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Analysis of Stable Expression of Chimeric *cry2AX1* Gene in Insect Resistant Cotton Progeny over Successive Generations

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The lepidopteran pests are a major threat to cotton production worldwide and are considered as a major constraint in cotton productivity. Stable inheritance and expression of transgenes in transgenic plants is of paramount importance in successful application of genetic engineering in crop improvement. The aim of producing transgenic crop is to obtain a durable protection which requires, in particular, the stability of gene expression during generation advancement and introgression of transgenes into other genetic backgrounds. Insect resistant cotton lines of a Coker310 event, CH12 expressing *cry2AX1* gene were evaluated in T_1 and T_2 generations for stable integration, expression and resistance against cotton bollworm, *Helicoverpa armigera*. The expression of Cry2AX1 protein ranged from 0.466 to 0.899 and 0.094 to 1.374 µg/g of fresh leaf tissue in the T_1 and T_2 progeny respectively. The detached leaf disc bioassay recorded mortality of 46.66 to 86.66 and 73.33 to 90.00 per cent in T_1 and T_2 progeny respectively against *H. armigera*. These results indicate that the expression of chimeric *cry2AX1* is stable and possesses insecticidal activity against *H. armigera* in transgenic cotton progeny.

Key words: *cry2AX1*; *Helicoverpa armigera*; *Bacillus thuringiensis*; Insect bioassay.

Cotton is the most important fibre crop in the world. India ranks number one in terms of area and second in production of cotton. Insect pests pose a major threat to cotton production worldwide and are considered as main factor limiting cotton productivity. More than fifteen economically important insect pests attack cotton crop, sucking as well as chewing pests, however lepidopterans such as *Helicoverpa armigera*, *Pectinophora gossypiella*, *Earias insulana/vitella* and *Spodoptera litura* are the most devastating ones. The application of biotechnological tools in agriculture has allowed scientists to genetically engineer plants, thus es-tablishing the possibility of producing new crop varieties rapidly with traits beneficial to non target organisms, human health and the environment. Crops have been successfully engineered to impart pest and disease resistance, herbicide tol-erance, nutritional qualities, and stress tolerance[1].

Bacillus thuringiensis (*Bt*) is perhaps, the most important source of insect resistance genes and cotton expressing insecticidal proteins from *Bt* has been one of the most rapidly adopted GE crops in the world. Insect resistant cotton is con-siderably effective in controlling lepidopteran pests, and is highly beneficial to grower and environment by reducing chemical insecticide sprays and preserving popu-lation of beneficial arthropods[2,3]. As a result of cultivation of *Bt* cotton hybrids in India, there has been increase in cotton production from 13.6 million bales in 2002-03 to 37.5 million bales in 2013-14[4]. Notably, there has been a very steep decline in insecticide usages to manage *H. armigera* in cotton from 71% in 2001 to

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3% in 2011[5]. Kathage and Qiam[6] reported that *Bt* has caused a 24% increase in cotton yield per acre through reduced pest damage and 50% gain in cotton profit among small farm holders in India. Nationwide *Bt* cotton cultivation of 26 million acres currently implies an annual net gain of almost 50 billion rupees in cotton profits.

The stability of transgene expression is an important issue in order to implement transgenic technology successfully for crop improvement. Inactivation of transgene and the expression has been extensively studied in dicots transformed by *Agrobacterium tumefaciens*. Factors which may affect the stability of transgene expression, including copy number, insertion site, co-suppression and gene methylation have been investigated[7,8]. The loss, inactivation or silencing of the introduced gene in the progenies of transgenic plants have also been reported by earlier workers[7,9,10,11]. Some researchers have shown that once foreign genes are incorporated into the host plant cells; are truly transferred to further progenies through sexual generations while retaining high meiotic and expression stability[12,13,14]. The present study was carried out to evaluate the stability of transgene expression in a transgenic cotton event over two generations.

MATERIALS AND METHODS

Plant material

The present study was conducted at Department of Plant Biotechnology, Centre for Plant Molecular Biology & Biotechnology, Tamil Nadu Agricultural University, Coimbatore. Event CH12, which was generated by transforming Coker310 with *cry2AX1* gene (comprising sequences of *cry2Aa* and *cry2Ac*) (Fig.1) was studied for the stability in expression and protection against target insect. To investigate the inheritance of *cry2AX1* gene and stability of expression in the subsequent generations, T_1 and T_2 progeny from fertile CH12-T_0 transgenic event along with control plants were grown in greenhouse.

Molecular analysis of transgene in progeny

The molecular approaches such as PCR and South-ern blot hybridisation analysis were performed to confirm stable integration of insecticidal gene, *cry2AX1* in progeny of transgenic event, CH12. PCR was carried out for transgenes, *cry2AX1* and *hptII* gene in T_1 and T_2 progeny using gene specific primers. The plasmid DNA was used as positive control and DNA isolated from untransformed plant was used as negative control. Genomic DNA from the leaves of transgenic and control plants were isolated following Stewart and Via[15] protocol. The primers used were as follows: *cry2AX1* FP 5'-AACGTTCTTAACTCTG GAAGGA-3'; RP 5'-GCAGAAATTCCCCAC TCATCAG-3': *hptII* FP 5'-GCTGTTATGC GGCCATTGGTC-3'; RP 5'-GACGTCTGTC GAGAAGTTTG-3'.

For Southern blot hybridisation analysis, 10 µg of genomic DNA was digested with *Eco*RI and fractionated in 0.8 % agarose gel. Gel was denatured, neutralised and blotted onto Hybond-N+ nylon membrane (Sigma-Aldrich, St. Louis, USA). Probes of 800 bp internal region of *cry2AX1* fragment were generated by PCR and purified. The probe DNA was labelled with $\pm^{32}$P dCTP by random priming using Decalabel DNA labelling kit (Thermo scientific Inc. Waltham). Hybridization was carried out for 16 h at 60° C. The blot was washed after hybridization and the membrane exposed to X-ray film.

Expression analysis of Cry2AX1 endotoxin in progeny

Expression of Cry2AX1 protein in the progeny was quantified by ELISA using Envirologix Cry2A Kit, Portland, USA. Negative and positive controls were added to wells of the ELISA plate along with test samples. ELISA was performed according to the procedure given in the kit and 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 per gram of fresh leaf tissue.

Detached leaf disc insect bioassay

The efficacy of Cry2AX1 protein in transgenic progeny against *H. armigera* was evaluated by insect bioassay. Fresh leaf discs (3 cm diameter) from transgenic and control plant were placed on a moist filter paper placed inside a petriplate and 10 neonates were released per replication. Three replications were maintained and the experiment was carried out at 25 ºC ±1, 60 % relative humidity. Larval growth and mortality was recorded every day upto five days.

RESULTS AND DISCUSSION

Transgene inheritance and molecular analysis of progeny

Genetic engineering of plants has resulted in development of novel plants expressing desirable traits with resistance to pests, herbicides, pathogens, and environmental stress[16]. Insect resistant transgenic cotton event, CH12 was generated by transforming Coker310 genotype with chimeric *cry2AX1* gene (comprising sequences of *cry2Aa* and *cry2Ac*). The present study was undertaken to determine the expression of *cry2AX1* gene in transgenic cotton progeny of CH12 event in two generations and evaluate the insecticidal activity of Cry2AX1 protein against neonates of *H. armigera*.

The stable inheritance of transgene in T_1 and T_2 progeny was analysed by PCR with gene specific primers, amplifying internal sequence of *cry2AX1* and *hptII* gene. Among twenty five plants tested in T_1 generation, nineteen plants were found positive for the presence of transgene. These PCR results indicated that the progeny of CH12 followed Mendelian segregation pattern of 3:1 (Detected: Non-detected, Pd" 0.05, Ç^2=3.841) (Table.1), which provides an evidence that the integration of *cry2AX1* is confined to a single locus (Fig.2). All the T_2 progeny tested were found positive for the presence of *cry2AX1* gene, confirming the homozygosity of T_1 plant (Fig.3).

To assess the integration pattern of transgene, the genomic DNA of selected T_1 and T_2 progeny from CH12 event were digested by *Eco*RI enzyme and probed with 800 bp of *cry2AX1* gene. The Southern hybridization analysis showed a hybridization signal of ~5 kb size, demonstrating single integration of *cry2AX1* gene in the cotton genome. The DNA from wild type plant did not show any signal (Fig. 4).

Expression of analysis of *cry2AX1* gene in progeny of two generations

The progeny of T_1 and T_2 which were found positive in PCR were subjected to ELISA.

Table 1. Segregation analysis of T1 progeny of CH12

Status (*cry2AX1)*	Observed (O)	Expected (E)	X ^{2}	Tabulate value (P≤0.05)
Detected Non detected	196	18.756.25	0.0066	3.841

Table 2. Expression of Cry2AX1 protein and leaf disc bioassay for H. armigera on transgenic T1 cotton progeny

S. No	Lines	Conc. of Cry2AX1 protein (µg/g)	Mortality (%)
1	CH12-36	0.549	46.66
2	CH12-38	0.466	NT
3	CH12-41	0.624	66.66
4	CH12-43	0.824	73.33
5	CH12-44	0.874	86.66
6	CH12-46	0.866	76.66
7	CH12-49	0.899	80.00
8	CH12-51	0.633	53.33
9	CH12-54	0.699	66.66
10	BG II	24.66	100.0
11	Control	0.00	0.00

NT – Not tested

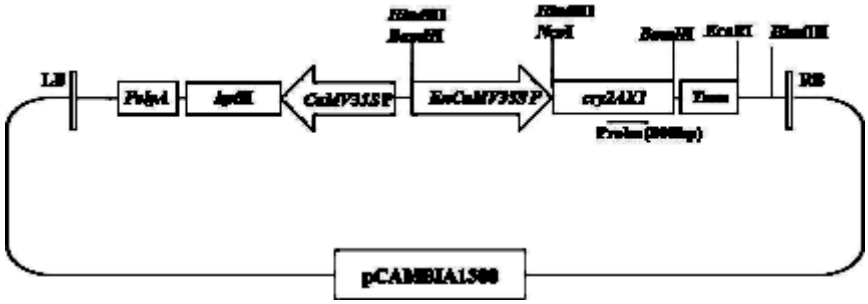
Table 3. Expression of Cry2AX1 protein and leaf disc bioassay for H. armigera on transgenic T2 cotton progeny

S.No	Lines	Conc. of Cry2AX1 protein (µg/g)	Mortality (%)
1	CH12-27-7	0.094	NT
2	CH12-27-11	0.616	NT
3	CH12-27-13	1.357	90.00
4	CH12-27-14	0.94	86.66
5	CH12-27-18	0.791	73.33
6	CH12-27-21	1.249	83.33
7	CH12-27-23	1.165	86.66
8	CH12-27-24	1.049	76.66
9	CH12-27-25	1.274	86.66
10	CH12-27-26	1.266	83.33
11	CH12-27-27	1.374	90.00
12	BGII	27.66	100.0
13	Control	0.00	0.00

NT – Not tested

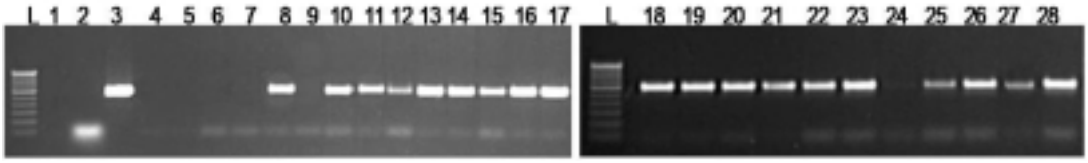
From nineteen PCR positive plants tested in ELISA, 18 plants have shown detectable level of Cry2AX1 protein in T_1 generation. All the 26 lines tested in T_2 progeny were found positive in ELISA. The concentration of Cry2AX1 protein in T_1 and T_2 progeny ranged from 0.466 to 0.899 and 0.094 to 1.374 µg/g of fresh leaf tissue, respectively. Of the twenty six T_2 plants analysed, twenty plants expressed Cry2AX1 protein in the range between 0.79 and 1.37 µg/g, whereas remaining plants are found to contain low levels, in the range of 0.094 to 0.616 µg/g of fresh tissue. Mehrotra *et al.* [17] observed that the level of Cry1Ab protein varied among T_1 progeny, ranging from 18.0 to 26.0 ng/mg TSP.

The control of transgene integration, structure and subsequent expression levels and stability remain key issues limiting transgenic studies. Transgene inactivation was clearly a dynamic process occurring at any step of plant development or generation. Study of transgene stability at the structural level is a prerequisite for characterising other forms of instability at the expression level, such as reduced expression levels or gene silencing as reported by Vain *et al.* [18]. Reduced expression of transgene is more often reported to be dependent on the presence of homologous sequences[19,20]. In homozygous plants, allelic interactions may contribute to silencing in a manner reminiscent of paramutation[21]. In the



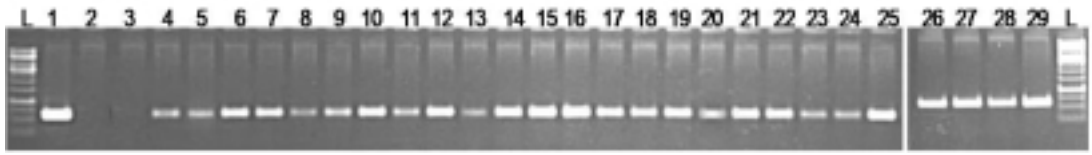
The *cry2AX1* gene is driven by a double enhancer CaMV35S promoter and terminated by the nopaline synthase (*nos*) terminator. The plant selectable marker gene, *hptII* is under the control of the duplicated CaMV35S promoter and tailed by the CaMV35S polyA. LB: left border of T-DNA region; RB: right border of T-DNA region.

Fig. 1. T-DNA region of plant transformation construct p1300-*2AX1*.



A 600 bp internal sequence of *cry2AX1* gene amplified by PCR from the DNA isolated from CH12-T_1 progeny. Lane L: 1 kb ladder, Lane 1: negative control, Lane 2: non-transformed plant, Lane 3: positive control (p1300-*2AX1* plasmid), Lane 4 – 28: T_1 progeny.

Fig. 2. PCR analysis for *cry2AX1* gene from T_1 cotton progeny



A 600 bp internal sequence of *cry2AX1* gene amplified by PCR from the DNA isolated from CH12-T_2 progeny. Lane L: 1 kb ladder, Lane 1: positive control (p1300-*2AX1* plasmid), Lane 3: non-transformed plant, Lane 4 – 29: T_2 progeny.

Fig. 3. PCR analysis for *cry2AX1* gene from T_2 cotton progeny

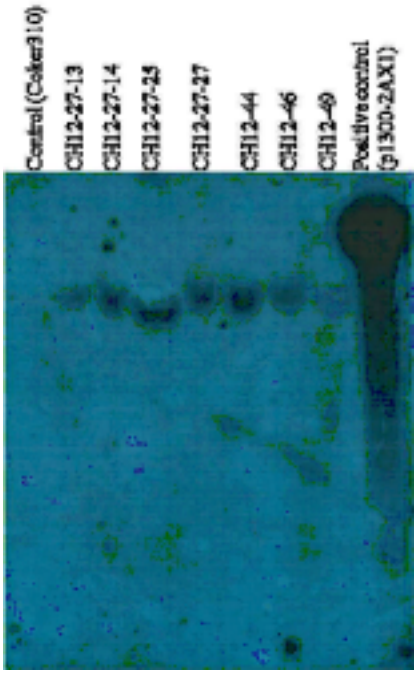
absence of homology-dependent interactions, it has been suggested that the silencing may result from the spreading of hypermethylation from genomic DNA at the integration site into the transgene sequence[22]. Bregitzer and Tonks[23] reported variability in transgene expression among the progeny of transgenic events in barley.

Variation in expression pattern among sibling plants carrying identical transgene insertions correlates with transgene methylation[24]. Emani *et al.* [25] observed the reduction in the *bar* gene expression in the homozygous T_2 progeny. In order to examine the reduction of transgene expression due to methylation, the immature embryo derived T_2 (homozygous) calli was treated with 5-azacytidine and the substantial increase in Phosphinothricin Acetyl Transferase activity was observed. Based on their results, they suggested that the partial methylation may have reduced the expression of *bar* gene. 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 *nptII* transcript level and was associated with hypermethylation of the integrated DNA[26].

Insect bioassay

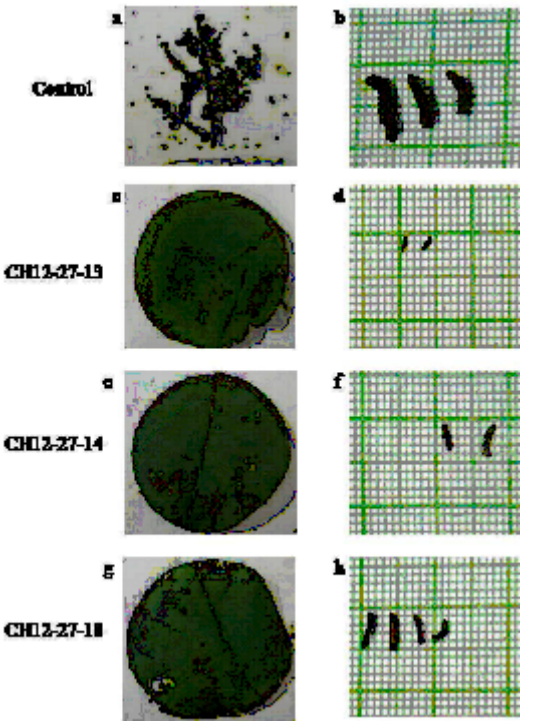
The selected progeny of T_1 and T_2 generation were tested to evaluate the efficacy of Cry2AX1 protein against the neonate larvae of *H. armigera*. The larval mortality of 46.66 to 86.66 per cent mortality was recorded in T_1 generation (Table 2) and 73.33 to 90 per cent mortality was observed in T_2 generation (Table 3). The growth of surviving larvae was severely inhibited and leaf area damage was also significantly low in insect resistant cotton progeny whereas the non-transformed control plant did not show mortality and larval growth inhibition.

Detached leaf disc bioassay of transgenic plants expressing *cry2AX1* gene under laboratory



Total DNA from selected T_1 and T_2 progeny were digested with *Eco*RI enzyme and probed with a 800 bp internal sequence of *cry2AX1* gene; DNA isolated from wild type plant used as negative control; pC1300-*2AX1* digested with *Eco*RI enzyme used as positive control.

Fig. 4. Southern hybridization of CH12 T_1 and T_2 cotton progeny with *Eco*RI enzyme



a, c, e, g. Comparison of feeding by cotton boll worm on non transformed control and T_2 transgenic progeny; b, d, f, h. Growth of surviving larvae on control and transgenic progeny.

Fig. 5. Detached leaf disc bioassay against cotton boll worm (*H. armigera*) in insect resistant T_2 cotton progeny expressing Cry2AX1 protein

conditions showed larval mortality against *H. armigera* compared to wild type (control) plants. Bioassay studies revealed that the highest larval mortality was 86.66 per cent with less leaf area damage in the T_1 generation. The maximum larval mortality of 90.00 per cent of *H. armigera* was recorded at the concentration of 1.3 µg/g of fresh tissue followed by 86.66 and 83.33 per cent mortality in T_2 generation. The minimum level mortality of 73.33 per cent was recorded with protein concentration of 0.791 µg/g of fresh tissue. No significant difference was observed in feeding pattern of larvae among transgenic progeny, but the surviving larvae fed on these progeny exhibited severe growth inhibition (Fig.5). The effect of varying concentration of Cry2AX1 protein was also seen on the surviving larvae as there was a difference in size of larvae that fed on transgenic progeny.

Changes in protein levels[27], reduced expression of the *cry1Ac* gene[28], instability of mRNA of Cry1Ac transcript[29], changes in tannin concentration[30], and now plant-toxin interactions, all play a role in altering the toxicity of Bt cotton to first instar *H. armigera.* Differences in the efficacy of Bt crops seen in the field suggest that environmental factors are also affecting the efficacy[31]. Interactions between Cry1Ac and plant proteases or the location and fate of the toxin protein within the plant cell could also influence the toxicity of the protein[32]. Even slight variations in amino acid sequence within a Cry protein class can dramatically impact insecticidal activity[33,34,35].

The level of protein expression and insect resistance observed in the present study was low. There is a necessity to increase the expression level significantly to achieve a desirable level of insect resistance against target insect. Though the level of Cry2AX1 protein expressed in insect resistant cotton progeny (T_2) was low (1.2 ¼g/g fresh leaf tissue), insect bioassay showed maximum of 90 per cent mortality against the tested target insect, *H. armigera*. *H. armigera* is a major polyphagous lepidopteron pest attacking different agriculturally important crops. Hence, the synthetic *cry2AX1* gene of the present study could be valuable gene to impart lepidopteran insect resistance in crop plants.

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