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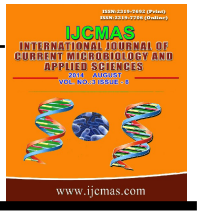

Original Research Article

Molecular characterization of Bt chickpea (*Cicer arietinum* L.) plants carrying *cry1Aa$_3$* gene

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K e y w o r d s

Transgenic chickpea plants· *Agrobacterium*· Genetic transformation· Bt · *Helicoverpa* · Cry toxin

A B S T R A C T

Transgenic chickpea plants expressing *cry1Aa$_3$* gene have been generated using a novel process of genetic transformation for resistance against pod borer (*Helicoverpa armigera*)-the major damage causing insect. Multiple evaluation strategies, such as PCR, ELISA and southern hybridization were employed for characterization of the integration and expression of *crylAa$_3$* gene. Quantitative assessment of Bt Cry1Aa$_3$ toxin by ELISA in leaves of transgenic chickpea plants showed toxin in range of 91.0 to 154.0 ng g^{-1} FW. Southern hybridization using gene specific probe confirmed single copy integration of the *cry1Aa$_3$* gene into the chickpea genome. The efficacy of transgenic chickpea plants expressing *cry1Aa$_3$* gene against the target pest showed retarded larval growth. The study demonstrated potentialities of developing insect resistant transgenic chickpea and provides an opportunity for further test and advancement to reach the field utility level.

Introduction

Chickpea (*Cicer arietinum* L.), world's second most widely grown legume crop after soybean, is cultivated in Asia, Mediterranean regions, Australia, Canada, the USA and Africa. It is a self-pollinating diploid (2n=2x=16) with genome size of 740 Mbp (Arumuganathan et al., 1991) and belongs to the family Leguminosae and subfamily Faboideae. India is the largest chickpea growing country; with 8.3mha of chickpea grown area and producing 7.7mt chickpea, more than 68% of the global production with an average yield of about 0.92 ton ha^{-1} (FAOSTAT, 2012). Gram pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is the most devastating insect pest and an important biotic constraint to chickpea production, which at times causes severe pod damage up to 90%, resulting in annual losses of over US $325 million (Yadav et al., 2006). Approaches to the generation of transgenic crops is an elegant and perhaps the most

effective delivery system for *Bacillus thuringiensis* (Bt) toxins, that apparently provides a relatively long lasting and seed borne solution for the management of the lepidopteran pest (Tabashnik et al., 2003), the success story of which is very well demonstrated in widely cultivated transgenic cotton expressing Bt insecticidal protein (Quaim et al., 2003).

Till date, transformed chickpea plants resistant to pod borer have been produced by *Agrobacterium*-mediated genetic transformation by several groups (Kar et al., 1997; Sanyal et al., 2005; Biradar et al., 2009; Acharjee et al., 2010; Asharani et al., 2011 and Mehrotra et al., 2011) by transferring different versions of *cry* genes. However we are unaware of any study on efficacy of *cry1Aa*$_3$ gene in chickpea for resistance against *Helicoverpa* (Acharjee and Sarmah, 2013). The *cry1Aa*$_3$ gene was used to transform sugarcane (Kalunke et al., 2009) and eggplant (Rai et al., 2013) to produce lepidopteran pest resistant plants. Bt chickpea plants (cv. C-235) carrying *cry1Aa*$_3$ gene generated successfully by Kharb et al (2012) have been characterized for the integration and expression of transgene in the present study.

Materials and Methods

Plant material

Progeny of a single transformation event of Bt chickpea plants (cv. C-235) carrying *cry1Aa*$_3$ gene, generated by Kharb et al (2012) using *Agrobacterium tumefaciens* strain EHA105 were used in the present study for molecular characterization. Transgenic chickpea plants expressing *cry1Aa*$_3$ gene have been generated using a novel process of genetic transformation without using tissue culture protocol.

PCR analysis

Total genomic DNA was isolated from young leaves of greenhouse grown putative transformed and control chickpea plants by CTAB method (Doyle, 1990). PCR amplification of the genomic DNA of chickpea plants using *cry1Aa*$_3$ gene specific primers were carried out in MY-CYCLER (Bio-Rad) to confirm the presence of the transgene in the 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 forward primer (5'-CCTCTGGCCAAACACGGAGACG-3'), 0.1µM reverse primer (5'-ACCCTGAGGTCGAGGTCCTTGGT-3'), 1 U *Taq* DNA polymerase (Banglore Genei) and template DNA (50ng). 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 1 min extension at 72°C for 1 min and final extension at 72°C for 7 min. The plasmid DNA was used as positive control and wild type non transformed chickpea plant genomic DNA sample was used as negative control for the PCR reaction. The products were run on a 1.5% agarose gel and analyzed on Alpha Innotech Gel Documentation system.

ELISA analysis

QuantiPlate Kit for Cry1Ab/1Ac (Envirologix, USA) was used for detection of $Cry1Aa_3$ toxin in the transgenic chickpea plants by sandwich ELISA using cell-free extracts of young leaf samples according to manufacturer's instructions. The results were read at wavelength of 450 nm using the plate reader (iMark Microplate Absorbance Reader, Bio-Rad, USA) and represented in ng g^{-1} FW of leaves.

Southern hybridization analysis

Genomic DNA (20 µg) samples from T_4 generation plants carrying *cry1Aa*$_3$ gene were digested with *Hind*III, separated on 0.8% agarose gel, and blotted on nylon membrane. The PCR amplified 492bp fragment of *cry1Aa*$_3$ 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 manufacturer's 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 leaflets, 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, 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.

Results and Discussion

PCR analysis

The T_4 generation progeny plants of a single transformation event were analyzed by PCR using the genomic DNA samples showed amplification of 492bp of *cry1Aa*$_3$ gene fragment using gene specific primers (Fig. 1), similar to that of their respective positive control, whereas, no amplification was observed in non-transformed control plants. The stability and inheritance of the *cry1Aa*$_3$ also confirmed by PCR analysis in T_5 to T_7 generation transgenic chickpea plants (Fig. 1).

ELISA analysis

The Cry1Aa$_3$ toxin detected by ELISA confirmed the expression of the transgene in the transgenic chickpea plants. The level of Cry1Aa$_3$ toxin in transgenic chickpea plants was in the range of 91.0 to 154.0 ng g^{-1} FW of leaves of 60 days old T_4 generation plants (Table 1). The high expressing lines were selected and used for raising the next generation plants for further molecular characterization. The level of Bt toxin in the next generation plants (up to T_7) was in the same range as in the parent plants (Fig. 2). The presence of Bt toxin in the transgenic plants expressing the toxin in their leaves was not observed in immature seeds of 120 days old plants. Further the toxin concentration in leaves declined in transgenic chickpea plants after pod formation in 120 days old plants (Fig. 2).

Southern hybridization analysis

Southern hybridization analysis of the genomic DNA of transgenic chickpea plants digested with *Hind*III, probed with *cry1Aa*$_3$ gene specific biotin labeled probe confirmed the integration of the target gene into the transgenic chickpea plants genome (Fig. 3, Lane 1-6). The single copy integration after hybridization of biotin labelled fragment of *cry1Aa*$_3$ gene from selected high expressing T_4 transgenic chickpea plants ($T_4$3-28, $T_4$3-43, $T_4$6-19, $T_4$6-41, $T_4$7-10 and $T_4$11-2) have been observed. The genomic DNA from non-transgenic control plant was used as a negative control and no hybridization

signal was detected (Fig. 3, lane C), while gene specific fragments generated hybridization signal as positive control (Fig. 3, lane P).

Insect bioassay

Insect bioassay was performed with some promising T_5 transgenic plants with moderate level of Cry1Aa$_3$ toxin (93 - 141 ng g^{-1} FW) with second instar larvae of *H. armigera*. Fresh twigs from 60 days old transgenic chickpea plants were fed to the larvae and response of their feeding on weight gain, life cycle and mortality of insect was monitored. Larvae challenged on leaves of transgenic plants showed retarded growth after 3 days of feeding but significant mortality was not observed (Table 1).

Transgenic chickpea plants generated by a novel process of genetic transformation in chickpea using *Agrobacterium* (Kharb et al., 2012) were characterized in the present study. The stability and inheritance of the *cry1Aa$_3$* gene was analyzed by PCR and ELISA up to T_7 generation of transgenic chickpea plants. We are unaware of any study on efficacy of *cry1Aa$_3$* gene in chickpea for resistance against *Helicoverpa* but similar results have been observed by other workers for *cry1Aa$_3$* gene in sugarcane (Kalunke et al., 2009) and eggplant (Rai et al., 2013) for resistance against lepidopteran insect pests. The Cry1Aa$_3$ toxin concentration declined in leaves of transgenic chickpea plants after flowering as analyzed by ELISA in 120 days old transgenic chickpea plants. Similar decline in concentration of Bt toxin has been reported earlier in Bt cotton crop (Olsen et al., 2005). Results obtained from Southern hybridization of transformed chickpea plants that were positive to PCR/ELISA tests confirmed the single copy integration of the target gene into the transgenic chickpea genome carrying *cry1Aa$_3$* gene. Since the *Hind*III restriction site is unique in the T-DNA, the probe detected a hybridization signal of T-DNA left border/plant junction fragment of 2.3 kb which revealed the single copy integration after hybridization of biotin labelled fragment of *cry1Aa$_3$* gene in selected T_4 transgenic chickpea plants. The same size of hybridizing genomic DNA fragments of T_4 transgenic plants with the probe indicated that they resulted from the same stable T-DNA integration event into the chickpea genome. Evaluation of the T_5 generation plants with moderate level of Cry1Aa$_3$ toxin (93 -141 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 level of Cry1Aa$_3$ toxin expression in transgenic chickpea plants was not enough to cause larval mortality. *H. armigera* is generally known to have low sensitivity to Cry1Aa3 toxin (Avilla et al., 2005). This suggests screening of a larger population of primary transformants for obtaining an event with higher expression of Bt-toxin in chickpea plant, a recalcitrant grain legume (Somers et al., 2003). The study demonstrated potentialities of developing insect resistant transgenic chickpea and provides an opportunity for further test and advancement to reach the field utility level.

Table.1 Expression of Bt-toxin Cry1Aa$_3$ (ng g^{-1} FW) analyzed by ELISA in 60 days old transgenic chickpea plants and toxicity of T_5 chickpea plants to 2nd instar *Helicoverpa* larvae.

Bt toxin in T_4 generation plants		Bt toxin in T_5 generation plants		
Plant #	Bt toxin (ng g^{-1} FW±SE)	Plant #	Bt toxin (ng g^{-1} FW±SE)	% average larval weight reduction ± SE
$T_4$3-28	154.0 ± 0.1	$T_5$3-28-7	136.0 ± 0.8	77 ± 0.1
$T_4$6-41	93.5 ± 0.2	$T_5$6-41-14	99.0 ± 0.3	55 ± 0.2
$T_4$7-10	91.0 ± 0.4	$T_5$7-10-10	119.0 ± 0.6	64 ± 0.1

Fig.1 PCR analysis of the transgenic chickpea plants using the *cry1Aa$_3$* gene specific primers. Lane 1-32: T_4 Transgenic plants; Lane 33-54: T_5 Transgenic plants; Lane 55-63: T_6 Transgenic plants; M: DNA marker; P: Plasmid control; NT: Non transformed control; NC: Negative control.

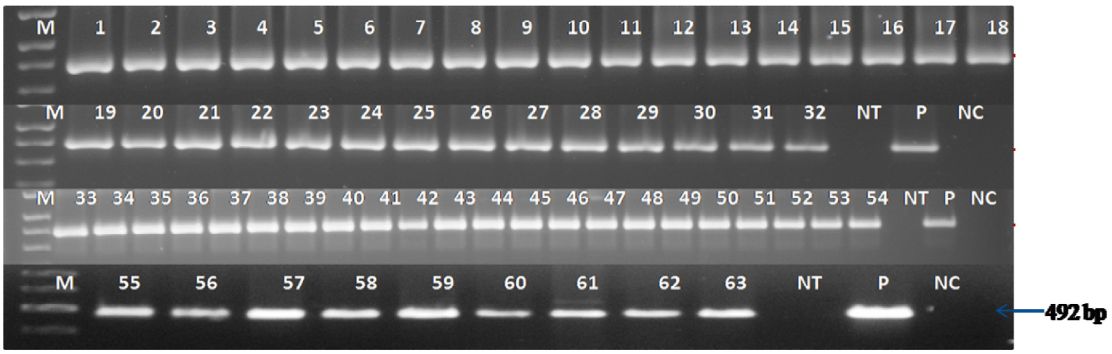


Fig.2 Representative graphs depicting the Cry1Aa$_3$ toxin (ng g^{-1} FW) analyzed by ELISA in 60 days and 120 days old T_7 transgenic chickpea plants.

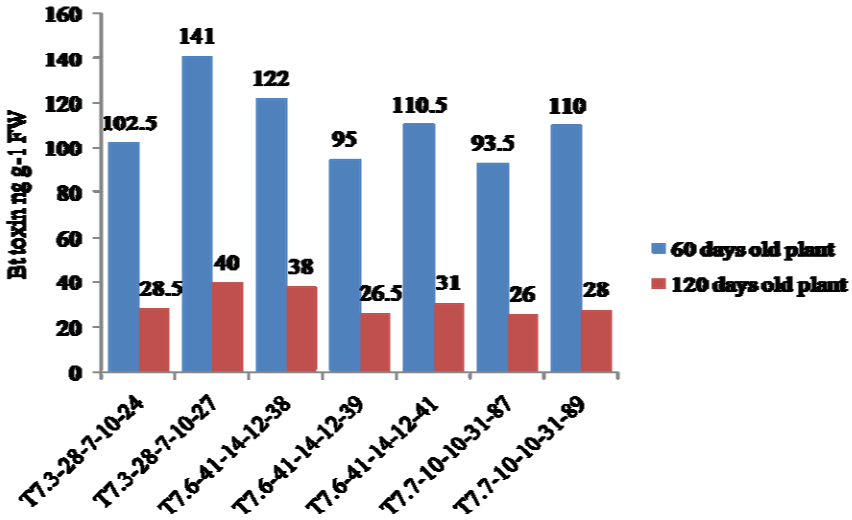
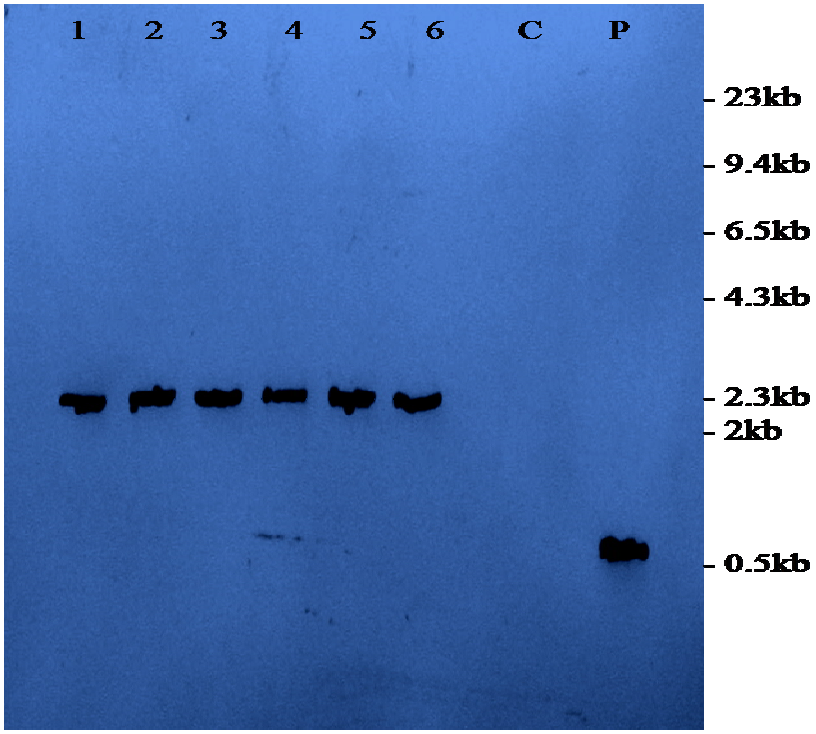


Fig.3 Southern analysis of the T_4 transgenic chickpea plants carrying *cry1Aa*$_3$ gene. The genomic DNA samples were digested with *Hind*III restriction enzyme and PCR amplified product of *cry1Aa*$_3$ was used as a biotin labelled probe. Lanes 1- $T_4$3-28, 2- $T_4$3-43, 3- $T_4$6-19, 4- $T_4$6-41, 5- $T_4$7-10 and 6- $T_4$11-2; C- non-transgenic chickpea plant control; P- positive control.



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.

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.

Avilla, C., Vargas-Osuna, E., González-Cabrera, J., Ferré, J. and González-Zamora, J.E. 2005. Toxicity of several δ-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Spain. J. Invertebr. Pathol. 90: 51-54.

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.

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.

Doyle, J.J. 1990. Isolation of plant DNA from fresh tissue. Focus. 12: 13-15.

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.

Kalunke, R.M., Kolge, A.M., Babu, K.H. and Prasad, D.T. 2009. *Agrobacterium* – mediated transformation of sugarcane for borer resistance using *cry1Aa3* gene and one-step regeneration of transgenic plants. Sugar Tech. 11(4): 355-359.

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.

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.

Olsen, K.M., Daly, J.C., Holt, H.E. and Finnegan, E.J. 2005. Season-long variation in expression of *cry1Ac* gene and efficiency of *Bacillus thuringiensis* toxin in transgenic cotton against *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol. 98: 1007-1017.

Qaim, M., Pray, C.E. and Zilbeman, D. 2008. Economic and social considerations in the adoption of Bt crops In: Integration of insect-resistant genetically modified crops within IPM programs. Springer, Dordrecht, The Netherlands pp. 329-356.

Rai, N.P., Rai, G.K., Kumar, S., Kumari, N. and Singh, M. 2013. Shoot and fruit borer resistant transgenic eggplant (*Solanum melongena* L.) expressing *cry1Aa3* gene: Development and bioassay. Crop Prot. 53: 37-45.

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.

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 Breed. 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.