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A Chimeric Protein Encoded by Synthetic Genes Shows Toxicity to *Helicoverpa armigera* and *Spodotera littoralis* Larvae

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Abstract

Insects have natural potential to develop resistance against chemical insecticides. Several resistance strategies have been suggested including biopesticides and use of two dissimilar toxins. Advances in molecular biology techniques have now allowed construction of chimeric proteins to delay the development of resistance in insect population, but still there are chances of developing resistance in insect population against them as these fusions are based on *Bacillus thuringiensis* (*Bt*) genes only, which have some homology in their amino acid sequences, having same mode of action and derived from same bacterial origin. In the present study ω-ACTX–Hv1a toxin gene (*Hvt)* as an insect calcium channel antagonist is fused with *Bt cry1Ac* to combine both strategies (biopesticides and two dissimilar toxins) and delay the resistance in insect population. The recombinant protein has been successfully expressed in prokaryotic system and was detected by SDS PAGE. Topical application of the 1.0 pmol purified recombinant protein to the thoracic region paralyzed and immobilized the *Helicoverpa armigera* and *Spodoptera littoralis* larvae within 2 h. 100% mortality was observed in insects after 24 h. The LD50 was found to be 4 and 2 pmol per gram of body weight for *H. armigera* and *S. littoralis* larvae, respectively. The present study clearly indicates that this recombinant protein is highly effective against agronomical important lepidopteron insects and is an excellent candidate for use as a biopesticides or expressed heterogeneously in agricultural crops to provide long lasting resistance to insect attacks.

Keywords: Recombinant toxin; Biopesticides; Insect resistance; Multiple genes; Insecticidal proteins; Spider toxin

Introduction

Chemical insecticides have become less effective as the target insect populations develop resistance and have killed non-target population of predators and parasites that otherwise keep herbivorous insects in balance (Zhao *et al*., 2010). Pesticides exposure also affected the human health and organochlorine pesticides residues have also been reported in the blood of workers occupationally engaged in agriculture (Dhananjayan *et al*., 2012). Biopesticides are attractive alternative to the broad spectrum organophosphate insecticides. Biopesticides are fairly specific, quite lethal, safe to most beneficial insects and animals, biodegradable and do not persist in the environment and hence can delay the onset of resistance in insects unlike chemical insecticides, which are persistent in environment and quite general in their effect. Biopesticides also suit integrated pest management and integrated crop management strategies (Gupta and Dikshit, 2010). It is well documented that many insects are susceptible to the toxic activity of *B. thuringiensis*. Among them lepidopterans have been exceptionally well studied, and many toxins have shown activity against them (Monerat *et al*., 2007). Today, a great variety of *B. thuringiensis*-based bioinsecticides are commercially available for the control of a wide variety of agriculture and forestry pests, including disease vectors (Sauka and Benintende, 2008). Commercial biopesticide namely XenTari consisting of *Bt aizawai* has been successfully used to control the larvae of *Galleria mellonella* (Basedow *et al*., 2012). Soberon and Bravo (2007); Abad *et al.* (2008) have described representative patents in which the insecticidal activity has been directed toward different insect pests, particularly to protect plants from pest damage. In recent years, hybrid delta-endotoxins have arisen as proteins with potential for enhanced toxic activity or improved properties. Recent advances in molecular methodologies have allowed gene fusions and chimeric protein construction. This construction can include alteration of amino acid sequences, fusion of portions of two or more proteins together into a single recombinant protein (Rosas-Garcia, 2009). But still there are chances of developing resistance in insect population against them because these fusions are based on *B. thuringiensis* genes only which have some homology in their amino acid

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sequences, having same mode of actions and derived from same bacterial origin. It is therefore the need of time to fuse two different genes with unique mode of actions in order to develop biopesticides with durable and long lasting resistance to insect pests. Most spider venoms are rich source of insecticidal compounds and their primary role is to kill or paralyze arthropod prey. Mukhtar *et al.* (2004) developed codon optimized ω- ACTX –Hv1a toxin gene (*Hvt)* for high level expression in plants. Hvt is a 37 amino acid, insect specific calcium channel antagonist from Australian funnel web spider. The peptide is toxic to a range of agriculturally important arthropods in the orders Coleoptera, Lepidoptera and Diptera but has been reported to have no effects on a number of mammals (Khan *et al*., 2006; Chong *et al*., 2007). Shah *et al.* (2011) cloned the *Hvt* gene under RSs1 and RoLC phloem specific promoters. The resulted transgenic tobacco confirmed resistance to *Heliothis armigera.*

In the present study the *Bt cry1Ac* and Spider *Hvt* (*ACTX)* genes with unique mode of actions have been recombined. It has been demonstrated that translational fusions of *Bt cry1Ac* and spider *Hvt* (*ACTX)* genes paralyzed and immobilized the *Helicoverpa armigera* and *Spodoptera littoralis* larvae and is an excellent candidate for use as biopesticides or expressed heterogeneously in agricultural crops to provide long lasting resistance to insect attacks.

Materials and Methods

Gene Designing and Plasmid Construct

Translational fusion of *Bt cry1Ac* (patent pending) and Spider *Hvt (ACTX)* gene (Khan *et al*., 2006), was commercially synthesized from Medigenomics, Germany. To facilitate the cloning of this gene under desirable expression cassette, few restriction sites were added to the flanking regions of the gene at the 5' as well as 3' ends of the gene. Resultant vector was named as pSAK-IV (Fig. 1a). The bacterial expression vector was developed at the Plant Molecular Biology and Transformation Lab at NIBGE which contained c*ry1Ac* fused with *Hvt* (*ACTX)* gene and was named as pSAK-V (Fig. 2f).

Development of Bacterial Expression Constructs

Synthetic *cry1Ac* fused with the *Hvt (ACTX*) was amplified (Fig. 1b) from plasmid pSAK-IV (Fig. 1a) using full-length primers. The forward primer was based on *cry1Ac* gene 5-' GCATGGATAATAACCCTGGA-3' and reverse primer was based on *Hvt* gene 5-' TTAATCGCATCTTTTTACGG-3'. Total volume for PCR reaction was 50 µL. The PCR profile was optimized for the amplification of synthetic *cry1Ac* gene fused with the *Hvt* (*ACTX*) gene as denaturation at 94^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 2.5 min and final extension of 72^{o}C for 10 min. Eppendorf thermal cycler was used for PCR and amplified products were analyzed by electrophoresis on 1% agarose gels along with 1 kbp DNA marker.

The amplified product was cloned with correct orientation in T/A cloning vector pTZ57R. The resultant vector was named as pZSTA (Fig. 1c). *Eco*R1-*Hin*dIII enzymes were used to lift the *cry1Ac* fused with *Hvt (ACTX)* gene from the pZSTA. The bacterial expression vector pET32a (+) was digested with *Eco*R1-*Hin*dIII and eluted from the gel and used as a vector for cloning. The *Eco*R1-*Hin*dIII fragment of pZSTA was ligated with the pET32a (+). The resultant bacterial expression construct was named as pSAK-V (Fig. 2f).

Expression of pSAK-V in *E. coli* Strain BL21

The BL21 (DE) pLysS (Novagen) host containing a chromosomal copy of T7RNA polymerase gene under *lacUV5* promoter was transformed with pSAK-V. The *lacUV5* promoter is responsible for driving the expression of T7RNA polymerase. It is inducible by isopropyl beta D thiogalactopyranoside (IPTG). pET32a (+) carries the target gene under T7 promoter. When IPTG is added it activate the *lacUV5* promoter and turn on synthesis of T7 RNA Polymerase. This in turn transcribes the gene in pET vector.

After transformation and confirmation of pSAK-V in BL21, the target gene was induced by the addition of 1 mM IPTG. Colony was picked from a freshly streaked plate and inoculated into 3 mL LB medium, in a 15 mL falcon tube, containing the ampicillin and chloramphenicol for the selection of plasmid and BL21 host strain, respectively. This 3 mL culture was incubated with shaking at 37°C for overnight and further inoculated into 50 mL of LB containing ampicillin and chloramphenicol and grown for overnight at 37°C until OD 600 nm reached 0.4–1.0 (0.6 recommended; about 3 h). Un-induced control sample was removed and to the remainder, IPTG was added from a 100 mM stock to a final concentration of 1 mM. Samples were taken at regular intervals after induction i.e., 0.5, 1.0, 1.5, 2.0 and 2.5 h. The cells were harvested by centrifugation at 13,400 rpm for 1.5 min. The cells were frozen at -20°C for 30 min. The harvested cells were allowed to thaw, sonicated and then BugBuster Protein Extraction method was used to gently disrupt the cell wall of *E. coli* resulting in the liberation of soluble protein. 10% SDS polyacrylamide (SDS-PAGE) gel was prepared. The expression of target genes was assessed quickly by analysis of total cell protein on an SDS-PAGE gel followed by Coomassie blue staining and destaining.

Biological Toxicity Assays

In order to conduct the biological toxicity assay, the larvae of *H. armigera and S. littoralis* were taken from fields in petri plates having moist whatmann filter papers. The larvae

were allowed to grow and feed on cotton leaves. Adults of larvae were then shifted to the jars having muslin cloth. Eggs of adults were then transferred from muslin cloth to the vials containing artificial insect diet. 2nd instar larvae of *H. armigera and S. littoralis* emerged, while incubating the vials at a temperature of 25±2ºC.

As recombinant protein is a fusion between *cry1Ac* and *Hvt (ACTX)* gene, two separate toxicity assays were carried out. In order to measure the toxicity of *cryIAc*, the recombinant protein was activated by the gut juice of silkworm following the procedure as described by Gringorten *et al.* (1990). The toxicity was determined by the force feeding technique as previously illustrated by Van *et al.* (1991). The control experiment was also carried out having only gut juice.

Toxicity of *Hvt* was measured by topical application of 2 µL of the recombinant protein in elution buffer to the thoracic region of 2nd instar *H. armigera and S. littorallis* larvae having an average weight of 3.2 ± 0.06 and 2.98±0.04 mg, respectively. Each of 0.02, 0.06, 0.12, 0.25, 0.5 or 1.0, 2.0, 4.0, 8.0, or 16.0 pmol of recombinant protein doses were applied, using a micropipette, to twenty larvae in order to determine the LD 50 values after twelve hours of application. Control experiment was also conducted having the thioredoxin protein only.

Results

Cloning of *Cry1Ac* and *HVT* Genes in Bacterial Expression Vector

The 1,968 bp DNA fragment containing the full length synthetic *cry1Ac* fused with the *Hvt* gene was amplified from plasmid pSAK-IV (Fig. 1a) using full-length primers. The forward primer was based on the *cry1Ac* 5' region while the reverse primer was designed based on the 3' region of the *Hvt* gene. These primers amplified 1,968 bp fragment as shown in Fig. 1b. This 1,968 bp fragment was cloned in T/A cloning vector pTZ57R and the orientation of the insert was confirmed using *Sac*I. Since there is a unique *Sac*I site in pTZ57R in addition to one *Sac*I site in the insert, therefore, the digestion with *Sac*I should produce two fragments of either 742 bp and 4,112 bp (orientation I) or 1,300 and 3,554 (Orientation II). Fig. 1d shows that upon digestion with *Sac*I, the resultant vector produced two fragments of 742 and 4,112 bp indicating that the insert has been cloned in correct orientation I. The resultant vector was named as pZSTA (Fig. 1c). pZSTA was digested with *Eco*R1-*Hin*dIII to clone the *cry1Ac-Hvt (ACTX)* genes in frame with the coding TAG sequence of the pET32a(+) as shown in Fig. 2a. The resultant vector was named as pSAK-V (Fig. 2b). The cloning was confirmed through digestion of pSAK-V with *Eco*R1 and *Hin*dIII, which should remove the insert (2,048 bp fragment) from the parent vector.

Upon digestion with *Eco*R1 and *Hin*dIII, pSAK-V released the 2,048 bp fragment along with the vector

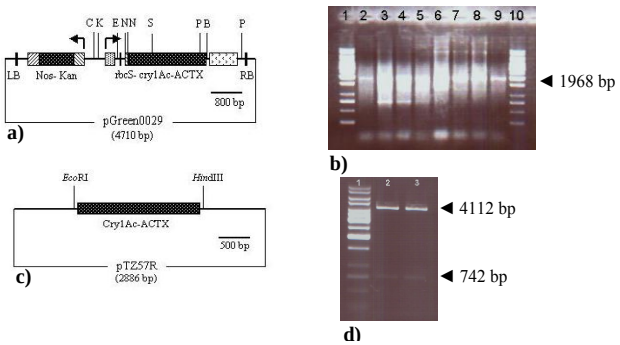

Fig. 1: Cloning of *Cry1Ac-Hvt* genes in pTZ57R
a) Physical map of plasmid pSAK-IV, (C, *Cla*1; K, *Kpn*1; E, *Eco*R1; N, *Nhe*1; S, *Sac*1; P, *Pst*1; B, *Bam*H1; RB, right border; LB, left border); **b)** PCR amplification of the full length synthetic *cry1Ac-Hvt (ACTX)* genes (1968 bp) from pSAK-IV; lane 1 and 10, 1 kb DNA ladder ; lanes 2-9 1968 bp fragment amplified from pSAK-1V; **c)** cloning of PCR amplified 1968 bp fragment in pTZ57R to from pZSTA; **d)** confirmation of the cloning through digestion of pZSTA with *Sac*1; lane 1, 1 kb DNA ladder ; lanes 2 and 3, restriction with *Sac*1

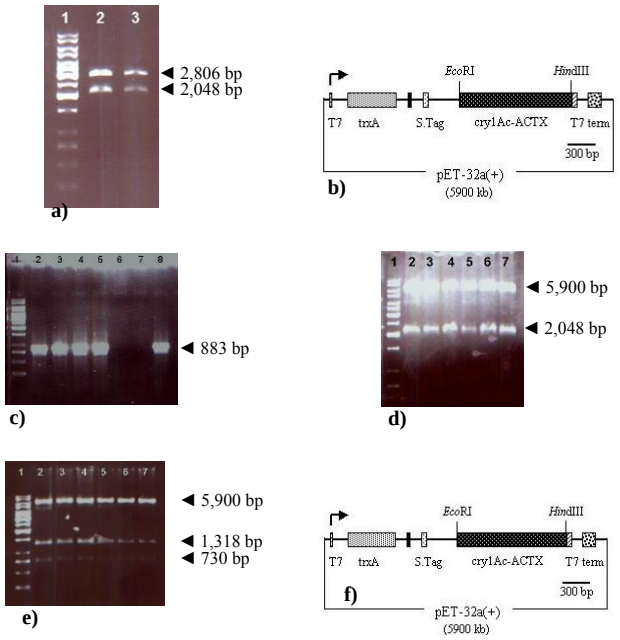

Fig. 2: Development of bacterial expression Construct
a)Digestion of pZSTA with *Eco*R1 and *Hin*dIII Lanes 2 and 3; lane 1, 1 kb DNA ladder; **b)** cloning of EcoR1-HindIII fragment (2048 bp) in pET32a(+) to from pSAK-V; **c)** PCR amplification for confirmation of pSAK-V, lane 1, 1 kb DNA ladder ; lanes 2-5, 883 bp fragment amplified from pSAK-V; lane 6, Negative control pET32a(+); lane 7, water control; lane 8, plasmid (pSAK-1V) control; **d)** confirmation of cloning of *cry1Ac –Hvt* (ACTX) genes in pET32a(+) through restriction analysis, lane 1, 1 kb DNA ladder; lanes 2-7, pSAK-V digested with *Eco*R1-*Hin*dIII; **e)** confirmation of pSAK-V through restriction analysis with *Sac*1-*Hin*dIII, lane 1, 1 kb DNA ladder; lanes 2-7, pSAK-V digested with *Sac*1-*Hin*dIII; **f)** physical map of the resultant vector pSAK-V

backbone (5,900 bp) as shown in Fig. 2d. This confirmed the cloning of *cry1Ac-Hvt* (ACTX) genes in pET32a (+). To further confirm the cloning in pET32a (+), pSAK-V was digested with *Sac*I-*Hin*dIII, which should produce three

fragments (i.e. vector backbone 5,900 bp, 1,318 bp and 730 bp). Fig. 2e shows that upon digestion with *Sac*I-*Hin*dIII, pSAK-V produced three fragments of the expected sizes thus confirming the cloning of *cry1Ac-Hvt* genes in pET32a (+). Further confirmation of the clone was made by PCR using *cry1Ac* specific primers, which produced 883 bp internal fragment of the *cry1Ac* gene as shown in Fig. 2c indicating that the insert has successfully been cloned into pET32a (+).

pSAK-V Transformation in *E. coli* Strain BL21

pSAK-V was introduced into *E. coli* strain BL21 through electroporation. The selected bacterial colonies were analysed for the presence of pSAK-V through PCR analysis using *cry1Ac* specific primers as given below:

5'-TGCCAACTTGCATCTCTCTG-3'and
5'-TCGGTGAATCCATGAGAACA-3'.

The amplification of 883 bp fragment (Fig. 3) confirmed the transformation of plasmid pSAK-V in *E. coli* strain BL21.

Expression of the Target Gene

After transformation and confirmation of pSAK-V in BL21, expression of the target gene was induced by the addition of 1 mM IPTG. Samples were taken at regular intervals after induction i.e., 0.5, 1.0, 1.5, 2.0 and 2.5 h. The samples were processed and analyzed on SDS PAGE. The intensity of desired protein (88 kDa) was increased with increase in time after induction i.e., 30, 60, 90, 120 and 150 minutes as shown in Fig. 4.

Cry1Ac Detection through Immuno-blot in Chimeric Protein

In another experiment after processing of samples, immuno-blot was done for Cry1Ac detection in translational fusion. In control sample, the total soluble protein was extracted from IPTG induced BL21 strain transformed with pET32a (+) and was used for immuno-blot. The Cry1Ac protein was not detected in control sample, while a strong signal appeared when total soluble protein from BL21 strain transformed with pSAK-V was used in the immuno-blot (Fig. 5). This shows that active domains of Cry1Ac remained intact in translational fusion.

Functional Analysis of Chimeric Protein

In order to determine the minimum toxic concentration of recombinant protein, topical application to the thoracic region of *H. armigera* and *S. littoralis* larvae was done. The minimum toxic concentration of recombinant protein was found to be 1.0 pmol for both types of insects. At 1.0 pmol the larvae showed lack of coordination among different organs, continuous head shaking, and stopped feeding and

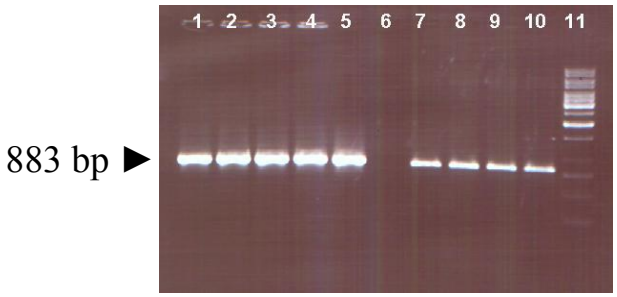

Fig. 3: Agarose gel electrophoresis of PCR amplified product of *E. coli* strain BL21 transformed with pSAK-V using cry1Ac specific primers. Lane 1-5, selected BL21 colonies; lane 6, non-transformed BL21 culture; lanes 7-10, selected BL21 colonies; lane 11, 1 kb DNA ladder

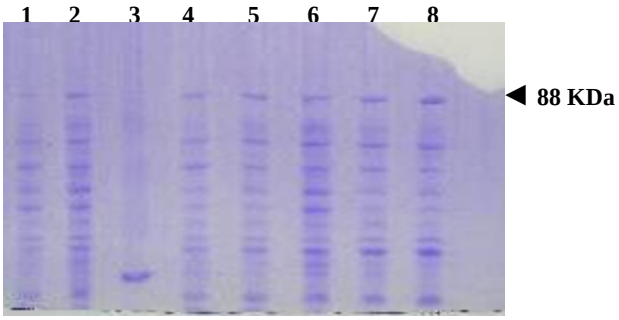

Fig. 4: SDS-PAGE analysis of total soluble protein isolated from bacterial strain BL21 transformed with pSAK-V. From left to right Lane 1-2, un-iduced protein; lane 3, protein marker (MBI Fermentas #SM 0441; lane 4, protein induced after 30 min; lane 5, protein induced after 60 min; lane 6, protein induced after 90 min; lane 7, protein induced after 120 min; lane 8, protein induced after 150 min.

finally paralysis was noticed within two hours which is the characteristic symptoms of *Hvt* (ACTX) protein which shows that active domain of *Hvt* also have not been disturbed in chimeric protein. The LD50 was found to be 4 and 2 pmol per gram of body weight for *H. armigera* and *S. littoralis* larvae, respectively. 100% mortality was observed after 24 h in both types of larvae. In control experiment both larvae remained unaffected, developed normally to adult stage.

In another experiment the activated recombinant protein was delivered into the midgut region of both larvae with a 0.25-mL syringe having blunted 30-gauge needle. The characteristic symptoms of the *Hvt* were again observed rather than the characteristic symptoms of *cry1Ac*, which suggested that *Hvt* has a more quick mode of action and more efficient to prevent damages to the agricultural crop comparatively to the *cry1Ac*. The larvae remained unaffected and developed normally in control experiment having gut juice solution only.

Fig. 5: Immuno blots showing the detection of expressed *Bacillus thuringiensis Cry1Ac* in translational fusion. A, BL21 having pET32-a with cloned *cry1Ac-Hvt* genes; B, BL21 having wild type vector (pET32-a)

Discussion

Biopesticides are eco-friendly alternative to chemical pesticides and can be derived from natural sources such as microorganisms (Gupta and Dikshit, 2010) and animals (Nicholson, 2007). Biopesticides derived from *Bacillus thuringiensis* have been widely used to control agronomical important insects (Sauka and Benintende, 2008). Besheli (2007) examined the efficacy of *Bt* for control of *Phylocnistis citrella* on detached citrus leaves. The results showed positive correlation between larval mortality and *Bt* concentrations. It has also been observed that *Bt* plus mineral oil reduced the Citrus leaf minor larvae to a level non-significantly different from treatment consisting of *Bt* alone. LRC3 strain of *B. thuringiensis* containing different crystal genes on its plasmid has been widely used to control lepidopteron insects; moreover, the genes that encode these proteins are subject of intensive research (Lysyk *et al*., 2006). The patented *Cry7Bal* from *B. thuringiensis* strain encoding novel toxic protein showed enhanced activity against lepidopteran insects (Sun *et al*., 2008). Synthetic *cryIAc* gene from *B. thuringiensis* is also used in the present study.

In order to delay the development of resistance in insect population novel chimeric protein has been expressed in the present study. Previously different chimerics have also been expressed to cover the broad host range and enhanced toxicity (Rosas-Garcia 2009). Bradfisch *et al.* (1998) have recombined *cry1F* and *cryIAc* for enhanced toxicity and activity against broader insect host range. Van *et al.* (2007) studied the functional analysis of synthetic proteins having Cry1C, Cry1B or Cry1D.

Although many chimeric proteins have been expressed but still there is a probability of developing resistance in insects against them because the genes encoding these proteins are only based on *B. thuringiensis* which have similar mode of actions (Schneph *et al*., 1998) and resistance to *Bt* toxins in insect population has been shown experimentally (Liu *et al*., 2001). The introduction of diverse and multiple genes in crop plants could reduce the resistance in insect arising from the single or similar group of genes (Zhao *et al*., 1997; Tabashnik *et al*., 2002). The present chimeric protein is based on the bacterial and spider genes which have unique mode of action from each other and expected to offer a long lasting resistance against insects. *Hvt* is toxic to a range of agriculturally important arthropods in the orders coleoptera, lepidoptera and diptera but has been reported to have no effects on a number of mammals (Khan *et al*., 2006; Chong *et al*., 2007). Unlike other spider toxins, Mukherjee *et al*. (2006) reported that *Hvt* has also the ability to cause toxicity when administrated orally to American lone star tick (*Amblyomma americanum*). Shah *et al*. (2011) also examined mortality of *Heliothis armigera* within 72 h on detached tobacco transgenic leaves expressing *Hvt* under phloem specific promoters. It has also been illustrated that fusion of *Hvt* with other carrier proteins such as garlic lectins or snowdrop lectins tremendously increases absorbtion of toxin by midgut of insects and increase toxicity through oral administration (Fitches *et al*., 2004, 2008).

The chimeric protein consisting of Bt Cry IAc and Hvt (ACTX) has been successfully expressed in prokaryotic system and was detected by SDS PAGE. Functional analysis clearly indicates that this recombinant protein is highly effective against agronomical important lepidopteron insects and is an excellent candidate for use as a biopesticides or when expressed in crop plants for transgenic protection.

In summary, we performed the functional analysis of new recombinant toxin protein developed from two distinctly unrelated *Bt cry1Ac* and spider *Hvt (ACTX)* genes. Recombinant protein showed the characteristic symptoms of the spider *Hvt* gene during the both force feeding and topical application experiment on *H. armigera* and *S. littoralis* possibly due to the quicker mode of action of spider Hvt protein than Cry1Ac protein. The expressed Cry1Ac protein in bacterial strain BL21 was detected by immuno-blot analysis, which shows that active domains of Cry1Ac protein remained unaffected in translational fusion. Our study shows that in translational fusion the active domains of both Cry1Ac and Hvt remained intact and this

translational fusion is highly effective chimeric molecule against agronomically important insects, which should be evaluated further for use as biopesticides or introduced in crop plants especially cotton for long lasting transgenic protection against herbivorous insects.

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