

Genetic Transformation of Cotton for Bollworm *Helicoverpa armigera* (Hubner) Resistance using Chimeric *cry2AX1* Gene

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Abstract

Cotton (*Gossypium hirsutum*) is the most important fiber crop and backbone of textile industry of the world. Cotton bollworm (*Helicoverpa armigera*) is one of the most destructive insect pests of cotton in India. Expression of chimeric or hybrid Bt protein has led to enhanced resistance to pests and added advantage with respect to insect resistance management. We report in this paper, expression of a chimeric *cry2AX1* gene (consisting part of *cry2Aa* and *cry2Ac*) in transgenic cotton and its resistance towards *H. armigera*. *Agrobacterium* strain LBA4404 harbouring chimeric *cry2AX1* gene construct driven by enhanced CaMV35S promoter in pCambia2300 backbone was used for co-cultivation of cotton cv. Cocker 310 embryogenic calli. A total of 11 putative T₀ transgenic plants were generated and all of them found PCR positive for the presence *cry2AX1* and *nptII* genes. Southern blot hybridization confirmed the T-DNA integration and intactness of *cry2AX1* gene. Quantitative assessment of *cry2AX1* protein by ELISA in transgenic T₀ cotton plants showed expression of protein in the range 0.015 to 0.080 µg/g fresh leaf tissue. Insect bioassay of transgenic T₀ cotton plants using *H. armigera* neonates recorded a mortality of 10 to 36.66 per cent and showed significant reduction in leaf feeding and inhibition of growth in surviving larvae. The results demonstrate potential of the chimeric *cry2AX1* gene in developing *H. armigera*-resistant transgenic cotton varieties.

Keywords: *Agrobacterium tumefaciens*, Cotton Transformation, Cry2AX1, ELISA, Insect Bioassay, *Helicoverpa armigera*

1. Introduction

Cotton is the leading fiber crop produced in the world and the most important crop in India. India is the largest producer of cotton in the world, the second largest exporter of raw cotton (www.cottoninc.com). The growth and productivity of cotton crop is often hampered by various biotic as well as abiotic stress factors which results in yield reduction and poor quality of cotton fibres. One of the major limiting factors, which affect cotton production in India, is the incidence of pests, especially bollworms, causing more than 50 per cent yield loss¹. *Helicoverpa armigera* is the most common bollworm of cotton in India and can result in yield loss ranging from 30-80%¹⁷.

The limited genetic variability for bollworm resistance in cotton germplasms makes the task of developing

bollworm-resistant genotypes very difficult¹⁵. However, developing improved cotton cultivars/hybrids with desirable traits by conventional cross breeding methods is time consuming and laborious. Under these circumstances traits from other organism could be horizontally transferred by use of genetic engineering techniques. Genetic engineering offers successful production of transgenic plant with desirable traits within stipulated period of time. The use of proteins encoded by *cry* genes of *Bacillus thuringiensis* (Bt) for insect pest management has emerged as a potent tool, being pesticide free, environment friendly and highly specific against target insects due to presence of specific receptor and non toxic to majority of beneficial insects and vertebrates, due to lack of specific receptor^{6,27}. Genetically modified (GM) crops expressing Bt toxin for imparting resistance against

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different insect pests have been commercially cultivated in several countries. Socio-economic benefit of GM crops has been well proven through meta-analysis studies on impact of GM crops. Adoption of GM technology has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%¹⁶. *Agrobacterium*-mediated gene transfer method has been used successfully for transformation of numerous dicot species. Cotton engineered with different insecticidal protein gene(s) from *Bt* has been reported by many authors to provide protection to the plants against different lepidopteran insect pests^{18,30,31,35,36}. Owing to the difference in structure and insecticidal mechanism, *cry2A* genes are promising candidates for the management of insects in crop plants¹². The chimeric *Cry2AX1* protein was found to be more toxic than its parental proteins (*Cry2Aa* and *Cry2Ac*)³⁴. In the light of the above developments, the present study was conducted to produce transgenic cotton plants expressing *cry2AX1* gene, using *Agrobacterium*-mediated transformation and to evaluate the resistance of the developed plants to *H. armigera*.

2. Material and Methods

2.1 Binary Vectors and Strain

The pC2300-*En35S-2AX1* construct containing *cry2AX1* gene¹⁴ (Figure 1 (a)) and pC2300-*En35S-ctp2AX1*²⁵ containing *cry2AX1* with chloroplast transit peptide sequence (Figure 1 (b)) (both were driven by *EnCaMV35S* promoter and *nos-polyA* in pCambia 2300 backbone⁹, were mobilized into *Agrobacterium* strain LBA4404.

Both these vectors harbor the neomycin phosphotransferase (*nptII*) gene (driven by *CaMV35S* promoter

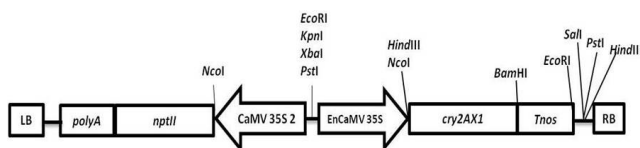


Figure 1. (a) Schematic representation of T-DNA region of the plant transformation construct pC2300-*En35S-2AX1*-*Tnos*.

LB: left border, poly A: *CaMV35S* terminator, *nptII*: neomycin phosphotransferase gene, *CaMV35S2*: Cauliflower mosaic virus 35S duplicated promoter, *EnCaMV35S*: Enhanced cauliflower mosaic virus 35S promoter, *cry2AX1*: gene of interest, *Tnos*: *nopaline synthase* terminator, RB: right border

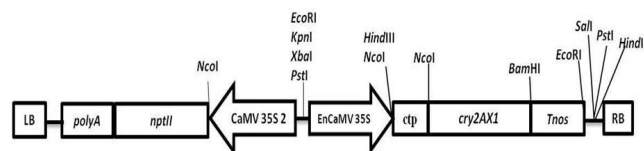


Figure 1. (b) Schematic representation of T-DNA region of the plant transformation construct pC2300-*En35S-ctp2AX1*-*Tnos*.

LB: left border, polyA: *CaMV35S*, *nptII*: neomycin phosphotransferase gene, *CaMV35S2*: Cauliflower mosaic virus 35S duplicated promoter, *EnCaMV35S*: Enhanced cauliflower mosaic virus 35S promoter, *ctp*: chloroplast transit peptide, *cry2AX1*: gene of interest, *Tnos*: *nopaline synthase* terminator; RB: right border

and polyA), which confers resistance to kanamycin used for plant selection. *Agrobacterium* strain LBA4404 harbouring these binary vectors was used for transformation of cotton; grown on YEP [1% Yeast extract, 1% Peptone and 0.5% NaCl pH 7.0] media containing kanamycin 50 µg/ml and rifampicin 20 µg/ml in an incubator at 28°C and utilized for transformation of embryogenic cotton calli.

2.2 *Agrobacterium* Mediated Transformation of Cotton

Sulphuric acid delinted seeds of Coker 310 were surface sterilized with 70% ethanol for 1 min and washed thrice with sterile distilled water. They were again surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 10 min, followed by three washes with sterile distilled water and the seeds were blot dried on sterile tissue paper. The sterile seeds were germinated on a half strength MS basal medium²³ in germination bottle, supplemented with 15 g/L sucrose and 8 g/L agar. The germination bottles were incubated for one week in the culture room at 25 ± 2°C under 16/8 hr photoperiod Cotyledon (~10 mm²) and hypo-cotyledon (8 mm long) sections were excised from seven day old cotton seedlings and inoculated in callus induction medium (CIM) supplemented with 0.1 mg/L 2, 4-D and 0.5 mg/L kinetin, 30 g/L maltose solidified with 3.6 g/L phytigel for callus induction. The explants were sub-cultured in CIM after 2 weeks. After one month, induced fresh calli were separated from the explants and sub-cultured in the same medium for another one month for calli proliferation. Highly proliferating calli of two to three months old were transferred to MS basal medium and maintained for two month on

the same medium to achieve callus maturation. After callus maturation (total age of callus-120 days) good proliferating callus (loose, friable and cream-coloured) were transferred to MS medium supplemented with 1.9 g/L KNO₃, 30 g/L maltose solidified with 3.6 g/L phytagel [referred to as somatic embryogenesis medium (SEM)] for the induction of embryogenic callus. After one month of culture, vigorously growing, friable, loose and white embryogenic calli were used for co-cultivation. The cultures were maintained at 26°C under a 16/8-h photo-period (white fluorescent bulb) at a light intensity of 100 $\mu\text{moles m}^{-2} \text{sec}^{-1}$ in a growth chamber (Sanyo Electric Co Ltd, Moriguchi, Japan).

2.3 Molecular and Biochemical Analyses

2.3.1 PCR Analysis

DNA was isolated from putative transgenic cotton plants using CTAB method²⁴. PCR screening of putative cotton transformants was performed using *cry2AX1* gene specific primers (Forward 5'CCTAACATTGGTGGACTTCCAG 3' and Reverse 5' GAGAAACGAGCTCCGTATTCGT 3') and *nptII* gene specific primers (forward primer 5'- AGAACTCGTCAAGAAGGCGA and reverse primer 5'-CAGACAATCGGCTGCTCTGA). The PCR for the both sets of samples was carried out using a Thermal Cycler (Eppendorf, Germany) in 25 μl reaction volume containing, 2.5 μl of 10X *Taq* buffer, 75 μM each of dNTPs, 50 ng each of forward and reverse primers and 1.5U of *Taq* DNA polymerase (Bangalore Genei, India) and incubated in a thermal cycler which was programmed for 5 min preheat at 94°C and then 30 cycles of denaturation at 94°C for 1 min, annealing temperature of 58°C for 45 s and extension time of 45 s at 72°C, with a final extension at 72°C for 7 min. The plasmid DNA was used as positive control and wild type non transformed cotton plant genomic DNA sample was used as negative control for the PCR reaction. The PCR products were run on 1.0 % agarose gel and analyzed on G: BOX F3 (Syngene) gel documentation system.

2.3.2 Southern Blot Analysis

Ten microgram of genomic DNA (isolated from T₀ transformed and non-transformed cotton plants) was digested overnight by *HindIII* enzyme, electrophoretically separated on 0.8 % agarose gel using 1X TAE, denatured with 0.25 M NaOH and transferred overnight to a positively charged membrane using 20X SSC following standard

upward capillary transfer protocol. The transferred DNA was cross linked by UV exposure at 1200 $\mu\text{J}/\text{min}$ for 1 min. For hybridization, an 800 bp internal region of *cry2AX1* gene was used as a probe. Probe DNA was labeled with α ³²P deoxycytidine triphosphate (dCTP) using the decalabel DNA labeling kit (Thermo Scientific Inc, Waltham, USA) and added into hybridization solution. Prehybridization was carried out for 1h and hybridization for 16 h at 60°C. After hybridization, the blot was washed with 2X SSC (saline sodium citrate) + 0.1% SDS (sodium dodecyl sulphate) for 15 min, followed by 10 min in 0.5X SSC + 0.1% SDS and final wash with 0.1X SSC+ 0.1% SDS for 5 minute. All washings were carried out at 60°C and the blot was exposed to X-ray film.

2.3.3 ELISA Analysis

A double-antibody sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) was used to detect the presence of the Cry2AX1 protein expressed in the leaves of transgenic cotton plants. Experiments were performed with double sandwich quantitative Cry2A ELISA kit (Envirologix, Portland, USA). Proteins from leaf samples of transformed and untransformed cotton plants were extracted using the protein extraction buffer provided in the kit. Each sample was replicated twice. Leaf extract was diluted to fit in the linear range of the provided Cry2A standards and steps were performed according to manufacturer's instructions. Optical density (O.D.) was read at 450 nanometres in an ELISA plate reader (Biotek, USA). The quantity of Cry2AX1 protein present in the sample was calculated by referring to standard graph generated with Cry2A calibration standards and represented in $\mu\text{g/g}$ fresh weight of leaves.

2.4 Insect Bioassay

Detached leaf bit bioassay was carried out to determine the degree of insect resistance in ELISA positive T₀ transgenic cotton plants under laboratory condition with *H. armigera* neonates. Leaf bit from T₀ transgenic and control cotton plants were placed in a wet filter paper in Petri plates and used for bioassay. One neonate larva of *H. armigera* was released on each leaf bit. Each treatment was replicated thrice and for each replication ten larvae were taken. The experiment was carried out at 27 $\pm$ 1°C with about 65 per cent relative humidity. Larval mortality was recorded after 48 hours at 24 hours interval for seven days.

3. Results

3.1 Plant Regeneration

A total of 11 putative transgenic plants were regenerated out of which six were generated with pC2300-*En35S-ctp-2AX1* construct and five with pC2300-*En35S-2AX1* construct.

3.2 PCR Analysis

Eleven putative T_0 transgenic cotton plants generated were screened for the presence of *cry2AX1* gene and *nptII* gene with specific primers targeting the coding region of *cry2AX1* and *nptII* gene, respectively. All the eleven plants were found positive for PCR and amplified expected size of 712 bp fragment for *nptII* specific primers (Figure 2 (a)) and 800 bp fragments for *cry2AX1* gene specific primers respectively (Figure 2 (b)).

3.3 Southern Hybridization Analysis

For southern hybridization analysis, the genomic DNA from selected four primary transformants were digested with *Hind*III enzyme and probed with internal fragment of *cry2AX1* gene specific probe. This confirmed the

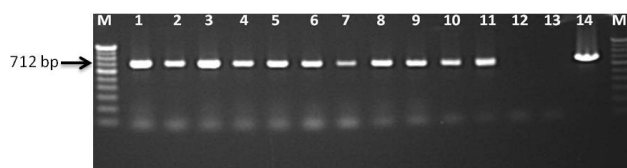


Figure 2. (a) Screening of putative transformants of cotton for the presence of *nptII* gene.

M: 100 bp DNA ladder, Lanes 1–11: putative transgenic plants, Lane 12 and 13: Negative control (water blank) and control plant respectively, Lane 14: positive control (pC2300-*En35S-2AX1*).

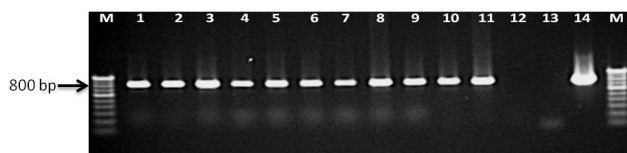


Figure 2. (b) Screening of putative transformants of cotton for the presence of *cry2AX1* gene.

M: 100 bp DNA ladder, Lanes 1–11: putative transgenic plants, Lane 12 and 13: Negative control (water blank) and control plant respectively, Lane 14: positive control (pC2300-*En35S-2AX1*).

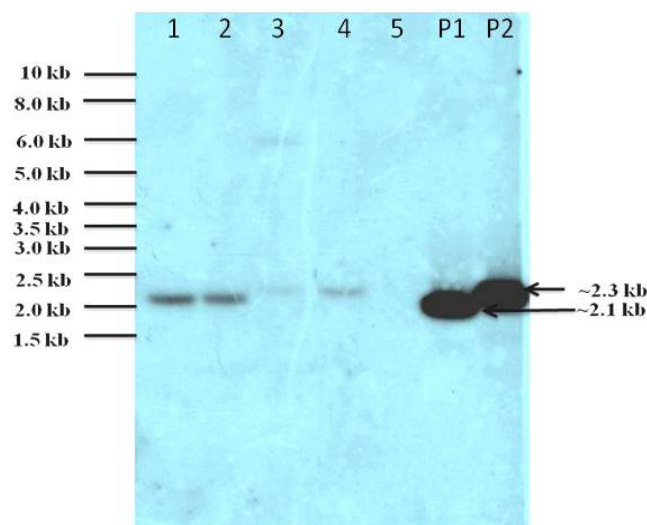


Figure 3. Southern blot hybridization analysis of transgenic T_0 cotton plants expressing *cry2AX1* gene.

Lane 1: CE-1, Lane 2: CE-3, Lane 3: CETP-M1, Lane 4: CETP-M4 (Lane 1-4: transgenic plants) Lane 5: Control plant, Lane P1: Positive control (pC2300-*En35S-2AX1*), Lane P2: Positive control (pC2300-*En35S-ctp2AX1*)

integration of *cry2AX1* gene in the genome of transgenic cotton plants (Figure 3 Lane 1-4).

Additional band of higher size was also observed in the transformant CETP-M1 (Figure 3 (lane 3)). The genomic DNA from non-transgenic control plant was used as a negative control and no hybridization signal was detected (Figure 3 (lane 5)), while gene specific fragments (positive control) generated hybridization signal of size ~2.1 and ~2.3 Kb, respectively as in positive control plasmids (Figure 3 (lane P1, lane P2)).

3.4 ELISA Analysis

PCR positive plants were further analyzed for the expression of *Cry2AX1* protein by quantitative ELISA. Out of 11 plants tested 7 were found positive for expression of *cry2AX1* and the concentration of protein ranged from 0.015 to 0.080 $\mu\text{g/g}$ of fresh leaf tissue (Table 1).

3.5 Insect Bioassay

The mortality of neonates on T_0 plants ranged from 10 to 36.66 per cent, whereas the control plants showed no mortality (Table 1). Even though, the level of mortality was less, growth inhibition in surviving larvae and reduction in leaf area feeding were observed in the ELISA positive plants (Figure 4).

Table 1. Expression of Cry2AX1 protein and mortality of *H. armigera* neonates in T₀ transgenic cotton events

* Mean of two replicates

Mean of three replications

CE: Transgenic plants generated with construct pC2300-*En35S-2AX1* and

CETP: Transgenic plants generated with construct pC2300-*En35S-ctp2AX1*

S. No.	T ₀ cotton Events	Concentration of Cry2AX1 protein in fresh leaf tissue (µg/g at 60 DAT)* Mean ± SD	Mortality of <i>H. armigera</i> neonates (%) (70 DAT) (Mean ± SD)#
1.	CE-1	0.035 ± 0.01	13.33 ± 4.71
2.	CE-2	0.030 ± 0.01	16.66 ± 4.71
3.	CE-3	0.080 ± 0.03	36.66 ± 4.71
4.	CETP-M1	0.041 ± 0.01	16.66 ± 4.71
5.	CETP-M2	0.039 ± 0.00	13.33 ± 4.71
6.	CETP-M3	0.015 ± 0.00	10.00 ± 0.00
7.	CETP-M4	0.041 ± 0.01	16.66 ± 4.71
8.	Control (Cocker 310)	0	0

4. Discussion

Transgenic crop plants expressing different Bt toxin have shown significant resistance to important agricultural insect pests in addition to reduced application of pesticides and improved yield^{13,26}. However, there is always a

risk that field insects could develop resistance to these Bt toxins after prolonged and consistent exposure³. Adaptation of insects to insecticidal toxins of *Bt* can reduce the efficacy of toxins and thereby the benefits of the technology. We have shown that the expression of a novel synthetic *Bt* gene encoding the Cry2AX1 protein could be an alternative *Bt* gene for protection against *H. armigera*. PCR positive cotton transformants of *cry2AX1* gene were analyzed for the level of expression of Cry2AX1 protein. Seven out of 11 PCR positive plants were found to be positive for the expression of Cry2AX1 protein, ranging from 0.015 to 0.080 µg/g fresh leaf tissue. Low and variable level of Bt protein expression in transgenic plants were reported by earlier workers. The variable level of Cry2AX1 protein observed among different transgenic lines could be envisaged for the relative location of the integrated T-DNA into the genome, so called position effects²¹; the chromatin structure; methylation state²²; post-transcriptional regulation²⁸ and copy numbers^{5,10,11}. Truncations and rearrangements have also been reported to influence the expression of the transgene⁷. The few transgenic lines which were positive by PCR but did not have expression of Cry2AX1 protein at all; this could be due to the complete inactivation of the gene because of its integration into highly repetitive DNA region of the plant's genome²⁸. Integration site of transgene in the genome may have a detrimental effect on its expression. Significant differences in the level of expression of the *Bt* protein among the transgenic T₀ plants have been reported earlier^{20,29}.

Southern blot analysis provided the additional evidence of the integration of *cry2AX1* gene in cotton genome. When the DNA was digested with *Hind*III, the hybridization was occurred at ~2.1 and ~2.3 Kb for the transformants generated with p2300-*En35S-2AX1* and p2300-*EnCaMV-ctp-2AX1* constructs, respectively. The ~2.1 Kb fragment includes whole coding sequence of the *cry2AX1* gene and the *nos* terminator whereas the ~2.3 Kb fragment includes *ctp-cry2AX1* fusion gene and *nos* terminator. This result indicated that the integrated *cry2AX1* gene is intact. Besides this transformant (Lane 3) showed extra band of higher size than the expected band size, (~2.3 Kb), which indicates truncation of the cassette and subsequent integration in the cotton genome, probably due to deletion at 3' *Hind*III site. Similar observations of truncation/deletion of integrated T-DNA have been reported in other studies in tobacco^{19,32,33}, in rice³⁷ and in barley². No hybridization signal could be detected from the untransformed plant.

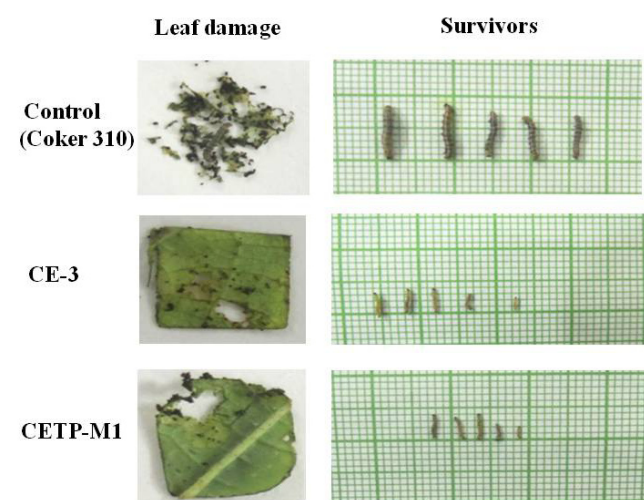


Figure 4. Detached leaf bit bioassay against *H. armigera* in transgenic cotton plants expressing Cry2AX1 protein.

The cotton transformants showed a mortality in the range 10 to 36.66 % against the neonates of *H. armigera* even with low level of Cry2AX1 protein. A positive correlation was established between the level of Cry2AX1 expression and per cent mortality and reduction in feeding against *H. armigera* larvae. Such positive correlation between expression level of Cry protein and insect mortality have been reported in many studies^{4,8,20,29}. However, we could not obtain T₀ transgenic cotton event expressing higher level of Cry protein as reported earlier in transgenic tobacco, maize, rice, cotton and tomato^{13,26}.

5. Conclusion

In the present study, we have introduced a synthetic chimeric gene, *cry2AX1* into cotton to study its insecticidal activity against *H. armigera*. The cotton transformants showed mortality up to 36 % in neonates of *H. armigera* even at the lower level expression of Cry2AX1 protein. This suggests screening of a larger population of primary transformants for obtaining an event with higher expression of Bt toxin in recalcitrant plant species like cotton is necessary.

6. Acknowledgement

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