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RESEARCH ARTICLE

Development and characterization of transgenic chickpea (*Cicer arietinum* L.) plants with *cry1Ac* gene using tissue culture independent protocol

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Abstract

Tissue culture independent *Agrobacterium*-mediated transformation with *cry1Ac* gene following the non-selective/PCR detection system using direct plant PCR screening indicated the putative transgenic nature of plants and represented transformation frequency of 13.4% and 41% in cvs. C-235 and HC-1, respectively. The putative transgenic chickpea plants were analyzed adopting multiple evaluation strategies, such as PCR, ELISA and Southern blotting, for selection of plants for further advancement. Quantitative assessment of Bt Cry toxin by ELISA in leaves of transgenic chickpea plants showed variation in expression of Cry1Ac toxin (106 to 364 ng g^{-1} FW), but high expressing events (> 200 ng g^{-1} FW) were found to exhibit phenotypic abnormalities. Results obtained from Southern blotting using gene specific probe confirmed the single copy integration of the *cry1Ac* gene into the chickpea genome. Evaluation of the T_2 generation progenies of few promising T_1 transgenic plants for entomocidal activity showed retarded larval growth but significant mortality was not observed. The present study offers a suitable approach for development of chickpea plants with novel traits in with high efficiency and short duration, with the possibility of developing marker free transgenic events, allows stacking of multiple genes and can be applicable across different genotypes/cultivars of chickpea.

Introduction

Chickpea (*Cicer arietinum* L.) a self-pollinating diploid (2n=16) with a genome size of 740 Mb (Arumuganathan et al., 1991), world's second most widely grown annual legume crop after soybean. Its cultivation is of particular importance to food providing in the developing world and in diversifying the cereal-based cropping system, owing to its capacity for symbiotic nitrogen fixation (Jukanti et al., 2012). Globally, chickpea is grown in an area of 12.2mha producing 11.3mt with an average yield of about 0.93 ton ha^{-1} (FAOSTAT, 2012). India is the largest chickpea growing country; with 8.3mha of chickpea grown area and producing 7.7mt with an average yield of about 0.92 ton ha^{-1} (FAOSTAT, 2012). The most devastating insect pest to chickpea production is Gram pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), which at times causes severe pod damage up to 90% accounting for about 10-33% yield failure (ICRISAT, 1992; Yadav et al., 2006). The limited genomic resources coupled with narrow genetic diversity in the elite gene pool of chickpea have hampered the genetic improvement, either by traditional or molecular methods (Varshney et al., 2010).

Agrobacterium-mediated genetic transformation of chickpea plants resistant to pod borer have been produced by several groups (Kar et al., 1997; Sanyal et al., 2005; Neelima et al., 2008; Biradar et al., 2009; Acharjee et al., 2010; Asharani et. al., 2011 and Mehrotra et. al., 2011) by transferring different versions of *cry* genes, but still Bt chickpea is India's most wanted genetically modified (GM) pulse crop (Acharjee and Sarmah, 2013). Kharb et al.

(2012) developed a novel process of genetic transformation in chickpea using *Agrobacterium* which works without involvement of any tissue culture procedure and does not require the complex steps for selection of the transgenic events. In view of the above, an effort was made in the present study to develop insect pest resistant transgenic chickpea lines of cv. C-235 and HC-1 carrying *cry1Ac* gene using the protocol developed by Kharb et al., (2012).

Materials and Methods

Plant material and *Agrobacterium* strain used

The seeds of two widely adaptable desi type chickpea cvs. C-235 and HC-1 were procured from the Pulses Section, Department of Genetics & Plant Breeding, CCS HAU, Hisar. *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pBinAR (Hofgen and Willmitzer, 1990) was procured from NRCPB, IARI, New Delhi and used for transformation (Fig. 1). The pBinAR vector harbors the synthetic *cry1Ac* gene (GenBank accession No. Y09787.1) driven by a CaMV35S constitutive promoter.

***Agrobacterium* mediated chickpea transformation**

For the *Agrobacterium* culture, a single colony from freshly grown bacterial culture was inoculated in 100 ml liquid LB medium containing 50 mg l^{-1} kanamycin (HiMedia, Mumbai, India) and 10 mg l^{-1} rifampicin (HiMedia, Mumbai, India), and grown overnight at 28°C on a rotary shaker (120 rpm). *A. tumefaciens* strain LBA4404 carrying *cry1Ac* gene was used for chickpea cvs. C-235 and HC-1 transformation using the protocol as described by Kharb et al. (2012) without using tissue culture procedure. Following co-cultivation, the seeds were washed briefly with sterile water and sown in potted soil to grow in a containment greenhouse along with the wild type plants used as negative non transformed controls.

Direct Plant PCR analysis of putative chickpea transformants

The putative transgenic events of T_0 chickpea plants (cvs. C-235 & HC-1) were identified for the presence of the *cry1Ac* gene by subjecting small leaf piece to Direct PCR using a 0.5 mm Harris Uni-Core puncher and Harris Cutting Mat using Phire Plant Direct PCR Kit. PCR amplification using the *cry1Ac* gene specific primer pair (Table 1) to obtain a 1068bp product was carried out in MY-CYCLER (Bio-Rad) as per kit manual's instruction. Total genomic DNA from young leaves of T_1 plants, offspring of T_0 with PCR proven *cry1Ac* presence, was isolated by CTAB method (Chakraborti et al., 2006). PCR amplification of the genomic DNA of chickpea plants using *cry1Ac* gene specific primers (Table 1) were carried out in MY-CYCLER (Bio-Rad) to confirm the presence of the transgene in the T_1 transgenic plants that were selected to be advanced further. PCR reaction was set up in total volume of 20µl containing, 1x PCR Buffer, 2.5mM $MgCl_2$,0.2mM dNTPs each, 0.1µM each primer, 1 U Taq DNA polymerase (Banglore Genei), Template DNA (50ng) and Thermal cycling conditions were Initial Denaturation at 94°C for 4 min then 32 cycles of Denaturation at 94°C for 1 min, Annealing at 58°C for longer and 53°C for shorter cry1Ac fragment for 1 min, Extension at 72°C for 1 min and Final Extension at 72°C for 7 min. The products were run on a 1.5% agarose gel and analyzed on Alpha Innotech Gel Documentation system.

ELISA analyis

QuantiPlate Kit for Cry1Ab/1Ac (Envirologix, USA) was used for detection of Cry1Ac toxin in the transgenic chickpea plants by sandwich ELISA using cell-free extracts of young leaf samples according to manufacturer's instructions. The results of the assay were visualized with a color development step; color development is proportional to Cry1Ac concentration in the sample extract. The absorbance of extracts was read at wavelength of 450 nm using the plate reader (iMark Microplate Absorbance Reader, Bio-Rad, USA) and results were represented in ng g^{-1} FW of leaves.

Southern Hybridization Analysis

Genomic DNA (20 µg) samples from T_2 generation progeny plants of two (cv. HC-1) (TH2-44 and TH2-166) and one cv. C-235 (TC2-33) independent lines carrying *cry1Ac* gene were digested with *Hind*III separated on 0.8% agarose gel, and blotted on nylon membrane. The PCR amplified 533bp fragment of *cry1Ac* gene was eluted from the gel using QIAquick Gel Extraction Kit (Qiagen Inc., USA) and was labelled by non-radioactive process using Biotin following the manufacturers manual (Biotin Decalabel™ DNA Labelling Kit, Fermentas). Biotin Chromogenic Detection Kit (Fermentas) was used to detect hybridized biotin labelled probe on the nylon membrane following the kit's manual.

Insect bioassay

Insect mortality bioassay was performed through no-choice test on detached twigs of T_2 plants screened for Cry1Ac Bt toxin content, by *H. armigera* larval feeding. Larvae of *H. armigera* were reared on artificial diet enriched with gram flour (Gupta *et al*., 2004) to obtain the adult moths for egg laying. Fresh twigs of transgenic

plants were placed in plastic magenta box on slanted agar to prevent desiccation and five second instar larvae from lab-reared moths were kept at 26± 1C for 16 h photoperiod and 70% relative humidity and allowed to feed for 3 days and thereafter observations were recorded for the number of dead/ live larvae and larval weight reduction in comparison with control plants fed larval weight calculated.

Results

Development of transgenic chickpea plants

Following co-cultivation with *A. tumefaciens* strain LBA4404 carrying *cry1Ac* gene, a total of 44 out of 100 (cv. HC-1) and 174 out of 200 (cv. C-235) seeds were germinated without using tissue culture procedure, after treatment were characterized for presence of the *cry1Ac* gene.

Direct plant PCR analysis of transformants

Non selective direct plant PCR screening rendered a total of 18 out of 44 (cv. HC-1) and 25 out of 174 (cv. C-235) T_0 putative transformants amplifying anticipated 1068bp amplicon similar to that of positive control whereas, no such amplicons were observed in non-transformed control chickpea plants (Fig. 2). This confirmed the integration of the *cry1Ac* gene in the chickpea genome and represented the transformation frequency of 41.0% and 13.4% for chickpea cvs. C-235 and HC-1 plants respectively.

PCR analysis of Transgenic plants

Seeds harvested from the selected T_0 transgenic chickpea plants were sown in the greenhouse for raising T_1 generation plants and analyzed by PCR screening using the genomic DNA samples of the T_1 generation chickpea plants (cvs. C-235 and HC-1). PCR analysis of T_1 generation chickpea plants (cvs. C-235 and HC-1) showed amplification of 533bp of *cry1Ac* gene fragment using gene specific primers (Fig. 3), similar to that of their respective positive control, whereas, no such amplicons were observed in non-transformed control plants. The stability and inheritance of the *cry1Ac* gene in the next generation was also confirmed by PCR analysis for the presence of *cry1Ac* gene in T_2 generation transgenic chickpea plants (cvs. C-235 and HC-1) (Fig. 3).

ELISA analysis

The Cry1Ac toxin was detected only in 24 % (cv. C-235) and 33.3 % (cv. HC-1) of the PCR-positive T_0 generation transgenic chickpea lines (Table 2). Quantitative assessment of Bt Cry1Ac toxin in T_0 chickpea plants by ELISA showed toxin in the range of 100.5 to 363.5 ng g^{-1} FW of leaves (Fig. 4a and 4b). More importantly, all the T_0 generation transgenic chickpea lines that showed levels of accumulation of Cry1Ac toxin higher than 200 ng g^{-1} FW were found to exhibit phenotypic abnormalities and these abnormalities ranged from extreme retardation in the growth of the plant, to no flowering, and no setting of seeds. Out of twelve ELISA positive independent events of chickpea carrying *cry1Ac* gene, only two of cv. HC-1 (TH-137 and TH-165) and one of cv. C-235 (TC-131) plants with moderate level of Cry1Ac toxin (100-130 ng g^{-1} FW) (Fig. 4a and 4b) were able to set seeds, which were used for raising the next generation plants. Randomly selected T_2 generation transgenic chickpea plants (cvs. HC-1 and C-235) carrying *cry1Ac* gene were analyzed by ELISA showed the Cry1Ac toxin in range of 82.5 to 141.2 ng g^{-1} FW of leaves (Table 2).

Southern hybridization analysis

Southern hybridization of genomic DNA samples from three independent T_2 transgenic chickpea lines digested with *Hind*III enzyme and probed with biotin labelled fragment of *cry1Ac* gene showed the integration of the *cry1Ac* gene in chickpea genome (Fig. 5). The non-identical hybridization patterns of different transformation events of T_2 chickpea plants of cv. HC-1 (TH2-44 and TH2-166) and cv. C-235 (TC2-33) showed independent single copy integration of the *cry1Ac* gene into the transgenic chickpea plants (cvs. HC-1 and C-235). The genomic DNA from non-transgenic control plant was used as a negative control and no hybridization signal was detected (Fig. 5, lane C), while gene specific fragments generated hybridization signal as positive control (Fig. 5, lane P).

Insect bioassay

The T_2 generation chickpea transgenic plants expressing Cry1Ac toxins were evaluated for entomocidal activity by insect feeding bioassays performed with second instar larvae of *H. armigera*. Larvae challenged on leaves of transgenic plants showed retarded growth after 3 days of feeding but significant mortality was not observed (Table 2).

Table 1 List of primer pairs used for PCR analysis of transgenic plants.

Primer Name	Sequence (5'- 3')	Amplicon size (bp)	Annealing temperature
cry1Ac F	CCCAGAAGTTGAAGTACTTGGTGG	1068	58°C
cry1Ac R	CCGATATTGAAGGGTCTTCTGTAC		
cry1Ac F2	TTCTGCCCAAGGTATCGAAG	533	53°C
cry1Ac R2	CAGAACGGTGAATCCAAGAG		

Table 2 Accumulation of Bt-toxin Cry1Ac (ng g^{-1} FW) analyzed by ELISA in 30-day-old transgenic chickpea plants (cvs. HC-1 and C-235) and toxicity of T_2 chickpea plants to 2nd instar *Helicoverpa* larvae.

Bt-toxin Cry1Ac in T_0 Plants		Bt-toxin Cry1Ac in T_2 Progeny		% average larval weight reduction
Plant #	Cry1Ac toxin (ng g^{-1} FW)	Plant #	Cry1Ac toxin (ng g^{-1} FW)	
TH-137	106.3 ± 0.3	TH2-44	95.0 ± 0.2	75 ± 0.3
		TH2-51	82.5 ± 0.5	73 ± 0.7
TH-165	100.5 ± 0.7	TH2-166	102.5 ± 0.4	74 ± 0.2
TC-131	127.8 ± 0.4	TC2-32	133.5 ± 0.1	54 ± 0.4
		TC2-33	95.0 ± 0.3	48 ± 0.4
		TC2-39	141.2 ± 0.2	68 ± 0.6

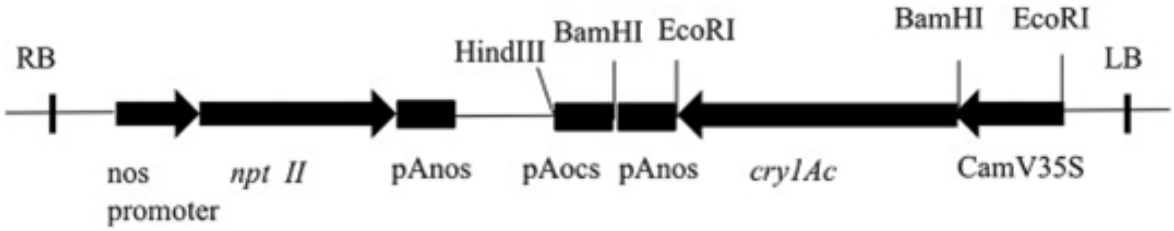

Figure 1 The synthetic *cry1Ac* gene T- DNA construct containing *cry1Ac* gene in pBinAR transformation vector. LB: left border; RB: right border; CaMV35S: cauliflower mosaic virus 35S promoter; OCS Ter: terminator sequence of octopine synthase; NOS: nopaline synthase promoter; *nptII*: neomycin phosphotransferase; Nos Ter: terminator sequence of nopaline synthase.

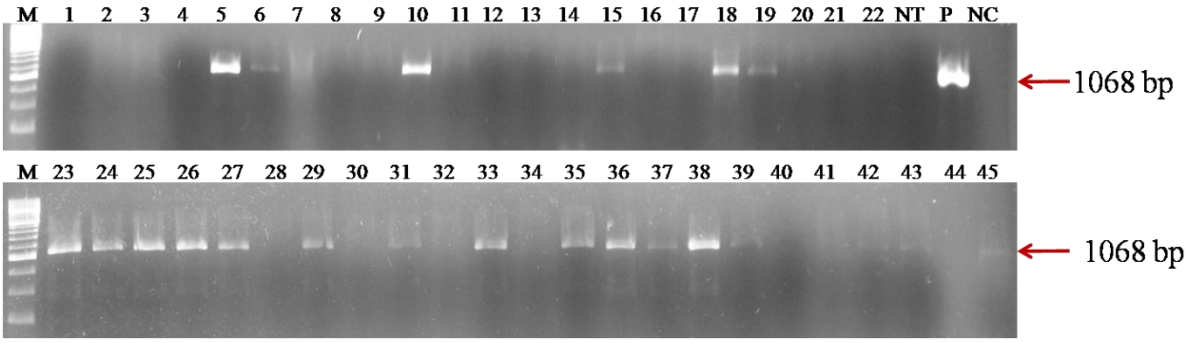

Figure 2 Direct plant PCR analysis of the putatively transformed chickpea plants (cv. C-235 and HC-1) using the *cry1Ac* gene specific primers. M: 200 bp DNA marker; P: Plasmid control; NT: Non transformed plant control; NC: Negative control and Lane 1-45, Seedling samples of putative transformants (T_0).

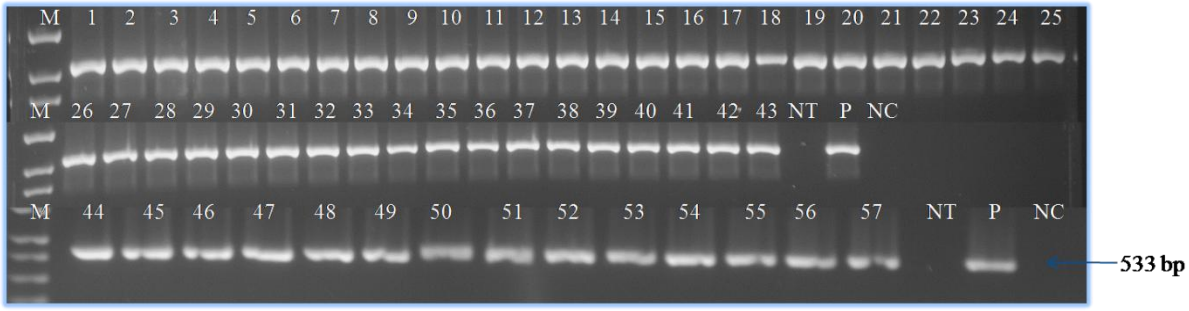

Figure 3 PCR analysis of the transgenic chickpea plants (cv. C-235 and HC-1) using the *cry1Ac* gene specific primers. Lane 1-43: T_1 Transgenic plants; Lane 44-57: T_2 Transgenic plants; M: DNA marker; P: Plasmid control; NT: Non transformed control; NC: Negative control.

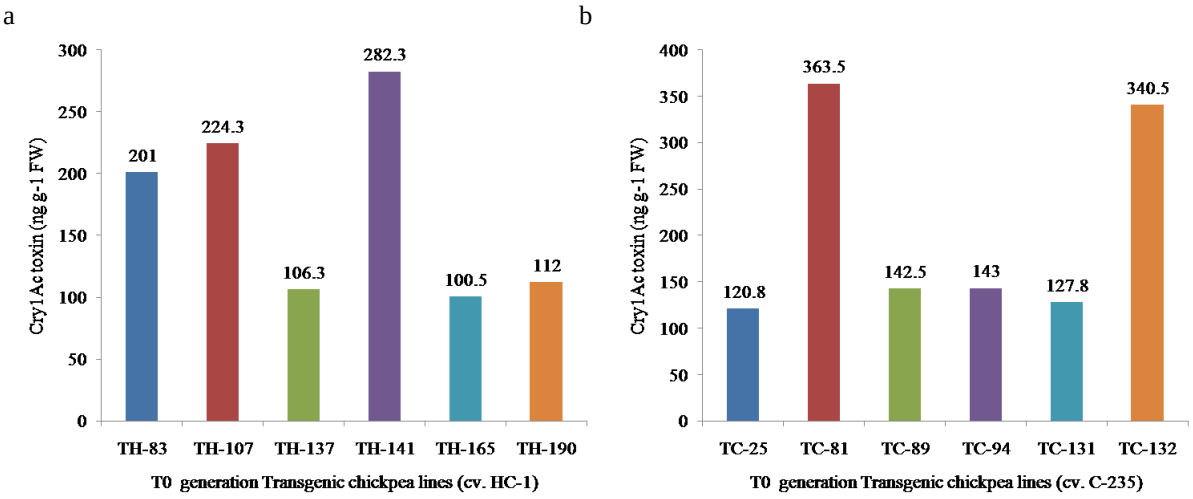

Figure 4 Representative graphs depicting the Cry1Ac toxin (ng g^{-1} FW) on x-axis, analyzed by ELISA in T_0 plants on y-axis, with PCR detected transgene. a) cv. HC-1, b) cv. C-235.

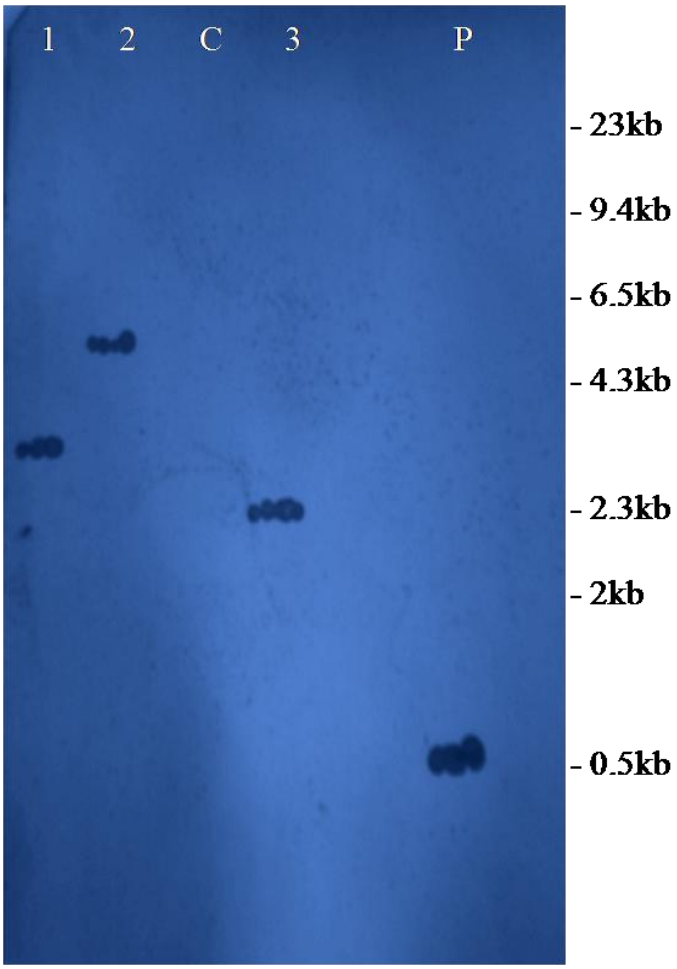

Figure 5 Southern analysis of the T_2 transgenic chickpea plants carrying *cry1Ac* gene. The genomic DNA samples were digested with *Hind*III restriction enzyme and PCR amplified product of *cry1Ac* was used as a biotin labelled probe. Lane 1-TH2-44, Lane 2- TH2-166 and Lane 3-TC2-33 transgenic chickpea plants; C- non-transgenic chickpea plant control; P- positive control.

Discussion

Transformation following the non-selective/PCR detection system using direct plant PCR screening represented transformation frequency of 13.4% and 41% in cv. C-235 and HC-1 respectively which is quite high as compared to the 2.77 % of a previous report of *Agrobacterium* – mediated transformation of chickpea with *cry1Ab* gene (Mehrotra et al., 2011). Similar high frequency transformation of 45.6% and 45.4% have been observed in brinjal (Subramanyam et al., 2013) and sugarcane (Mayavan et al., 2013) respectively using in planta seed transformation. Our results of ELISA for Bt-Cry1Ac toxin in T_0 transgenic population developed with *cry1Ac* gene have revealed variation in expression in population which is consistent with earlier observations for differential expression of Bt-endotoxin in T_0 plants (Perlak et al., 1991; Husnain et al., 2002; Mehrotra et al., 2011). This variation in expression of Bt-endotoxin amongst population of primary T_0 transgenic plants may be attributed to position effect of gene integration, flanking sequences, chromatin context of the locus, and increase in DNA methylation and physiological changes of the Bt protein in the plant tissues (Peach and Velten 1991; Down et al., 2001; Husnain et al., 2002).

The abnormal phenotype of transgenic lines with high levels of Cry1Ac protein could be due to accumulation of high levels of Cry1Ac protein which could be detrimental to chickpea plant development. In a study, based on the genetic transformation of cotton and tobacco, Rawat et al. (2010) showed that the expression of the Cry1Ac endotoxin had detrimental effects on both the *in vitro* and *in vivo* growth and development of transgenic plants resulting in phenotypical abnormalities. Acharjee et al. (2010) found significant reduction in the growth rate and seed production in chickpea lines expressing high levels of Cry2Aa protein when compared to the parental line. It

appeared that the high level of Cry protein was causing growth reduction in chickpea. Southern hybridization using independent plant transformants indicated different sizes of hybridizing genomic DNA fragments, which could be due to random independent stable T-DNA integration event in chickpea genome as also reported by other workers (Acharjee et al., 2010; Mehrotra et al., 2011). However, presence of the expected hybridization signals with genomic DNA fragments (>2.6 kb) in the transformed plants, showed that the probed gene *cry1Ac* remained intact when integrated into the chickpea genome. Evaluation of the T_2 generation progenies of few promising T_1 transgenic plants with moderate level of Cry1Ac toxin (100-130 ng g^{-1} FW) for entomocidal activity showed retarded growth after 3 days of larval feeding but significant mortality was not observed. These results demonstrate that the low level of Cry1Ac toxin expression in transgenic chickpea plants was not enough which could cause larval mortality. The *Agrobacterium*-mediated transformation and recovery of transgenics of grain legumes are restricted due their complex genome (Dita et al., 2006; Eapen, 2008). Obtaining an event with higher expression of Bt-toxin in chickpea plant, a recalcitrant grain legume will require screening of a larger population of primary transformants (Somers et al., 2003).

In conclusion, the protocol ensured not genotype dependent generation of transgenic plants with considerable ease in a short time with high frequency and does not require the complex steps for selection of the transgenic events. These findings will certainly accelerate the development of chickpea plants with novel traits with the possibility of developing marker free transgenic events using binary vectors that are devoid of the marker gene/s upfront, which allows stacking of multiple genes.

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References

Acharjee, S., Sarmah, B.K., Kumar, P.A., Olsen, K., Mahon, R., Moar, W. J., Moore, A. and Higgins, T. J. V. (2010): Transgenic chickpeas (*Cicer arietinum* L.) expressing a sequence-modified *cry2Aa* gene. Plant Sci., 178: 333-339.

Acharjee, S. and Sarmah, B.K. (2013): Transgenic *Bacillus thuringiensis* (Bt) chickpea: India's most wanted genetically modified (GM) pulse crop. Afr. J. Biotechnol., 12(39): 5709-5713.

Anwar, F., Sharmila, P. and Saradhi, P. P. (2010): No more recalcitrant: Chickpea regeneration and genetic transformation. Afr. J. Biotechnol., 9(6): 782-797.

Arumuganathan, K. and Earle, E.D. (1991): Nuclear DNA content of some important plant species. Plant Mol Biol Rep., 9(3): 208-218.

Asharani, B.M., Ganeshaiah, K.N., Raja, A., Kumar, V. and Makarla, U. (2011): Transformation of chickpea lines with *cry1X* using *in planta* transformation and characterization of putative transformants T1 lines for molecular and biochemical characters. J. Plant Breeding Crop. Sci., 3(16): 413-423.

Biradar, S.S., Sridevi, O. and Salimath, P.M. (2009): Genetic enhancement of chickpea for pod borer resistance through expression of CryIAc protein. Karnataka Journal of Agricultural Science., 22(3): 467-470.

Chakraborti, D., Sarkar, A., Gupta, S. and Das, S. (2006): Small and large scale genomic DNA isolation protocol for chickpea (*Cicer arietinum* L.) suitable for molecular marker and transgenic analyses. Afr. J. Biotechnol., 5(8): 585-589.

Dita, M.A., Rispail, N., Prats, E., Rubiales, D. and Singh, K.B. (2006): Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. *Euphytica.*, 147: 1–24.

Down, R.E., Ford, L., Bedford, S.J., Gatehouse, L.N., Newell, C., Gatehouse, J.A. and Gatehouse, A.M.R. (2001): Influence of plant development and environment on transgene expression in potato and consequences for insect resistance. Transgenic Res., 10: 223-236.

Eapen, S. (2008): Advances in development of transgenic pulse crops. Biotechnol Adv., 26: 162–168.

FAOSTAT, (2012): Agricultural Data. http://faostat3.fao.org/faostat-gateway/go/to/download/Q/QC/E

Gupta, G.P., Birah, A. and Ravi, S. (2004): Development of artificial diet for mass rearing of American bollworms (*Helicoverpa armigera*). Indian J. Agr. Sci., 74: 548-557.

Hofgen, R., and Willmitzer, Z., (1990): Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum*). Plant Sci., 66: 221-230.

Husnain, T., Jan, A., Maqbool, S.B., Datta, S.K. and Riazuddin, S. (2002): Variability in expression of insecticidal *cry1Ab* gene in Indica basmati rice. Euphytica., 128: 121-128.

ICRISAT, (1992); The medium term plan. International Crops Research Institute for Semi-Arid Tropics. Patancheru. India.

Jukanti, A.K., Gaur, P.M., Gowda, C.L. and Chibbar, R.N. (2012): Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br. J. Nutr., 108: S11-S26.

Kar, S., Basu, D., Das, S., Ramakrishnan, N.A., Mukherjee, P., Nayak, P. and Sen, S.K. (1997): Expression of *cryIA(c)* gene of *Bacillus thurigenesis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. Transgenic Res., 6: 177-185.

Kharb, P., Batra, P. and Chowdhury, V.K. (2012): A novel process of genetic transformation in chickpea using *Agrobacterium*. Indian Patent No. 252590.

Mayavan, S., Subramanyam, K., Arun, M., Rajesh M., Dev, G.K., Sivanandhan, G., Jaganath, B., Manickavasagam, M., Selvaraj, N. and Ganapathi, A. (2013): *Agrobacterium tumefaciens*-mediated in planta seed transformation strategy in sugarcane. Plant Cell Rep. DOI 10.1007/s00299-013-1467-5.

Mehrotra, M., Sanyal, I. and Amla, D.V. (2011): High-efficiency *Agrobacterium*-mediated transformation of chickpea *(Cicer arietinum* L.) and regeneration of insect-resistant transgenic plants. Plant Cell Rep., 30: 1603-1616.

Neelima, M.G., Ramu, S.V., Sreevathsa, R., Asharani, B.M., Kumar, A.R.V. and Gayatri, M.C. (2008): *In planta* transformation strategy to generate transgenic plants in chickpea: Proof of concept with a cry gene. J. Plant Biol., 35(3): 201-206.

Peach, C. and Velten, J. (1991): Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. Plant Mol. Biol., 17: 49-60.

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L. and Fischoff, D.A. (1991): Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA, 88: 3324-3328.

Rawat, P., Singh, A.K., Ray, K., Chaudhary, B., Kumar, S., Gautam, T., Kanoria, S., Kaur, G., Kumar, P., Pental, D. and Burma, P.K. (2011): Detrimental effect of expression of Bt endotoxin Cry1Ac on in vitro regeneration, in vivo growth and development of tobacco and cotton transgenics. J. Biosci., 36: 363-376.

Sanyal, I., Singh, A.K., Kaushik, M. and Amla, D.V. (2005): *Agrobacterium* – mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis cry1Ac* gene for resistance against pod borer insect *Helicoverpa armigera*. Plant Sci., 168: 1135-1146.

Somers, D.A., Samac, D.A. and Olhoft, P.M. (2003): Recent advances in legume transformation. Plant Physiol., 131: 892–899.

Subramanyam, K., Rajesh, M., Jaganath, B., Vasuki, A., Theboral, J., Elayaraja, D., Karthik, S., Manickavasagam, M. and Ganapathi, A. (2013): Assessment of factors influencing the *Agrobacterium* mediated in planta seed transformation of brinjal (*Solanum melongena* L.). Appl. Biochem. Biotechnol. DOI 10.1007/s12010-013-0359-z.

Tabashnik, B.E., Dennehy, T.J., Carrière, Y., Liu, Y.B., Meyer, S.K., Patin, A., Sims, M.A. and Ellers-kirk, C. (2003): Resistance management: slowing pest adaptation to transgenic crops. Acta. Agric. Scand., Sect. B., Soil and Plant Sci., 53(1): 51-56.

Varshney, R.K., Thudi, M., May, G.D. and Jackson, S.A. (2010): Legume genomics and breeding. Plant Breeding Rev., 33: 257-304.

Yadav, S.S., Kumar, J., Yadav, S.K., Singh, S., Yadav, V.S., Turner, N.C. and Redden, R. (2006): Evaluation of *Helicoverpa* and drought resistance in desi and kabuli chickpea. Plant Genetic Resources: Characterization and Utilization, 4: 198-203.