditions for immature cotyledonary segments

The plant growth regulators viz., 2-iP, BA, kinetin, thidiazuron, zeatin, IAA and α-NOA, were used either alone or in different combinations for inducing morphogenesis in immature cotyledons (Table 1). Fifty excised immature cotyledon explants, collected at periodic intervals were incubated adaxially (Fig. 3a) on semi-solid agar plates in three replicates, for 45 days under culture room conditions and examined microscopically for morphogenic development (Fig. 3b). Globular, cylindrical, flat, cotyledon-like and elongated structures developed after 30 days of culture, were sub-cultured on MS medium containing 4.4 μM 6-benzyladenine (BA) for further proliferation and shoot development. The individual shoots, 2–3 cm in length were rooted in MS medium containing 2.46 μM indole-3-butyric acid (IBA), 1 % (w/v) sucrose and 0.5 % (w/v) agar. The rooted plantlets were acclimatized and hardened for further development as described previously (Sanyal et al. 2005).

Histological examination of regenerating explants

For histological examination, tissues were fixed periodically starting from the initial stage to completely differentiated structures after 45 days of culture. Histology was performed according to the procedure described by Steeves and Sussex (1989). The blocks were sectioned longitudinally (L.S.) at 10 μm thickness and permanently mounted with Canada balsam for observation under the light microscope (Leica DMIL, Germany).

Table 1 Morphogenic responses of adaxially placed immature cotyledons 21 DAP on various combinations of growth regulators after 6 weeks of culture

Murashige and Skoog (MS) medium supplemented with different PGR's in µM concentration								Response frequency (%)	Nature of response
BA	IAA	Zeatin	2-iP	Thidiazuron	NOA	NAA	Kinetin		
0	0	0	0	0	0	0	0	90.39	NM
4.44	0	0	0	0	0	0	0	45.90	CS
8.88	0	0	0	0	0	0	0	64.25	CS
8.88	0	0	0	0	0	0.27	0	88.69	CA
22.2	0.29	0	0	0	0	0	0	53.23	CA
4.44	0	0	0	0	0	0	4.65	89.06	CA
0	0.29	0	0	0	0	0	9.3	92.29	NM
0	0	0	9.84	0	0	0	0	40.55	MD + SP
0	0	0	24.6	0	0	0	0	68.26	MD + SP
0	0	0	49.2	0	0	0	0	56.42	MD + SP
0	0	0	24.6	0	0.25	0	0	85.52	SP
0	0	0	49.2	0	0.25	0	0	80.40	SP
0	0	0	0	4.54	0	0	0	83.34	CA
0	0	0	0	13.62	0	0	0	85.73	CA
0	0	0	0	22.7	0	0	0	88.53	CA
0	0	4.56	0	0	0	0	0	58.85	MG + SP
0	0	13.68	0	0	0	0	0	75.58	MG + SP
0	0	22.8	0	0	0	0	0	66.57	MG + SP
0	0.29	13.68	0	0	0	0	0	83.76	MG + SP
0	0.29	13.68	24.6	0	0.25	0	0	83.34	SH
0	0.29	13.68	49.2	0	0.25	0	0	80.58	SH

CA callus, *CS* callus with shoot buds, *MD* morphogenesis with differentiated structures, *MG* morphogenesis and differentiation, *NM* no morphogenesis, *SP* shoot primordia, *SH* shoots

The values presented were average of 50 explants used in three independent experiments

Cytokinin oxidase-dehydrogenase (CKX) assay and determination of endogenous zeatin

The cell-free extracts of different explants of chickpea, were prepared by homogenization with liquid nitrogen in extraction buffer consisting of 50 mM potassium acetate, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.5 mM dithiothreitol (DTT). Cytokinin oxidase/dehydrogenase activity in immature cotyledons at various stages of development was assayed in the cell-free extracts according to the procedure of Libreros-Minotta and Tipton (1995). The endogenous level of zeatin in the immature cotyledons of 7, 14, 21, and 28 DAP was estimated with Phytodetek t-ZR test kit, as per the instructions of the manufacturer (Agdia Inc., USA).

Bacterial strain, chimeric plant vector and culture conditions

A. tumefaciens strain LBA 4404 harbouring the chimeric vector p35SGUS-INT (Fig. 4a) having an intron in the *uid*A gene (Vancanneyt et al. 1990) was used for optimization of parameters crucial for *Agrobacterium*-mediated transformation of chickpea by the co-cultivation of 21 DAP immature cotyledons. The culture conditions for growth of *Agrobacterium* were the same as described previously (Mehrotra et al. 2011a, b).

Agrobacterium inoculation of immature cotyledons and regeneration of transformed shoots

Ten immature cotyledon explants suspended in 10 ml *Agrobacterium* suspension ($OD_{600} \approx 0.3$) were sonicated in 30 ml borosilicate glass tubes at 42 kHz for 2–10 s in a water bath sonicator (Branson 2510, USA). The explants after 30 min of incubation with *Agrobacterium* cells were blotted on sterilized filter paper to remove excess *Agrobacterium* and incubated for 48 h in dark on MS co-cultivation medium containing, B5 vitamins, 13.68 µM zeatin, 24.6 µM 2-iP, 0.29 µM IAA and 0.25 µM α-NOA (ZPIN medium), 50 µM acetosyringone, 3 % (w/v) sucrose and gelled with 0.8 % (w/v) agar at 24 ± 1 °C. After co-cultivation, the explants were subjected to screening and

selection cycles each of 15 days duration on ZPIN medium fortified with 500 mg l^{-1} cefotaxime for 1 week followed by two cycles (15 days each) of sub-culturing on ZPIN medium fortified with 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. The shoots surviving the antibiotic selection pressure were cultured for 10 days on MS basal medium for elongation followed by root induction on MS medium supplemented with 2.46 μM IBA and 1 % (w/v) sucrose. Root initiation commenced after 10 days of culture and plantlets with 0.8–1.0 cm long roots were transferred to pots filled with soilrite, followed by hardening and acclimatization in culture room prior to transfer to the glasshouse as described previously (Mehrotra et al. 2011a).

Histochemical and fluorometric assay for β-glucuronidase (GUS)

Transient histochemical and fluorometric GUS assays to check the expression of *uid*A gene in transformed chickpea explants or plantlets were performed according to the procedure described by Jefferson et al. (1987) with some modifications. GUS foci on the transformed cotyledon surface was viewed under a stereo zoom microscope (Leica WILD M3Z, Germany) and the GUS activity expressed as pmoles MU per mg protein per min was measured using a fluorometer by excitation at 365 nm and emission at 455 nm (DyNA Quant 200, Hoefer, USA).

Electron microscopy

The explants were fixed in 200 mM potassium phosphate buffer (pH 7.0) containing 3 % (v/v) glutaraldehyde and 2 % (v/v) paraformaldehyde for 3 h at room temperature. Samples were dehydrated in an ascending ethanol series (50–100 %) for 10 min each, critical point dried, sputter-coated with platinum and viewed on XL-20 scanning electron microscope (Philips, Holland).

Polymerase chain reaction (PCR) and Southern blot hybridization

Chickpea genomic DNA was isolated as described previously (Sanyal et al. 2005). Regenerated shoots were screened by the specific amplification of 506, 678 and 450 bp fragments of *uid*A, *npt*II and *vir*G genes respectively in the GeneAmp® PCR system 9700 (PE Biosystems, USA) using the set of specific primers for 30 cycles, each cycle comprising of denaturation at 92 °C for 1.5 min, annealing at 58, 55 and 53 °C for *uid*A, *npt*II and *vir* G genes respectively for 1 min followed by extension at 68 °C for 3 min. The set of specific primers for *uid* A was F-5′ TTT AAC TAT GCC GGG ATC CAT CGC-3′ and R-5′ CCA CTC GAG CAT CTC TTC AGC GTA-3′, for *npt*II was F-5′ TAT TCG GCT

ATG ACT TGG CC-3′ and R-5′ GCC AAC GCT ATG TCC TGA TA-3′ and for *vir*G was F-5′ CTG GCG GCA AAG TCT GAT-3′ and R-5′ TGT CGT AAA CCT CCT CGT-3′. PCR cocktail in 10 μl final volume was prepared as described previously (Sanyal et al. 2005). The amplified products were electrophoresed on 1.5 % (w/v) agarose gel and visualized after staining in 0.5 mg l^{-1} ethidium bromide solution on a UV transilluminator. Southern blot hybridization was performed to confirm the stable integration of transgene in the putative transformants according to the procedure of Sambrook and Russell (2001). Approximately 15 μg of chickpea genomic DNA from the transgenic plants, was digested with 50 units of *Eco*RI (New England Biolabs, USA) for 16–24 h, resolved in 0.8 % (w/v) agarose gel and immobilized on Zeta probe-GT nylon membrane (BIO-RAD, USA) by vacuum transfer (Vacuum blotter-model 785, BIO-RAD, USA). The blots were hybridized with radiolabelled probes generated by random priming method using 2.06 kb *Bam*HI-*Sac*I fragment for *gus* and 1.6 kb *Pst*I fragment of *npt*II from p35SGUS-INT labeled with α^{32}P dCTP (BRIT Mumbai, India).

Reverse transcriptase PCR (RT-PCR) analysis

Total RNA was isolated from leaf and seed tissues using TRI reagent (Sigma, USA) and treated with RNase-free DNaseI (Sigma, USA). The cDNA first-strand was synthesized by enhanced Avian RT-PCR kit (Sigma, USA). Reverse transcription was performed at 50 °C for 10 min, initial denaturation at 95 °C for 5 min followed by 40 amplification cycles comprising of 10 s denaturation at 95 °C and combined annealing and extension for 30 s at 60 °C in 25 μl reaction volume, according to manufacturer's instructions. PCR amplification of cDNA was performed using the specific primers of *uid*A, *npt*II and *vir*G genes as mentioned earlier.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and means were compared for level of significance ($p \leq 0.0001$) by Duncan's Multiple Range Test using Statistical Package for Social Sciences (SPSS 14.0, SPSS Inc. USA).

Results

Effect of age, orientation, and genotype on regeneration from immature cotyledon explants

Chickpea pods were collected at periodic intervals for obtaining immature cotyledon explants. The pods harvested at 7 and 14 DAP were partitioned into pod wall,

seed coat, juvenile immature embryo with cotyledons having endospermic fluid, reflecting the commencement of seed filling stage and having minimal CKX activity. Pods of 21, 28, and 35 DAP showed well-developed pod wall, seed coat, young cotyledons, distinct immature embryo axes, and revealed maximum seed filling and CKX activity, while pods harvested after 35 days were partitioned into hard pod wall, seed coat, mature cotyledons and embryonic axes, with relatively lower CKX activity (Figs. 1, 2c). The morphogenic response of immature cotyledons collected at periodic intervals of all the genotypes were highly dependent on age, size and orientation of explants cultured on MS medium supplemented with 13.68 μM zeatin and 0.29 μM IAA. The adaxially placed explants of 21 DAP showed maximum morphogenic response followed by 28 and 14 day old explants in all the genotypes of chickpea (Fig. 2a). Response of various combinations of plant growth regulators on the induction of morphogenic dedifferentiation in 21 DAP immature cotyledons of chickpea is summarized in Table 1. Statistical analysis showed culture of explants on medium supplemented with 13.68 μM zeatin and 24.6 μM 2-iP either alone or in different combinations with low levels of auxins 0.29 μM IAA or 0.25 μM α-NOA, resulted in the initiation and development of morphogenic structures or shoot primordia from the proximal end of the cotyledonary explants.

Statistical analysis showed that the optimum morphogenic response was achieved with adaxially placed cotyledons of 6.0 mm in size, obtained from 21 DAP developing pods cultured on medium containing 13.68 μM zeatin and 0.29 μM IAA resulted into development of morphogenic structures in 83.76 % of responding explants (Table 1). While 13.68 μM zeatin, 0.29 μM IAA, 24.6 μM 2-iP and 0.25 μM α-NOA combination showed a maximum induction of shoots (5.64 shoots per responding explant) in variety P 362, followed by 4.38, 3.35 and 2.39 shoots per responding explant in variety BG 256, C 235 and P 372 respectively (Table 2; Fig. 2b). Whereas explants excised from developing pods at 7, 14, 28, 35, and 42 DAP did not reflect a comparable morphogenic response (Table 2). Culture of 21 DAP immature cotyledons on medium supplemented with 13.68 μM zeatin, 24.6 μM 2-iP, 0.29 μM IAA and 0.25 μM α-NOA resulted in the development of morphogenic structures. Sub-culturing of the individual morphogenic structures on MS medium fortified with 4.4 μM BA resulted in 5.64 shoots in 76.19 % responding explants in variety P 362, whereas 4.38 shoots in 54.80 % responding explants in variety BG 256, 3.35 shoots in 67.65 % responding explants in variety C 235 and 2.39 shoots in 43.45 % responding explants in variety P 372 (Table 2).

The individual shoots after 2 weeks of culture were rooted with 88.54 % response frequency, in half-strength
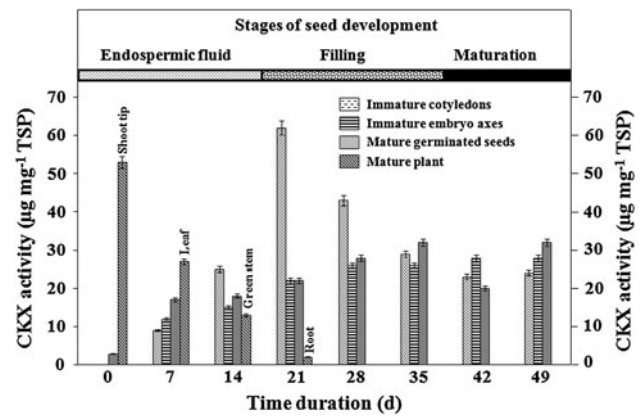
Fig. 1 Cytokinin oxidase-dehydrogenase (CKX) activity at various time intervals in different developmental stages of seed and in various plant parts of chickpea such as shoot, root and leaf. The assay has been performed in cell-free extracts of chickpea genotype P 362

MS medium containing 2.46 μM IBA. Plantlets with primary roots were acclimatized and hardened in the culture room for 14 days before transfer to the glasshouse for further development and seed setting. The plantlets showed flowering, pod development, and seed setting, except that the number of pods ranged from 6 to 14 pods per plant, compared to 14–22 pods in seed-grown control plants under identical conditions.

Assay of cytokinin oxidase-dehydrogenase (CKX) activity

Activity of CKX, an important enzyme involved in plant growth and development, was monitored at periodic intervals in the immature cotyledons as well as developing explants of chickpea. The results showed relatively higher levels of CKX activity in immature developing cotyledons, as compared to other explants like leaf, shoot, root and flower (Fig. 1). Statistical analysis revealed that immature cotyledons excised up to 14 DAP exhibited a consistent level of CKX activity between 10 and 28 μg mg^{-1} soluble protein that significantly increased several fold (61.67 μg mg^{-1} soluble protein) in developing cotyledons 21 DAP and remained consistent for the next 6–8 days (Table 2). The enzymatic activity decreased with maturation of cotyledons, while CKX activity showed more or less consistent profile in developing embryo axes (14–24 μg mg^{-1} soluble protein) until maturation of pods (Fig. 2c). Explants with maximum CKX activity also revealed maximum response for morphogenesis, dedifferentiation, and development of adventitious shoots (Table 2).

The estimation of endogenous zeatin concentration in the immature cotyledons of chickpea varieties revealed maximum level in genotype P 362 (0.54 pmol μg^{-1} fresh weight of total protein) in 21 DAP explants which was
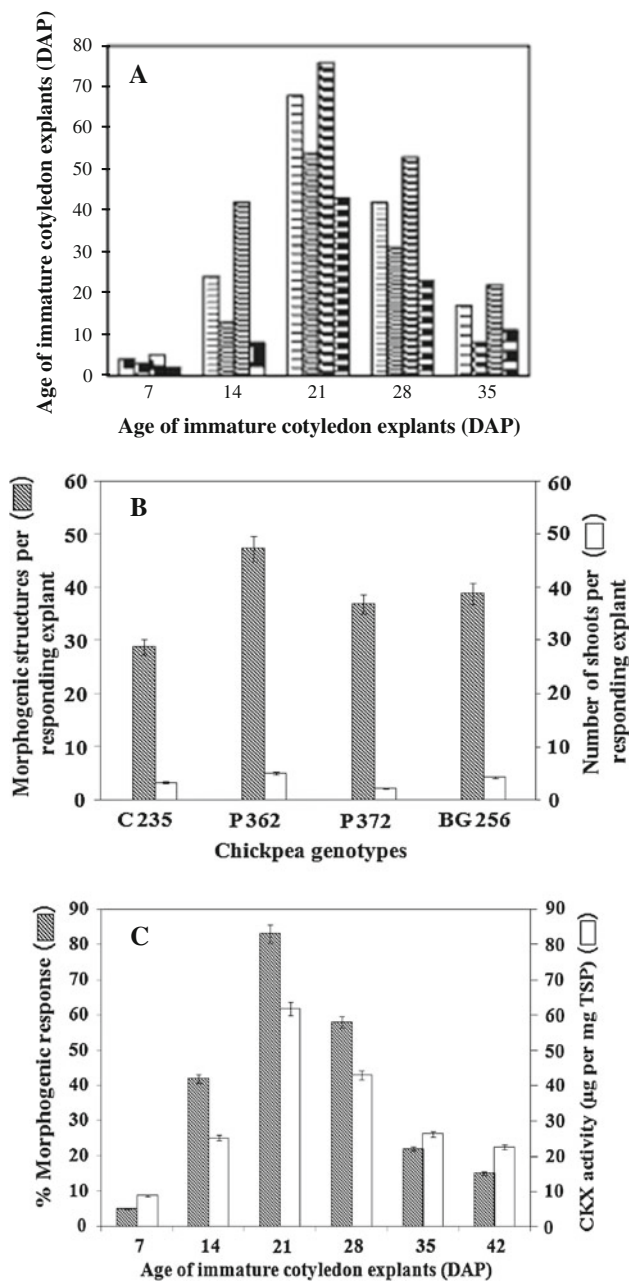
Fig. 2 Effect of explant age, morphogenic response and CKX activity in immature explants of chickpea. **a** Effect of age and genotypes C 235 (*dashed bar*), BG 256 (*striped bar*), P 362 (*cross striped bar*), P 372 (*dotted bar*) of immature cotyledons of chickpea on morphogenic response, **b** Genotypic response on the development of morphogenic structures per responding explant (*cross striped bar*) and number of shoots per responding explant (*open bar*) in four genotypes of chickpea, **c** Correlation between age of explant, % morphogenic response (*cross striped bar*) and CKX activity (*open bar*) expressed as $\mu g \ mg^{-1}$ total soluble protein (TSP)

immature cotyledons of 21 DAP ranging between 0.40 and 0.46 pmol mg^{-1} fresh weight and showed identical pattern for endogenous level of zeatin in explants of different age similar to variety P 362. The high-level of endogenous zeatin in 21 DAP of immature cotyledons coupled with maximum CKX activity in all the genotypes of chickpea suggests that rapid metabolism of exogenous cytokinin is responsible for maximum induction of morphogenic development.

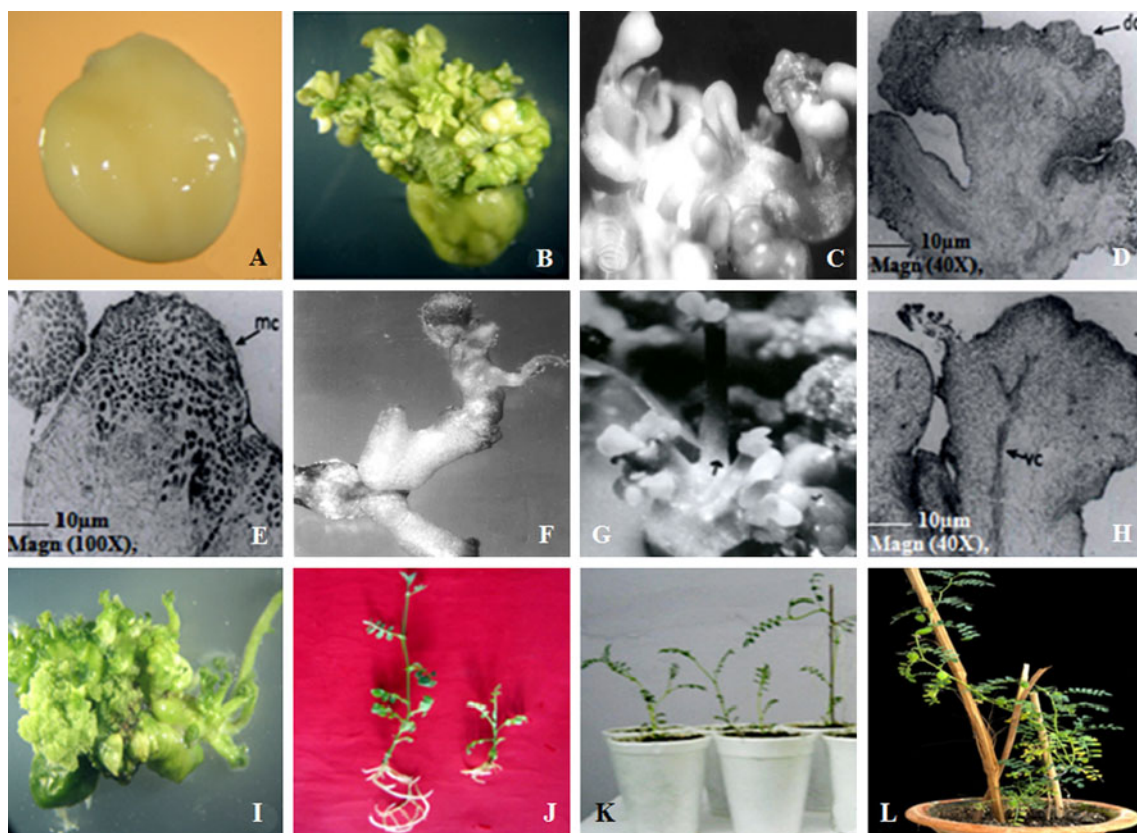Histological studies of morphogenic immature cotyledon tissues

The histological preparations of excised 21 DAP immature cotyledons showed several layers of actively meristematic, sub-epidermal cells along with internal parenchymatous cells in the expansion phase (Fig. 3d–e). Incubation of the explants on 13.68 μM zeatin supplemented medium resulted in the stimulation of cell division into sub-epidermal cells leading to initiation of morphogenic growth after 15–18 days of culture (Fig. 3c–e). Initial stages of dedifferentiation, regeneration, and development were marked with callusing at the sites from where the embryo axes were detached. Higher concentrations of zeatin (>13.68 μM) restricted the development of non-morphogenic callus and provoked rapid cell division, both in periclinal and anticlinal planes, in the sub-epidermal cells. This led to the formation of small protuberances with distinct tunica and corpus layers (Fig. 3e). Subsequently, after 28 days of culture, development of differentiated morphogenic structures such as globular, cylindrical, flat, cotyledon-like and stalked-crescent types were visible. The distribution pattern of these structures varied from single to many in clusters, occasionally stalked and laterally fused (Fig. 3g). These cotyledon-like structures (CLS) were efficiently germinated on 4.4 μM BA supplemented medium (Fig. 3f).

Longitudinal and transverse sections of the explants incubated on zeatin-supplemented medium, during the course of morphogenic development, showed intense meristematic activity in the cells below the epidermal and sub-epidermal layers, distributed throughout the proximal part of immature cotyledons while most of the internal parenchymatous cells remained undifferentiated with stored reserve material (Fig. 3e). The region of morphogenic activity in the explants can be well characterized in having compact, densely stained and actively dividing cells (mc) that can be clearly distinguished from the parenchymatous somatic cells (sc) (Fig. 3e). These sectors of morphogenic activity eventually led to development of morphogenic structures, shoot primordia, or shoot bud-like structures. Development of organized pro-vascular tissues having vascular tracheary elements (vc) connected with the

maximum amongst four genotypes and declined significantly to 0.20 pmol μg^{-1} fresh weight in 28 DAP immature cotyledons whereas it was undetectable in explants of 7 and 14 DAP. The zeatin level in remaining three genotypes of chickpea showed maximum concentration in

Table 2 Morphogenic response frequencies and shoot development of adaxially incubated immature cotyledons from different genotypes of chickpea excised at periodic intervals and cultured on MS medium supplemented with 13.68 μM zeatin, 24.6 μM 2-iP, 0.29 μM IAA and 0.25 μM α-NOA

Explant characteristics			Chickpea genotype showing response frequencies (%) on ZPIN medium				Average number of shoots per responding explants in chickpea genotypes			
Age DAPS	Size (mm)	CKX-activity	C 235	BG 256	P 362	P 372	C 235	BG 256	P 362	P 372
7	1.8–2.2	8.80	4.38	3.26	5.63	2.90	–	–	–	–
14	2.5–3.5	25.01	24.27	13.76	42.94	8.60	1.0	1.5	2.2	1.2
21	3.5–6.0	61.67	67.65	54.80	76.19	43.45	3.35	4.38	5.64	2.39
28	6.0–7.2	42.73	42.65	31.66	54.16	23.61	1.5	1.8	2.8	1.0
35	6.0–8.0	26.50	17.45	7.86	30.71	12.00	–	≅1.0	≅1.0	–
42	7.0–8.5	25.01	11.36	7.52	22.28	6.30	–	–	–	–

CKX activity expressed as average of three assays (μg product per mg of soluble protein); morphogenic response values are expressed as average of 50 explants used in three independent experiments on ZPIN medium consisting of zeatin, 2-iP, IAA and α-NOA and number of shoots are average of all responding explants used in three independent experiments

Fig. 3 In vitro regeneration and histological studies of immature cotyledonary explants of chickpea during morphogenic development. **a** 21 DAP immature cotyledon placed abaxially on the culture medium, **b** Emergence of morphogenic structures, **c** Morphogenic response at proximal end of immature cotyledon on 13.68 μM zeatin + 0.29 μM IAA after 6 weeks showing the development of CLS, **d** Longitudinal section (L.S.) through CLS showing rapidly dividing cells (dc), **e** L.S. through CLS showing meristematic cells (mc), **f** CLS germinated on MS medium supplemented with 4.4 μM BA, **g** Stalked CLS on 13.68 μM zeatin + 0.29 μM IAA, **h** L.S. passing through the CLS structures showing rapidly dividing cells and differentiation of internal vascular cells (vc) in the morphogenic structures, **i** Shoot primordia emerging after 6 weeks from morphogenic structures on 13.68 μM zeatin + 0.29 μM IAA + 24.6 μM 2-iP + 0.25 μM α-NOA, **j–l** Hardening, acclimatization and establishment of in vitro grown plantlets for seed setting in glasshouse

maternal tissues were seen after 28–35 days of culture (Fig. 3h). The morphogenic structures cultured on MS medium supplemented with 13.68 μM zeatin + 0.29 μM IAA + 24.6 μM 2-iP + 0.25 μM α-NOA regenerated into shoots after 6 weeks of culture (Fig. 3i). The regenerated individual shoots were rooted and acclimatized in the

culture room before transfer to earthen pots filled with soil in the glasshouse (Fig. 3j–l).

Agrobacterium-mediated transformation and selection of putative transformants

Experiments were performed with *Agrobacterium* suspension of varying cell densities on transient GUS expression with 21 DAP immature cotyledon explants. The number of responding explants increased from 7.06 ± 2.48 % (OD_{600} 0.1) to 88.30 ± 2.46 %, when the cell density was increased from $OD_{600} \approx 0.1$ to $OD_{600} \approx 0.3$, having approximately $2–5 \times 10^9$ cfu ml^{-1}. Highest number of GUS foci (79.36 ± 2.34) per responding explant appeared at $OD_{600} \approx 0.3$, while an enhanced density of bacterial cells ($OD_{600} \approx 0.5–1.0$, 2×10^{12} cfu ml^{-1}) resulted in the development of large sectors showing GUS expression (Figs. 4b, 5e). Co-cultivation of explants with *Agrobacterium* for 48 h in the dark, resulted in maximum GUS expression (78.66 ± 2.40 foci) in 78.30 ± 2.68 % responding explants to the treatment (Fig. 4c). Higher duration (72 h) of co-cultivation resulted to explants turning white, while lower duration led to the decrease in the number of GUS foci and the percentage of responding explants showing GUS foci per explant. Temperature of co-cultivation also showed variable response as evident from percent explants expressing GUS. Co-cultivation of explants with optimum cell density at 20, 25, 30, 35 and 40 °C showed maximum responding frequency of explants with GUS was in the order 35 ± 2.28, 88 ± 3.56, 75 ± 4.84, 27 ± 2.12 and 15 ± 3.88 % respectively. Maximum frequency of co-cultivation of explants was achieved at 25 °C (data not shown).

Supplementation of 50 μM acetosyringone in the co-cultivation medium increased the efficiency of T-DNA transfer in terms of explants responding to the treatment showing more than four fold increment (88.38 ± 2.64 %) as compared to its absence in the medium (20.04 ± 0.08 %) as shown in Fig. 4d. Higher concentration of acetosyringone in the co-cultivation medium led to the decrease in the number of explants responding to regeneration after co-cultivation and showing corresponding high numbers of GUS foci. Different concentrations of kanamycin ranging from 0, 10, 25, 50, 75, and 100 mg l^{-1} were tested with 21 DAP immature cotyledon explants, for ascertaining the optimal threshold level at which regeneration was inhibited completely (data not shown). The concentration of kanamycin at 50 mg l^{-1} showed complete inhibition of regeneration in control explants and was used thereafter for selection of putative transformants following co-cultivation, whereas, kanamycin concentration of 75 and 100 mg l^{-1} led to complete cessation of growth and development of the explant, followed by extensive browning and necrosis after 2–3 weeks of incubation.

Fig. 4 Effect of various parameters on *Agrobacterium*-mediated ▶ genetic transformation in immature cotyledon explants in chickpea. **a** T-DNA region of p35SGUS-INT construct showing the internal fragments of the corresponding genes used for preparation of radiolabelled probes and the sites for PCR amplification. *Bold lines* showing fragments used for DNA probe and *arrows* showing primer sites used for PCR amplification, G1, G2 for *uid*A; N1, N2 for *npt*II forward and reverse primers, respectively, **b** *Agrobacterium* cell density OD_{600}, showing percent explants with GUS expression (*cross striped bar*) and the number of GUS foci per explant (*open bar*). **c** Duration of co-cultivation, showing percent explants with GUS expression (*cross striped bar*) and the number of GUS foci per explant (*open bar*). **d** Acetosyringone concentration (in μM), showing percent explants with regeneration (*cross striped bar*) and the percent explants showing GUS expression (*open bar*). **e** Sonication treatment to the explants with *Agrobacterium* cells (in seconds) showing percent explants with regeneration (*cross striped bar*) and the percent explants showing GUS expression (*open bar*). **f** GUS expression in four genotypes of chickpea showing percent explants with regeneration (*cross striped bar*) and the percent explants showing GUS expression (*open bar*)

Mild sonication of 21 DAP immature cotyledon explants for 1, 2, 3, 5, and 10 s enhanced the percentage of explants showing GUS expression from 15 ± 0.42 % in unsonicated control to 80 ± 1.66 % in sonicated explants for 3 s. However, sonication beyond 3 s affected the regeneration significantly resulting into 3.08 ± 2 % regeneration of explants sonicated for 10 s, in comparison to 80 ± 2.29 % in unsonicated control (Fig. 4e). Scanning electron microscopy of unsonicated immature cotyledon explants co-cultivated with *Agrobacterium*, revealed an intact smooth surface of the explant (Fig. 5a), while sonicated immature cotyledons showed micro wounds of 8–10 μm in diameter (Fig. 5b). These micro wounds provide niches for colonization of *Agrobacterium* and thereby facilitating their interaction with sub-epidermal cells of host plant. Prolific colonization, growth of bacterial cells in and around the wounded sites, their multiplication and formation of cellulose fibrils were clearly visible in sonicated explants (Fig. 5c).

The 21 DAP immature cotyledons of all the four genotypes of chickpea were co-cultivated with *Agrobacterium* cells on the optimized parameters and evaluated for comparative GUS expression. Chickpea genotype P362 responded favourably, both in terms of explants showing GUS expression (84.34 ± 2.64 %) and the number of GUS foci per responding explant (96.88 ± 1.12) followed by comparable response in the order C 235 > BG 256 > P 372 genotypes, respectively (Fig. 4f). GUS expression was observed on the adaxial surface and localized primarily on the proximal parts of the co-cultivated explants, while explants that were not co-cultivated (control) did not show any GUS expression (Fig. 5d). The regenerating explant developed intense blue colour in the areas destined to give rise to shoots (Fig. 5e, f). Longitudinal section (L.S.) of the developing shoot primordia showed blue GUS foci in and
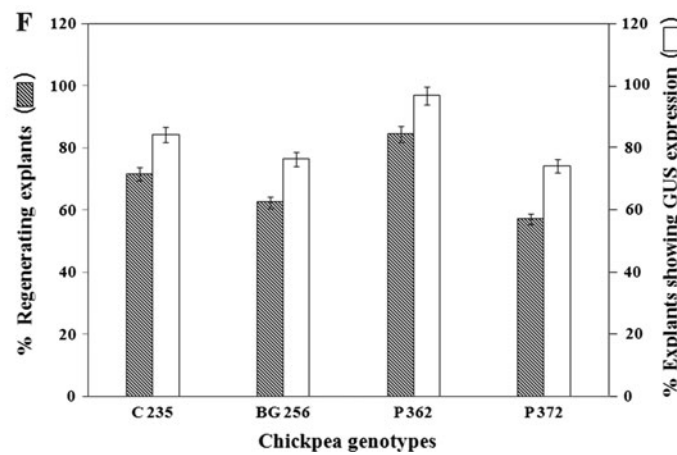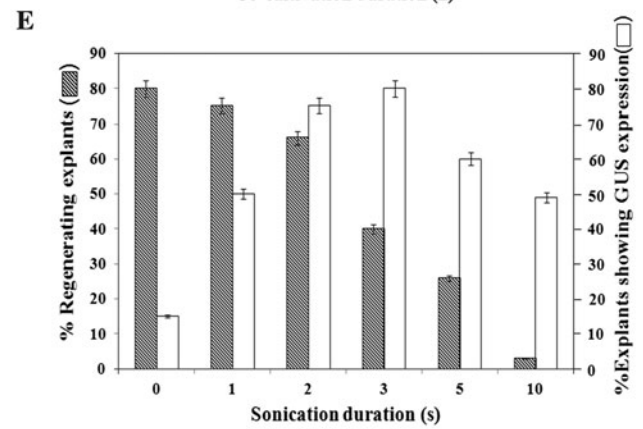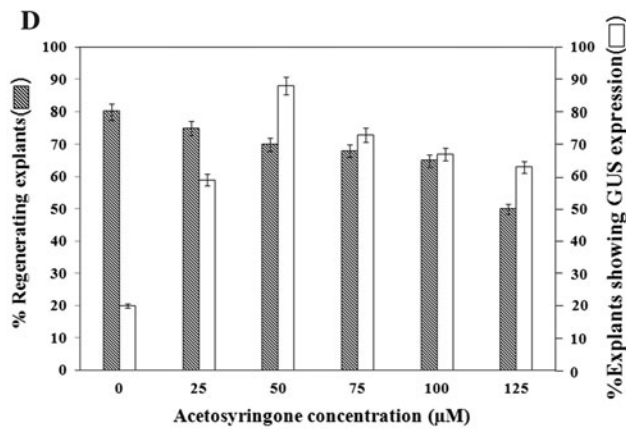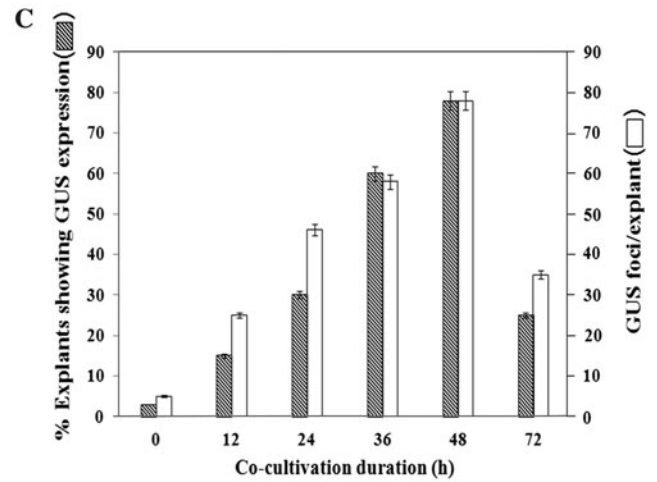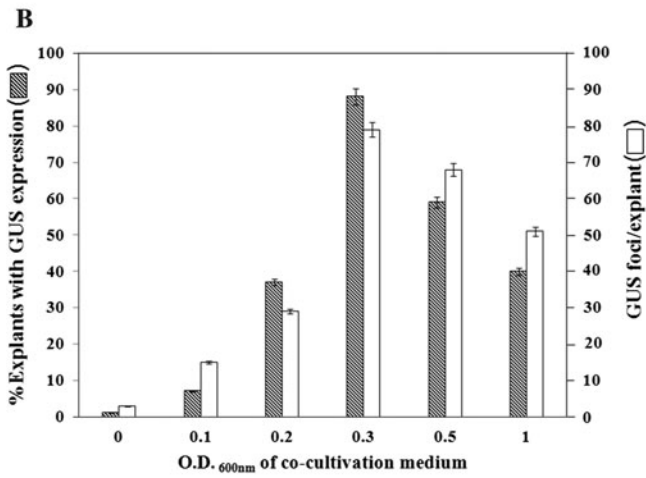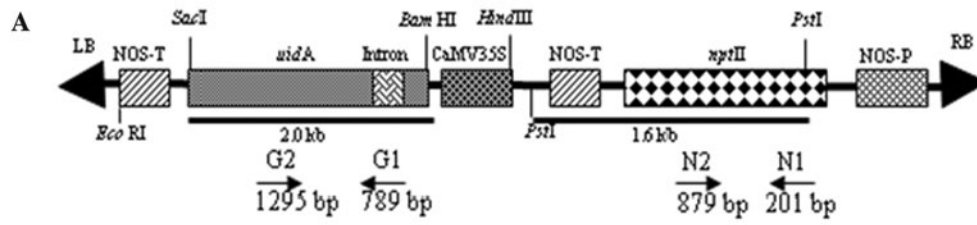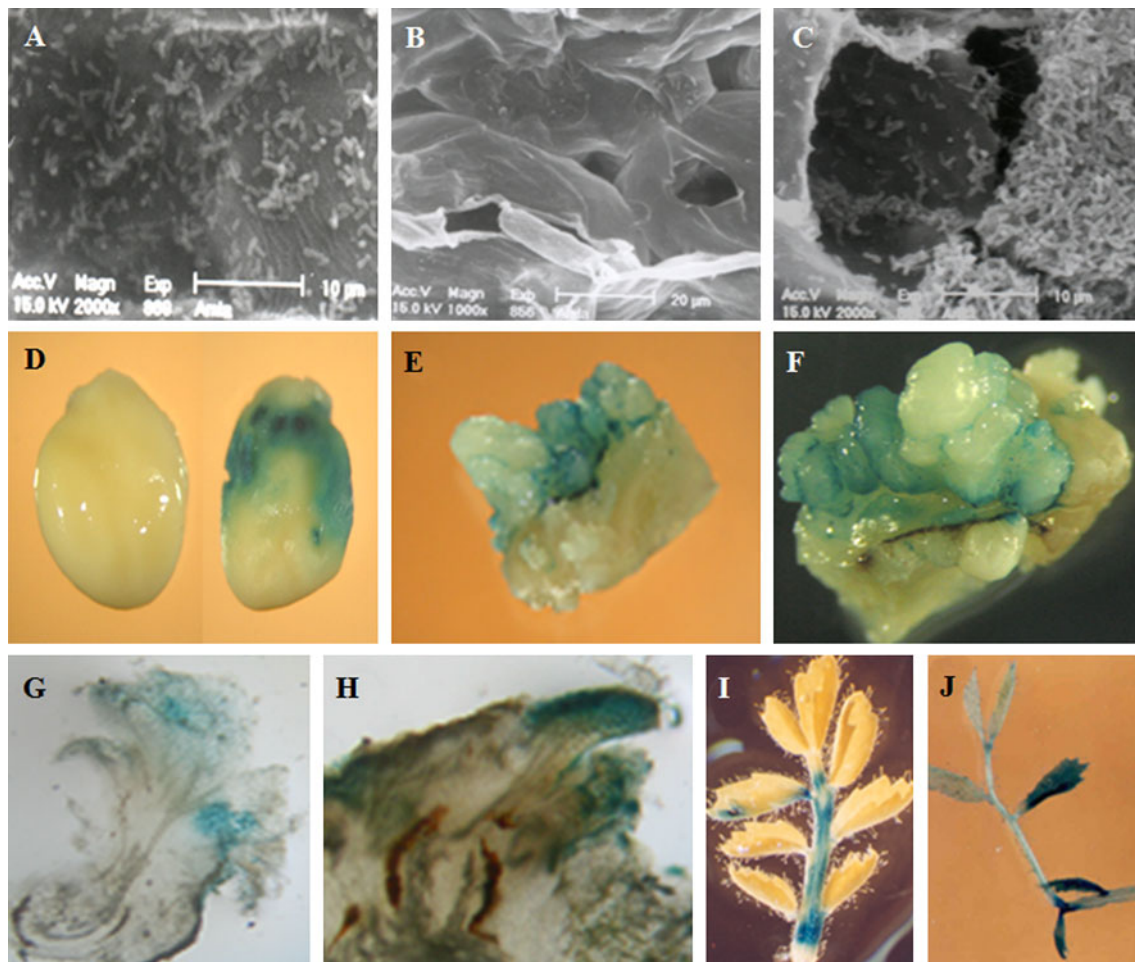
Fig. 5 *Agrobacterium*-mediated genetic transformation of immature cotyledons of chickpea. **a** Scanning electron microscopy of an unsonicated explant co-cultivated in the presence of *Agrobacterium*, **b** Sonicated explants showing micro wounds, **c** Attachment of *Agrobacterium* cells on the surface of explants and colonization around micro wounds created by sonication, **d** GUS expression in untransformed immature cotyledon and co-cultivated explant showing GUS foci after 10 days of agroinoculation, **e** Stable GUS expression in developing shoot primordia after 4 weeks on antibiotic-supplemented media, **f** Stable GUS expression after 6 weeks of co-cultivation, **g** L.S. of morphogenic structures (C.L.S.) showing stable GUS expression in shoot primordia after 12 weeks of co-cultivation, **h** L.S. of shoot tip showing stable GUS expression in emerging shoots, **i** Stable GUS expression in petiole, **j** Stable GUS expression in mature leaflets

around the meristematic areas destined to give rise to elongated shoots (Fig. 5g, h). The putative transgenic shoots regenerated after antibiotic selections were assayed for stable GUS expression by histochemical and fluorometric assays for qualitative and quantitative estimation of β-glucuronidase activity. Blue colour was visible on the leaf lamina and petioles of the transformed shoots as shown in Fig. 5i, j.

Agrobacterium-mediated transformation of 21 DAP immature cotyledons of all the four genotypes of chickpea with vector p35SGUS-INT using the optimized parameters resulted into generation of 79 number of T_0 putative transgenic shoots after two cycles of antibiotic selection. The PCR analysis of these 79 numbers of T_0 plantlets for *npt*II and *uid*A genes showed that 73 were positive for *npt*II

genes and 67 of them were positive for both *uid*A and *npt*II gene. The overall transformation frequency for both the genes in genotypes C 235, BG 256, P 362 and P 372 was ranging from 1.60 to 2.08 % and the results are summarized in Table 3.

Molecular analysis of transformants

The 67 T_0 transgenic chickpea plants showing co-transformation of *npt*II and *uid*A genes out of 79 putative transgenic plants selected on antibiotics were checked for quantitative GUS expression, PCR, RT-PCR, Southern blot hybridization and the results are shown in Fig. 6. The quantitative GUS estimation by fluorometric method of 67 T_0 transgenic plants showed GUS expression ranging from 100 to

Table 3 Transformation frequency of chickpea genotypes co-cultivated with *A. tumefaciens* strain LBA 4404 harboring the chimeric vector p35SGUS-INT

Chickpea genotypes	Total no. of explants	Total no. of shoots expected	No. of shoots after two cycles of antibiotic selection	Molecular analysis of putative transformants						Co-transformation frequency (%)
				PCR amplification				Southern blot hybridization analysis of PCR positive shoots		
				gus^+	$nptII^+$	gus^-	$nptII^-$	gus^+	$nptII^+$	
C 235	250	750	18	15	18	3	–	15	14	1.86
BG 256	250	1,000	21	16	21	5	–	16	16	1.60
P 362	250	1,250	28	26	25	2	3	26	25	2.08
P 372	250	500	12	10	9	2	3	10	9	1.80

2,000 pmol MU mg^{-1} protein min^{-1} and the results for individual transgenic plant is shown in Fig. 6a. Considering the data on GUS expression profile, nine highly expressing plants (GUS activity >1,500 pmol MU mg^{-1} protein min^{-1}) # G1, G9, G16, G26, G33, G41, G47, G48 and G59 were selected as the promising transgenic plants and their molecular analysis data are shown. The results of PCR and RT PCR assays performed for *uid*A, *npt*II and *vir*G genes are shown in Fig. 6b, c, d, e and f respectively. Amplification of expected DNA fragment of 506 and 678 bp for *uid*A and *npt*II genes respectively was achieved with set of specific primers while all the plants showed negative results upon amplification of *vir*G gene. Southern blot hybridization with specific probes performed with DNA of the nine promising T_0 transgenic plants showed possibility of two copies of *uid*A gene in three transgenic plants (# G1, G33 and G47), while remaining six plants (# G9, G16, G26, G41, G48 and G59) showing single copy integration (Fig. 6g). Whereas single copy of *npt*II gene was evident in seven transgenic plants (# G9, G26, G33, G41, G47, G48 and G59), while two plants (# G1 and G16) are showing two copies of *npt*II gene (Fig. 6h).

Discussion

High-frequency and genotype-independent procedure for efficient regeneration of fertile plantlets from immature cotyledons of *Cicer arietinum* (L.) without an intervening callus phase reflects the morphogenic competence of these explants for *Agrobacterium*-mediated transformation. These results were in agreement with earlier reports for induction of shoots by direct organogenesis from the cotyledonary nodes, developing embryo axes, cotyledons, and leaflets on different combinations of cytokinin-supplemented medium (Mehrotra et al. 2011a, b; Parrott et al. 1992; Maheshwaran and Williams 1984; Angelini and Allavena 1989). However, maximum morphogenic response in chickpea was observed with immature cotyledons of 21 DAP cultured on 13.68 μM zeatin, 24.6 μM 2-iP, 0.29 μM IAA, and 0.25 μM α-NOA, while all other combinations of cytokinins including BA, kinetin and thidiazuron, that are well-documented for exhibiting efficient induction of organogenesis in a number of grain legumes, did not evoke significant morphogenic response in immature cotyledons of chickpea (Tetu et al. 1990; Malik and Saxena 1992; Murthy et al. 1996; Mohan and Krishnamurthy 2002). These results also differed significantly from auxin-induced direct somatic embryogenesis or organogenesis as reported in soybean (Hartweck et al. 1988), peanut (Ozias-Akins 1989), chickpea (Suhasini et al. 1994; Sagare et al. 1995), clover (Parrott et al. 1992, 1995), and almond (Ainsley et al. 2001).

The efficacy and effectiveness of zeatin, 2-iP and IAA combination for efficient induction of morphogenesis in 21 DAP immature cotyledons of chickpea over other cytokinins may be attributed to the age of explant, orientation of explant on the culture medium and maximum CKX activity for efficient metabolism of zeatin (O'Keefe et al. 2011; Hoque and Mansfield 2004). It is reported that most CKX gene family members respond favourably to exogenous cytokinin application by an increase in expression (Vyroubalova et al. 2009) and higher expression in developing seeds (O'Keefe et al. 2011). These results correspond well with our study where the maximum activity was observed in immature cotyledons of 21 DAP followed by shoot tip, leaves, stem, and roots. Maximum activity of CKX has been demonstrated in developing seeds in a variety of plant species including maize (Bilyeu et al. 2001) and chickpea (Emery et al. 1998) and has been attributed for the rapid metabolism of exogenous natural cytokinins (Jones and Schreiber 1997).

Stimulated cell division, as a consequence of culture of immature cotyledons of chickpea on zeatin and 2-iP resulted in the development of morphologically distinct monopolar structures in clusters, around the proximal end of the cotyledon as evident from histological sections. These morphogenic structures, later on showed differentiation of vascular tissues, a situation analogous to the
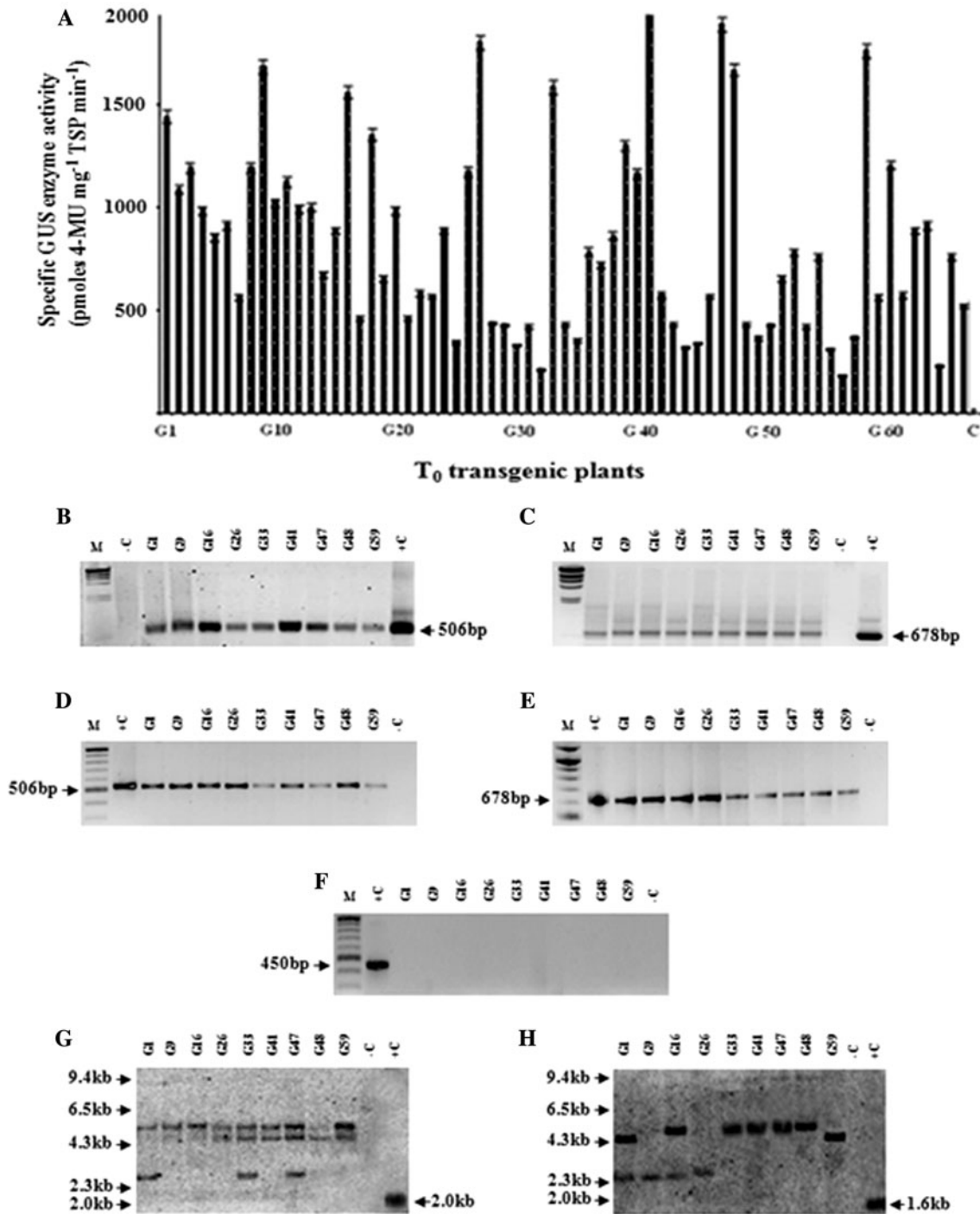
Fig. 6 Molecular characterization of T$_0$ transgenic chickpea plants. **a** Quantitative GUS expression in 67 numbers of T$_0$ transgenic chickpea plants transformed with vector p35SGUS-INT and transgenic plants developed with different genotypes; # 1–15 are of variety C 235, # 16–31 of variety BG 256, # 32–57 of variety P 362, # 58–67 of variety P 372 respectively and 'C' denotes the untransformed control plant, **b** PCR amplification in nine promising transgenic plants showing higher expression of GUS (>1,500 pmol MU produced) with GUS gene-specific primers and −C and +C denote the negative and positive controls respectively, **c** PCR amplification with *npt*II gene-specific primers, **d** RT-PCR with GUS-specific primers, **e** RT-PCR with *npt*II-specific primers, **f** PCR with *vir*G gene-specific primers, **g** Southern blot hybridization of the nine promising transgenic plants probed with 2.0 kb *Bam*HI/*Eco*RI fragment of GUS gene, **h** Southern blot hybridization of the transgenic plants probed with 1.6 kb *Pst*I fragment of *npt*II gene

development of adventitious shoot buds via direct organogenesis (Vernoux et al. 2000). We have consistently observed significantly high morphogenic response of immature cotyledons in all the four genotypes of chickpea, comparable to the earlier report of Shri and Davis (1992), for induction of direct organogenesis using immature cotyledons. Moreover, because of the lack of histological and physiological data, Shri and Davis (1992) could not ascertain the development and exact nature of cotyledon-like structures. Resemblance of cotyledon like structures with abnormal embryoids in soybean (Lazzeri et al. 1985) and chickpea (Suhasini et al. 1996) and possibility of shoot development from the shoot apices associated with abnormal embryoids have been suggested earlier. However, our results have clearly demonstrated a two-step process of plantlet regeneration, stimulated by zeatin and IAA and shoot organogenesis on media supplemented with zeatin, 2-iP, IAA, and α-NOA from immature chickpea cotyledons. The regeneration procedure of 21 DAP excised immature cotyledons of chickpea seems to be highly amenable to Agrobacterium-mediated transformation.

In the present study, we have considered various physiochemical and physical factors that were crucial for Agrobacterium inoculation of the regenerative cells or tissues embedded in the sub-epidermal zones of immature cotyledons of chickpea that tend to differentiate and regenerate into shoots under specific culture conditions. Sonication has been reported to enhance Agrobacterium-mediated T-DNA delivery by creating micro wounds in the cells of soybean, wheat, Medicago, barley and chickpea (Trick and Finer 1997; Santarem et al. 1998; Amoah et al. 2001; Kumlehn et al. 2006; Sanyal et al. 2005; Mehrotra et al. 2011a, b). Micro wounding provides niches for colonization of Agrobacterium and thereby facilitating the interaction with deeply-embedded, competent regenerative cells for genetic transformation. Whereas sonication for longer duration has a negative impact on the explants owing to extensive and irreparable physical damage to the tissues. Mild sonication to explants in presence of 50 μM acetosyringone enhanced the transformation frequency as a result of synergistic impact, while treatment beyond optimal levels seems to induce hypersensitive response and induction of polyphenol oxidase activity resulting in the necrosis of explants (Angleton and Flurkey 1984). Increased bacterial cell density during co-cultivation beyond the optimum threshold levels led to decrease in transformation frequency and regeneration of shoots due to extensive colonization of bacterial cells leading to the over-expression of defense-related genes against abiotic and biotic stresses (Dita et al. 2006; Veena et al. 2003). The observed variability for co-transfer of uidA and nptII genes amongst 79 putative transgenic plants selected after two cycles of antibiotic selection reflects the possibility of

escapes due to complexity of direct in vitro regeneration of shoots involving group of cells for shoot differentiation and development in grain legumes. Our results for GUS expression have shown different levels and extent of β-glucuronidase activity amongst the population of T₀ transgenic plants. This variation in activity might be as result of random integration and possible rearrangement of transgene in the host genome of primary transformants. Histochemical GUS assay in mature leaflet of transgenic plant and results with PCR, RT-PCR and Southern blot hybridization of T₀ transgenic plants have shown stable integration of uidA and nptII genes.

On the basis of molecular analyses of transgenic shoots, transformation frequency up to 2.08 % was achieved among various chickpea genotypes suggesting the feasibility of using immature cotyledons for high-frequency Agrobacterium-mediated transformation. In conclusion, an appropriate stage of immature cotyledons has been characterized in chickpea where a proportion of deeply embedded sub-epidermal cells on zeatin and 2-iP supplemented medium that could be induced for high-frequency morphogenesis and differentiation into shoots. The optimized parameters for high-frequency, genotype-independent, T-DNA mediated transformation achieved in recalcitrant large-seeded grain legume chickpea can be used for routine genetic transformation studies.

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