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Study of Expression of Indigenous *Bt cry2AX1* Gene in T_3 Progeny of Cotton and its Efficacy Against *Helicoverpa armigera* (Hubner)

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HIGHLIGHTS

- Bt cotton event 'CH12' T_3 progeny showed stable inheritance and expression of transgene.
- Single locus integration of *cry2AX1* gene in T_3 progeny reveals stable inheritance of transgene.
- Insect bioassay using *Helicoverpa armigera* larvae showed more than 90 per cent mortality in T_3 plants.

Abstract: Development of transgenic *Bt* crops with stable and high level of Bt protein expression over generations under different environmental conditions is critical for successful deployment at field level. In the present study, progenies of transgenic cotton Coker310 event, CH12 expressing novel *cry2AX1* gene were evaluated in T_3 generation for stable integration, expression and resistance against cotton bollworm, *Helicoverpa armigera*. The *cry2AX1* gene showed stable inheritance and integration in the T_3 progeny plants as revealed by PCR and Southern blot hybridization. The expression of Cry2AX1 protein on 90 days after sowing (DAS) was in the range of 1.055 to 1.5 µg/g of fresh leaf tissue except one plant which showed 0.806 µg/g of fresh leaf tissue and after 30 days (i.e., on 120 DAS) three plants recorded in between 0.69 to 0.82 µg/g and other plants are in range of 0.918 to 1.058 µg/g of fresh leaf tissue. Detached leaf bit bioassay in T_3 progeny on 110 DAS recorded mortality of 73.33 to 93.33 per cent against *H. armigera* and severe growth retardation in surviving larvae. These results indicate that the expression of chimeric *cry2AX1* is stable and exhibits insecticidal activity against *H. armigera* in T_3 progeny of CH12 event of transgenic cotton.

Keywords: *cry2AX1*; *Helicoverpa armigera*; *Bacillus thuringiensis*; Insect bioassay

INTRODUCTION

Cotton is the leading crop among fibre crops produced in the world and the most important cash crop in India. India is the largest producer of cotton in the world, the second largest exporter of raw cotton (www.cottoninc.com). Various biotic as well as abiotic stress factors affect the growth and productivity of cotton crop which results in significant reduction in quantity and quality of fibre yield. Among the biotic stresses, insect pests, particularly cotton bollworm, *Helicoverpa armigera* (Hubner) causes severe yield loss ranging from 30-80% [1].

Genetic engineering offers successful production of plants with novel traits which are unavailable through conventional plant breeding. The use of insecticidal proteins encoded by cry genes of *Bacillus thuringiensis* (Berliner) (*Bt*) for insect pest management has emerged as a potent tool, being highly specific against target insects and it doesn't have harmful effects on environment [2,3]. Development of *Bt* transgenic plants resistant against insects through plant genetic engineering technology was successful in agriculture and has revolutionized global cotton and corn cultivation. Besides increased yield and profit, the benefits of growing *Bt* crops include reduction in usage of chemical pesticides by effective control of target pests. Socio-economic benefits of such crops have been well proven through meta-analysis studies on impact of genetically modified (GM) crops. The adoption of GM technology has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmers profits by 68% [4]. However, the effectiveness of transgenic crops was reduced by evolution of resistance against Cry protein in insects and reduced efficacy of transgenic plants caused by field-evolved insect resistance has been reported [5].

Evolution of insect resistance against Cry1A protein is not cross-resistant to another Cry protein (Cry2A) protein [6]. Hence, expression of *Bt* genes with different modes of action in a single plant is an effective alternative strategy to improve the protective efficacy and durability of resistance against target pest(s). Owing to the difference in structure and insecticidal mechanism, *cry2A* genes are the most promising candidates for the management of pests in crop plants [7]. The first-generation insect resistant transgenic plants possess single *Bt* toxin whereas second-generation transgenic crops produces more than one *Bt* toxins with an objective to delay the development of insect resistance and to improve protective efficacy against additional insect pests [8]. The evolution of insect resistance can be delayed by pyramiding one or more *Bt* gene producing distinct toxins which binds to different receptor in insect gut or the same pest can be controlled using other traits [8,9]. Based on experimental evidence, resistance development in insects are delayed using *Bt* gene pyramids rather temporal alternations/spatial mosaics of transgenic crop with different *Bt* toxin [10]. Several new *Bt* genes and various versions of *cry2A* gene were developed and the toxicity analysis was carried out at laboratory and field level [11-15]. Pyramiding of *Bt* genes is an effective strategy to combat evolving insect resistance.

A chimeric *cry2AX1* gene was derived from *cry2Aa* and *cry2Ac* genes, using 585 N-terminal and 48 C-terminal amino acids of these two genes, respectively and the Cry2AX1 protein was found to be more toxic than its parental proteins [16]. A synthetic *cry2AX1* gene (NCBI accession number GQ332539.1) was codon optimized to express in plants and it was used to generate insect resistant transgenic cotton plants. Expression of Cry2AX1 protein in rice and tomato showed significant mortality of target insect species [17-19]. In T_0 generation, the *cry2AX1* transgenic cotton event, CH12 (one of the best performing event) showed 88 % mortality in *H. armigera* with the Cry2AX1 concentration of 1.0 µg/g of fresh leaf tissue [20].

Before commercialization of transgenic plants, it is very important to study the inheritance, segregation, stability of transgene expression over the generation of a transgenic event. Loss of gene expression would inevitably reduce the value of the variety to the end user [21]. Early generation transformants are less suitable for studying the transgene behaviour since in these populations, the inheritance of transgenes is likely to be affected by factors like tissue culture induced variations, multicopy insertions of the transgenes, hemizygosity [22], transgene rearrangements, transformation methods, and selection strategy [23, 24]. Therefore, it is necessary to characterize transgenic plants of advanced generation for gene expression, trait evaluation and inheritance pattern. In general, the concentration of protein decreases gradually throughout the growing season among all the genotypes i.e., with the age of the plant and also with active plant growth. In the present study, T_3 progenies derived from the transgenic cotton event, CH12 were analysed for the stable expression of Cry2AX1 protein and the bioefficacy was studied at flowering stage.

MATERIAL AND METHODS

Plant Material

Seeds of two T_2 Progenies (CH12-27-13 and CH12-27-25) derived from an event, CH12, which was generated by transforming Coker310 with *cry2AX1*, using *Agrobacterium tumefaciens* [20] were used to raise the T_3 progeny along with control (non-transgenic plants) in greenhouse conditions.

Molecular Analysis of Transgene

Polymerase chain reaction using gene specific primers and Southern blot hybridisation analysis were performed to confirm stable inheritance of insecticidal protein gene, *cry2AX1* in the progeny of event CH12.

PCR Analysis

Young leaves of greenhouse grown T_3 progeny and control cotton plants were used for the isolation of total genomic DNA using CTAB method [25]. PCR amplification of genomic DNA from cotton plants using *cry2AX1* gene specific primers were carried out in thermal cycler (Eppendorf, Hamburg, Germany) to confirm the presence of *cry2AX1* gene in T_3 progeny. The plasmid DNA and DNA isolated from untransformed plant were used as positive and negative control, respectively. The *cry2AX1* primers used were as follows: *cry2AX1* FP 5'-AACGTTCTTAACTCTGGAAGGA-3'; RP 5'-GCAGAAATTCCCCACTCATCAG-3'. The gene specific primer amplifies 800 bp of internal sequence of *cry2AX1* gene. The PCR reaction was set up in 25 µl volume containing, 2.5 µl of 10X Taq buffer, 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5 U 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 at 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 amplified PCR products were run on 1.0 % agarose gel and analyzed on G: BOX F3 (Syngene, USA) gel documentation system.

Southern Blot Analysis

Ten micrograms of genomic DNA samples from four selected T_3 progenies (27-13-10, 27-13-16, 27-25-13 and 27-25-24) were digested with *Eco*RI or *Nco*I enzymes, which have single recognition site within the T-DNA and fractionated in 0.8 % agarose gel. Gel was denatured, neutralised and the digested genomic DNA was transferred onto Hybond-N+ nylon membrane (Sigma-Aldrich, St. Louis, USA). The transferred DNA was cross-linked at 1200 µJ min-1 for 1 min by using UV crosslinker. Amplicons of internal region of *cry2AX1* fragment (800 bp) were generated by PCR and used as probe. The probe DNA was labelled with radioactive α32P dCTP by random priming using Decalabel DNA labelling kit (Thermo scientific Inc. Waltham). Hybridization was carried out for 16 hr at 60° C. After hybridization, the blot was washed for 15 min with 3X SSC + 0.1 % SDS and 2X SSC + 0.1 % SDS. Another washing with 0.5X SSC + 0.1% SDS was given for 10 min. All washings were carried out at 60° C. The blot was washed after hybridization to remove excess radioactive material and the membrane was exposed to X-ray film.

Temporal Expression Profiling of Cry2AX1 Protein

Qualitative Assay

For qualitative detection of Cry2AX1 protein in the T_3 progeny, DesiGen Cry2Ab qualitative kit (DesiGen Diagnostics, Jalna, India) based on alkaline phosphatase was used according to manufactures instruction.

Quantification of Cry2AX1 Endotoxin in The T_3 Progeny

The PCR positive progenies showing expression of Cry2AX1 protein in qualitative assay were used for quantification at two different growth stages for determining temporal variation. Expression of Cry2AX1 protein in the progeny was quantified by ELISA using a Cry2A Kit (Envirologix inc, Portland, USA). Thirty mg of leaf sample was collected from T_3 progenies and homogenised with extraction buffer using tissue lyser. One hundred microlitre of supernatant from homogenised sample was loaded into the wells in ELISA plate. The Cry2A protein standards were added to the wells of ELISA plate along with test samples and the ELISA assay was performed according to the manufacturer's instructions. Quantification of Cry2AX1 endotoxin was done by plotting the absorbance values of Cry2AX1 test samples on the standard curve generated with

positive standards and expressed as microgram of Cry2AX1 protein per gram of fresh leaf tissue. Each experiment included three replicates. The variation in expression of Cry2AX1 protein from leaf tissue was analysed at two stages viz., 90 and 120 days after sowing.

Bioefficacy Analysis of Cry2AX1 Protein

The efficacy of Cry2AX1 protein expressed in transgenic progeny against *H. armigera* was evaluated at 110 days after sowing. Fresh leaf bits (1.5 cm^2 size) from transgenic and control plants were placed on a Petri plate lined with moist filter paper. One neonate larva of *H. armigera* was released on each leaf bit overlaid on filter paper using a fine hair brush. Each treatment was replicated thrice and for each replication ten larvae were used. The experiment was carried out at a temperature of 25 ºC ±1, 60 % relative humidity. Larval growth and mortality of neonate larvae were recorded after 48 hr at 24 hr interval for five days.

Statistical Analysis

The experiment was conducted in a completely randomized design (CRD). The mortality percentage data were converted to arcsine values and concentration of protein data were converted to square root values prior to analysis. Data were analyzed using the AGRES statistical package (Version 7.01, Pascal International Software Solutions).

RESULTS

Molecular Analysis

The present study was undertaken to determine the expression pattern of *cry2AX1* gene in progeny of CH12 event in the advanced T_3 generation at two different growth stages and to evaluate the insecticidal activity of Cry2AX1 protein against neonates of *H. armigera*. The stable inheritance of transgene in T_3 progeny was analysed by using PCR with gene specific primer, amplifying internal sequence of *cry2AX1* gene. Among sixty two plants tested in T_3 generation, all the 26 progenies of T2-13 and 36 progenies of T2-25 were found positive for the presence of transgene *cry2AX1*, confirming the homozygosity of T_3 plants studied in this investigation. There was no amplification in control plant (Figure 1).

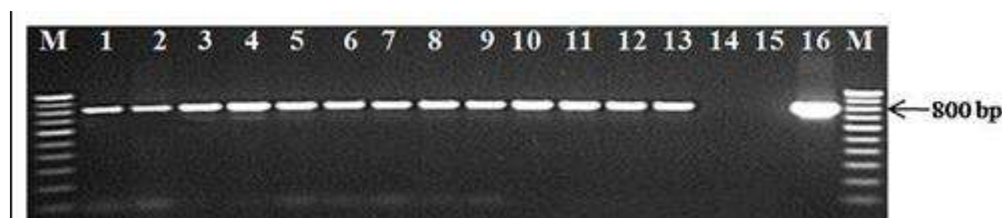

Figure 1. PCR analysis for cry2AX1 gene in T_3 progeny plants.
A 800 bp internal sequence of cry2AX1 gene amplified by PCR from the DNA isolated from T_3 progeny. Lane M:100 bp ladder, Lane 1 – 13: T_3 progeny, Lane 14: Water blank, Lane 15: Non-transformed plant, Lane 16:Positive control (pC1300-En35S-2AX1 plasmid)

To assess the integration pattern of transgene, the genomic DNA of four selected T_3 progenies (27-13-10, 27-13-16, 27-25-13 and 27-25-24) was digested by *Eco*RI and *Nco*I enzymes separately, where recognition sites are present on either side of gene of interest (Figure 2a). All the four T_3 progenies showed single hybridization signal of ~6.0 kbp size when digested with *Eco*RI (Figure 2b) and of ~5.0 kbp size when digested with *Nco*I (Figure 2c), demonstrating inheritance of single integration of *cry2AX1* gene in the cotton genome and it is similar to the integration pattern of T_0 event CH12. The DNA from control plant did not show any signal.

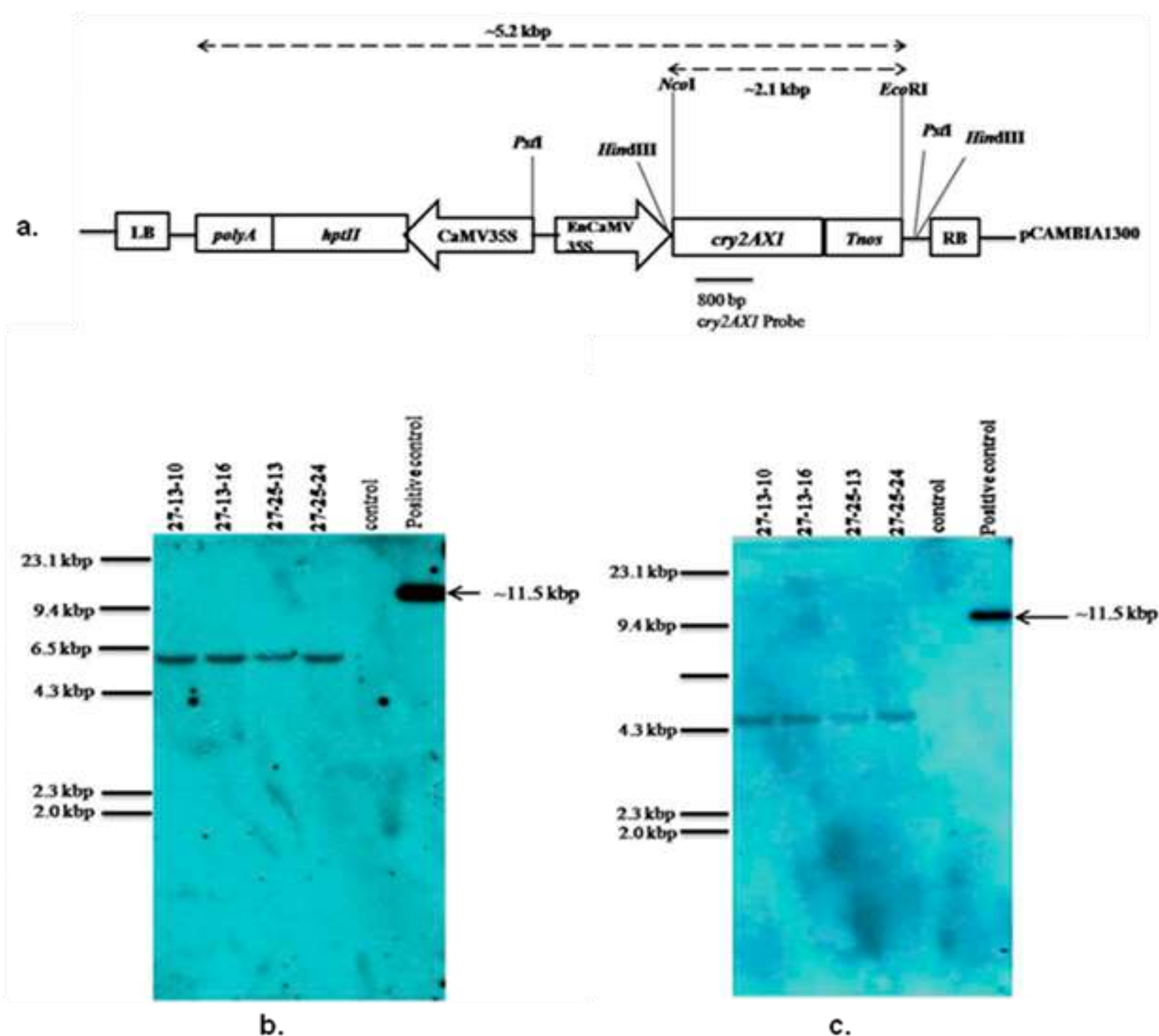

Figure 2. Southern hybridization analysis of T_3 cotton progeny

Total genomic DNA from selected T_3 progeny were digested with *Eco*RI or *Nco*I enzyme. DNA isolated from wild type plant used as control; pC1300-En35S-2AX1 plasmid digested with respective enzymes used as positive control. Southern blot was probed with α32P dCTP labelled 800 bp internal fragment of *cry2AX1* gene

a. T-DNA region of plant transformation construct
b. Plant genomic DNA restricted with *Eco*RI enzyme
c. Plant genomic DNA restricted with *Nco*I enzyme

Expression of Cry2AX1 Protein In The T_3 Progeny

All the 26 T_3 progenies derived from CH12-27-13 and 36 T_3 progenies derived from CH12-27-25 were analysed by qualitative ELISA for gene expression. All the T_3 progenies tested were found positive for expression of Cry2AX1 protein, whereas control plant was found negative.

Temporal Expression of Cry2AX1 Protein

Five plants from CH12-27-13 and four plants from CH12-27-25 lines, respectively, were used for quantitative estimation of Cry2AX1 protein. Expression analyses of Cry2AX1 protein in fresh leaf tissue were carried out at two different growth periods 90 and 120 days after sowing. Among nine plants tested for expression of Cry2AX1 protein, only one plant showed Cry2AX1 protein concentration 0.806 μg/g of fresh leaf tissue whereas other plants expressed in range between 1.055 to 1.5 μg/g of fresh leaf tissue at 90 days after sowing. After 30 days (i.e., 120 DAS), in three plants the concentration of Cry2AX1 protein ranged from 0.69 to 0.82 μg/g and the remaining six plants recorded 0.918 to 1.058 μg/g of fresh leaf tissue (Table 1).

This reveals that the expression level of Cry2AX1 protein in leaf tissue declines on 120 DAS compared to 90 DAS.

Table 1. Temporal expression of Cry2AX1 protein and leaf bit bioassay for *H. armigera* on transgenic T_3 cotton progeny

S. No	**T_3 Progeny**	**Cry2AX1 concentration in fresh leaf tissue (µg/g)* Mean± SD**		**Mortality of *H. armigera* (%)**Mean ± SD**
		90 DAS	**120 DAS**	**110 DAS**
1.	27-13-10	1.026 ±0.034 (1.235)	0.929 ±0.005 (1.195)	83.33± 4.71 (66.15)
2.	27-13-16	1.565 ±0.001 (1.437)	1.014 ±0.024 (1.230)	86.67± 4.71 (68.86)
3.	27-13-18	1.219±0.009 (1.311)	0.966 ±0.000 (1.211)	86.67±4.71 (68.86)
4.	27-13-19	1.238 ±0.021 (1.318)	1.058 ±0.045 (1.248)	93.33±4.71 (77.41)
5.	27-13-24	1.055 ±0.008 (1.247)	0.918 ±0.027 (1.191)	83.33±4.71 (66.15)
6.	27-25-5	1.066 ±0.027 (1.251)	0.933 ±0.027 (1.197)	90.00± 0.00 (71.57)
7.	27-25-13	0.806 ±0.007 (1.143)	0.692 ±0.004 (1.092)	73.33±4.71 (59.01)
8.	27-25-24	1.334 ±0.026 (1.354)	0.826 ±0.004 (1.151)	76.67± 4.71 (61.22)
9.	27-25-27	1.245 ±0.046 (1.328)	0.725 ±0.007 (1.107)	73.33± 4.71 (59.01)
10.	Control	0.0±0.00 (0.707)	0.0±0.00 (0.707)	0.00±0.00 (0.91)
11.	BGII (Positive control)	23.507±0.285 (4.900)	21.347±0.116 (4.674)	100.00±0.00 (89.10)
		LSD = 0.0253	LSD = 0.0182	LSD = 7.822
		SEd = 0.0122	SEd = 0.0088	SEd = 3.771
		CV = 0.95%	CV = 0.74%	CV = 7.38%

Table[1] The statistical significant difference among the mean values at $p \leq 0.05$ was determined by least significant difference test. The values expressed are Mean ± SD based on three replications.
*Values given in bracket, in this column, are square root of x+0.5 transformed
** Values given in bracket, in this column, are arcsine transformed

Insect Bioassay Of T_3 Progenies

All the nine T_3 plants analyzed for temporal gene expression (at 90 and 120 DAS) were subjected to bioassay against the neonate larvae of *H. armigera* on 110 DAS. Insect bioassay carried out under laboratory conditions in T_3 progenies expressing the *cry2AX1* gene showed larval mortality of 73.33 to 93.33 per cent (Table 1). The growth of surviving larvae was severely inhibited and leaf area damage was also significantly less in insect resistant transgenic cotton progeny in comparison to the non-transformed control plant (Figure 3).

The transgenic plants expressing Cry2AX1 protein concentration ranging from 0.918 to 1.058 µg/g of fresh leaf tissue (on 120 DAS) recorded insect mortality above 80 per cent (on 110 DAS) whereas the plants expressing 0.692 to 0.826 µg of Cry2AX1 protein per g of fresh leaf tissue showed insect mortality of 73 to 76 per cent. Surviving larvae fed on any of the tested transgenic plant exhibited severe growth inhibition (Figure 3)

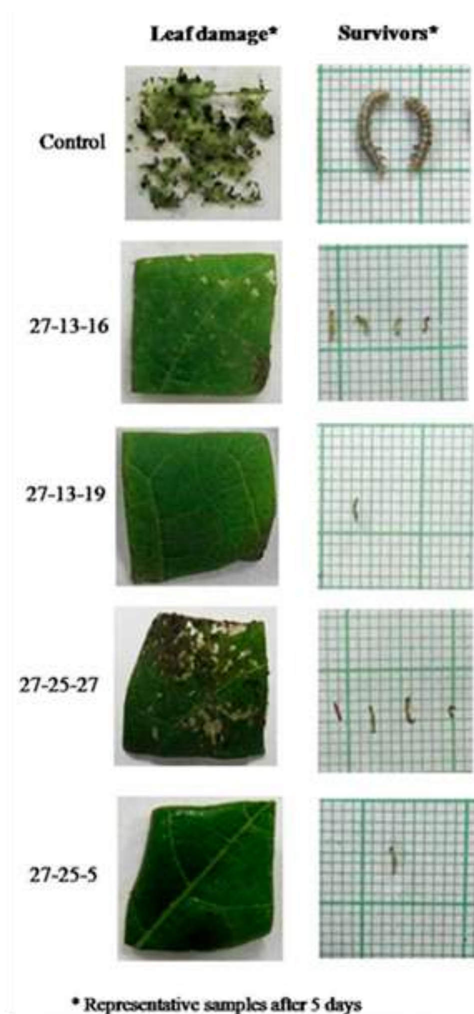

Figure 3. Detached leaf bit bioassay against *H. armigera* in T_3 cotton progeny expressing Cry2AX1 protein

DISCUSSION

After introduction of GM crops in 1996, the global hectarage of those crops has increased 100-fold and has been the fastest adopted crop technology in recent times. The introduction and commercial planting of *Bt* cotton has made India the number one producer of cotton globally which is attributed to the adoption of *Bt* cotton by Indian farmers. The area under *Bt* cotton increased from 10.8 mha in 2016 to 11.4 ha in 2017 which is equivalent to 93% of the total cotton area of 12.24 million hectares grown in the country [26]. The discovery of new *Bt* genes is very important for *Bt* crop development, since new *Bt* genes with different insecticidal mechanisms can be used to target resistant pests and also to control other important hemipteran pests such as mirids, plant hopper, aphids rather than lepidopteran and coleopteran insects. Stable inheritance and expression of transgenes in transgenic plants is of paramount importance in the successful application of genetic engineering in crop improvement.

The present study was undertaken to demonstrate stable integration of transgene and evaluate temporal expression of *cry2AX1* gene and its toxicity against *H. armigera* in T_3 progeny plants derived from the event CH12. Earlier studies on CH12 event of *cry2AX1* transgenic cotton in T_1 and T_2 generation showed Cry2AX1 expression of about ~1.0 to 1.2 µg/g of fresh leaf tissue and mortality of *H. armigera* from 86 to 90 per cent [27,20] and found to be promising candidate for insect resistance. Molecular analysis of T_3 progeny showed that gene integration remained stable in subsequent generations as revealed by PCR and Southern blot analyses of the transgenic plants. In PCR analysis and qualitative ELISA, all the sixty-two progeny (26 plants from CH12-27-13 and 36 plants from CH12-27-25) tested were found positive for the presence of *cry2AX1* gene and expression of protein, indicating homozygosity of the transgene in progeny plants. Southern hybridization analysis with *Eco*RI revealed single locus integration as it was found in T_0 and T_1 progeny. The result of another experiment of Southern hybridization analysis with *Nco*I enzyme also provided additional support for single locus integration (Figure 2c).

Temporal expression analysis by ELISA revealed that there is a reduction in the amount of Cry2AX1 protein in leaves. An explanation for these results would be the total protein production goes down towards the latter part of plant growth. These results are in concurrence with the earlier studies in *Bt* cotton [28-39] which reported a decline in expression of other Bt protein(s) during the crop growth period. Besides, decrease in expression of *Bt* protein gene at later developmental stages was also correlated with changes in the methylation state of CaMV35S promoter region [28, 40]. The temporal variation in *Bt* gene expression may also be due to developmental regulation of CaMV35S promoter [34, 41]. The spatio temporal variation was observed in expression of *cry1Ac* gene in different *Bt* cotton genotypes [42]. Most of the genotypes lost their expression after 90 DAS and their expression was reduced to critical level (1.9 µg/g). In contrary to this trend, the level of *Bt* (Cry1Ac/Cry1Ab fusion) protein expression in case of GK 19 cotton cultivar was high in early season (529.45 ng/g), declined in mid-season to 65.42 ng/g and again increased in late season to 509.37 ng/g [43]. Reason for slight variation in the level of Cry2AX1 protein among the sibling progeny of both CH12-27-13 and CH12-27-25 derived from single event under the homozygous condition is not known. It might be due to the greenhouse environmental condition [44] or otherwise the expression of transgene may be affected by epistatic gene interaction which might have occurred among the integrated transgenic locus and endogenous genes.

Early generation transgenic plants such as T_2 or T_3 are undergoing conversion to homozygosity and are more prone to silencing [45]. These variations between clonal replicates or sibling plant carrying identical transgene may be a consequence of the variations in the methylation state, which is known to increase as the plant age [46,47]. A reduction or loss of marker gene in 50% of the clonal cell line showed that the variability in marker gene expression occurred due to a reduction in the *npt*II transcript level and was associated with hypermethylation of the integrated DNA [48]. The variable transgene expression among the advanced generation transgenic lines derived from single event may be due to the complicated locus structure which appears to be located at single locus [49, 50].

The increased level of Cry2AX1 expression shows higher level of insect mortality and reduction in feeding against *H. armigera* larvae. Similarly, correlation between expression level of Cry protein and insect mortality have been reported in earlier studies [17,51,52,53]. Expression level of Cry2AX1 in the leaves could kill majority of the *H. armigera* larvae which feeds on leaves. Further studies on insect bioassay using different plant parts fed by *H. armigera* are needed to be carried out.

CONCLUSION

Screening of T_3 progenies of the *cry2AX1* transgenic *Bt* cotton event, CH12 using PCR and Southern blotting reveals presence of *cry2AX1* gene in all the plants and inheritance of intact gene cassette to T_3 generation indicating homozygous nature of transgene. The transgenic plants expressing 0.9 to 1.0 (µg/g of fresh leaf tissue) Cry2AX1 protein showed mortality of more than 80 per cent of *H. armigera* larvae. Significant difference was observed between surviving larvae from control and transgenic plants. Surviving larvae fed on any of the tested transgenic plant exhibited severe growth inhibition. This study has proved the potential of indigenous *cry2AX1* gene and success in developing bollworm-resistant transgenic cotton plants. Further this gene can be effectively deployed in developing pyramids of insect-resistant transgenic crop plants.

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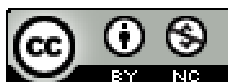
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