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A fusion gene encoding two different insecticidal proteins of *Bacillus thuringiensis*

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A chimeric fusion gene was constructed with the coding regions of insecticidal crystal protein (Cry1Ac) and vegetative insecticidal protein (Vip3Aa14) of *Bacillus thuringiensis (Bt)*. Overexpression of the fusion gene in *Escherichia coli* resulted in the synthesis of a protein of ~140 kDa size, as revealed by SDS-PAGE analysis. Western hybridization analysis using polyclonal antisera raised against Cry1Ac showed the presence of a band corresponding to ~140 kDa. Stability of the fusion protein was studied by trypsin digestion. Insect bioassays of the fusion protein on three insect species viz., *Helicoverpa armigera*, *Spodoptera litura* and *Plutella xylostella* showed that the fusion protein retained the toxicity of Cry1Ac, but partially lost that of Vip3Aa14.

Keywords: *Bacillus thuringiensis,* protein engineering, vegetative insecticidal protein, transgenic plants, insecticidal crystal protein

Introduction

Insect pest management in transgenic crops by expressing insecticidal proteins of *Bacillus thuringiensis (Bt)* has found commercial application in many countries. A variety of δ-endotoxin genes have been cloned and transferred to economically important crop species to impart insect resistance[1]. The high specificity of *Bt* insecticidal crystal proteins towards harmful insects, which arises due to the need of high alkaline *p*H (>9.5) for solubilisation, presence of serine proteases in midgut for processing and most importantly the presence of highly specific receptors in the insect midgut epithelial cells for binding, narrow down the host range of *Bt*[2]. It makes the useful insects such as pollinators and other organisms feed safely on *Bt*-transgenic plants. This high margin of safety recommends its use in food crops or in other sensitive sites where chemical pesticides can cause adverse effects. Ironically, the limitation of *Bt*-transgenic plants arises directly from its strength. Different *Bt* genes are highly insect-specific, for instance Cry1Ac is active against *Helicovepa armigera* and *Plutella xylostella* but it shows no activity against *Spodoptera litura*[3]. On the other hand, recently discovered Vip3Aa14 is highly toxic against *S. litura* but not to *H. armigera* or *P. xylostella*[4]. These insects are serious pests on economically important crops like cotton, tomato, cabbage, and cauliflower[5]. Due to the constraints imposed by high specificity of *Bt* genes it is not possible to control these insects using single gene. Expressing multiple genes in plants has its limitations, as till date only a few promoters such as CaMV 35S are available to provide high levels of constitutive expression in plants. However, using the same promoter to express more than one gene may lead to homology-based gene silencing[6]. A viable option is to use construct fusion genes and express them under the control of a powerful promoter.

Recent advances in protein engineering facilitated creation of multi-functional chimeric proteins containing modules from various proteins typically joined via an oligopeptide linker. These artificial fusion proteins have proven to be very useful in molecular biology[7]. Designing of artificial multifunctional proteins may have important and potential applications in the development of insect resistant *Bt*-transgenic plants. *Bt* toxins with different or overlapping specificities can be fused together to obtain fusion proteins, which will be toxic to multiple insect pest species and also function as a strategy to prevent resistance development in insects. A translational fusion of two *Bt* genes *cry1Ac* (toxic to *H. armigera* and *P. xylostella*)[3] and *Vip3Aa14* (active

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against *S. litura*)[8,9] may yield a protein that retains the toxicity against these three insects. However, this requires that the fusion protein should exhibit the biological activities of both the partner proteins.

Bt proteins have been expressed as fusion proteins viz., Cry1Ab with Cry1B[10,11], Cry1Ac with Cry1Ab[12], Cry1Ac with GFP[13], and Cry1Ac with NUS[14]. All these reports indicate the suitability of Cry proteins with other fusion tags. Hofte *et al* demonstrated that the addition of long protein fragment to the C-terminus of *Bt* protein did not disturb the activity of fused proteins[15]. Hence, in the present study, Vip3Aa14 toxin was fused at the C-terminus of the Cry1Ac toxin.

The present study demonstrates the feasibility of construction and overexpression of *Bt* Cry1Ac and Vip fusion protein. Insect bioassays of the fusion protein on three insect species viz., *H. armigera*, *S. litura* and *P. xylostella* showed that the fusion protein retained the toxicity of Cry1Ac, but partially lost that of Vip3Aa14.

Materials and Methods

Construction of *Cry1Ac-Vip3* Fusion Gene

Plasmid pET29 (Stratagene, USA) was used as cloning and expression vector. *E. coli* strain DH5α was used for cloning and BL21 (DE3) strain was used as the expression host. *cry1Ac* gene was PCR amplified using *Pfu* polymerase (MBI Fermantas) using forward primer (TGccatggATGGAT AACAATCCGAACATC) and reverse primer (CCTaatattCAGCCTCGAGTG). Forward and reverse primers carry *Nco* I *and Bam*H I restriction sites, respectively. These sites are shown in small letters in primer sequences. PCR amplified *cry1Ac* gene was cloned in *Nco* I and *Bam*H I sites of pET29 vector already having *Vip3Aa14* gene in *Bam*H I and *Sal* I sites.

Expression of Recombinant Proteins in *E. coli*

The pET-*cry1Ac, vip* and *cry1Ac-vip3Aa14* gene constructs were introduced in *E. coli* strain BL-21 DE3. The transformants were grown in 500 mL Luria Broth medium at 37°C for 12 h. The protein expression was induced by 1 m*M* Isopropyl-β–D-thiogalactopyranoside (IPTG) and cells were grown at 15°C for another 16-18 h. Cells were harvested by centrifugation at 6000 x g for 10 min at 4°C and resuspended in sonication buffer containing 50 m*M* Tris-Cl (*p*H 8.0), 5 m*M* EDTA and 5 mg of protease inhibitor (Complete mini, Boehringer Mannheim). Sonication was carried out at a power output of 100W three times for 1 min each. The lysate was centrifuged at 14000 x g for 20 min at 4°C. The pellet was processed for the extraction of expressed protein.

The pellet obtained after the centrifugation of lysate was washed thrice with wash buffer I (0.5 M NaCl, 2% Triton X 100), 5 times with wash buffer II (0.5 M NaCl) and thrice with sterile distilled water. Each washing step was followed by centrifugation at 16000 x *g* for 10 min at 4°C. The pellet was lyophilized and stored at -20°C until use. The total protein concentration was estimated by the Bradford assay. Total proteins from *E. coli* cells expressing *cry1Ac*, *vip* or *cry1Ac-vip* fusion gene were resolved on 10% SDS-PAGE using Laemmli procedure[16].

Immunoblotting Analysis

Proteins were separated by SDS-PAGE according to standard protocol[17]. Transfer of proteins from the gel to PVDF membrane was carried out using a Trans-Blot apparatus (BioRad) and transfer buffer (39 m*M* Glycine, 48 m*M* Tris buffered saline (50 m*M* Tris-Cl, *p*H 7.50 and 150 m*M* NaCl) containing 0.1% tween 20 (TBST). The membrane was incubated with polyclonal antisera of Cry1Ac in 1:10,000 dilutions in TBST containing 0.2% BSA at 4°C for overnight. The unbound antibodies were removed by three washes with TBST. After three washes with TBST, the blot was incubated with alkaline phosphatase-conjugated anti rabbit IgG for 1 h and developed using p-nitroblue tetrazolium chloride (NBT and 5-bromo-4-chloro 3-indoylphosphate toluidine (BCIP) substrate as recommended by the manufacturer (Boehringer Mannheim).

Protein Toxicity Assays

The toxicity of these three proteins was tested against neonate larvae of *H. armigera*, *S. litura*, and *P. xylostella*. Different concentrations of toxins were either incorporated in the diet (for *H. armigera*) or spread on leaf disks of respective host plants (for *P. xylostella*, and *S. litura*). LC_{50} values were calculated by Probit analysis[18].

Diet Incorporation Assays for Protein Toxicity

The diet of *H. armigera* was prepared by the method of Singh and Rembold[19]. Molten diet mix was

cooled to 50°C and mixed thoroughly with desired concentrations of toxins. The diet was then immediately poured in the 24-well culture plate (1 mL/well) (Cellstar, Greiner Labortechnik, Germany) and allowed to solidify. One first instar larva was released in each well. A set of 10 neonate larvae was tested for each concentration at one time. The plates were covered with Saran wrap and kept in the insect culture room at 28±2°C, 60±5% humidity and 14L:10D photoperiod. Mortality was recorded after 3 d. The experiment was replicated five times.

Leaf Disk Assays

Leaf disk bioassays were performed in a 6-well macroplate (Cellstar, Greiner Labortechnik, Germany). Disks of 3 cm diameter were cut using a cork borer from cabbage leaves for *P. xylostella* and from castor leaves for *S. litura,* respectively. Stock solutions toxins were prepared in autoclaved distilled water and desired concentrations were spread on the leaf disks and air-dried. A single disk was placed on moist Whatman filter paper in each well. Ten first instar larvae were released on each leaf disk with a paintbrush and the plates were then tightly sealed with Saran wrap. The plates were kept at 28±2°C, 60±5% humidity and 14 h photophase. Mortality was recorded after 3 d. Each treatment was replicated five times.

Results and Discussion

Construction of Cry1Ac-Vip Fusion Protein

A translation fusion gene encoding two different *Bt* toxins Cry1Ac and Vip, having different toxicity spectrum was constructed. Cry1Ac and Vip proteins have different modes of action for their toxicity towards insects. So, to act independently, the gut proteases must release these toxins from one another. To ensure the release of independent toxin molecules by gut protease, one trypsin site present just after the third domain and at the beginning of C-terminal half of Cry1Ac protoxin, was included in the linker peptide (DRFEFIPVTATLEAE) between the protease resistant Cry1Ac and Vip proteins. The fusion gene was over expressed in *E. coli* and evaluated for its toxicity against target insects.

SDS-PAGE analysis of IPTG induced *E. coli* harboring *cry1Ac-vip3Aa14* fusion gene showed the overexpression of protein corresponding to approximately 140 kDa, which is prominent in IPTG induced culture and absent in un-induced control (Fig. 1). Densitometric analysis showed that the fusion protein amounted approximately 5% of the total protein, indicating the accumulation of expressed protein without much degradation.

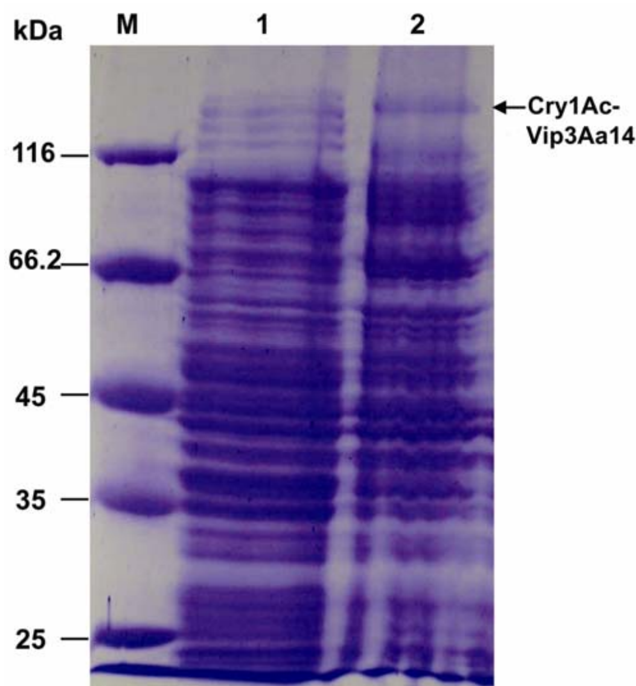

Fig. 1—Cry1Ac-Vip3Aa14 fusion protein expressed in *E. coli*. Lane 1: Un-induced (control). Lane 2: IPTG induced *E. coli*; the position of the molecular marker (in kilo Daltons) is indicated on the left.

The presence of full-length polypeptide was further confirmed by Western blot analysis, using the polyclonal antisera raised against Cry1Ac protein (Fig. 2). These results confirmed the presence of antigenic polypeptide at approximately 140 kDa, comparable to the migrating position of over expressed protein band in the SDS-PAGE. The presence of the signal of expected size against Cry1Ac antibodies indicated that the entire sequence of the fusion gene is translated and accumulated as a stable protein.

Trypsin Digestion Analysis

B. thuringiensis Cry proteins over expressed in *E. coli* accumulate in inclusion bodies while most of the over expressed Vip protein remains in soluble fraction[4]. Like other Cry proteins Cry1Ac-Vip fusion protein was also expressed in inclusion bodies. The Inclusion bodies are the aggregates of unfolded and partially folded protein molecules[20]. These inclusion bodies were solubilized in carbonate buffer (*p*H 10.5). Trypsin digestion for different time periods was carried out to confirm the stability of *in vitro* solubilized protein structure and

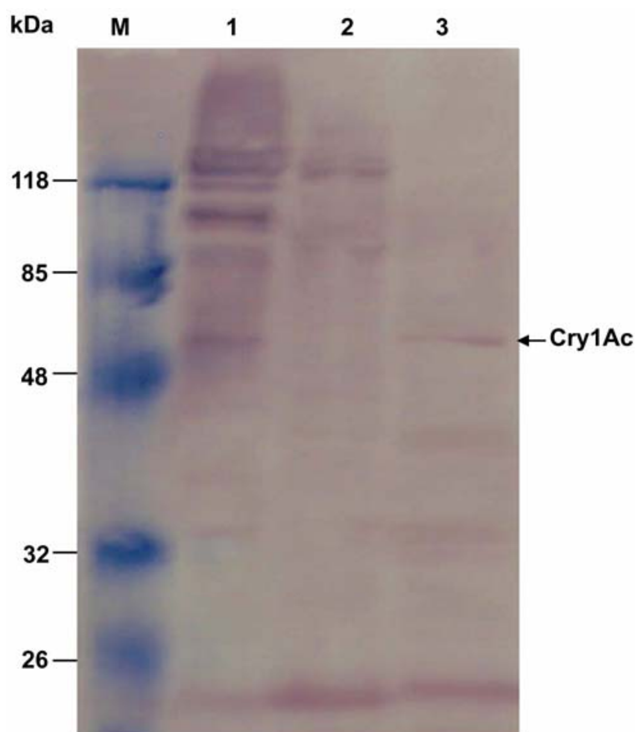

Fig. 2—Immunoblot of Cry1Ac-Vip3Aa14 fusion protein with anti Cry1Ac polyclonal antibodies. Lane 1: Total protein from Cry1Ac-Vip3Aa14 fusion protein expressing *E. coli*. Lane 2: Partially purified Cry1Ac-Vip3Aa14 fusion protein. Lane 3: Trypsin digested Cry1Ac-Vip3Aa14 fusion protein.

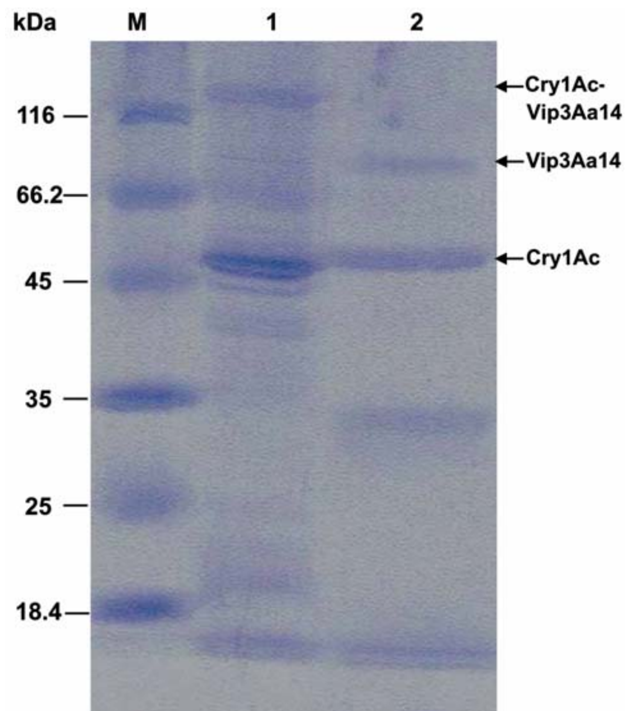

Fig. 3—SDS-PAGE analysis of trypsin digested Cry1Ac-Vip3Aa14 fusion protein. Lane 1: Partially purified Cry1Ac-Vip3Aa14 fusion protein. Lane 2: Trypsin digested Cry1Ac-Vip3Aa14 fusion protein. The position of the molecular weight marker (in kilo Daltons) is indicated on the left.

digestion of fusion protein to release independent functional Cry1Ac and Vip toxin molecules.

SDS-PAGE analysis of *in vitro* folded fusion protein digested with trypsin showed that the 140 kDa protein gradually got digested to ~80 and ~60 kDa protein bands, corresponding to Vip and Cry1Ac proteins (Fig. 3). Western blot analysis of trypsin digested Vip-Cry1Ac fusion protein with polyclonal antisera raised against Cry1Ac protein showed a positive signal at approximately ~60 kDa (Fig. 2 Lane 3), corresponding to Cry1Ac band on the SDS-PAGE. It is evident that trypsin site included in the linker region of fusion protein remained accessible. Moreover, the presence of ~80 and ~60 kDa trypsin resistant bands suggests that the 3-D structure of the proteins is not disturbed by the fusion.

Effect of Cry1Ac-Vip Fusion Protein on Target Pests

In order to determine the entomocidal activity of the fusion protein, insect bioassays were performed on the larvae of *H. armigera*, *S. litura* and *P. xylostela*. Toxicity data recorded for Cry1Ac, Vip3Aa14 and Cry1Ac-Vip3A fusion proteins are given in Table 1. Since the fusion protein was showing stability and correct processing by trypsin, it was expected that the fusion protein would be effective against all the three-target pests tested. But the larval bioassay data showed that fusion

Table 1—LC_{50} (ng/cm^2) values for Cry1Ac, Vip3Aa14 and Cry1Ac-Vip3Aa14 fusion toxins against three different lepidopteron insect pests

Toxin	LC_{50} (ng/cm^2) (95% fiducial limits)		
	H. armigera	*S. litura*	*P. xylostella*
Cry1Ac	0.089 (0.1258-0.070)	NA	0.169 (0.223-0.126)
Vip3Aa14	NA	0.144 (0.177-0.114)	NA
Cry1Ac-Vip3Aa14 fusion	0.123 (0.154-0.095)	NA*	0.138 (0.223-0.083)

**S. litura* showed remarkable difference in weight loss but no mortality ad hence LC_{50} could not be observed.

Table 2—Effect of Cry1Ac-Vip3Aa14 fusion protein on the growth of *S. litura* larva

Toxin concentration (ng/cm^2)	Average weight of the larvae (g)	% Growth reduction
Control	0.103	-
100	0.083	19.73
200	0.041	60.85
400	0.014	66.47
800	0.007	93.24
1000	0.004	96.14

protein is toxic to *H. armigera* and *P. xylostella*, which is similar to Cry1Ac toxicity. The toxicity of the fusion protein on *S. litura* was not up to the level of Vip3Aa14; instead it caused only growth reduction (Table 2).

The loss of Vip toxicity may be attributed to limitations posed by insufficient folding of the recombinant protein expressed in the inclusion bodies. Proteins over expressed in the inclusion bodies are subjected to refolding to their native state by solubilization, by providing high *p*H and suitable ion concentration. This *in vitro* solubilization and refolding process never leads to complete folding of the toxin, so only a fraction of molecules are converted to properly folded active toxins. This limitation of incomplete *in vitro* folding is clearly visible in experiments for calculation of lethal concentrations of *Bt* toxins. LC_{50} of the toxin, that is dependant on fraction of toxin molecules folded to active toxin, vary from one experiment to another. The LC_{50} of Cry1Ac has been reported to be 115 ng/cm^2, 5.6 ng/cm^2, 20 ng/mL and 240 ng/mL[21-24]. However, when the Cry1Ac was expressed as a soluble protein using NUS fusion tag, LC_{50} of Cry1Ac toxin decreased to 0.10 ng/cm^2, it may be because in the soluble form all the proteins will get properly folded[14]. In another report, Cry1EC gene when expressed in *E. coli* inclusion bodies, did not show any toxicity towards *S. litura,* however, expression of same gene in plants showed high toxicity[25]. So, in the present study the partial loss of Vip toxicity may be due to limitations posed by *in vitro* folding of the toxin.

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