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Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*

Ritu Bhalla [a], Monika Dalal [a], Siva K. Panguluri [a], Borra Jagadish [a], Ajin D. Mandaokar [a], A.K. Singh [b], Polumetla A. Kumar [a,*]

[a] *National Research Center for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India*
[b] *Department of Zoology, Delhi University, Delhi 110 007, India*

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Abstract

Twenty-four serovars of *Bacillus thuringiensis* (Bt) were screened by polymerase chain reaction to detect the presence of vegetative insecticidal protein gene (*vip*)-like sequences by using *vip3Aa1*-specific primers. *vip*-like gene sequences were identified in eight serovars. These genes were cloned and sequenced. The deduced amino acid sequence of the *vip3Aa14* gene from *Bacillus thuringiensis tolworthi* showed considerable differences as compared to those of *Vip*s reported so far. The *vip3Aa14* gene from *Bt tolwarthi* was expressed in *Escherichia coli* using expression vector pET29a. The expressed Vip3Aa14 protein was found in cytosolic supernatant as well as pellet fraction, but the protein was more abundant in the cytosolic supernatant fraction. Both full-length and truncated (devoid of signal sequence) Vips were highly toxic to the larvae of *Spodoptera litura* and *Plutella xylostella*. Truncation of Vip3Aa14 protein at N-terminus did not affect its insecticidal activity.

Keywords: *Bacillus thuringiensis*; Vegetative insecticidal protein

1. Introduction

Bacillus thuringiensis (Bt) has proved to be a remarkable source of the insecticidal protein genes used for transgenic expression in crop species [1]. During the sporulation phase, Bt produces crystalline inclusions that consist of about 130 kDa proteins known as δ-endotoxins or crystal (Cry) toxins. Bt δ-endotoxins are a part of large and still growing family of homologous proteins differing in insect specificities [2]. In addition to these, several strains of Bt are known to produce insecticidal proteins during the vegetative phase of growth. These proteins are called vegetative insecticidal proteins (Vip). As a family, *Vip*s do not show any similarity to δ-endotoxins of *Bt*. The first *vip* genes described were *vip3Aa1* and *vip3Aa2* isolated from Bt strain AB88 and AB424 [3]. Vips possess toxicity of the same magnitude as that of Bt δ-endotoxins against the susceptible insects. The insecticidal spectrum of Vips includes certain important pests, which have shown insensitivity to the Bt δ-endotoxins.

In the present study, we analysed twenty-four serovars of Bt for the presence of *vip*-like genes. The *vip* genes amplified from different serovars were sequenced to determine their homology. A novel *vip3Aa14* (www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/intro.html)

* Corresponding author. Fax: +91 11 2584 3984.
E-mail addresses: polumetla@hotmail.com (P.A. Kumar).

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gene, which differs from vip3Aa1, has been identified. Full-length and truncated *vip3Aa14* genes were cloned and expressed in *Escherichia coli* and the expressed proteins were used for toxicity assays against laboratory-reared populations of several lepidopteran pests.

2. Materials and methods

2.1. Screening of Bt serovars

Twenty-four strains representing different serovars of Bt obtained from *Bacillus* Genetic Stock Center, Columbus, OH, USA (Table 1) were screened for the presence of *vip* homologs using Polymerase Chain Reaction (PCR). The total genomic DNA of different Bt serovars was isolated following the procedure of Ausubel et al. [4] and used as template for the PCR analysis. Primers used for screening were designed on the basis of published sequence of *vip3Aa1* gene [3].

Forward primer: 5′-ATGAACAAGAATAATAC-TAAATTAAGC-3′
Reverse primer: 5′-GGTCGACTTACTTAATAGA-GACATCG-3′

The PCR products (2.4 kb) were isolated, cloned separately in the vector pBluescript SK+ (Stratagene) and sequenced as described by Sanger [5]. The nucleotide sequences of the inserts were compared with the published sequence of *vip3Aa1* (NCBI Accession No. L48811).

Table 1
Analysis of *Bacillus thuringiensis* serovars for presence of *vip*-like genes

Serovar designation	Subspecies	PCR result
4Q2-72	*israelensis*	+
1715	*thuringiensis*	+
HD3	*finitimus*	−
OATS 43	*dakota*	−
HD542	*thompsoni*	−
HD537	*tolworthi*	+
HD146	*darmstadiensis*	−
HD168	*galleriae*	+
HD867	*kumamotoensis*	−
HD201	*toumanoffi*	−
HD395	*pakistani*	+
HD4	*alesti*	+
78-FS-29-17	*tohokuensis*	−
HD11	*aizawai*	−
HD521	*indiana*	−
HD868	*tochigiensis*	−
HD541	*kyushuensis*	−
HD293	*kenyae*	−
HD1	*kurstaki*	+
SOTTO	*sottoldendrolimus*	−
HD525	*wuhanensis*	+
HD12	*morrisoni*	−
HD10	*entomocidus*	−
HD224	*canadensis*	−

PCR positive Bt serovars were reconfirmed by Southern hybridisation [6]. The genomic DNA of each serovar was completely digested with *Eco*RI, electrophoresed on 0.8% agarose gel and transferred to Hybond N^+ membrane (Amersham). The PCR amplified *vip3Aa1* gene fragment was labelled with [α^{32}P] dCTP and was used as probe for Southern hybridisation. Truncation of the *vip3Aa14* gene was carried out using a forward primer starting at the nucleotide 99 downstream from the ATG codon (5′-CGGATCCATGAACATGATTT-TTAAAACGG-3′).

2.2. Expression of vip3Aa14 in E. coli

The full-length (PVF) and truncated (PVT) *vip3Aa14* fragments were individually cloned in pET29a and 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 *vip3Aa14* expression was induced by 1 mM Isopropyl-β-D-thiogalactopyranoside and cells were grown at 15 °C for another 16–18 h. Cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C and resuspended in sonication buffer containing 50 mM Tris–Cl (pH 8.0), 5 mM EDTA and 5 mg of protease inhibitor (Complete mini, Boehringer Mannheim). Sonication was carried out at a power output of 100 W three times for 1 min each. The lysate was centrifuged at 14000*g* for 20 min at 4 °C. The pellet and supernatant were processed separately for the extraction of expressed protein.

The proteins in the supernatant were precipitated by 70% ammonium sulphate (w/v) at 4 °C overnight and subsequently centrifuged at 5000*g* for 15 min. The resulting protein pellet was resuspended in 20 mM Tris–Cl buffer (pH 8.0) and dialyzed against distilled water at 4 °C overnight. The protein was lyophilized and stored at −20 °C until use.

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 16,000*g* for 10 min at 4 °C. The pellet was lyophilized and stored at −20 °C until use. The total protein concentration of both supernatant and pellet fraction was estimated by the Bradford assay. Total proteins of both the fractions from *E. coli* cells expressing PVF and PVT were resolved on 10% SDS–PAGE and western analysis of Vip3Aa14 was carried out using standard protocol [6]. The amount of Vip3Aa14 was estimated by ELISA using anti-Vip3Aa1 antibodies [7].

2.3. Protein toxicity assays

The toxicity of full-length and truncated Vip3Aa14 was tested against neonate larvae of *Helicoverpa armi-*

gera, *Spodoptera litura*, *Earias vitella*, *Pieris brassicae* and *Plutella xylostella*. Different concentrations of full-length and truncated toxins were either incorporated in the diet (for *H. armigera*) or spread on leaf disks of respective host plants (for *P. xylostella*, *E. vitella*, *P. brassicae* and *S. litura*). LC_{50} values were calculated by Probit analysis [8].

2.4. Diet incorporation assays

The diet of *H. armigera* was prepared by the method described by Singh and Rembold [9]. Molten diet mix was cooled to 50 °C and mixed thoroughly with desired concentrations of Vip3Aa14. 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 of full-length and truncated Vip3Aa14 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 days. The experiment was replicated five times.

2.5. 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 *P. brassicae* and from cotton and castor leaves for *E. vitella* and *S. litura*, respectively. Stock solutions of full-length and truncated Vip3Aa14 toxin 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 days. Each treatment was replicated five times.

3. Results

PCR analysis of the genomic DNAs of 24 serovars of *Bt* showed amplification of 2.4 kb fragment of *vip* in 8 serovars (Table 1). The PCR was repeated thrice to confirm the results. Southern analysis also confirmed the presence of *vip* in the eight PCR positive Bt serovars (Fig. 1) and absence of the same in one PCR negative Bt serovar (*Bacillus thuringiensis canadensis*) (Fig. 1).

The PCR products were individually cloned in pBluescript SK+ and partial sequencing of the inserts from either end was done using T7 and M13 sequencing primers. The *vip* nucleotide sequence of the 8 Bt serovars were compared with that of the published sequence of *vip3Aa1*. Only the *vip* from *Bacillus thuringiensis tolworthi* showed a few differences in nucleotide sequence, while *vip* from other servovars were similar to that of *vip3Aa1*. The *vip* from *Bt tolworthi* (referred to as *vip3Aa14* in this study) was differed from *vip3Aa1* at 31 nucleotides. The predicted protein sequence of Vip3Aa14 with those of reported Vips [3,7,10] revealed a difference of 18 amino acid residues (Table 2). The nucleotide sequence of *vip3Aa14* gene was deposited in GenBank (Accession No. AF548629).

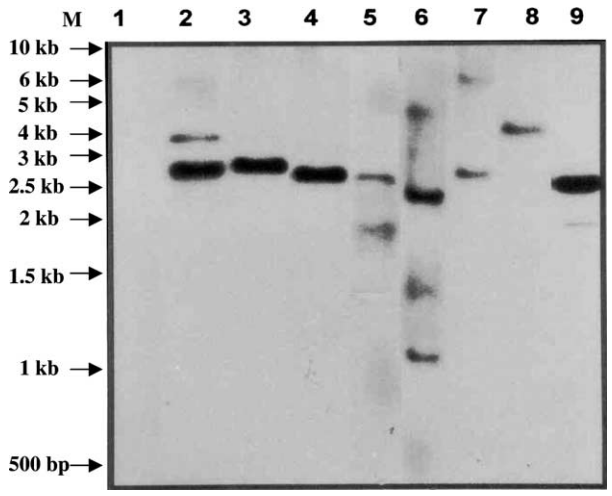

Fig. 1. Southern blot analysis of *vip* in eight PCR positive Bt serovars. Lanes: 2–9, Bt *israelensis*, Bt *thuringiensis*, Bt *tolworthi*, *Bacillus thuringiensis galleriae*, *Bacillus thuringiensis Pakistani*, *Bacillus thuringiensis alesti*, Bt *kurstaki* and *Bacillus thuringiensis wuhanensis*; lane: 1, PCR negative Bt serovar (Bt *canadensis*).

In order to conduct the functional analysis of Vip toxin, *vip3Aa14* gene was expressed in *E. coli*. Double antibody sandwich ELISA of total protein extracted from cytosolic supernatant and pellet fractions of *E. coli* clones (PVF and PVT) using rabbit anti-Vip antibodies was done to determine the proportion of Vip toxin in the total protein of both fractions. The amount of Vip3Aa14 was determined by extrapolating ELISA data to a BSA standard curve. ELISA results showed that in the supernatant extracts, Vip3Aa14 was approximately 1/9 of the total protein and in the pellet extracts, Vip3Aa14 was approximately 1/20 of the total protein. Total protein from both the fractions was resolved on 10% SDS gel (Fig. 2(a)). Western analysis confirmed that the 88 kDa band as Vip3Aa14 protein (Fig. 2(b)). Analysis of total protein (5 μg) of *E. coli* cells expressing PVF and PVT on 10% SDS–PAGE revealed the presence of full-length and truncated Vip3Aa14 proteins (Fig. 2(c)). Lane 2 shows the presence of an approximately 88-kDa band of full-length Vip3Aa14 and lane 3 shows the truncated Vip3Aa14 protein band with

Table 2
Differences in amino acid residues in the Vips

Amino acid position	Amino acid residue in toxin				
	Vip3Aa1	Vip3Aa2	Vip3Aa9	Vip3Aa10	Vip3Aa14
214	E	E	E	E	**G**
220	T	T	T	T	**P**
274	F	F	F	F	**S**
280	A	A	A	A	**L**
284	Q	**K**	**K**	**K**	**K**
291	T	**P**	T	T	T
323	L	L	L	L	**P**
358	I	I	I	I	**V**
401	C	C	C	C	**G**
406	E	**G**	E	E	**G**
407	Q	Q	Q	Q	**P**
411	T	T	T	T	**P**
536	S	S	S	S	**K**
633	N	N	N	N	**T**
742	K	**E**	**G**	**E**	K
755	M	M	M	M	**I**
760	F	F	F	F	**L**
761	E	E	E	E	**G**
770	P	**S**	**S**	P	P
776	Y	Y	Y	Y	**N**
781	H	H	H	H	**K**

MW slightly less than that of full-length Vip3Aa14 as expected (Fig. 2(c)). The efficacy of full-length and truncated Vip3Aa14 toxins was evaluated against a few common lepidopteron pests. With both the proteins, the maximum mortality was observed in *S. litura* followed by *P. xylostella* and *E. vitella* (Table 3). Larvae of *H. armigera* and *P. brassicae* showed insensitivity even at high dosages. The truncation of Vip3Aa14 protein did not have any apparent effect on the toxicity of the Vip3Aa14 protein. The LC_{50} values for Vip3Aa14 toxin against susceptible insects were calculated by Probit analysis (Table 3).

4. Discussion

The molecular and biological properties of Vips established them as distinct class of insecticidal toxins that are different from Bt δ-endotoxin family. They represent second generation of insecticidal toxins that can be used to target important insect pests that are not susceptible to Bt δ-endotoxins [11]. In order to clone new

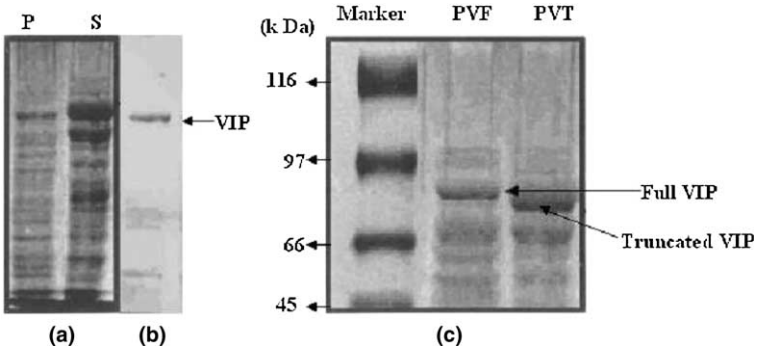

Fig. 2. SDS–PAGE analysis and immunoblot of Vip3Aa14 expressed in *E. coli*. (a) Sub cellular localization of Vip3Aa14 in pellet (P) and supernatant (S) fractions. (b) Western analysis of Vip3Aa14 using anti-Vip antibody. (c) Coomassie stained gel for full-length Vip3Aa14 (PVF) and truncated Vip3Aa14 (PVT).

Table 3
LC_{50} values for full-length and truncated Vip toxins against different lepidopteran insect pests

Insect	LC_{50} (ng/cm^2)			
	Vip3Aa14		Vip3Aa10[c]	Vip3Aa9[b]
	Full-length	Truncated		
Spodoptera litura	12 (8–74)[a]	11 (6–81)	45.41 (37.4–55.2)	5
Plutella xylostella	120 (25–320)	132 (37–356)	220.73 (99–494)	36
Earias vitella	794 (380–980)	830 (420–980)	NT	NT
Helicoverpa armigera	NA	NA	325.2 (119–881)	NT
Pieris brassicae	NA	NA	NT	NT

NA, not active; NT, not tested.
[a] Values in all parentheses indicate 95% fiducial (confidence) limits.
[b] Selvapandiyan et al. [7].
[c] Doss et al. [10].

members of *vip* family, we screened 24 different Bt serovars by PCR analysis. Out of the 24 serovars, only 8 serovars showed PCR amplification of Vip gene, which was also confirmed by Southern hybridisation (Fig. 1). The southern hybridization showed different banding patterns in the serovars. This may be due to the presence of *vips* at different locations in genome and there may be more than one copy of the gene. Estruch et al. [3] reported the presence of *vip3Aa1*-insecticidal gene homologues in 15% of *Bacillus* strains analyzed. In a similar study, Doss et al. [10] observed the presence of *vip3Aa1*-like genes in two out of 11 *Bacillus thuringiensis kurstaki* strains and absence in *Bacillus thuringiensis israelensis* strains.

Cloning and partial sequencing of these genes established that seven of them were identical at the 5′ and 3′ ends to the *vip3Aa1* [3]. The predicted protein sequence of the *vip* gene amplified from Bt *tolworthi (vip3Aa14)* showed difference in 18 amino acid residues with that of the *vip3Aa1* (Table 2). Selvapandiyan et al. [7] reported a Vip toxin with a difference of three amino acid residues from Vip3Aa1 and Vip3Aa2. This Vip toxin on expression in *E. coli* showed lack of insecticidal activity against *Agrotis ipsilon*. Therefore, a difference of 18 amino acids observed in Vip3Aa14 may be of considerable significance in terms of its toxicity to insects.

Expression of Vip3Aa14 using pET 29a expression vector in *E. coli* strain BL21 DE3 showed presence of higher amounts of protein as soluble fraction in the cytosolic supernatant (1/9 of the total *E. coli* protein) than as inclusion bodies in the pellet (1/20 of the total *E. coli* protein (Fig. 2(a)). We confirmed that the overexpressed protein is Vip3Aa14 by Western analysis with anti-Vip3Aa1 antibody (Fig. 2(b)). Similarly, Doss et al. [10] have also reported the presence of expressed Vip in the soluble periplasmic fraction as well as in the inclusion bodies. However, Selvapandiyan et al. [7] reported presence of expressed Vip only in the soluble cytosolic fraction.

Vip3Aa14 toxin showed very high toxicity against *S. litura* followed by *P. xylostella* (Table 3). Our results are in good agreement with the earlier reports by Selvapandiyan et al. [7] and Doss et al. [10], who also reported Vip toxicity to *S. litura* and *P. xylostella*. However, in each of these cases there is considerable difference between the respective LC_{50} values. Vip3Aa14 toxin showed very low levels of toxicity to *E. vitella* and no toxicity against *H. armigera* and *P. brassicae* (Table 3). Doss et al. [10] reported activity of Vip against *H. armigera* (LC_{50} 325.20 ng). The absence of toxicity of Vip3Aa14 protein against certain insects such as *H. armigera* and *P. brassicae* suggests that Vips are specific in their action. Specificity in case of Cry toxins has been attributed to toxin–receptor interaction [12]. The criteria that determine insect specificity in the case of Vips are not completely understood although some evidence points towards the involvement of Vip3Aa13 binding to 80 and 100-kDa proteins [13,14].

Deletion of nucleotide sequence corresponding to 33 amino acids at the N-terminal end of Vip3Aa14 toxin did not alter the toxicity of the protein (Table 3). The overlapping 95% fiducial limits indicate that the difference in levels of toxicity induced by full-length and truncated proteins is not significant. The N-terminal sequence possesses structural features typically found in signal peptides reported for *Bacillus* species [3] and is therefore predicted to play a role in secretion of Vips across the *Bacillus* cell wall. Selvapandiyan et al. [7] reported the N-terminal deletion of 39 amino acids caused total loss of toxicity of Vip toxin against *S. litura* but not against *Chilo partellus*. However, in the present study, a N-terminal truncation of Vip3Aa14 toxin up to 33 amino acid residues did not alter its toxicity against any of the pests tested in the bioassays including *S. litura*. Thus, it is clear that the amino acid residues 1–33 of Vip protein sequence do not play any role in the toxicity against these insects.

Thus we have cloned and characterized a novel *vip* from Bt *tolworthi*, which can be used either alone or in gene pyramiding with other insecticidal protein genes for durable resistance against lepidopteran insect pests.

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