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Expression of a Novel Synthetic *cry* gene of *Bacillus thuringiensis* in Transgenic Tobacco Confers Resistance to *Helicoverpa armigera* and *Spodoptera litura*

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The *cry2AX1* is a chimeric gene consisting sequences of *cry2Aa* and *cry2Ac* of *Bacillus thuringiensis*. The Cry2AX1 is more potent than its parental proteins. A newly constructed, codon optimized synthetic *cry2AX1* gene was validated for expression in transgenic tobacco as a model plant and analyzed for its insecticidal efficacy against larvae of *Helicoverpa armigera* and *Spodoptera litura*. The presence of gene and its transcripts was confirmed by PCR and RT-PCR analysis, respectively, in T_0 transgenic tobacco leaves. Expression of Cry2AX1 protein in T_1 generation plants of two events (PT4 and PT7) was 1.2 μg/g of fresh leaf tissue. T_2 generation plants exhibited sustained significant level of mortality, 100 per cent in *H. armigera* and 91 to 98 per cent in *S. litura*. Southern analysis of T_2 progenies revealed single locus integration of *cry2AX1* gene in the event PT7. The synthetic *cry2AX1* gene could be effectively used for developing transgenic crop plants resistant to lepidopteran insect pests.

Key words: *Bacillus thuringiensis*, *cry2AX1*, *Helicoverpa armigera*, *Spodoptera litura*, Transgenic tobacco.

The *cry* genes of *Bacillus thuringiensis* (Bt) mediated insect resistance is one of the most widely used strategies in developing insect resistant transgenic crops. During 2012, insect resistant Bt crops were cultivated in over 60 million ha across the globe. The technology has provided effective control of target pests besides significantly reducing insecticide applications[6]. However, the target insects may develop resistance to Bt proteins expressed in plants if transgenic plants with a single Bt gene is grown continuously on a long term basis[15]. Insects that developed resistance against Cry1A proteins were not cross-resistant to Cry2A proteins[14]. Pyramiding two or more Bt genes with different modes of action is one of the strategies advocated to delay the resistance development in insects. The combination of Cry1Ac and Cry2Ab in Bollgard II Bt-cotton and its commercialization resulted in superior control of lepidopteran pests and expected to have positive implication in resistance management in insects[9]. With a view to develop strategies for improvement of toxicity and/or resistance management, fusion toxin genes have been used to impart insect resistance in crops[13]. The *cry2AX1* is a novel chimeric gene constructed in Tamil Nadu Agricultural University, Coimbatore, India, using the sequences of *cry2Aa* and *cry2Ac*, cloned from Indian strains of Bt. Amino acid

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sequence of the Cry2AX1 differ from that of the Cry2Aa at 10 positions. The Cry2AX1 isolated from recombinant bacterium was found to be more effective against *H. armigera* than Cry2Aa, Cry2Ab and Cry2Ac proteins[16]. Synthetic gene or DNA sequences are required for enhanced expression of a foreign protein in a transgenic plant system, because the codon frequency of the foreign gene need to be adjusted to the preferred codon usage frequency of the plant system. This paper describes for the first time the expression of codon optimized synthetic *cry2AX1* gene (Accession No. GQ332539.1) in tobacco as a model plant and the gene's efficacy in controlling two important lepidopteran pests, *H. armigera* and *S. litura***.**

MATERIALS AND METHODS

Binary vector and plant transformation

The codon optimized synthetic *cry2AX1* gene driven by double enhancer version of CaMV35S promoter was used for construction of a plant transformation vector using the binary vector, pCAMBIA1300. It was mobilized into *Agrobacterium tumefaciens*, LBA4404 through triparental mating and the transconjugants were confirmed by PCR and restriction digestion analysis.

Leaf explants were collected from tobacco (*Nicotiana tabacum* L. cv Petite Havana) plants which were grown axenically on half strength MS medium with 1.5 % sucrose (w/v) and 0.8 % agar at pH 5.8. The leaf bits were precultured on plant regeneration medium (MS + 1.0 mg BAP/l + 0.1 mg NAA/l + 3 % sucrose (w/v) and 0.8 % agar, pH 5.8) for two days and the pre-cultured explants were infected with *Agrobacterium*. The infected leaf bits were co-cultivated for two days at 25°C on plant regeneration medium with 100 µM acetosyringone to allow efficient T-DNA transfer. The leaf bits were washed with cefotaxime (250 mg/l) and placed on plant regeneration medium containing 20 mg hygromycin/l. Subsequent sub-culturing was done at two week intervals and individual shoots which were actively growing on selection plates were transferred to ½ strength MS medium with 1.0 mg IBA/l and 20 mg hygromycin/l. The plants which produced profuse roots were transferred to small paper cups with pot mixture containing soil + sand + vermicompost at the ratio of 1:1:1. Gradually plants were acclimatized to greenhouse condition and then transferred to 7" diameter pots containing above mentioned pot mixture. The matured tobacco capsules were harvested and the seeds were sown for further advancement into next generations, *viz.*, T_1 and T_2.

PCR

The genomic DNA isolated by CTAB method[2] from young leaves was subjected to PCR analysis using primers specific to *cry2AX1* gene (forward 5'-CCTAACATTGGTGGACTTCCAG-3' and reverse 5'- GAGAAACGAGCTCCGTTATCG to obtain an amplicon of 800 bp internal fragment. The amplified PCR products were resolved on 1.2 % agarose gel, visualized on UV transilluminator upon ethidium bromide staining.

Southern blot analysis

Ten microgram of genomic DNA (isolated from T_2 progenies and non-transformed tobacco plants) was digested overnight by *Eco*RI enzyme, electroporetically separated on 0.8 % agarose gel using 1X TBE, denatured with 0.25 M NaOH and transferred overnight to a positively charged Zeta Probe membrane (Bio-Rad, USA) using 20X SSC following standard upward capillary transfer protocol. The transferred DNA was cross linked by UV exposure at 1200 µJ/min for 1 min. The 1.9 kb *Nco*I and *Bam*HI fragment of *cry2AX1* gene purified from pC1300 binary vector was used for preparing probe. The probe DNA was labeled with α-[^{32}P]-dCTP using Random Primer Labeling Kit (Amersham, USA). The denatured probe was hybridized with membrane at 65°C for 16 h in hybridization oven. The hybridized membrane was subjected to high stringency wash at 65°C and exposed to X-ray film for 48 h at -80°C and the film was developed as per the manufacturer's instruction (Konica).

Expression of *cry2AX1* gene in transgenic tobacco Reverse Transcription-PCR analysis

Total RNA was isolated from PCR positive (T_0) plants along with a non-transgenic control plant using SV Total RNA Isolation System (Promega, USA) following manufacturer's instructions. The first strand cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (MBI Fementas, UK). To confirm the expression of *cry2AX1* gene, 100 ng of cDNA was used as template for PCR amplification of 600 bp internal fragment using *cry2AX1* specific primers

(forward 5'-AACGTTCTTAACTCTGGAAGGA-3' and reverse 5'-GCAGAAATTCCCCACTCA TCAG-3').

Detection of Cry2AX1 protein by ELISA

Quantitative estimation of Cry2AX1 protein in transgenic tobacco leaves was done by ELISA using commercially available Quantiplate Kit for Cry2A (EnviroLogix Inc., USA) according to the manufacturer's instructions. Each sample was replicated thrice. The samples which were reading 'out of the range' of the instrument were diluted to get the OD within the reading range of the instrument (<3.0 OD). The quantity of Cry2AX1 protein present in the sample was calculated by referring to standard graph generated with Cry2Aa calibration standards provided along with the kit.

Insect bioassay of transgenic plants

Detached leaf disc bioassay was carried out on the ELISA positive plants with neonate larvae of *H. armigera* and *S. litura*. The neonate larvae were released on to fresh leaf discs (in *H. armigera* 3–4 larvae per disc; 10 larvae per disc in case of *S. litura*) placed in 90 mm petriplates lined with wet Whatmann No.1 filter paper. Three replications were maintained and for each replication ten larvae were taken in case of both the insects. A control was also maintained by using leaf discs from non-transformed tobacco plants of same age compared to transgenic plants. The larvae were observed carefully using a 10x magnified lens and which did not show any movement even after probing with thread of camel hair brush were considered as dead. Final mortality data were recorded on seventh day of the experiment.

Statistical analysis

The experiment was conducted in a completely randomized design (CRD). All percentage data were converted to arcsine square-root values prior to analysis. Data were subjected to analysis of variance (ANOVA) following the AGRES statistical package (Version 7.01, Pascal International Software Solutions). Values reported are mean ± SD.

RESULTS

Transformation of tobacco with *cry2AX1* gene and stable inheritance of transgene in transgenic progenies

Agrobacterium-mediated transformation of tobacco with *cry2AX1* gene resulted in generation of 12 independent transgenic plants on selection medium and these plants were established in transgenic greenhouse. Out of twelve plants screened by PCR, eight were found positive with expected amplicon (~800 bp) of *cry2AX1* gene. Based on the level of *cry2AX1* expression, insecticidal efficacy and seed germination per cent (fertility), two events (PT4 and PT7) were forwarded to T_1 generation; and two T_1 lines (PT4-8 and PT7-

Table 1. Expression of Cry2AX1 protein and its efficacy against neonates of *H. armigera* and *S. litura* in T_0 transformants of tobacco

Tobacco events	Cry2AX1 conc. in fresh leaf tissue (µg/g)*	Larval mortality (%)*	
		H. armigera	*S. litura*
PT1	0.008 ± 0.003	13.33 ± 5.77	6.67 ± 5.77
PT2	1.094 ± 0.012	100.0 ± 0.0	63.33 ± 5.77
PT3	0.009 ± 0.003	16.67 ± 5.77	6.67 ± 5.77
PT4	0.712 ± 0.022	73.33 ± 5.77	36.67 ± 5.77
PT5	0.011 ± 0.004	33.33 ± 5.77	13.33 ± 5.77
PT6	0.102 ± 0.009	53.33 ± 5.77	23.33 ± 5.77
PT7	0.853 ± 0.007	86.67 ± 5.77	46.67 ± 5.77
PT8	0.004 ± 0.003	6.67 ± 5.77	6.67 ± 5.77
Control	0.000 ± 0.000	0.0 ± 0.0	0 .0 ± 0.0
	LSD = 0.017	LSD = 8.21	LSD = 11.13
	SEd = 0.008	SEd = 3.91	SEd = 5.30

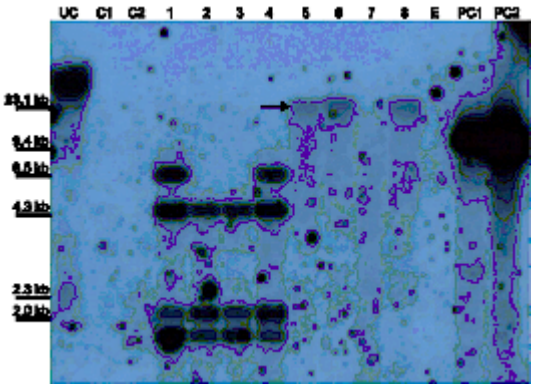
*The values expressed are Mean ± SD based on three replications
In T_0, ELISA and bioassay were carried out on 55 and 59 days after planting, respectively
The statistical significant difference among the mean values at $p \leq 0.05$ was determined by Least Significant Difference (LSD) test

69) were further forwarded to T_2 generation for demonstrating stable integration, expression and efficacy of the transgene. The presence of *cry2AX1* gene was confirmed by PCR analysis; all the sixty plants tested in each of the two T_1 lines were found positive with amplification of ~800 bp internal fragment of *cry2AX1* gene. The number of transgene integration was confirmed by Southern blot analysis in four progenies in each of two T_1 lines, wherein progenies of PT4-8 showed a complex integration pattern and the progenies of PT7-69 showed single locus integration of *cry2AX1* gene (Fig. 1).

Expression of *cry2AX1* gene in transgenic tobacco plants

The presence of Cry2AX1 transcripts in all the PCR positive T_0 plants was confirmed by RT-PCR analysis which showed an expected size amplicon of ~600 bp. The expression of Cry2AX1 protein in these plants was analyzed by quantitative ELISA and the concentration of protein ranged from 0.004 to 1.094 µg/g of fresh leaf tissue. Among the eight events, three (PT2, PT4, PT7) performed significantly superior than others regarding the concentration of Cry2AX1 in fresh leaf tissue (Table 1). The expression of Cry2AX1 protein in T_1 progenies of PT4 and PT7 was 1.2 µg/g of fresh leaf tissue.

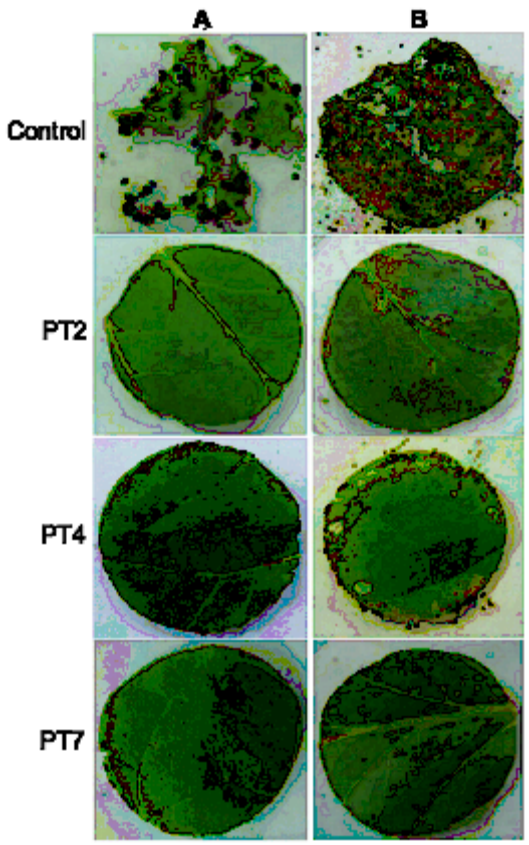
Efficacy of Cry2AX1 protein against *H. armigera* and *S. litura*



UC: Undigested genomic DNA from PT4-8-1, C1 & C2: Genomic DNA from control plants, Lanes 1 to 4: T2 progenies from the event PT4-8 (1, 3, 7 & 9), Lanes 5 to 8: T2 progenies from the event PT7-69 (5, 7, 8 & 9), Arrow indicates the signal. E: Empty lane, PC1: pC1300-2AX1 plasmid 50 pg digested with EcoRI and PC2: pC1300-2AX1/EcoRI (250pg)

Fig. 1. Southern blot analysis of T_2 progenies of tobacco expressing *cry2AX1* gene

The mortality of neonates on T_0 plants ranged from 6.7 to 100 and 6.7 to 63.3 per cent in *H. armigera* and *S. litura*, respectively. Among eight T_0 events, three (PT2, PT7 and PT4) performed significantly superior than others by recording a mortality of 100, 86.7 and 73.3 per cent in case of *H. armigera* and 63.3, 46.7 and 36.7 per cent in *S. litura*, respectively (Table 1). In all these events, the mortality was higher in *H. armigera* than *S. litura*. The surviving larvae in the transgenic lines showed severe inhibition in larval growth and size than in non-transformed plants (Fig. 2). In leaf disc bioassay performed on T_1 progenies derived from PT4 and PT7 exhibited 100 per cent mortality in neonates of both the insects. The T_2 progenies *viz.*, PT4-8-7 and PT7-69-8 recorded 100 per cent mortality against *H. armigera* while they recorded 98.3 and 91.7 per cent mortality against *S. litura*, respectively.



Difference between three transformants of tobacco, PT2, PT4 and PT7 in comparison to control for feeding after five days by neonates of *H. armigera* (A) and *S. litura* (B)

Fig. 2. Bioassay against *H. armigera* and *S. litura* in T_0 transgenic tobacco plants expressing Cry2AX1

DISCUSSION

Globally, the area devoted to growing Bt transgenic crops is increasing and will continue to increase. Continuous exposure of insect pests to a single Bt protein may lead to development of resistance in insects[7]. Rotation of *cry* genes or stacking of *cry* genes which encode protein with different mode of action could be useful for managing resistance development in insects. Due to the difference in structure[8] and insecticidal mechanism[3], the Cry2A proteins are promising candidates for management of resistance-development in insects against the first generation Bt-gene such as *cry1Ac*. Hence, the combination of Cry1Ac and Cry2Ab is used in the second version of Bt-cotton, Bollgard II[9]. Besides, this will also broaden the spectrum of insect-resistance. The *cry2Ab* is more toxic than *cry1Ac* for *S. litura* and *vice-versa* for *H. armigera*[10]. Variation of a single amino acid residue at certain positions of Cry proteins can remarkably influence the level of toxicity[11]. Therefore, efforts were made in our earlier study to improve the level of toxicity of Cry2A by constructing a chimeric *cry2AX1* gene consisting sequences of *cry2Aa* and *cry2Ac* genes which were isolated from indigenous strains[16]. During the transfer of a gene from prokaryotic to a eukaryotic genome, the prokaryote adapted sequence motifs that are not appropriate for expression in plant system were changed to the preferred sequence of the plant system. Hence codon optimized *cry2AX1* gene was synthesized for efficient expression in plants by modifying 427 of 634 codons. The GC content of *cry2AX1* was increased from 35 to 42 per cent in the codon optimized synthetic gene (Accession No.GQ332539.1). In the present study we expressed the synthetic *cry2AX1* gene in tobacco and studied its transformants for gene integration, protein expression and efficacy for insect control.

Presence of *cry2AX1* gene in T_0, T_1 and T_2 progenies by PCR indicated stable integration of transgene. With respect to the number of transgene integration, T_2 progenies of PT4 showed multicopy integration of different size and intensities indicating complex integration pattern, whereas the progenies of PT7 showed a single locus integration of *cry2AX1* gene in tobacco genome. However, no hybridization signal was detected in one of the progenies due to some artifact in the gel.

Expression analysis by RT-PCR and ELISA in T_0 tobacco plants indicated the presence of *cry2AX1* transcripts and Cry2AX1 protein, respectively in transgenic plants. Further quantification of Cry2AX1 protein by ELISA in T_1 generation confirmed stable expression (1.2 µg/g fresh leaf tissue) of *cry2AX1* gene.

Detached leaf disc method of bioassay was performed to assess the efficiency of Cry2AX1 expressed in transgenic tobacco plants against *H. armigera* and *S. litura*. The Cry2AX1 expressed in transgenic tobacco was found effective against both *H. armigera* and *S. litura.* Surviving larvae on transgenic lines, if any, exhibited severe growth inhibition. The non-transformed control plant did not show mortality and larval growth inhibition. In general, the expression of Cry2AX1 Bt protein had a direct relation to insect-resistance in transgenic tobacco plants. In an earlier study, It was observed that the concentration of a different Bt protein (Cry2A*) was directly related to the level of insect-resistance[1]. In the present study, the T_1 progenies derived from the events PT4 and PT7 expressing Cry2AX1 protein at a concentration of 1.2 µg/g of fresh leaf tissue showed 100 percent mortality in both *H. armigera* and *S. litura*. The T_2 progenies of Cry2AX1 tobacco recorded 100 and near 100 percent mortality in *H. armigera* and *S. litura*, respectively. In general, the *cry2AX1* transgenic tobacco plants recorded higher level of mortality in *H. armigera* than *S. litura.*

Even though the level of Cry2AX1 protein expressed in tobacco was low (1.2 µg/g fresh leaf tissue) when compared to the expression of Cry2Ab protein (23 µg/g fresh leaf weight) in a commercialized transgenic cotton *i.e.,* BG II, insect bioassays in T_1 progenies of *cry2AX1* transgenic tobacco showed 100 percent mortality in both *H. armigera* and *S. litura.* Whereas, the BG II version of transgenic Bt cotton hybrids showed up to 73 per cent mortality only against *S. litura*[4]. The *H. armigera* and *S. litura* are major polyphagous lepidopteron pests attacking different agriculturally important crops[5,12]. Hence, the synthetic *cry2AX1* gene of the present study could be valuable to impart lepidopteran insect resistance in crop plants. Higher level expression of *cry2AX1* in crop plants

will be useful for effective control of target pests in the field condition. The expression of higher level of Cry2AX1 protein can possibly be achieved by generating and screening large number of events.

In the present study, stable transgenic tobacco plants expressing synthetic *cry2AX1* gene of Bt were generated through *Agrobacterium tumefaciens* mediated transformation. Two T_1 progenies recorded Cry2AX1 expression of 1.2 µg/g fresh leaf tissue and showed 100 per cent mortality against both *H. armigera* and *S.litura*. The transgene expression and efficacy was also sustained in T_2 generation progenies. Thus the *cry2AX1* is a potential gene for developing transgenic crops resistant to lepidopteran insect pests.

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