

## TRANSGENIC EXPRESSION OF TRANSLATIONAL FUSION OF SYNTHETIC *CryIAc* AND *Hvt* GENES IN TOBACCO CONFERS RESISTANCE TO *Helicoverpa armigera* AND *Spodoptera littoralis* LARVAE

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Single toxin in crops to defend them from herbivorous insects has proven an effective mean of reducing farmer's dependence on environmentally unsafe chemical insecticides. On the other hand insects have great natural ability to develop resistance against these toxins. The incorporation of multiple genes in a plant that produces proteins with different insecticidal mechanisms has the potential to delay insect resistance more effectively than a single gene, or two or more genes that produce proteins with similar mechanism. In the present investigation the translational fusion of two unrelated genes i.e. omega atracotoxin (*Hvt*) and *CryIAc* derived from an Australian spider (*Hadronyche versuta*) and *Bacillus thuringiensis*, respectively was transformed and characterized in *Nicotiana tabacum* L. cv. *spade*. Transgene expression level was confirmed to transcriptional level by reverse transcriptase PCR (RT-PCR) and to protein level by western blotting and immunological detection. Leaf bioassays demonstrate the effectiveness of this translational fusion with 100% mortality in *Spodoptera littoralis* and *Helicoverpa armigera* larvae within 48 and 72 h, respectively. The present study shows that new chimeric protein with two different insecticidal mechanisms retained its biological activity in plant system against agronomically important insects and is an exceptional candidate for long lasting transgenic protection of crop plants.

**Keywords:** *Bt* toxin, chimeric toxin, insect resistance, synthetic genes, spider toxin.

### INTRODUCTION

The chemical insecticides become less effective when the target insects develop resistance, plus beneficial insects that keep herbivorous insects in balance are often killed by insecticides (Zhao *et al.*, 2010). Biopesticides based on living organisms are attractive alternative to the chemical insecticides but the disadvantages of using biopesticides as a foliar spray are that its effectiveness is usually reduced by wind, rain and radiation. Furthermore pests that feed on the underside of leaves or that burrow into the plant can escape *Bt* sprays (Whalon *et al.*, 2003). With the introduction of genetic transformation techniques, it is now possible to incorporate genes into the plant genome that confer resistance to targeted pest insects (Shelton *et al.*, 2002). Insect resistant cotton containing *Bt* toxins is a rapidly adopted technology in the world (James, 2012; Qayyum *et al.*, 2015). The natural ability of insects to develop resistance threatens the benefits achieved by growing the *Bt* crops (Tabashnik, 1994). Development of insect resistance in transgenic plants with a single toxin is sometimes problematic, thus more durable resistance management strategies have been proposed that include the expression of multiple toxins in the same plant. *Bt* toxins that target different receptors in the same insect species can be used in this strategy so that the phenomenon of cross-resistance may

not occur (Cohen *et al.*, 2000; Ullah *et al.*, 2014). Theoretical models also suggest that pyramiding two dissimilar toxins have the ability to delay resistance in insect population much more effectively than a single-toxin (Zhao *et al.*, 2003).

Bollgard II genotype (Monsanto 15985) expressing dual toxin (*CryIAc*+*Cry2Ab*) was found highly effective against lepidopteron cotton pests that were not adequately controlled by the Bollgard varieties having single *CryIAc* toxin (Chitkowski *et al.*, 2003). Bollgard II and WideStrike (*CryIAc* + *Cry1F*) are effective genetically engineered plants against major lepidopteron pests and were introduced to raise the control level of crops (Jackson *et al.*, 2003, Gahan *et al.*, 2005). The *CryIAc* and *Cry2Ab* toxins in Bollgard II have different receptor sites in the target species. This two-gene pyramid should be effective in delaying insect resistance because theoretically two independent mutations are required in genes encoding the receptor sites of the target insect (Jackson *et al.*, 2003).

Pyramiding different toxins from *B. thuringiensis* has been an effective strategy so far (Jackson *et al.*, 2003). However, there still is a need to discover new toxins with completely different modes of action to equip crop plants with more durable resistance against lepidopteron pests (Abbas *et al.*, 2013).

Keeping this in view, there is a need to look for alternates to *B. thuringiensis* (*Bt*) toxins so that the new toxins with unique

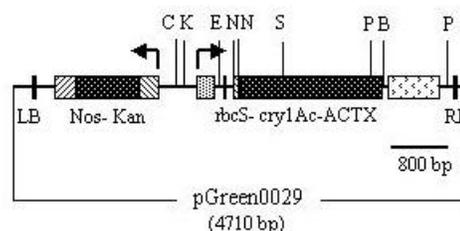
mechanism from pre-existing toxins can be used alone or in combination with *Bt* toxins to develop broad spectrum and long lasting transgenic plants against herbivorous insects. The majority of spider venoms are an excellent source of toxins that kill or paralyze specific arthropod prey (King *et al.*, 2002). Atkinson *et al.* (1996) recognized the insecticidal activity of crude venoms of seven taxonomically distinct Australian funnel-web spiders against members of six different insect orders. During the investigation it was found that crude venoms were most lethal to *Helicoverpa armigera*, which is a refractory agricultural pest found in Indian Sub-Continent. Fletcher *et al.* (1997) identified 37-residue peptide among others from the venom of *Hadronyche versuta*. This peptide inhibits the insect voltage-gated calcium channels currents and was referred to as  $\omega$ -ACTX-Hv1a. The great phylogenetic specificity of  $\omega$ -atracotoxins positions them for controlling different insects of interest (Wang *et al.*, 1999). The possibility of oral toxicity of  $\omega$ -ACTX-Hv1a to insects came from the work of Mukhtar and his co-researchers who designed *Hvt* gene with codon specific to plant genome from  $\omega$ -ACTX-Hv1a toxin protein (Mukhtar *et al.*, 2004). Tobacco transformed with this gene was lethal to two economically important pests of cotton, *Helicoverpa armigera* and *Spodoptera littoralis*, but did not show any deleterious effects on mammals (Khan *et al.*, 2006, Shah *et al.*, 2011). Keeping in mind the mechanism of cross resistance and to develop long lasting resistance against lepidopteron pests, Abbas *et al.* (2013) fused the domains of both neurotoxic *Hvt* and *Bt CryIAc* to combine two different mechanisms in a single chimeric protein. The translational fusion was expressed in prokaryotic system, which was lethal to two lepidopteron pests. The study suggests that the new chimeric protein is a good candidate for development of biopesticide or transgenic plant. In the present study the same domains from both *Hvt* and *Bt CryIAc* are expressed in tobacco to evaluate the biological activity of this chimeric protein in plant system.

## MATERIALS AND METHODS

### Gene designing, plasmid construction and its transformation in *A. tumefaciens* strain LBA4404:

The amino acid sequence of the *Bt CryIAc* gene available in the Genbank database was modified according to the plant preferred codons. The sequence of the synthetic *Hvt* gene was retrieved from the nucleotide database (GenBank ID: AJ938032.1). The translational fusion of *Bt CryIAc* and *Hvt* genes was commercially synthesized from Medigenomics, Germany. The plant expression vector was developed which contained the 2,477 bp synthetic fragment containing RbcS (Rubisco small subunit) promoter, transit peptide from tobacco, intron from catalase gene of castor bean, *Bt CryIAc* fused with *Hvt* and ocs terminator. This vector was named as pSAK-IV (Fig.1). pSAK-IV and pSOUP were introduced

into *A. tumefaciens* strain LBA4404 through electroporation. The few bacterial colonies were confirmed for the presence of pSAK-IV through PCR analysis using (5'-TGCCAACTTGCATCTCTCTG-3' and 5'-TCGGTGAATCCATGAGAACA-3') primers.



**Figure 1. Physical map of plasmid pSAK-IV, (C, *Cla*I; K, *Kpn*I; E, *Eco*RI; N, *Nhe*I; S, *Sac*I; P, *Pst*I; B, *Bam*HI; RB, right border; LB, left border; transit peptide is located between E and N while intron is flanked by N.**

### Stable transformation of pSAK-IV through *Agrobacterium* mediated transformation in *Nicotiana tabacum* cv. Spade:

Single transformed colony of *A. tumefaciens* having pSAK-IV and pSOUP was inoculated in 25 ml liquid LB medium containing 50  $\mu\text{g ml}^{-1}$  rifampicin, 50  $\mu\text{g ml}^{-1}$  kanamycin and 10  $\mu\text{g ml}^{-1}$  tetracycline in 50 ml centrifuge tube and shaken at 250 rpm for 2 days in dark. Leaf discs from *in vitro* grown tobacco plants were placed upside down on MSO medium for 2 days at 16/8 light and dark cycle at  $25 \pm 1^\circ\text{C}$  in growth room. Leaf discs were then immersed in *Agrobacterium* suspension in sterile petriplate for 25-30 min with gentle periodic shaking. Leaf discs were dried on autoclaved filter paper and then transferred to co-cultivation medium for 48 h at  $25^\circ\text{C}$ . After co-cultivation period, the leaf discs were transferred to the selection medium with cut edges in contact with the selection medium (MS medium containing B5 vitamins; sucrose, 30  $\text{g L}^{-1}$ ; kanamycin, 50  $\text{mg L}^{-1}$ ; cefotaxime, 250  $\text{mg L}^{-1}$ ; BAP, 1  $\text{mg L}^{-1}$ ; NAA, 1  $\text{mg L}^{-1}$ ; and phytagar, 15  $\text{g L}^{-1}$ ) after washing with cefotaxime (250  $\text{mg L}^{-1}$ ) to remove the excess of *Agrobacterium* cells. Regenerated leaf discs were shifted to fresh selection medium after 2-3 weeks. After another 2 weeks, shoots were transferred to magenta jars. Shoots having at least 2-3 internodes were shifted to rooting medium (MS medium containing B5 vitamins; sucrose, 30  $\text{g L}^{-1}$ ; kanamycin, 25  $\text{mg L}^{-1}$ ; phytagar, 15  $\text{g L}^{-1}$ ). After vigorous root development, plants were shifted to sand while covered with polyethylene bags to retain humidity. Polyethylene bags were backed off gradually within 7-10 days until plants were acclimatized to ambient temperature and humidity conditions. Control experiments were also carried out in which non-transformed leaf discs were cultured on different media, one having kanamycin and other without kanamycin, to observe the effect of selection agent.

**Molecular analysis of transgenic plants:** PCR analysis was carried out for the confirmation of transgenic plants using already mentioned primers. The PCR profile was optimized as initial denaturation for 5 min at 95°C, followed by 35 cycles of 94°C for 1 min, 55°C for 40 sec, 72°C for 1 min and final extension of 72°C for 7 min. The amplified products were analyzed by electrophoresis on 0.8% agarose gels along with 1kb DNA marker. Trizol reagent (Life Technologies Cat # 15596-018) was used for total genomic DNA extraction.

In order to carry out the reverse transcriptase PCR (RT-PCR), the total RNA was isolated from the leaves of four different transformed and one non transformed line using Trizol reagent (Life Technologies Cat # 15596-018). 10 ng of RNA from each transformed event was used to synthesize first-strand of complementary DNA using the specific reverse primer of *CryIAC* by AMV Reverse transcriptase # EP0641. 3µl of reverse transcription reaction product of each transformed event was used to amplify the internal portion of *CryIAC* gene with earlier mentioned internal primers of *CryIAC* and conditions in 50 µl PCR reaction using premix taq (Takara Ex Taq version 2.0 code # RR003A).

SDS PAGE was carried out to analyze protein expression of transgenic and control plants. Leaf tissues were ground to fine powder with the help of liquid nitrogen and the total soluble proteins were extracted using the Trizol reagent. The 50 µg of protein from both transformed and non-transformed plants was run per lane on 10% SDS-PAGE gels. Proteins were transferred to nylon membrane at lower voltage of 50 volts in the cold room. A lower acrylamide concentration was initially used for better transfer of higher molecular weight proteins. The protein transfer on nylon membrane was confirmed by using the pre stained protein marker. Western blot analysis was carried out using already raised polyclonal antibodies against chimeric protein as a primary antibody. Goat anti-rabbit antibodies conjugated with alkaline phosphatase were used as secondary antibodies to detect primary antibodies. The blot was developed using the Nitro Blue Tetrazolium (NBT) and Bromo Chloro Indolyl Phosphate (BCIP) as substrate for the alkaline phosphatase.

**Leaf bioassays:** Leaf bioassays of transformed and non-transformed tobacco plants were compared to test the effectiveness of expressed chimeric protein in transformed plants. Four leaves were detached from each transgenic event and non-transgenic plant. Each detached leaf was exposed to five, 2<sup>nd</sup> instar larvae of *Spodoptera littoralis* in petri dishes containing filter papers. Similar leaf bioassays were also set

up for *Helicoverpa armigera* separately. The Petri dishes were incubated at 25±2°C and a relative humidity of 50-70%. Mean weight and mortality percentage of both insect species were statistically compared for all treatments. Data on mortality after exposure of leaves to both species was recorded after 24, 48 and 72 h. Weights of larvae from both species were recorded before and after the bioassays. Two factor factorial completely randomized design (CRD) was used to analyze the data using statistix 8.1. The means were compared using least significant difference test (LSD). The area of leaf damage of both transformed and non-transformed plants were recorded 4 days after the introduction of *H. armigera*.

**RESULTS**

**Confirmation of transformed *A. tumefaciens* strain LBA4404:** The plant expression vector pSAK-IV (Fig. 1) and helper plasmid pSOUP were transformed into *Agrobacterium tumefaciens* strain LBA 4404 through electroporation at the same time. The few transformed colonies were analyzed by PCR for the presence of plasmid using *cryIAC* specific primers. The amplification of 883 bp fragment (Fig. 2) from the bacterial colonies indicated that pSAK-IV was successfully transformed into *A. tumefaciens* strain LBA 4404.



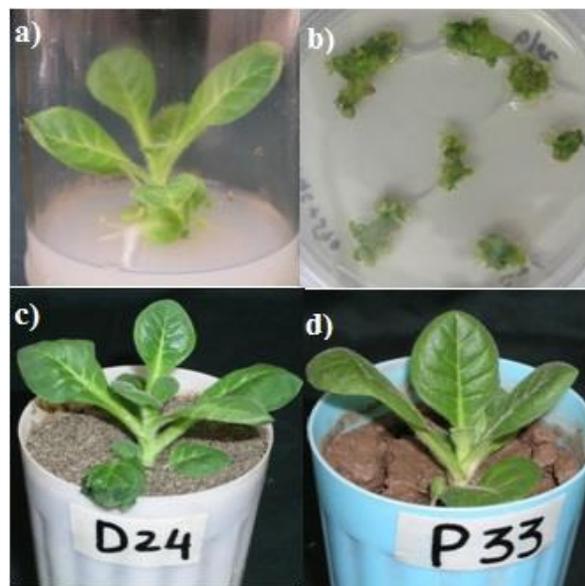
**Figure 2. Agarose gel electrophoresis of PCR amplified products from *Agrobacterium tumefaciens* strain LBA4404.** Lane 1 & 11, 1Kb DNA ladder; lane 2-9, 883 bp fragment amplification from transformed colonies; lane 10, non-transformed colony (negative control).

**Stable transformation of *Nicotiana tabacum* cv spade:** About 300 explants of *N. tabacum* L. cv. Spade 28 were transformed with *A. tumefaciens* having pSAK-IV in three different attempts. Out of 300 explants, only 110 explants could survive on selection media and regenerated plants with

**Table 1. Different experiments showing number of kanamycin resistant plants and transformation efficiencies.**

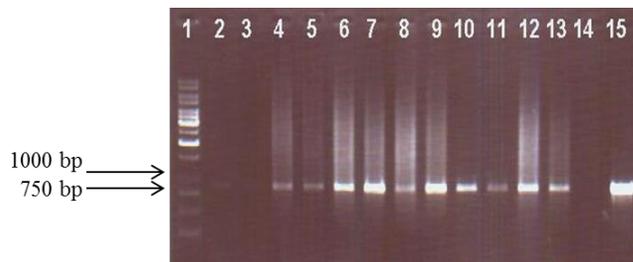
Exp. No.	Explants used	Kanamycin resistant explants	Transformation efficiency (%)	Plants produced	plants transferred to soil
1	95	16	16.8	28	6
2	109	49	44.6	95	18
3	96	45	46.9	137	28
Total	300	110	36.1	260	52

an average transformation efficiency of 36.1% as shown in Table 1. The rest of the explants bleached out indicating that these were not transformed. The first signs of regeneration of plantlets were evident after 35 days from the date of infection with the bacteria. A large number of kanamycin resistant plants were recovered (Table. 1) and successfully established in soil. The different steps involved in transformation of plants through *Agrobacterium* are shown in Figure 3.

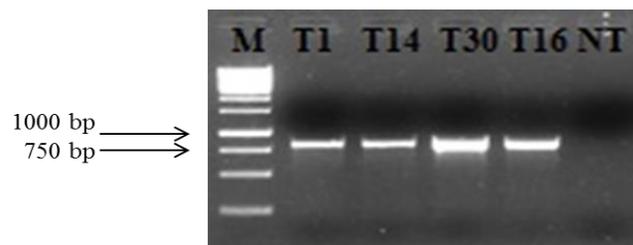


**Figure 3.** Different steps in transformation of plants through *Agrobacterium*. a, In-vitro plants used for cuttings leaf discs; b, plant regeneration from leaf discs on selection media; c, putative transgenic plants in sand for root growth and development; d, putative transgenic plants established in soil.

**Molecular analysis of transgenic plants:** A large number of putative transgenic plants were recovered but it was not possible to screen all the regenerated plants. Therefore, only few plants representing each experiment, from individual transformation events were randomly selected and analyzed by PCR. The DNA isolated from 12 putative transgenic plants and a non-transformed control was subjected to PCR analysis using *CryIAc* specific forward and reverse primers. The result of PCR analysis of the putative transgenic plants is shown in Figure 4. The figure shows that all the 12 putative transgenic plants were positive for the transgene. No amplification from the non-transformed control authenticates our results. One non-transformed and four transformed lines were subjected to the RT-PCR analysis. The expression of translational fusion of *Bt cryIAc* and *Spider Hvt* to transcriptional level was confirmed in transformed lines while no such transcript was found in non-transformed line (Fig. 5).



**Figure 4.** Agarose gel electrophoresis of PCR amplified products from DNA extracted from putative transgenic and non-transgenic (control) plants, using *CryIAc* specific primers. Lane 1, 1 Kb DNA ladder; lanes 2-13, 883 bp fragment amplified from DNA of putative transgenic plants; lane 14, DNA from a non-transformed plant (negative control) and lane 15, plasmid control (pSAK-IV).



**Figure 5.** RT-PCR analysis of transformed and non-transformed plants. Lane M, 1kb DNA marker; lanes T1, T14, T30 and T16 are the amplifications from the reverse transcription reactions product from four transformed lines; lane NT, non-transformed line (negative control).

Western blot was developed using the NBT and BCIP as substrate for the alkaline phosphatase, which gave the purple color for the positive samples and 74 KDa chimeric toxin was detected in T1, T14, T16 and T30 transgenic lines while non-transformed line (NT) lacked chimeric toxin and there was no purple signal in corresponding lane as shown in the Figure 6.



**Figure 6.** Western blot analysis of total soluble protein isolated from transgenic tobacco transformed with pSAK-IV expressing *CryIAc*-Hvt fusion protein. Lane M, Pre stained marker SM0441; lanes T1, T14, T16 and T30 proteins from putative transgenic plants and lane NT, protein from a non-transformed plant (negative control).

**Table 2. Weight and mortality of 2<sup>nd</sup> instar larvae of *S. littoralis* after exposure to *CryIAC-Hvt* transformed and non-transformed detached leaves of *N. tabacum* plants.**

Lines	Larvae weight (mg)*		Mortality percentage			Mean
	Time after introduction (h)		Time after introduction (h)			
	0	72	24	48	72	
NT	0.19±0.006	1.67±0.045 a	0	0	0	0.00 c
T1	0.20± 0.008	0.09±0.004 c	45	85	100	76.67 a
T14	0.22±0.004	0.44±0.188 bc	0	40	80	40.00 b
T16	0.23±0.003	0.44±0.177 b	10	50	75	45.00 b
T30	0.23±0.009	0.11±0.005 bc	55	100	---	77.50 a
	LSD	0.3536	22.0±5.6 c	55.0±8.4b	63.8±10.0a	8.1307

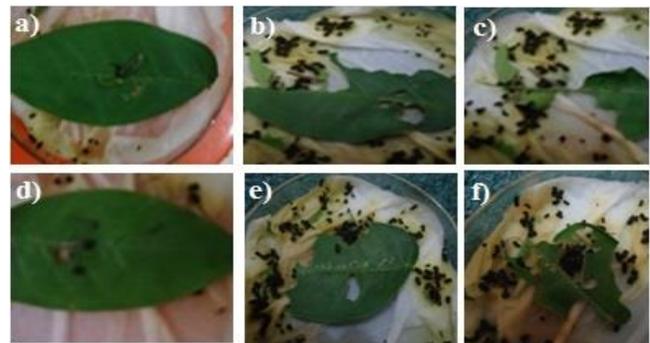
\*Mean of four replicates and each replicate consist of 5 insects; Means followed by same letters are not significantly different at  $p=0.05$ .

**Table 3. Weight and mortality of 2<sup>nd</sup> instar larvae of *H. armigera* after exposure to *CryIAC-Hvt* transformed and non-transformed detached leaves of *N. tabacum* plants.**

Lines	Larvae weight (mg)*		Mortality percentage			Mean
	Time after introduction (h)		Time after introduction (h)			
	0	72	24	48	72	
NT	0.32± 0.003	3.57±0.024 a	0	0	0	0.00 d
T1	0.30± 0.003	0.24±0.001bc	30	80	100	70.00 b
T14	0.30 ±0.004	0.27±0.018 b	10	45	80	45.00 c
T16	0.31±0.003	0.27±0.021 b	5	40	80	41.67 c
T30	0.31±0.009	0.22±0.001 c	55	95	100	83.33 a
	LSD	0.049	20±5.0 c	52±7.9 b	72±8.6 a	7.354

\*Mean of four replicates and each replicate consist of 5 insects; Means followed by same letters are not significantly different at  $p=0.05$ .

**Leaf bioassays:** Detached leaves exposed to 2<sup>nd</sup> instar larvae of *S. littoralis* and *H. armigera* are shown in Fig. 7. Data on insect mortality was collected after 24, 48 and 72 h. The different lines showed variable resistance to second instar larvae of *S. littoralis* and *H. armigera* (Table 2, 3). Statistical analysis revealed that significant differences exist ( $P<0.05$ ) among different transgenic events to control *S. littoralis* and *H. armigera* larvae. 24, 48 and 72 h of treatments were also significantly different for both insect species at 5% probability level ( $P=0$ ) (Table 2, 3). The transgenic line T30 showed 100% mortality of *S. littoralis* larvae within 48 hours while same larvae died within 72 hours when placed on detached leaves of transgenic line T1. Statistical analysis showed that transgenic lines T1 and T30 were non-significantly different from each other with respect to insect mortality at 5% probability level. The transgenic lines T14 and T16 showed reduced mortality of 2<sup>nd</sup> instar larvae of *S. littoralis* compared to the T1 and T30 (Table 2). The reduced weight of *S. littoralis* larvae was also observed on T1 and T30 detached leaves compared to T14, T16 and non-transformed detached leaves after 72 h of introduction (Table 2).



**Figure 7. Detached leaf bio-assays of transgenic tobacco line (T30).** a and d, detached leaves of transgenic line (T30) exposed to 2<sup>nd</sup> instar larvae of *S. littoralis* and *H. armigera* respectively (photographs were taken after 48 h of exposure); b and c, non-transformed detached leaves exposed to 2<sup>nd</sup> instar larvae of *S. littoralis* (photographs were taken after 48 h and 72 h of exposure respectively); e and f, non-transformed detached leaves exposed to 2<sup>nd</sup> instar larvae of *H. armigera* (photographs were taken after 48 h and 72h of exposure respectively).

Similar trend was also observed for *H. armigera* during detached leaf bioassays. The same transgenic lines T1 and T30 were found more toxic to 2<sup>nd</sup> instar larvae of *H. armigera* compared to other transgenic and non-transformed control line (Table 3). During this experiment the extent of damage to transformed (T30) line and non-transformed (NT) line was calculated after 4 days of the introduction of *H. armigera*. Average leaf damage to transformed leaves was 143.9 mm<sup>2</sup> compared to 546.7 mm<sup>2</sup> on non-transformed leaves in four replications. The leaf damages on both treatments were found significantly different (P=0). Least significant difference was found 17.5 for comparisons of means for significance. A total of 575.55 mm<sup>2</sup> area of leaf was consumed in four replicates on transgenic line T30 compared to 2186.67 mm<sup>2</sup> area of non-transformed leaves.

## DISCUSSION

Insects have the potential to develop resistance to *Bt* toxins. Field-evolved resistance has been reported in *Busseola fusca*, in South Africa to Cry1Ab toxin in corn (Kruger *et al.*, 2009), *Spodoptera frugiperda*, in Puerto Rico to transgenic corn containing Cry1F toxin (Matten *et al.*, 2008), *Pectinophora gossypiella*, in western India to cotton producing Cry1Ac (Bagla, 2010), *Helicoverpa zea*, to cotton expressing Cry1Ac in the south eastern United States (Tabashnik *et al.*, 2008). An exponential increase in the frequency of alleles conferring resistance to Cry2Ab was noticed in *Helicoverpa punctigera* population with the adaptation of dual toxin Bollguad II in Australia (Downes *et al.*, 2010). It has also been reported that larvae of *Helicoverpa armigera* not only completed their life cycle but also reproduced on commercial *Bt*-cotton hybrids containing single Cry1Ac toxin and double Cry1Ac and Cry2Ab toxins in experimental field plots (Ranjith *et al.*, 2010). The development of resistance to *Bt* toxins by herbivorous insects threatens the worth of *Bt* crops (Tabashnik and Carrière, 2009).

So it is highly advisable to discover novel genes that act at new or under exploited targets of the insects. Recently synthetic *Hvt* gene from Australian funnel web spider was codon optimized for high level expression in plants (Mukhtar *et al.*, 2004), that proved itself as an attractive small molecule for the development of genetically engineered crop against agriculturally important insect pests (Khan *et al.*, 2006). Transformed *N. tabacum L* containing *Hvt* gene under two phloem specific RSs1 and RolC promoters showed 93-100% mortality of 1<sup>st</sup> instar larvae of *Heliothis armigera* within 72 h on detached transgenic leaves (Shah *et al.*, 2011). Omega-atracotoxin-Hv1a (*Hvt*) is equally effective whether the toxin is orally fed or injected to *A. americanum* (Mukherjee *et al.*, 2006). Xia *et al.* (2009) constructed a fusion gene by combining the *cry1Ac* gene with a neurotoxin gene, hwtx-1.

Fusion crystals were more toxic than the original Cry1Ac crystal protein against third-instar larvae of *Plutella xylostella*. 92% mortality was noticed in *L. disper* larvae fed with transgenic poplar expressing fusion protein consisting of spider  $\omega$ -ACTX-Ar1 and *Bt*-toxin C-peptide compared to 16.7% on non-transgenic leaves (Cao *et al.*, 2010).

Li *et al.* (2012) expressed and characterized recombinant Cry1Ac crystal protein fused with spider  $\omega$ -ACTX-Hv1a in *B. thuringiensis*. Toxicity of the fusion inclusions was found at least five fold more towards the larvae of *Spodoptera exigua*. Abbas *et al.* (2013) performed the functional analysis of translational fusion between *Bt* Cry1Ac and spider  $\omega$ -ACTX-Hv1a (*Hvt*) genes. In both topical application and force feeding experiment, the *H. armigera* and *S. littoralis* showed the characteristic symptoms of the spider *Hvt* gene (Abbas *et al.*, 2013).

In the present study the same translational fusion was transformed in tobacco plant through *Agrobacterium* mediated transformation. Results showed that translational fusion retained its biological activity in plant system with 100% mortality in *Spodoptera littoralis* and *Helicoverpa armigera* within 48 and 72 h respectively. Reduced consumption of foliage was noticed in all transformed lines compared to non-transformed plants. Nearly 4 times more leaf consumption were noticed in non-transformed plant compared to the transformed lines.

Intron from bean catalase gene was included in the transcription region between RbcS promoter and coding region of fused *Cry1Ac* and *Hvt* genes for enhanced expression of the fusion protein. Callis *et al.* (1987) previously observed the functional importance of the introns in the expression of dehydrogenase-1 (*Adh1-S*) gene. Tanaka *et al.* (1990) also observed 10 to 40-fold more expression of beta-glucuronidase gene (*gusA*) containing the first intron of castor bean catalase gene in transgenic rice compared to intronless *gusA* transgenic rice.

Globally CaMV 35S promoter is usually used for transgene expression in genetically modified crops but many greenhouse and field studies have suggested that CaMV 35S promoter is not robust and its utility is influenced by undefined physiological and environmental factors (Sunilkumar *et al.*, 2002). Amarasinghe *et al.* (2006) observed equal expression of *Bt* gene under RbcS (Rubisco small subunit) promoter in greenhouse as well as in field studies. In the present study the expression of fusion protein was carried out with 217 bp RbcS (Rubisco small subunit) promoter in order to target the encoded chimeric protein to green tissues and to get consistent expression in greenhouse and as well as in field condition in future investigations.

**Conclusions:** In summary, the synthetic translational fusion of *Bt* *Cry1Ac* and spider *Hvt* genes has been characterized in

tobacco. The intron from castor bean catalase gene and transit peptide from tobacco have been added in transcriptional cassette to drive enhanced expression in green tissues of plants under the control of RbcS promoter. Our study clearly indicates that encoded chimeric protein is highly effective against agronomically important insects and is an excellent candidate for long lasting transgenic protection of crop plants that will help maximize the benefits of future transgenic crops.

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