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ORIGINAL PAPER

Cloning, characterization and evaluation of toxicity of newly identified Vip3Aa proteins from *Bacillus thuringiensis* recovered from diverse environments for biological control of *Helicoverpa armigera*

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Abstract

Ten full-length *vip3*-type genes amplified from the *Bacillus thuringiensis* (*Bt*) isolates recovered from various Indian agro-climatic zones and two from known *Bt* strains were cloned, sequenced and confirmed as *vip3Aa*-type genes based on sequence homology. Comparison of deduced amino acid sequences of these genes compared with previously reported *vip3Aa*-type genes and other lepidopteran-specific *vip3Aa* genes revealed new amino acid substitutions, indicating the diversity of Indian *Bt* isolates. These genes have been grouped into six phylogenetic clusters and assigned names as *vip3Aa67–vip3Aa78* by *Bt* nomenclature committee (www.bpprc.com). Interestingly, the substitution of lysine in the holotype Vip3Aa1 with glutamine at amino acid position 284 was uniformly found in all our Vip3Aa-type proteins, suggesting sequence conservation as well as diversity among these proteins. For evaluation of toxicity, *vip3Aa67–vip3Aa72* genes were cloned in pET-29a(+) *Escherichia coli* expression vector. The immunostrip assays, SDS-PAGE and western blot analysis with Vip3A antibodies confirmed the presence of Vip3Aa67, Vip3Aa68, Vip3Aa69, Vip3Aa70, Vip3Aa71 and Vip3Aa72 proteins of *ca*. 89 kDa size. The toxicity of these Vip3Aa proteins along with the Vip3Aa44 protein used as a positive control was evaluated against *Helicoverpa armigera.* LC_{50} ranged from 0.921 to 8.513 ppm indicating their insecticidal potential. The impact of Vip3A proteins on the life cycle characteristics of *H. armigera* was studied, and the results showed toxic and sublethal effects on the growth and fecundity of the insect population. Computational analysis of the three-dimensional structure of Vip3Aa proteins revealed the effect of amino acid substitutions on stability, highlighting the importance of flexibility in protein function. This study reveals the potential of the newly identified Vip3Aa proteins in the biological control of *H. armigera*.

Keywords *Bacillus thuringiensis* · *vip3A* genes · Amino acids substitutions · *Helicopverpa armigera* · Indian agroclimatic zones · Lepidoptera

Key message

- Vip proteins are highly specific and effective against pests that are less susceptible to Cry proteins
- Vip3Aa67-78 proteins have new amino acid substitutions compared to known Vip proteins
- The newly identified genes *vip3Aa67- vip3Aa72* have toxicity potential against *H. armigera*
- Our results showed the toxic and sublethal effects of these proteins on the growth and fecundity of the population of *H. armigera* during its life cycle

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Introduction

Food security is a key global challenge due to the rapidly increasing human population throughout the world (Hunter et al. 2017). The human population is expected to reach 8.6 billion by 2030, 9.8 billion by 2050 and 11.2 billion by 2100. To keep pace with this growth, it has been projected that it will be necessary to enhance world food production by 40% by 2030 and 70% by 2050 (FAO 2017). The threat posed to agricultural production by insect pests is among the key factors that threaten the world's food security. Ironically, a minimum of one-third of crop production is lost because of damage caused by insect pests. If insect pests keep spreading at existing rates, many of the world's biggest crop-producing countries will be inundated by the middle of the century (Bebber et al. 2014). Insect pests attack almost every crop and they are adaptable to diverse places and environmental conditions (Imms 1964). The insects are called major pests if they cause damage above 10% of the total crop production, whereas minor pests cause damage between 5 and 10% (Dhaliwal et al. 2010). Insect pests belonging to the order Lepidoptera are among the most detrimental pests. *Helicoverpa armigera*, a most harmful lepidopteran pest, causes severe economic losses to more than 180 types of crop plants (Pimentel 2009; Smith-Pardo 2014).

There are various ways of insect pest control. The two most important and widespread methods are chemical and biological (Oerke et al. 1994). Various chemical insecticides (carbamates, organophosphates, pyrethroids, organochlorine compounds, formamides, etc.) have been used over the last many decades for the control of insect pest populations. Although chemical insecticides are toxic to a wide spectrum of insect pests and generally provide a faster means of killing them, their excessive use has adversely affected wildlife, beneficial insects, predators and parasites (Yadav et al. 2015; Lacey et al. 2001; Aktar et al. 2009). As an alternative to the chemical method, biological methods, especially microbial insecticides, have gained popularity in the last few decades as an efficient insect pest management strategy because they are very specific against their target pests and harmless to other organisms. Thus, devastating insect populations can be controlled in an ecologically safe manner (Lacey et al. 2001; Chattopadhyay et al. 2017). A few entomopathogenic bacteria have been explored for use in controlling insect pests commercially. These include various subspecies of *Bacillus thuringiensis* (*Bt*), *Lysinibacillus* (*Bacillus*) *sphaericus*, *Paenibacillus* spp., and *Serratia entomophila*. Among these, *B. thuringiensis* subspecies *kurstaki* is the most commonly utilized for lepidopteran pest control in crops and forests due to their safety towards non-target species and the environment. Therefore, *Bt* is considered as best insect-pathogenic bacteria (Lacey et al. 2015; Deka et al. 2021; Kallaf et al. 2021; Tabashnik et al. 2021; Meissle et al. 2022). The potential toxicity of the spore-crystal mixtures from different *Bt* strains has been reported against various pests, viz. *Bt*K2 strain against *Culex pipiens* and *Spodoptera exigua* (Lee et al. 2001), *Bt*-BLB1 strain against *Ephestia kuehniella* (Saadaoui et al. 2009), *Bt*-BLB459 strain against *S. littoralis* and *E. kuehniella* (Boukedi et al. 2016) and *Bt*-14 isolate against *E. kuehniella* (Kallaf et al. 2021) demonstrating the utility of *Bt* in the management of different pests. Recently, the HearNPV (Nucleopolyhedrovirus from *H. armigera*) has also been employed to manage its attack in tomatoes under temperate climatic conditions (Manzoor et al. 2023).

Bt, which secretes Cry toxins, has been successfully employed to develop insect pest-resistant transgenic crops since 1996. Despite increasing commercial cultivation of *Bt* transgenic crops in several countries, continuous expression of *cry* genes in transgenic crops has led to the emergence of a resistant field population of insects, which is a major challenge for agricultural scientists (Tabashnik and Carrière 2019), Furthermore, Cry proteins currently available in the global database are not effective to control a significant number of insects (Noguera and Ibarra 2010). Besides these crystalline proteins, many researchers demonstrated that *Bt* strains secrete two more proteins during the vegetative growth phase, referred to as vegetative insecticidal proteins (Vips) and secreted insecticidal protein (Sips) (Estruch et al. 1996; Donovan et al. 2006).

Vip proteins are gaining importance as second-generation insecticidal proteins in resistance management programs because they do not share homology and receptor binding sites with Cry proteins and are highly specific and effective against pests that are less susceptible to Cry proteins (Liu et al. 2011; Chakroun et al. 2016; Gupta et al. 2021). Unlike Cry proteins, Vips are secreted as soluble proteins rather than crystal inclusions (Hernández-Martínez et al. 2013). These secreted proteins display insecticidal activity against lepidopterans (Chen et al. 2017; Wang et al. 2018), coleopterans (Warren 1997) and some sap-sucking insect pests (Sattar and Maiti 2011), expanding the overall host range of *Bt*. The action mechanism of Vip3 proteins is not fully understood, but it is thought to involve the disruption of the insect gut epithelium and disintegration of cells, which leads to the loss of nutrient absorption and ultimately death. These proteins may also affect physiological processes in insects, such as the regulation of ion and water balance, disturbance in transmembrane potential, leakage and swelling of cells, etc. (Yu et al. 1997; Lee et al. 2003; Chakroun and Ferré, 2014; Gupta et al. 2021). Two models have been proposed for Vip proteins: (a) pore formation (Lee et al. 2003; Chakroun et al. 2016) and (b) apoptosis (Hernández-Martínez

et al. 2013; Jiang et al. 2016; Nimsanor et al. 2020) with different receptor binding sites (Osman et al. 2019; Jiang et al. 2018a, 2018b). The same binding sites have been shared in *S. littoralis* between different members of Vip3A class (Vip3Ad, Vip3Ae and Vip3Af) which implicates the utility of various categories of Vip3A proteins in insect pest control (Boukedi et al. 2018b). The hemolytic activity of Vip3 proteins has also been demonstrated which may be responsible for the interaction between Vip3 and membrane lipids and ultimately pore formation (Boukedi et al. 2017b).

The proteins Vip3Aa11 and Vip3Aa19 were reported to have insecticidal properties against larvae of *H. armigera*, *S. exigua*, and *P. xylostella* (Liu et al. 2004, 2007). Another gene *vip3Aa14* gene was isolated, and protein was expressed (both truncated and full length) from *B. thuringiensis* subsp. *tolworthi* which was reported to be toxic against *S. litura* and *Plutella xylostella* (Bhalla et al. 2005). In addition, vip3LB (Vip3Aa16), derived from a Bt-BUMP95 strain, was reported to be toxic to *E. kuehniella* (Mesrati et al. 2005). Boukedi and group discovered two new proteins Vip3 (459) from *Bt*-BLB459 strain, and expressed protein was found to be toxic against *Agrotis segetum*, *S. littoralis*, *E. kuehniella* (Boukedi et al. 2017a) and *Ectomyelois ceratoniae* (Boukedi et al. 2018a). Another protein Vip3 (427) from the *Bt*-BLB427 strain was reported to be toxic against *S. littoralis* (Boukedi et al. 2020). Similarly, the toxicity potential of Vip3Aa61 (Lone et al. 2018) and Vip3Aa65 (Şahin et al. 2018) against *H. armigera* has been reported. Therefore, Vip proteins are promising candidates for deployment against agronomically important pests either alone or along with Cry toxins in gene pyramided mode (Kaur 2012; Chakroun et al. 2016; Chen et al. 2017; Gupta et al. 2021). Transgenic cotton cultivar VipCot™ stacked through Vip3Aa/Cry1Ab proteins and corn Agrisure Viptera and Agrisure Viptera 3 expressing Vip3Aa20 alone and a combination of Vip3Aa20/Cry1Ab, respectively, have been commercialized against major lepidopteran pests and have shown significant toxicity (Kurtz et al. 2007). The commercially important toxin genes have been identified by high throughput sequence analysis of native *Bt* isolates recovered from diverse habitats of India (Panwar et al. 2018). The *vip3Aa44* gene (NCBI GenBank accession number HQ650163) from *Bt* BGSC strain 4A6 was earlier cloned in our laboratory and insecticidal efficacy of its synthetic, plant-preferred codon-optimized version toward *H. armigera* (cotton bollworm) and *S. litura* (cotton leafworm) in transgenic tobacco has been reported (Anupama et al. 2019). Among all Vip proteins, Vip3 protein family is extensively studied. But the full potential of Vip3 proteins is relatively lesser-known, although around 130 Vip3 proteins have been identified and cloned to date (https://www.bpprc.org/pesticidal-proteins; accessed on 15. 09. 2022). Therefore, identification of Vip proteins, which are highly toxic against *H. armigera,* is need of hour. The present study was initiated with the objective to evaluate the toxicity of Vip3Aa proteins newly identified in our laboratory and their impact on the life cycle of *H. armigera*. The sequences of these have been proteins submitted to NCBI and *Bt* toxin nomenclature committee (Crickmore et al. https://www.bpprc.org/pesticidal-proteins). In order to clone novel alleles of *vip3A*-type genes, 155 *Bt* isolates recovered from diverse habitats of different agro-climatic zones in India and 10 reference *Bt* strains have been explored to determine the frequency distribution of *vip3A*-type genes (our unpublished data). In present study, PCR-amplified genes were cloned to assess genetic variability among them and the newly identified genes were further investigated for protein expression and toxicity potential against *H. armigera*.

Materials and methods

Cloning of full-length genes for sequence identification

Plasmid DNA isolation

Petri plates poured with autoclaved Luria Agar (LA, HiMedia, India) media with penicillin (10 µg/ml) were streaked with *Bt* isolates and incubated overnight at 28 °C. Next day, 5 ml of autoclaved Luria Bertani (LB) broth with penicillin was inoculated with a single colony and incubated at 28 °C for 3–4 h at 200 rpm (Kuhner, Basel, Switzerland). Thereafter, LB medium with penicillin was inoculated with 1 ml of starter culture and incubated at 28 °C, overnight at 200 rpm. The overnight culture was used for plasmid DNA extraction by modified alkaline lysis miniprep protocol (Birnboim and Doly 1979), including the lysozyme treatment step. Plasmid DNA purity and quality were checked by 0.8% agarose gel electrophoresis using a 1 kb ladder (MBI Fermentas, St. Leon-Rot, Germany) as a DNA marker.

PCR amplification, purification, cloning and transformation

A total of 10 full-length *vip3*-type genes amplified (unpublished data) by primers designed as per Mesrati et al. (2005) from isolates SK-306, SK-671, SK-792, SK-851, SK-986, SK-1065, SK-1328, SK-1340, SK-1386, SK-1401, belonging to 8 different agro-climatic zones (Table S1) and 2 reference *Bt* strains BGSC 4E3 (*B. thuringiensis* subsp. *sotto*) and BGSC 4K1 (*B. thuringiensis* subsp. *morrisoni*) were selected for cloning. The full-length amplified PCR products were purified from agarose gel with QIAquick Gel Extraction kit (Qiagen, GmbH, Hilden, Germany). The eluted products of 12 full-length 2.37 kb *vip3*-type genes having 3′-A overhangs were used for ligation with linear form of cloning

vector pGEM-T Easy having T overhangs (Promega, Madison, USA). The ligation mixture of 10 µl was prepared including 2X Rapid ligation Buffer (5 µl); 1 µl pGEM-T Easy Vector (50 ng); eluted product of insert (100 ng/µl); and 1 µl T4 DNA Ligase (3 Weiss units/µl), and deionized water supplied with the kit was added, to make final volume. The ligation mixture was mixed and kept for overnight incubation at 4 °C. Thereafter, NEB 5-alpha Competent *E. coli* (High Efficiency) cells (New England Biolabs, Ipswich, USA) were transformed with the ligation mixture, and 50 µl of cultured cells was spread on LA plates containing ampicillin/X-gal/IPTG and incubated overnight at 37 °C (Kuhner, Basel, Switzerland).

Selection, validation of clones and sequencing

The overnight incubated plates having selection ampicillin/X-gal/IPTG developed blue and white-colored colonies. The white-colored colonies represented recombinant clones having genes of interest. Recombinant clones of each gene were confirmed by colony PCR, restriction analysis and amplification of plasmid DNA with gene-specific primers. Every single colony from the plate of each isolate was picked and resuspended in 34.5 µl nuclease-free water (VWR, Life Sciences). A reaction of 50 µl with addition of 2.5 µl gene-specific primers (1 µM), 10X PCR buffer (5 µl), 2 mM dNTPs (5 µl), 0.2 of Taq DNA polymerase 1.0 U (MBI, Fermentas, Germany) was prepared. The reaction was performed in a thermal cycler (BioRad Laboratories, Inc. USA) with an amplification profile: initial denaturation at 94 °C for 1 min; 30 cycles of steps including denaturation at 94 °C for 1 min, annealing at 46 °C, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. Plasmid DNA (~ 5 to 10 µg) isolated from each colony was digested with different restriction enzymes (New England Biolabs, Ipswich, USA): *Sal*1 (single digestion); a combination of *Nco*1 and *Sal*1 (double digestion); and in some cases *Pst*1 (to confirm the right orientation of inserted gene in the vector). The restriction enzymes and buffer in each restriction reaction were used according to manufacturer's instructions. Total 10 µl volume was made with nuclease-free sterile water. The reaction was kept for overnight incubation at 37 °C. The restricted DNA was analyzed on 0.8% agarose gel using a 1 kb DNA ladder (MBI Fermentas, St. Leon-Rot, Germany). The sequencing was performed by AgriGenome Labs, India, through primer walking.

Protein expression

Bacterial strains and constructs for protein expression

The competent cells of *E. coli* DH5-alpha strain (New England Biolabs, Ipswich, MA, USA) for initial cloning and *E. coli* BL21(DE3) cells (New England Biolabs, Ipswich, MA, USA) for protein expression studies were used. *E. coli* strains were grown in LB broth or on LA plates at 37 °C with shaking at 180–220 rpm. Stock solutions of antibiotics, *i.e.*, ampicillin (100 mg/ml) and kanamycin (50 mg/ml), were supplemented to their working concentrations whenever required. The six genes cloned in pGEMT-Easy vector, namely pGEMT-SK792, pGEMT-BGSC4K1, pGEMT-SK986, pGEMT-SK851, pGEMT-SK306 and pGEMT-BGSC4E3, were used for protein expression studies. The pET-29a(+) expression vector (5.371 kb) contained T7 IPTG inducible promoter and S-tag (Novagen) for expression of complete ORFs (2.37 kb). An expression vector pET29a (+) cloned with *vip3Aa44* gene, previously cloned in our laboratory, was used as a positive control.

Plasmid DNA extraction and electrophoresis

The Qiagen Miniprep/Midiprep kit following instructions from the manufacturer was used for plasmid DNA extraction. The gel electrophoresis was carried out (Sambrook 1989) using agarose gels (0.7–1.0%). The 1 kb DNA ladder (MBI, Fermentas, Germany) was used. Electrophoresis was performed in a horizontal electrophoresis tank (Genetix, India) filled with 1 × TAE buffer for 45–90 min at constant voltage (50 V). The DNA bands were visualized, and images were captured using the gel documentation system (SynGene, UK).

Cloning and transformation

The vector and plasmid DNA of pGEMT-Easy clones were digested with restriction enzymes *Nco*1 and *Sal*1. The restricted fragments were eluted (QIAquick® Gel Extraction Kit), and resultant eluted products were ligated with restriction enzyme-digested pET-29a(+) vector in insert/vector ratio of 2:1 using T4 DNA Ligase. The ligation mixture was kept overnight at 4 °C for incubation. Five microliters of ligation mix was added to 50 µl *E. coli* DH-alpha competent cells for transformation following basic steps. The transformed cells were spread on LA plates containing 50 µg/ml kanamycin, 50 µg/ml X-gal and 1 mM IPTG and incubated overnight at 37 °C. The LA plates were screened for blue and white colonies for the selection of recombinant clones. Further, plasmid DNA isolated from pET29a(+)-DH5-alpha clones was used for the transformation of *E.coli* BL21(DE3) cells following the same procedure for protein expression. All the six clones were selected on LA plates containing kanamycin as a selection marker and incubated overnight at 37 °C.

Validation of recombinant clones

The plasmid DNA (~ 5 to 10 µg) isolated from each colony was digested with single restriction enzyme *Sal*1, double digestion with *Nco*1 and *Sal*1, and to confirm the orientation of gene in vector, *Pst*1 was used for some clones using appropriate buffers, following the manufacturer's instructions. The restriction mixture was analyzed on 0.8% agarose gel.

Protein expression, extraction and estimation

A single colony of recombinant *E. coli* BL21(DE3)-pET29a(+) having target gene and *E. coli* BL21(DE3) alone was pre-cultured by inoculation of the particular colony into 10 ml LB medium with kanamycin (50 µg/ml) and incubated overnight at 37 °C, 200 rpm at incubator shaker. The next day, 100 ml LB media was inoculated with 1% (100 µl) of freshly grown primary culture. The secondary culture was allowed to grow for 2 h (till OD value 0.6). Thereafter, 1 mM IPTG was added in this actively growing culture to induce the expression of the particular target protein. The samples were collected after 2 and 4 h of induction at 3 °C, 200 rpm. The uninduced samples of respective clones were also allowed to grow for 2 and 4 h without IPTG induction and collected for protein isolation. The protein from induced samples, uninduced samples and negative control *E. coli* BL21(DE3) was isolated. The collected cell cultures were centrifuged (BRK5424, Centurion, Scientific Limited, UK) at 7000 rpm, 4 °C for 15 min. The resultant pellet was resuspended in 50 ml lysis buffer containing lysozyme (2 mg/ml) and 100 µM of protease inhibitor PMSF (phenyl methyl sulfonyl fluoride). These samples were incubated at 37 °C, 150 rpm for 2 h. Each sample was sonicated on ice two times at a constant cycle at 60 watts for 1 min with a break of 10 s for sample cooling using Sonicator (Fisher Scientific, Sonic Dismembrator 500, UK). The sonicated samples were further centrifuged at 7000 rpm for 5 min, 4 °C to collect supernatant/lysate fraction of Vip3 proteins. The supernatant was filtered through 0.45 µm sterile filter followed by a filtration through a 0.2 µm sterile filter as described by de Escudero et al. (2014) and stored at – 20 °C. The protein concentration of the supernatant fraction was estimated following the Bradford method (Bradford 1976), and the readings were taken on ELISA Reader (SPECTRA max PLUS with SoftMax Pro 5 Software) at 595 nm. The standard curve was generated using BSA protein, and the protein concentration in the supernatant fraction was deduced with regression equation.

Validation of protein expression

The preliminary immunoassay was performed to confirm the presence of Vip3A-specific protein in the samples. This qualitative detection was performed using a Vip3A-specific rapid LFS kit (Amar Immunodiagnostics Hyderabad, India) according to manufacturer's instructions. The harvested samples were resolved on a vertical gel electrophoresis unit (Atto Corporation, Japan, Model AE-6210) using the method modified from Laemmli (1970) for SDS-PAGE analysis. The size of expressed protein was determined using 8% SDS-PAGE gel along with a pre-stained protein marker (Puregene, Genetix, India). The pictures of the gels were captured on a UV light convertor whiteboard in the Gel doc system (SynGene, UK). The samples resolved on 8% SDS-PAGE gel were transferred to PVDF membrane (Amersham Hybond P 0.45 µm) using a Semi-Dry blotting system (Bio-Rad, USA) at 25 V for 60 min to perform Western Blotting using Vip3A-specific antibodies (Amar Immunodiagnostics Hyderabad, India). The blotting procedure was followed according to the manufacturer's instructions using Western Breeze® Chromogenic Kit (Invitrogen, USA).

Bioassays and statistical analyses of mortality data to study the responses of *H. armigera*

Neonate (first instar) larvae of *H. armigera* were reared at National Facility for Insect Rearing and Xenobiotic-cum-Transgenic Bioassay, Division of Entomology, ICAR-IARI, New Delhi. The diet incorporation method (Dulmage et al. 1971) was used to evaluate toxicity of expressed proteins. Preliminary, laboratory bioassays were conducted with neonate larvae treated with Vip3A proteins in 10 ppm concentration in semi-synthetic diet. The corrected mortality data of 10 ppm was analyzed using Analysis of variance (ANOVA) at a 5% significance level to compare the insecticidal activities of different proteins. ANOVA analysis was performed using SAS (Statistical Analysis System) package. The dose–mortality response was calculated using probit analysis. Subsequently, bioassays were performed for determination of LC_{50} at five different concentrations, viz. 0.1 ppm; 1 ppm; 5 ppm; 10 ppm; and 50 ppm. An appropriate concentration of toxin was mixed thoroughly with 10 g of semi-synthetic diet and transferred to 3 Petri plates equally to maintain 3 replications for each concentration. Ten neonate larvae were carefully placed in each plate containing treated diet. Mortality was assessed each day up to 4th, 7th and 10th day (d). The bioassay with diet treated with sterile double distilled water and Vip3Aa44 protein was considered as negative and positive control, respectively. The bioassays were conducted in controlled conditions, at temperature 25 ± 2 °C, relative humidity 50 ± 10%, with a 14 h photoperiod. Percent mortality was considered as the criterion to

differentiate among toxins (Kaur and Singh 2000) as highly toxic (65–100%); moderately toxic (50–70%); low toxicity (20–50%); and negligible toxicity (< 20%). The mortality data were used to determine LC_{50} and 95% fiducial limits with PoloPlus Version 2.0 based on Probit analysis (Finney 1971). The various stages of larvae were monitored, and the weight and length of 7 d old larvae were taken. The impact of toxicity of expressed proteins over the course of whole life cycle of *H. armigera* was also assessed.

Molecular docking of Vip3Aa protein model with *H. armigera* receptor and its analysis

The three-dimensional model of Vip3Aa was retrieved from Protein Data Bank (Code: 6TFK). This represents the active configuration of the Vip molecule after trypsin digestion (Núñez-Ramírez et al. 2020). *H. armigera* fibroblast growth factor receptor (FGFR) was modeled using SwissModel (Waterhouse et al. 2018). Molecular docking was performed via **H**igh **A**mbiguity-**D**riven protein–protein **DOCK**ing, HADDOCK algorithm (Van Zundert et al. 2016). Flexibility of the protein was predicted through 'Multiclass flexibility prediction from sequences of amino acids' (MEDUSA) algorithm (Vander Meersche et al. 2021), which calculates protein flexibility on the basis of amino acid sequence through deep learning. Multiple sequence alignment of amino acid sequences was done using EMBOSS. Analysis of the effect of amino acid mutations on protein stability was predicted through SAAFEC-SEQ algorithm (Li et al. 2021).

Results

Cloning of full-length *vip3*-type genes

Cloning and screening of recombinant clones

The amplified PCR products were purified from the gel (Fig. S1), and all ligation reactions having recombinant vectors were transformed in competent *E. coli* cells. The white colonies of recombinant clones were selected from selection plates. Every single clone was validated for the presence of the desired gene through colony PCR (Fig. S2); PCR amplification (Fig. S3) and restriction analysis. The pGEM-T Easy vector has unique sites for restriction enzymes *Nco*1 at 5' end and *Sal*1 at 3' end, which are not present within known *vip3-*type genes. Therefore, these enzymes may cut the vector once from both ends but not the inserted gene. Single digestion was performed with *Sal*1 enzyme, which showed band for linearized recombinant pGEM-T Easy clone of 5.385 kb size. Double digestion of all recombinant vectors with *Nco*1 and *Sal*1 showed the expected bands of 2.37 kb and 3.01 kb for inserted gene and pGEM-T Easy vector, respectively. Since *Pst*1 has a single restriction site in the conserved region of known *vip3*-type genes at 605 bp position, the plasmid DNA of pGEMT-SK671, pGEMT-SK1065, pGEMT-SK1328, pGEMT-SK1340, pGEMT-SK1386 and pGEMT-SK1401 clones was restricted with *Pst*1 enzyme to confirm the correct orientation from 5′ to 3′ end of insert in pGEMT-Easy vector, before sequencing. The correct orientation was validated, as 1.77 kb and 3.62 kb bands were detected after *Pst*1 restriction analysis, as expected (Figs. S4; S5). These analyses confirmed that full-length *vip3*-type genes from 10 native *Bt* isolates and 2 *Bt* strains used as reference were successfully cloned in pGEM-T Easy vector for further sequence analysis. The respective clones were designated as pGEMT-SK306, pGEMT-SK671, pGEMT-SK792, pGEMT-SK851, pGEMT-SK986, pGEMT-SK1034, pGEMT-SK1065, pGEMT-SK1328, pGEMT-SK1340, pGEMT-SK1386, pGEMT-SK1401, pGEMT-BGSC4E3 and pGEMT-BGSC4K1.

Sequence determination and analysis

Primer walking approach was used to determine the sequences of genes cloned in pGEM-T Easy vector. The sequencing procedure was performed by service provider M/s ABA Biotech, India. Sequencing of resultant recombinant pGEM-T Easy clones of each gene yielded full-length complete ORFs of 2.37 kb with an initiation codon ATG at 5' end and termination codon TAA at 3' end, which encoded proteins of 789 amino acid residues. The deduced amino acid sequence of each gene was deduced from https://web.expasy.org/translate/ and also confirmed with http://insilico.ehu.es/translate/. The predicted protein molecular mass was 89 kDa. The BLASTp analysis revealed that these proteins were Vip3Aa-type proteins and had identity with Vip3Aa1 protein varying from 98.86 to 99.75% (Table 1). We designated these genes as Vip3A_792, Vip3A_986, Vip3A_851, Vip3A_306, Vip3A_4E3, Vip3A_4K1, Vip3A_671, Vip3A_1065, Vip3A_1340, Vip3A_1386, Vip3A_1401 and Vip3A_1328.

Comparison of deduced amino acids sequence of our *vip3Aa*-type genes with the holotype *vip3Aa1*

The deduced amino acid sequences of all the genes cloned in this study, and the *vip3Aa44* (NCBI accession number HQ650163) used as positive control, were compared with *vip3Aa1* (NCBI accession number AAC37036). The multiple sequence alignment of the deduced amino acid sequence of all *vip3A*_genes from 10 native *Bt* isolates, 2 reference *Bt* strains, one positive control Vip3Aa44 and holotype Vip3Aa1 was performed by BioEdit software using Clustal W alignment (Fig. S6). The divergent amino acids and their positions in the deduced amino acid sequence of all

Table 1 Amino acid substitutions identified in 12 cloned Vip3A genes and their position in comparison to Vip3Aa1

S. No.	Gene Name	NCBI Accession number	Percent (%) Similarity with Vip3Aa1	Number of amino acid substitutions	Amino acid position	Amino acid in Vip3Aa1	Amino acid in cloned gene
1	Vip3A_792 **(Vip3Aa67)**	MN120477	99.75	2	284	Q	K
					570	T	A
2	Vip3A_4K1 **(Vip3Aa68)**	MN120478	98.86	9	284	Q	K
					358	I	V
					536	S	K
					633	N	T
					755	M	I
					760	F	L
					761	E	G
					776	Y	N
					782	H	K
3	Vip3A_SK-986 **(Vip3Aa69)**	MN120479	99.62	3	31	K	R
					110	I	M
					284	Q	K
4	Vip3A_SK-851 **(Vip3Aa70)**	MN120481	99.24	6	179	V	F
					183	F	L
					284	Q	K
					520	L	S
					605	P	L
					667	I	T
5	Vip3A_SK-306 **(Vip3Aa71)**	MN120482	99.75	2	270	N	K
					284	Q	K
6	Vip3A_4E3 **(Vip3Aa72)**	MN120480	99.49	4	176	I	T
					284	Q	K
					615	G	E
					708	K	E
7	Vip3A_671 **(Vip3Aa73)**	MT468480	99.37	4	270	N	K
					284	Q	K
					501	R	G
					675	S	G
8	Vip3A_1065 **(Vip3Aa74)**	MT468481	99.37	5	160	V	A
					270	N	K
					284	Q	K
					465	R	G
					537	N	D
9	Vip3A_1340	MT468482	99.11	7	224	V	A
	(Vip3Aa75)				270	N	K
					284	Q	K
					358	I	V
					548	N	D
					671	E	K
					747	S	G
10	Vip3A_1386 **(Vip3Aa76)**	MT468483	99.62	3	45	D	G
					270	N	K
					284	Q	K
11	Vip3A_1401 **(Vip3Aa77)**	MT468484	99.75	2	270	N	K
					284	Q	K
12	Vip3A_1328 **(Vip3Aa78)**	MT681751	99.86	9	213	T	S
					280	A	T
					284	Q	K
					289	L	V
					309	E	G
					469	A	T
					526	K	R
					570	T	A
					629	Y	N

Table 1 (continued)

*Gene name in parenthesis assigned by *Bt* nomenclature committee (www.bpprc.org)

#Red color indicates similar amino acid substitution in all genes with reference to Vip3Aa1. Purple, green and brown colors indicate similar amino acid substitutions among cloned genes

the genes with reference to Vip3Aa1 were identified with MEGA-X software using Muscle Alignment. The amino acids substitutions were maximum (9) for Vip3A_4K1 and Vip3A_1386, followed by 7 for Vip3A_1340; 6 for Vip3A_851; 5 for Vip3A_1065; 4 for Vip3A_4E3 and Vip3A_671; 3 for Vip3A_986 and Vip3A_1386; and 2 for Vip3A_792, Vip3A_306 and Vip3A_1401. The deduced amino acid sequence of previously reported Vip3Aa65 (Şahin et al. 2018) was also included in the study for comparison with that of Vip3A_4K1, which was found to be 100% identical in terms of amino acid substitutions (Fig. 1, Table 1).

Detailed analysis revealed that there were differences in the deduced amino acid sequence of all the genes cloned in this study with that of the holotype Vip3Aa1 and were found to encompass region near to both N and C-terminus, starting from position 31 to 782. The deduced amino acid sequence of all the genes was found to be more than 95% similar, indicating that these *vip3Aa*-type genes differ from each other in the quaternary rank as per criteria of *Bt* Nomenclature Committee. Vip3Aa44 (NCBI accession number HQ650163) cloned in a previous study from our laboratory and used as a positive control is different in one amino acid at position 284 as compared with Vip3Aa1 protein, wherein Q (Glutamine) was substituted with K (Lysine). However, the *vip3Aa*-type genes cloned in our study showed divergent amino acids at two or more positions in their deduced amino acids sequence, in addition to the substitution at position 284, which was observed to be common in the deduced amino acid sequence of all genes except Vip3Aa1. The substitution of N (Asparagine) with K (Lysine) at position 270 was common among six, viz. Vip3A_306, Vip3A_671, Vip3A_1065, Vip3A_1340, Vip3A_1386 and Vip3A_1401.

Furthermore, diversity in the deduced amino acids sequences of *vip3A*-type genes, in that, same amino acid substitution present in *vip3A*-type genes cloned from *Bt* isolates from different agro-climatic zones as well as different amino acid substitutions occurring in *vip3* genes cloned from *Bt* isolates from same agro-climatic zone, has been observed. Although Vip3A_306 and Vip3A_1401 were cloned from *Bt* isolates from different agro-climatic zones, similar substitutions were present at positions 270 and 284 in the deduced amino acids sequence. Similarly, Vip3A_792 and Vip3A_1328 also had similarity at position 570 with A (Alanine). Both Vip3A_1386 and Vip3A_1401 were from *Bt* isolates recovered from Eastern Himalayan Region, but were different from each other at position 45 wherein D (Aspartic Acid) was substituted by G (Glycine). This indicates diversity of *Vip* genes in *Bt* isolates within an agro-climatic zone. Differences in deduced amino acid sequences for each of the 12 genes cloned in this study, except Vip3A_306 and Vip3A_1401, were observed. Thus, diverse *vip3Aa* type of genes from different agro-climatic

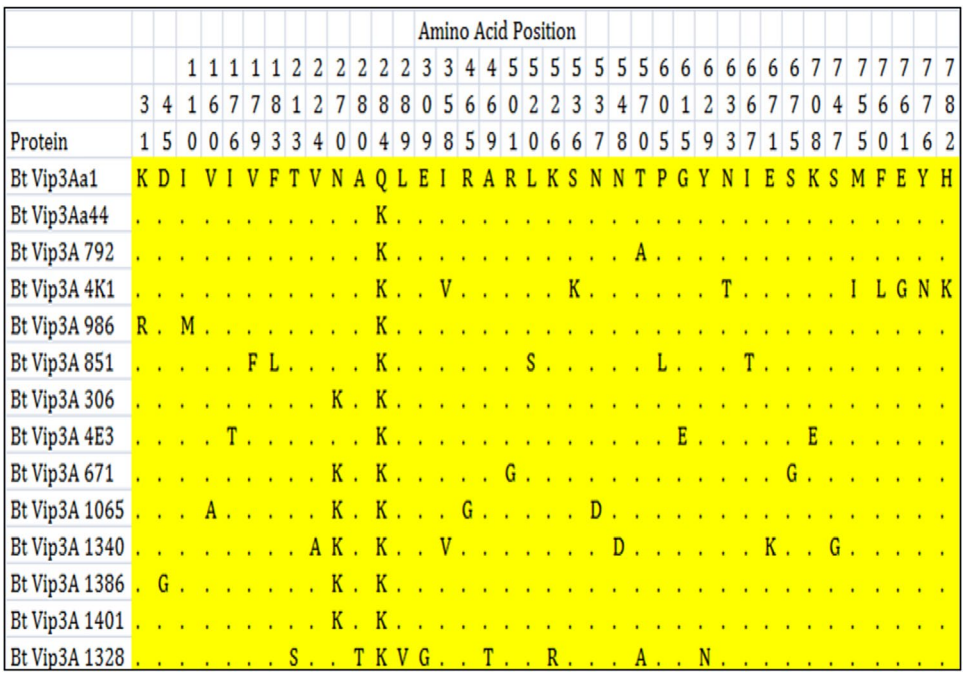

Fig. 1 The picture depicts differences in the deduced amino acids sequence and their positions in 12 *vip3Aa*-type genes cloned in this study, and protein Vip3Aa44 was used as a positive control with reference to the amino acid sequence of representative Vip3Aa1. The vertical column represents the names of the respective proteins and the horizontal column at the top depicts amino acid positions 31–782

zones have been discovered in this study, which indicates that Indian *Bt* isolates are a potential source of diverse range of *vip3* genes (Table 1).

Comparative analysis of identified genes with other *vip3Aa*-type genes reported to be toxic to Lepidopteran pests

Deduced amino acid sequences of 18 lepidopteran-specific Vip3 toxins including Vip3Aa1 listed by (Chakroun et al. 2016), Vip3Aa61 specific to *H. armigera* (Lone et al. 2018) and Vip3Aa65 (Şahin et al. 2018) were used for comparative analysis with Vip3Aa44 previously cloned in our laboratory and 12 genes identified in this study. The lepidopteran-specific toxins listed by Chakroun et al. 2016 were: Vip3Aa7, Vip3Aa9, Vip3Aa10, Vip3Aa11, Vip3Aa13, Vip3Aa14, Vip3Aa16, Vip3Aa18, Vip3Aa19, Vip3Aa29, Vip3Aa43, Vip3Aa45, Vip3Aa50, Vip3Aa58, Vip3Aa59 and Vip3Aa64. Deduced amino acid sequences of these genes were downloaded from NCBI database.

The multiple sequence alignment of deduced amino acid sequences of these 32 genes was performed for identification of variations as compared with Vip3Aa1 using BioEdit (Fig. S7), and divergent amino acid substitutions were identified using MEGA-X software (Fig. 2). The amino acid substitutions were found to be between positions 2 to 784. A consistently similar substitution was found at position 284 (Q/K), which was same in all except Vip3Aa1. The substitutions present in Vip3A_4K1 and Vip3Aa65 were also found to be similar to Vip3Aa59 having one extra substitution at 784 and with Vip3Aa14 having 9 more substitutions. Vip3Aa19 and Vip3Aa65 also had similar substitutions at 358 (I/V) and 633 (N/T). Vip3Aa61 gene had substitutions which are present in Vip3Aa19 except for one additional substitution at position 35 (N/Q). Thus, except Vip3A_4K1, all genes discovered in our study had new kinds of substitutions in their deduced amino acids sequences as compared with the genes already reported in the NCBI database and may exhibit different toxicity and specificity against Lepidopteran pests.

A phylogenetic analysis was also performed for grouping of all these 32 Vip3Aa proteins based on amino acid sequence using MEGA 6.0 following MUSCLE alignment. This evolutionary analysis formed different combinations of these proteins based on amino acid variations, with Vip3Aa29 being shown as an out-group in the phylogenetic tree (Fig. 3). These six combinations were: 1) Vip3A_851 and Vip3Aa18; 2) Vip3A_792 and Vip3A_1328; 3) Vip3A_1386, Vip3A_306, Vip3A_671, Vip3A_1401 and Vip3A_1065 were grouped together and Vip3A_1340 was closely related with them; 4) Vip3A_4K1 grouped with Vip3Aa65, Vip3Aa59, Vip3Aa14; 5) Vip3Aa19 and Vip3Aa61; and 6) Vip3A_986 and Vip3A_4E3 were grouped with rest of lepidopteran-specific proteins Vip3Aa1, Vip3Aa7, Vip3Aa9, Vip3Aa10, Vip3Aa11, Vip3Aa13, Vip3Aa16, Vip3Aa43, Vip3Aa44, Vip3Aa45, Vip3Aa50, Vip3Aa58, Vip3Aa64. Furthermore, the groups 4 and 5 were closely related to each other.

The nucleotide and deduced amino acid sequences of all the genes cloned in this study were submitted to NCBI GenBank database and Vip3 protein database. Their names were

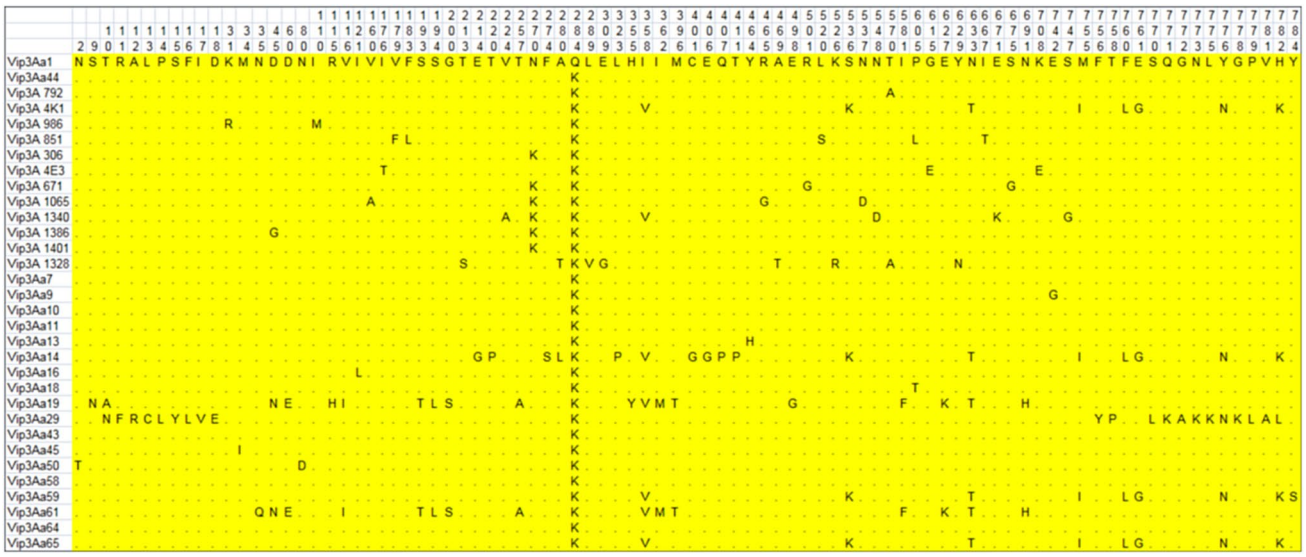

Fig. 2 Picture indicates divergent amino acids and their positions in amino acid sequences of 32 Vip3Aa-type toxins with reference to Vip3Aa1 protein. In the vertical column, Vip3A represents deduced proteins of *vip3Aa*-type genes discovered in this study and lepidopteran-specific Vip3Aa proteins available in the Vip3 protein database. The horizontal column at the top depicts amino acid positions 2 to 784

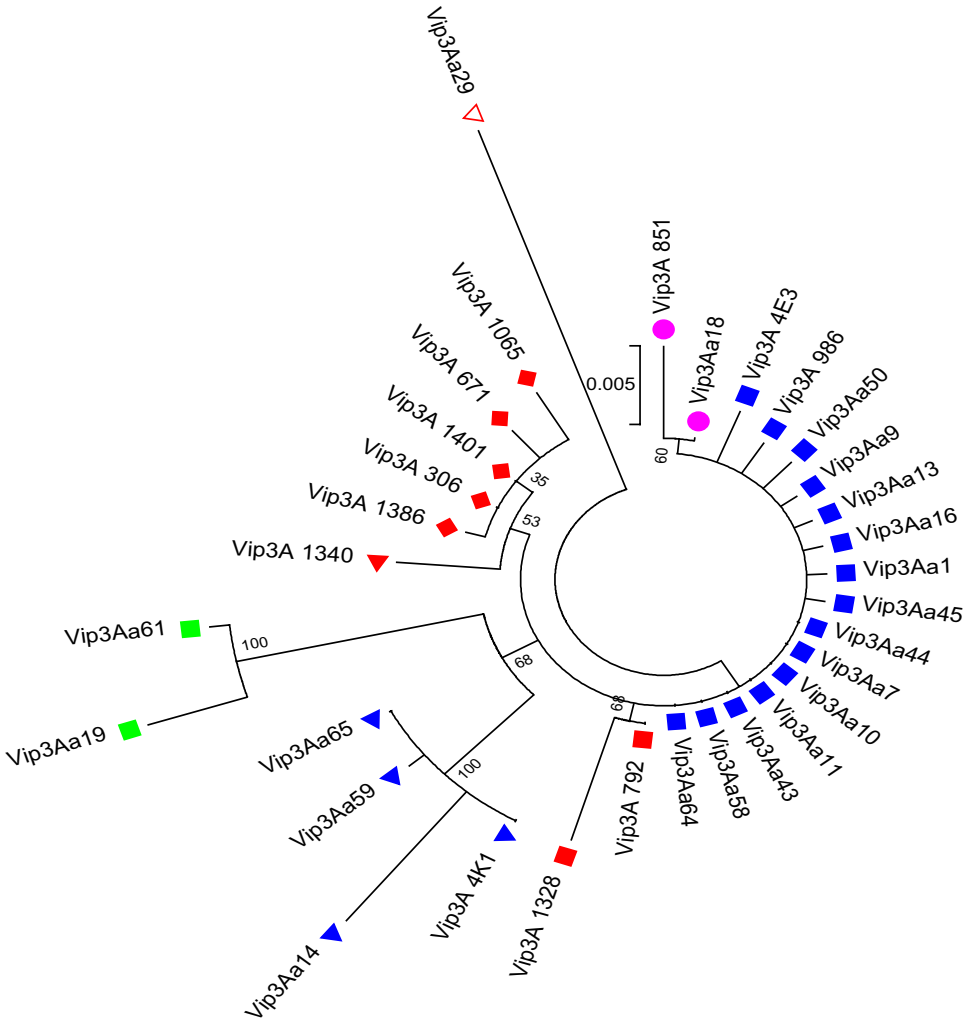

Fig. 3 Phylogenetic tree based on deduced amino acids sequence of 32 *vip3Aa* genes. Vip3A represents proteins of *vip3Aa*-type genes discovered in this study and Vip3Aa represents all proteins of other genes available in the Vip3 protein database. The phylogenetic relationship was analyzed by MEGA 6.0 using the Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The same group was represented by the same shape and color. There was a total of 789 positions in the final dataset

designated by *Bt* nomenclature committee and information regarding their nomenclature, and NCBI accession number is available at https://camtech-bpp.ifas.ufl.edu/categorize_database_vip as Vip3Aa67 (Vip3A_792: MN120477), Vip3Aa68 (Vip3A_4K1: MN120478), Vip3Aa69 (Vip3A_986: MN120479), Vip3Aa70 (Vip3A_851: MN120481), Vip3Aa71 (Vip3A_306: MN120482), Vip3Aa72 (Vip3A_4E3: MN120480), Vip3Aa73 (Vip3A_671: MT468480), Vip3Aa74 (Vip3A_1065: MT468481), Vip3Aa75 (Vip3A_1340: MT468482), Vip3Aa76 (Vip3A_1386: MT468483), Vip3Aa77 (Vip3A_1401: MT468484), Vip3Aa78 (Vip3A_1328: MT681751) as entry numbers 113 and 116 to 126. Thus, these genes became new members of Vip3A protein database (Table 1).

Protein expression

Construction of recombinant pET29a(+) vector, transformation and validation

The six newly identified genes, viz. *vip3A*_792, *vip3A*_4K1, *vip3A*_986, *vip3A*_851, *vip3A*_306 and *vip3A*_4E3, were cloned into expression vector pET-29a(+) individually for protein expression. The complete ORFs (2.37 kb) of six genes were amplified from the plasmid DNA of recombinant clones pGEMT-SK792, pGEMT-BGSC4K1, pGEMT-SK986, pGEMT-SK851, pGEMT-SK306 and pGEMT-BGSC4E3 using gene-specific primers. The plasmid DNA of pET-29a (+) and amplified products from these genes were digested with restriction enzymes *Nco*1 and *Sal*1 (Fig. S8). The eluted bands were ligated to digested pET29a(+)

vector to create recombinant pET29a(+)–*vip3A*_792, pET29a(+)–*vip3A*_4K1, pET29a(+)–*vip3A*_986, pET29a(+)–*vip3A*_851, pET29a(+)–*vip3A*_306 and pET29a(+)–*vip3A*_4E3 clones. Thereafter, these constructs were successfully transformed into competent *E. coli* BL21(DE3) cells (New England Biolabs, USA) for their protein expression studies and toxicity analysis. All positive clones of *E. coli* BL21(DE3) having recombinant pET29a(+) construct were confirmed on LA plates having kanamycin (50 µg/ml) as a selection marker.

The pET29a(+) vector with a size of 5371 bp has unique sites for *Nco*1 at the 5' end and *Sal*1 at the 3' end, while the gene sequences lack these restriction sites. Therefore, restriction digestion of each recombinant construct separated the insert from the pET-29a(+) vector and demonstrated two bands of 2.37 kb and 5.371 kb for insert and pET-29a(+) vector, respectively. *Sal*1 treatment gave the linearized recombinant expression vector band of 7.741 kb size. All recombinant pET29a(+)-DH5α clones, viz. pET29a (+)-SK792-DH5α, pET29a(+)–4K1-DH5α, pET29a(+)–SK986-DH5α, pET29a(+)–S,K851-DH5α, pET29a(+)–SK306-DH5α and pET29a(+)–4E3-DH5α, were analyzed through restriction analysis. The plasmid DNA of these clones was extracted (Fig. S9), and restriction analysis was performed using *Sal*1 and *Nco*1 (Fig. S10). It was also confirmed from sequence determination that *Pst1* restriction enzyme has only a single site in these genes at 605 bp from the 5′ end. Therefore, the orientation check of inserted genes was also performed with *Pst*1 restriction before the expression of proteins from recombinant constructs to ensure that there would not be any problems in subsequent protein expression studies. *Pst*1 treatment resulted in two digested fragments of 1.765 kb and 5.976 kb, as expected, for the correct orientation. Thus, the plasmid DNA extraction and restriction analysis of six recombinant constructs transformed in *E. coli* BL21(DE3), viz. pET29a(+)-SK792-BL21(DE3), pET29a(+)–4K1-BL21(DE3), pET29a(+)–SK986-BL21(DE3), pET29a(+)–SK851-BL21(DE3), pET29a(+)–SK306-BL21(DE3) and pET29a(+)–4E3-BL21(DE3), validated the cloning into an expression vector and correct orientation of a particular gene (Figs. S11 and S12).

Protein expression and validation

E. coli BL21(DE3) having Vip3A_792, Vip3A_4K1, Vip3A_986, Vip3A_851, Vip3A_306, Vip3A_4E3 and positive control pET29a(+)-Vip3Aa44 were induced with the treatment of 1 mM IPTG at 37 °C for 4 h, and *E. coli* BL21(DE3) cells without recombinant construct were used as a negative control for protein extraction and expression for SDS-PAGE analysis and immunoassays.

LFS immunoassay

The samples of negative control (protein extract from wild-type BL21(DE3), a protein expressed from Vip3Aa44 (used as positive control) and other six recombinant cells after IPTG induction and protein extracted from uninduced recombinant cells were analyzed. All protein samples from induced recombinant cells were found to be positive as these showed both control and test lines on the immunoassay strip. But, both uninduced samples and negative control showed only a control line on the immunoassay strip. This strip immunoassay confirmed the presence of Vip3A-specific proteins in the expressed protein samples and the absence of leaky expression of genes in uninduced samples (Fig. S13).

SDS-PAGE analysis of expressed Vip3A-type protein

The SDS-PAGE analysis confirmed the presence of approximate 89 kDa size protein band in all IPTG-induced supernatant protein samples of Vip3Aa44, Vip3A_792, Vip3A_986, Vip3A_4E3 and Vip3A_4K1, and the same pattern was obtained for uninduced samples (Fig. S14). The band of *ca.* 89 kDa was also observed in the uninduced sample, which may be a protein produced by wild-type BL21(DE3) cells. Further, the induced supernatant fraction of all six proteins (Vip3Aa67 to Vip3Aa72) were analyzed with positive (Vip3Aa44) and negative (BL21(DE3) sample) control on SDS-PAGE gel (Fig. S15).

Western blot analysis of expressed Vip3A-type proteins

The protein bands from SDS-PAGE (Fig. S16A) were transferred on the PVDF membrane, and a band of *ca.*89 kDa gave a positive signal with anti-Vip3A-polyclonal antibodies during the western blotting experiment. However, there was some non-specific binding due to the polyclonal nature of Vip3A antibodies. Initially, the antibody titer 1: 50, 000 was used and it was observed that there was non-specific binding in uninduced samples. Then, polyclonal Vip3A antibodies were adsorbed with a 2 ml overnight culture of an uninduced sample of Vip3A_792. In the second experiment, adsorbed polyclonal antibodies were used with titer 1: 100, 000 to reduce non-specific binding. In this western blot, non-specific binding was not observed in uninduced samples. However, some non-specific binding was still present in induced samples. Nevertheless, the intensity of 89 kDa was higher than any other band (Fig. S16B). Thus, western blot results confirmed that Vip3A_792, Vip3A_4K1, Vip3A_986, Vip3A_851, Vip3A_306, Vip3A_4E3 genes along with positive control Vip3Aa44 were expressed successfully in *E. coli* after IPTG induction.

Bioassays with *H. armigera*

Toxicity analysis of Vip3-type proteins at 10 ppm concentration against *H. armigera*

Preliminary bioassay was performed using filtered supernatant protein fraction (lysate) of four recombinant clones Vip3A_792, Vip3A_986, Vip3A_4E3 and Vip3A_4K1 at 10 ppm concentration with positive control as Vip3Aa44 and negative control as sterile double distilled water. Mortality data of the 7th d showed that all four genes were more toxic than positive control at a single concentration of 10 ppm. The observed percent corrected mortality after 7^{th} d was similar (65.39%) for Vip3A_4K1 and Vip3A_986, followed by 50.00% (Vip3A_4E3), 46.16% (Vip3A_792) and 38.46% (Vip3Aa44). However, there was a difference in percent mortality on the 4th and 7th days. The 4th d mortality of Vip3Aa44 treatment was similar to Vip3A_792. While 4th d mortality of Vip3A_986 was less than that of Vip3A_4K1, the 7th d mortality of Vip3A_986 and Vip3A_4K1 was found to be similar. The data also demonstrated that mortality caused by Vip3A_792 was not significantly different from that of Vip3Aa44 and Vip3A_4E3 (Table S2). In addition to this, the weight and length of larvae were observed to study the effect of proteins on the growth of larvae. Significant growth reduction was found after treatment with proteins as compared with control. The observed average weight and length of larvae from negative control treatment were 85.13 ± 4.41 mg and 17.1 ± 1.02 mm, respectively. The results demonstrated that the average weight and length of larvae treated with 10 ppm concentration of all toxins were significantly reduced as compared with negative control. The remarkable reduction in weight and length was shown by Vip3A_4K1 (2.7 ± 1.33 mg/4.44 ± 0.89 mm) and Vip3A_986 (3.58 ± 1.59 mg/4.88 ± 0.79 mm), but these values were not significantly different from each other (Table S3; Fig. S17). Similarly, there was no significant difference observed in mortality between Vip3A_792 and Vip3A_4E3. However, Vip3A_792 was significantly more effective in reduction of weight (6.4 ± 2.49) as compared to Vip3A_4E3 (12.95 ± 2.77). This data revealed that Vip3A_986, Vip3A_4K1 and Vip3A_792 were comparatively more toxic and were affecting insect growth than Vip3A_4E3. Therefore, determination of LC_{50} of these four toxic proteins against *H. armigera* along with Vip3Aa44 used as a positive control was carried out.

In addition to these four toxins, a bioassay was also performed with a 10 ppm concentration of Vip3A_851 and Vip3A_306. Both these toxins showed similar mortality after the 4th d, and Vip3A_306 showed more percent mortality than Vip3A_851 after the 7th d. Compared to previously tested toxins, Vip3A_306 showed higher mortality than Vip3A_4K1 and Vip3A_986 (data not shown) and was also taken for LC_{50} determination.

Determination of LC_{50} of Vip3A proteins

Five proteins, Vip3A_792, Vip3A_986, Vip3A_4K1, Vip3A_851 and Vip3A_306 from our study, along with Vip3Aa44 used as positive control and one negative control (double distilled sterile water) have been taken for LC_{50} determination. To determine LC_{50}, bioassay was performed with 5 concentrations, viz. 0.1 ppm; 1 ppm; 5 ppm; 10 ppm; and 50 ppm. The LC_{50} values (Table 2) ranged between 0.921 and 8.513 ppm for different Vip3A-type proteins. Based on LC_{50} values, this data showed higher toxicity of Vip3A_986, followed by Vip3A_306, Vip3A_851, Vip3A_4K1, Vip3A_792 and Vip3Aa44. Further the toxicity of Vip3A_986 was significantly higher than the other proteins except Vip3A_306, which was at par. Least toxicity was observed in Vip3Aa44 and Vip3a_792 compared to other proteins. The overlapping 95% fiducial limits clearly indicated that some of the proteins were not significantly different in terms of their toxicity against *H. armigera.* The dose–mortality regression analysis showed significant relation with high value of coefficient of determination ($R^2 > 0.8$) between the dose and the mortality in case of all the proteins (Fig. 4). The non-rejection of hypothesis of parallelism further confirmed the variable levels of susceptibility *H. armigera* to different Vip proteins as indicated by the bioassays. The bioassays with different Vip proteins indicated ninefold

Table 2 Mean lethal concentration LC_{50} in ppm of Vip3A-type proteins along with Vip3Aa44 used as positive control against *H. armigera* evaluated after 7 days in laboratory bioassay under controlled conditions

S.No	Gene name	Accession No	LC_{50} ppm	95% Fiducial limits	Slope ± SE	Chi-square (χ^2)
1	Vip3Aa44	HQ650163	8.513[a]	2.755–21.193	0.863 ± 0.230	1.486
2	Vip3A_792	MN120477	7.131[a]	0.814–44.029	0.504 ± 0.171	2.815
3	Vip3A_4K1	MN120478	2.362[b]	0.175–10.241	0.484 ± 0.153	1.072
4	Vip3A_986	MN120479	0.921[c]	0.011–4.847	0.393 ± 0.138	0.314
5	Vip3A_851	MN120481	2.106[b]	0.790–4.937	0.591 ± 0.123	2.545
6	Vip3A_306	MN120482	1.461[c]	0.508–3.317	0.601 ± 0.123	2.744

The LC_{50} values with different letters are significantly different from each other. The difference has been considered at par when the 95% Fiducial limits overlapped

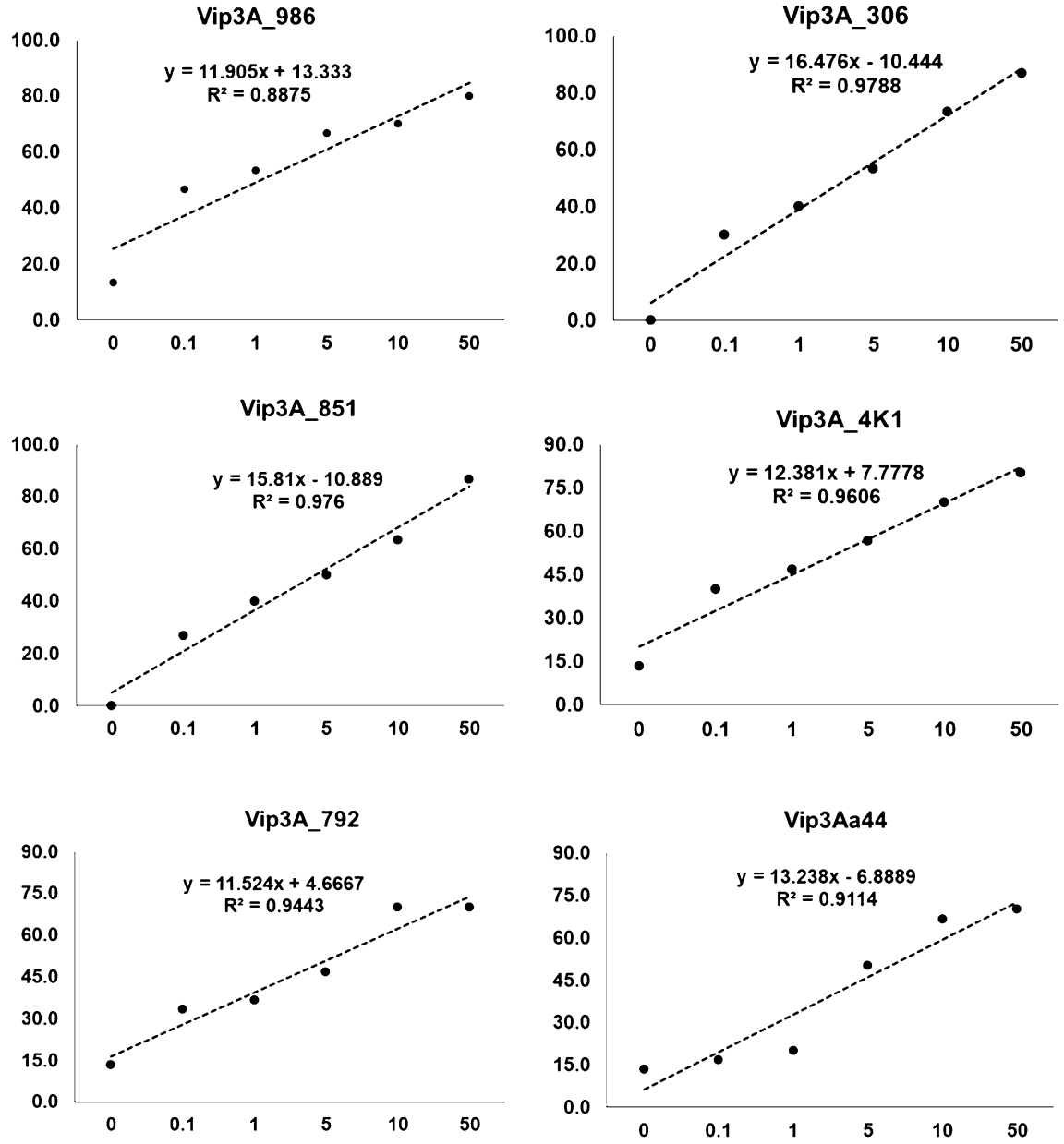

Fig. 4 Dose to mortality regression analysis of different Vip proteins. The high value of the Coefficient of determination ($R^2 > 0.8$) is indicative of the strong relationship between the dose and the toxicity of the respective proteins. The comparatively flat lines of the Vip3Aa44 and Vip3A_792 indicate their low sensitivity to the *H. armigera*

and sevenfold higher toxicity of Vip3A_986 compared to Vip3a44 and Vip3A_792, respectively. The narrow slope values of the various Vip proteins (Table 2) and parallel regression lines further supported the rejection of hypothesis of equality, indicating difference in toxicities of these proteins (Fig. 5).

Observations for cumulative mortality

The cumulative mortality including larval and pupal death was also recorded at each concentration for three proteins Vip3A_792, Vip3A_986 and Vip3A_4K1 along with positive control (Vip3Aa44). Larval mortality was found to be 100% at 50 ppm with majority of larvae remaining in the 1st or 2nd instar stage even after 7th or 10th d. Cumulative mortality with 10 ppm concentration of each toxin was also observed to be 100%. There was a clear trend of higher

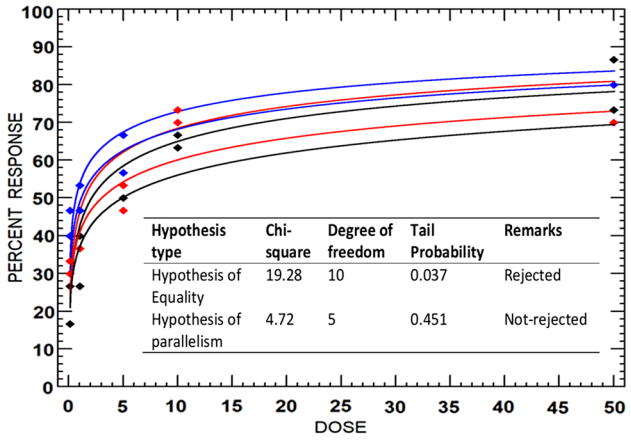

Fig. 5 Test of the hypothesis of Parallelism and Equality. The parallel lines of the figure clearly indicate that all the toxin proteins have the same mode of action and thus not-rejecting the Hypothesis of Parallelism. The Hypothesis of Equality stands rejected indicating differential toxicity levels of the toxin proteins

cumulative mortality with 50 ppm concentration of toxins, followed by 10 ppm, 5 ppm, 1 ppm and 0.1 ppm. This data represents that Vip3A_986, Vip3A_4K1 and Vip3A_792 proteins showed more mortality than Vip3Aa44 at 0.1 ppm and 1 ppm. The cumulative mortality was significantly different than the negative control. However, there was no significant difference in cumulative mortality between treatments and positive control at 5 ppm, 10 ppm and 50 ppm concentrations. Pupal mortality was seen at each concentration with maximum with Vip3A_4K1 followed by Vip3A_986, Vip3A792 and Vip3Aa44 (Table S4).

Effect of toxic proteins on the life cycle and different growth stages of *H. armigera*

Effect on larval growth and development

The weight and length of the larvae after 7th d were measured, and it was observed that there was a significant reduction in average weight and length of insect larvae with

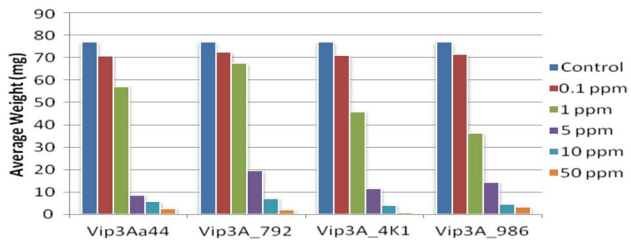

Fig. 6 Effect of 0.1 ppm, 1.0 ppm, 5 ppm, 10 ppm, and 50 ppm concentration of proteins on average larval weight (mg) in comparison to water control in laboratory bioassays under controlled conditions

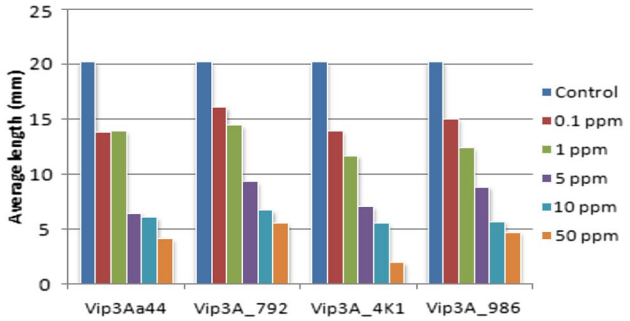

Fig. 7 Effect of 0.1 ppm, 1.0 ppm, 5 ppm, 10 ppm, and 50 ppm concentration of Vip3A proteins on average larval length (mm) in comparison to water control in laboratory bioassays under controlled conditions

each concentration of proteins Vip3A_792, Vip3A_4K1, Vip3A_986 and positive control Vip3Aa44 as compared to the negative control (data not shown). The most significant reduction was observed in larvae that consumed the diet incorporated with 50 ppm concentration of each protein, followed by 10 ppm, 5 ppm, 1 ppm and 0.1 ppm. There was normal weight and height of larvae surviving on a negative control diet (Figs. 6, 7). The larval growth was affected by the treatment of each protein, with the maximum reduction in growth in larvae treated with Vip3A_4K1, followed by Vip3A_986, Vip3Aa44 and Vip3A_792 (Fig. S18).

Effect on pupal and adult development and their morphological characters

Further, the developmental behavior of the treated insects, which survived, was analyzed in comparison with the control. Changes were observed at pupal and adult stages, in terms of relative growth inhibition as compared with control. All treatments showed a reduction in percent pupation and adult emergence with delay in larval and pupal periods. Surviving larvae (86.67%) in the water control treatment showed pupation and adult emergence. In contrast, almost all insects were dead at 10 ppm and 50 ppm concentrations of toxin treatments and larvae did not proceed beyond the 2nd instar stage. They did not reach up to 3rd instar after 7 d of treatment. Only 6.67%, 3.33%, 3.33% pupae were developed at 10 ppm concentration of Vip3Aa44; Vip3Aa_4K1; and Vip3A_986, respectively, and adults did not emerge from these pupae subsequently. Malformed larvae, pupae and adults were observed in all remaining treatments at 0.1 ppm, 1 ppm and 5 ppm. The miniature pupae having reduced weight and size as compared to control pupae were also detected during the life cycle. Their observed weights ranged between 11 and 148 mg (148 mg, 106.9 mg, 82.6 mg, 144.5 mg, 18.6 mg and 11 mg)). These weights were significantly lower than

the average weight (284.5 mg) of control pupa. Although adult emergence was higher in Vip3Aa44 treatments, the percentage of malformed adults was also maximum in larvae treated with this protein. A significant reduction in percent pupation and adult emergence was observed in insects treated with Vip3A_986. Although Vip3A_4K1 was more toxic than Vip3A_792, we found more pupation and adult emergence in insects treated with Vip3A_4K1 treatments at 0.1 ppm (66.67% and 56.67%, respectively) and 1 ppm (63.33% and 53.33%, respectively) as compared to Vip3A_792 at 0.1 ppm (50% and 36.67%, respectively) and 1 ppm (36.67% and 23.33%, respectively) (Table S5). This data indicates that despite being less toxic than other Vip3A_proteins in terms of mortality, Vip3A_792 and Vip3Aa44 affected the development stages of insects. This kind of toxic behavior can be termed as sublethal effect of the toxin.

There was no adult emergence from pupa formed at 10 ppm up to 48 d of life cycle, and the larval period was delayed from 31 ± 0 to 41 ± 0 d in treatments as compared to the larval period (15 ± 0.40 d) and pupal period (10 ± 0.68 d) of control. There was also delay in larval period ranging from 16.5 ± 0.60 to 28.5 ± 1.43 d and pupal period ranging from 10.5 ± 0.76 to 14.5 ± 0.64 d in treatments at 0.1 ppm, 1 ppm and 5 ppm. Furthermore, delay in the larval period was more as compared to the pupal period at these three concentrations. Also, there was an increase in delay in days to larval and pupal stages with increasing concentrations of Vip3A_proteins (Table S6). Therefore, it is inferred that these Vip3A_proteins have toxic effects and sublethal effects on the growth and fecundity of the insect population.

The photographs of normal growth with water control (Fig. S19); non-viable pupal-adult intermediates (Fig. S20); and the effect of different concentrations of proteins on different stages of growth were taken (Figs. S21; S22; S23; S24).

Molecular docking of Vip3Aa protein with receptor and its analysis

The crystallography model of Vip3Aa16 (6TFJ) is available (Núñez-Ramírez et al. 2020). It was used for in silico docking with *H. armigera* FGFR (Accession number: XP_021186745.1) since the interaction between Vip3 proteins and fibroblast growth factor receptor (FGFR) from *Sf*9 cells of *S. frugiperda,* which initiates apoptosis, has been confirmed (Jiang et al. 2018a, b). Figure 8A shows the interaction of Vip3Aa monomer with FGFR monomer. The activated model of Vip3Aa interacts with receptor at mainly N- and C-terminal. This was used to model the protein–protein interaction in the tetramer form (Fig. 8B). Analysis of protein sequence revealed that 36% of protein consists of flexible residues. Most of the mutations noted in the newly identified vegetative insecticidal proteins are located in the rigid portion (Fig. S25). All the mutations lead to destabilization of the protein, ranging from -0.6 to -1.8 ddG (Table S7). Destabilization of the backbone may impart flexibility to the protein molecule, which is necessary for the functional dynamics that Vip proteins may require to get activated and interact with receptors to initiate further processing.

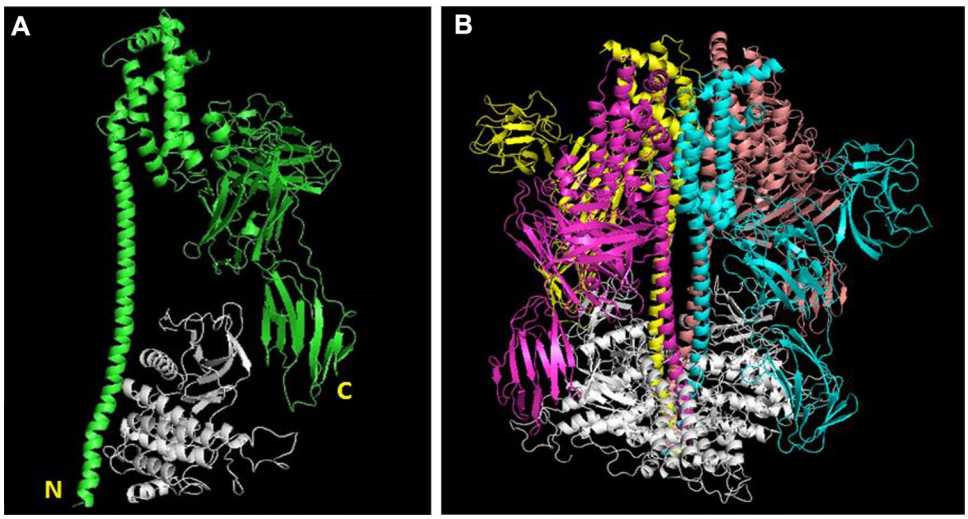

Fig. 8 Molecular docking of Vip3Aa protein three-dimensional model with *H. armigera* FGFR **A** Interaction of Vip3Aa monomer with FGFR monomer. N- and C-terminal residues of Vip monomer are marked **B** Quaternary representation of Vip3Aa tetramer with FGFR receptor. Vip monomers are shown in pink, cyan, yellow and magenta, while the receptor is shown in white color. The flexibility of the protein was predicted through 'Multiclass flexibility prediction from sequences of amino acids (MEDUSA) algorithm

Discussion

Comparative analysis of deduced amino acids sequence of all *vip3Aa*-type genes cloned in this study with that of previously cloned *vip3Aa44* and with holotype *vip3Aa1*

Comparative analysis of deduced amino acid sequence of 12 *vip3A*-type genes cloned in this study with *vip3Aa44* (previously cloned in our laboratory and used as positive control in this study) and holotype *vip3Aa1* genes revealed that amino acid substitutions were found throughout the genes at positions between amino acids 31 to 782 as shown in Table 1. Maximum (9) substitutions were found in Vip3A_4K1 **(Vip3Aa68)** and Vip3A_1328 **(Vip3Aa78),** followed by 7 in Vip3A_1340 **(Vip3Aa75)**, 6 in Vip3A_851 (**Vip3Aa70)**, 5 in Vip3A_1065 **(Vip3Aa74)**, 4 in Vip3A_4E3 **(Vip3Aa72)** and Vip3A_671 **(Vip3Aa73)**; 3 in Vip3A_986 **(Vip3Aa69)** and Vip3A_1386 **(Vip3Aa76)**; 2 in Vip3A_792 **(Vip3Aa67)**, Vip3A_306 **(Vip3Aa71)** and Vip3A_1401 **(Vip3Aa77)**. Vip3Aa44 protein, used as positive control, has only one substitution at amino acid position 284, as compared with the holotype Vip3Aa1 protein (Q/K). This substitution at position 284 was common in all the genes.

The substitution of N (Asparagine) with K (Lysine) at amino acid position 270 was common in 6 proteins, namely Vip3A_306, Vip3A_671, Vip3A_1065, Vip3A_1340, Vip3A_1386 and Vip3A_1401. Although Vip3A_306 and Vip3A_1401 were cloned from *Bt* isolates recovered from different agro-climatic zones, we found the same kind of substitutions at the same positions, viz. 270 and 284. The genes Vip3A_1386 and Vip3A_1401 were from *Bt* isolates from Eastern Himalayan Region, but these two genes were different from each other in that; D (Aspartic Acid) was substituted by G (Glycine) at position 45 in the deduced amino acids sequence. The nucleotide sequence of all the 12 genes was submitted to NCBI GenBank, and accession numbers were obtained (Table 1). It is already known for Cry proteins that slight variation in amino acid residues can significantly affect insecticidal action (Schnepf et al. 1998). Previous studies support this fact in the case of Vip proteins also, as it has been reported that variations at one or more amino acid positions could influence their toxicity (Estruch et al. 1996; Doss et al. 2002; Liu et al. 2007). Each *Bt* isolate may have the potential to encode Vip proteins with significant variation and toxicity (Güney et al. 2019). Thus, investigation of the presence of new *Vip* genes, variation at amino acids level and their toxicity analysis against specific target insects are important to evaluate the potential of *Bt* isolates belonging to different habitats and geographical locations. In our study, we have successfully cloned 12 *vip3A*-type genes with different amino acids substitutions in their protein sequences, from *Bt* isolates recovered from diverse habitats and agro-climatic zones.

Comparative analysis of genes identified in our study with other lepidopteran-specific *vip3Aa*-type genes available in the database

The analysis of the deduced amino acid sequence of 32 *vip3A*a genes was performed through MEGA-X. It revealed that all proteins have common amino acid residue lysine (K) at position 284 except Vip3Aa1. The amino acid substitutions among proteins ranged between positions 2 to 784. The proteins in the present study had new variations at amino acid positions, as compared with those reported earlier by other workers, except for Vip3A_4K1, which had 9 amino acids substitutions, that were same as already reported in Vip3Aa65. The gene Vip3Aa59 is also similar to these two genes except at one position, viz. 784, and Vip3Aa14 contains 18 amino acid substitutions including 9 substitutions which are the same as identified in this study. Two similar substitutions are also present in Vip3Aa19 at positions 358 (I/V) and 633 (N/T). The substitutions of Vip3Aa61 are also present in Vip3Aa19, except for one position. Phylogenetic analysis also clustered these five genes, viz. Vip3A_4K1, Vip3Aa65, Vip3Aa59 and Vip3Aa14, in one group (Fig. 3). Vip3A_851 was grouped with Vip3Aa18. Vip3A_792 and Vip3A_1328 also had similarity at position 570 with A (Alanine) despite two and nine substitutions, respectively, and were clustered together. The third group was formed by Vip3A_1386, Vip3A_306, Vip3A_671, Vip3A_1401 and Vip3A_1065, because all these have one similar substitution at 270 (N/K). Vip3A_1340 was also found to be closely related to this combination. The reason may be that despite having variations at 7 sites, one site at position 270 was also common in Vip3A_1340. Vip3Aa29 was out grouped from the tree because this gene had variations only at N and C- terminus and at 284 position which was entirely different from other genes. Two genes, Vip3A_986 and Vip3A_4E3, had dissimilar substitutions with each other and the rest of the genes. All these genes: Vip3A_986 and Vip3A_4E3, along with Vip3Aa1, Vip3Aa7, Vip3Aa9, Vip3Aa10, Vip3Aa11, Vip3Aa13, Vip3Aa16, Vip3Aa43, Vip3Aa44, Vip3Aa45, Vip3Aa 50, Vip3Aa58, Vip3Aa64, were clustered in one group.

Overall, new substitutions were found for each gene in our study except Vip_4K1, as compared with other lepidopteran-specific *vip3Aa* genes. Banyuls et al. (2018) have reported 19 critical amino acids positions, whose substitutions significantly affect the insecticidal activity of Vip3Af proteins. These include positions near to N-terminus [167(T), 168 (E), 171 (P), 209 (L), 229 (F), 238 (M), 242 (N), 244 (F),

246 (R), 255 (L), 272 (Y)]; positions 483 (E) and 552 (W) in the middle; and positions [(689(G), 699 (I), 711 (L), 719 (Y), 727 (G), 741 (F)] near to C-terminus. These positions and amino acids residues are also similar in Vip3Aa1, except 689 (G/S) and 699 (I/L). Therefore, these positions can also be considered critical for the activity of Vip3Aa-type proteins. It is noteworthy that no substitutions were found in these sites in our genes, indicating that critical residues were retained. Nevertheless, the new substitutions in our genes may exhibit effective insecticidal toxicity against diverse kinds of lepidopteran pests.

Protein expression and immunoassays

In present study, six new Vip proteins were investigated for their potential in biological control of *H. armigera*. The extracellular fraction of culture of recombinant clones was used for protein expression, as reported in several other reports related to Vip3 proteins, wherein supernatant fraction has been used for protein expression studies. In some other studies, it has been reported that although Vip3Aa protein expression was found in both insoluble fraction and pellet (Liu et al. 2007; Palma et al. 2013), but the expression was found to be more in the supernatant fraction (Liu et al. 2007). This has the advantage of ease in protein production, since extracellular medium does not require elaborate downstream handling for protein purification like the intracellular fraction. The Vip3 proteins were stable as these were detected in culture supernatants even after 15 h of inoculation to sporulation phase (Estruch et al. 1996; Mesrati et al. 2005). Initially, the presence of Vip3A protein in induced samples was confirmed through LFS immunoassay, which confirmed that leaky expression of the protein of interest that is Vip3A protein, was absent in uninduced samples (Fig. S13). The protein expression was induced with 1 mM IPTG induction and protein concentration increased after 4 h induction at 37 °C. An increase in the concentration of Vip3A protein over time has been reported by other workers as well (Ben Hamadou-Charfi et al. 2015; Lone et al. 2016). Further, the *ca* 89 kDa band with increased intensity with time was observed on SDS-PAGE gel (Fig. S14). The presence of particular proteins was confirmed by western blotting using anti-Vip3A-polyclonal antibodies. One of the methods to increase the specificity of the antibody is the pre-adsorption test (Swaab et al. 1978; Burry 2000; 2011) which has also been used to enhance the specificity of Vip3A polyclonal antibody by reduction of cross-reactivity in the present research study. The polyclonal serum was pre-adsorbed with supernatant of negative control which resulted in the removal of non-specific bindings from uninduced samples of recombinant clones and also reduced the cross-reactivity in induced samples (Fig. S16A and S16B). The non-specific binding with polyclonal Vip3A antiserum has been reported by other researchers as well, possibly due to the presence of proteins that resemble Vip3A (Li et al. 2007; Ben Hamadou-Charfi et al. 2015). The protein which was expressed constitutively in *E. coli* cells and which was recognized by antiserum of Vip3 along with specific 89 kDa band was considered as an internal control for sample loading as mentioned in previous study (Sellami et al. 2013). Similarly, there may be some constitutively expressed proteins in our sample, which may also have similar kinds of epitopes and thereby resemble Vip3A proteins. Thus, owing to these characteristics, these proteins might be recognized by the Vip3-polyclonal antibodies. This may explain non-specific cross-reactions observed in the western blot in our study.

Effect of toxins in bioassays with H. armigera

The lepidopteran pest *H. armigera,* a polyphagous pest of several important crops, was selected for the determination of the toxicity potential of expressed proteins. This insect has become a serious pest as it causes severe economic loss, has developed resistance to chemical pesticides and has even exhibited resistance to some of the *Bt* crystalline proteins (Tabashnik and Gould 2012; Warren 2013; Yang et al. 2013). In this study, the data showed the difference in the toxicity of Vip3Aa proteins toward *H. armigera* at a 5% probability level. Nevertheless, all the tested proteins were toxic toward *H. armigera,* as indicated by their LC_{50} values. The toxicity results found in this study were similar to the toxicity data reported by other researchers. The toxicity results of Vip3A_4K1 (2.36 ppm) were also similar to toxicity results of affinity-purified Vip3Aa65 (1650 ng/cm^2 or 1.65 µg/cm^2) against *H. armigera* (Şahin et al. 2018) which are 100% similar to each other in respect to amino acid substitutions. Thus, the almost similar toxicity results support our toxicity data and confirm that the supernatant fraction is similarly effective as the purified protein. Another protein Vip3Aa16 was also reported to be toxic against *H. armigera* with LC_{50} 1330 ng/cm^2 or 1.33 µg/cm^2 (Şahin et al. 2018). A range of LC_{50} 161.80 ng/cm^2 to 178.60 ng/cm^2 for 4 *vip3A* genes isolated from *Bt* isolates along with 190.42 ng/cm^2 for *Bt* HD-1 reference strain has been reported (Shingote et al. 2013). Another study reported the LC_{50} values for 700 bp long partial Vip3A toxin isolated from 7 Indian *Bt* isolates varied between 115.96 µg/ml and 246.60 µg/ml in comparison with LC_{50} value (105.75 µg/ml) for HD-1 (Lone et al. 2016). The potentiality of crude protein extract has been discussed, and LC_{50} values ranging between 9.09 and 42.02 µg/ml have been determined against *H. armigera* for full-length Vip3A toxins identified from 8 *Bt* isolates (Rangeshwaran et al. 2016), which also support our findings. Similarly mean lethal concentrations were reported against *H. armigera* as 325 ng/cm^2 or 0.325 µg/cm^2 for Vip3Aa10 (Doss et al. 2002); 160 ng/ml or 0.16 µg/ml for Vip3Aa13

(Chen et al. 2003); 89.06 ng/ml (Sattar et al. 2008); 25.7 ng/mg for Vip3Aa11, 24.1 ng/mg for Vip3Aa19 (Liu et al. 2007); 22.6 µg/ml for Vip3Aa29 (Yu et al. 2012); 1660 ng/cm^2 for Vip3Aa1 (de Escudero et al. 2014); and 169.63 ng/cm^2 or 0.169 µg/g for Vip3Aa61 (Lone et al. 2018). These Vip3A proteins exhibited significant differences in toxicity against *H. armigera* despite having only a little variation at the amino acid level (Chakroun et al. 2016). The same situation has also been observed with our proteins as there was variation at 2 sites in Vip3A_306, 3 sites in Vip3A_986, 6 sites in Vip3A_851 and 9 sites in Vip3A_4K1 but they also exhibited varying degree of toxicity against *H. armigera*. The Vip3A_986 and Vip3A_1386 proteins have three different amino acids compared to Vip3Aa16, which has previously been shown to have toxicity against *S. littoralis* and *E. kuehniella* according to Abdelkefi-Mesrati et al. (2011). As per the study of Zack et al. (2017), the majority of sequence diversity was found to be located at the C-terminal and further, both N and C-terminal were found to be important for insecticidal activity and specificity. It is noteworthy that the sequence diversity was found to be at N-terminal in Vip3A_986, Vip3A_1386, Vip3A_306 and Vip3A_1401 proteins and was observed to be at the C-terminal in other remaining Vip3Aa proteins identified in our study.

All studies have reported distinct variations in LC_{50} values of Vip3A proteins against *H. armigera*. While it may corroborate the view that changes of only a few amino acids may considerably impact toxicity, the observed differences could also be due to different experimental conditions. These conditions might be the use of different insect strains and stages, the sensitivity of geologically separated insect populations, diverse toxin properties, the difference in amino acid residues and processing of toxic protein, diet components, bioassay technique, protein estimation method, the temperature of the bioassay chamber, etc. (Avilla et al. 2005; Bird and Akhurst 2007; de Escudero et al. 2014). Different populations of *H. armigera* in India have been reported to have different susceptibility towards Cry toxins (Jalali et al. 2010).

The variation in insecticidal activities against different insects observed by various authors could also be due to difference in protoxin-hydrolysis rates in the insect's midgut (Chakroun et al. 2012; Boukedi et al. 2015). According to an experiment where BBMV ligand blotting was performed with Vip3 (459) toxin, it was suggested that the differences in the number of potential receptors recognized by these toxins could be a contributing factor to the varying levels of effectiveness of the toxins against the same lepidopteran host (Boukedi et al. 2017a). Subsequently, it has been observed that Vip3Aa and Vip3Af demonstrated comparable toxicity despite binding to distinct putative receptors. While Vip3Aa was found to specifically bind to BBMV of susceptible insect *S. littoralis*, it was also reported to bind specifically to the non-susceptible insect *O. nubilalis*, indicating that insecticidal activity cannot be attributed solely to specific binding. This variability could be ascribed to irreversible binding effects and binding affinity, which have been recognized as significant factors in determining insecticidal specificity and activity (Boukedi et al. 2018b).

An exhaustive study related to Vip3Aa1 protein recovery at individual steps of the purification process provided useful evidence about an average recovery of purified protein of only up to $50.4 \pm 4.0\%$, which is much lower than the recovery of other kind of proteins after purification (Hernández-Martínez et al. 2013). There were also reports of co-purification of other proteins with histidine-tagged proteins present in *E. coli* supernatant fractions. There may be various reasons for this co-precipitation, such as the existence of histidine residues on particular proteins, some have natural metal kind of motifs, and some proteins also have an affinity for agarose-based columns and histidine-tagged proteins (Bolanos-garcia and Davies 2006). Two different research groups have reported the loss of toxicity of Vip3A proteins due to purification through metal-chelate chromatography (Hernández-Martínez et al. 2013; Baranek et al. 2017). It was demonstrated that the toxicity of Vip3Ae protein against *S. frugiperda* and *Agrotis ipsilon* was significantly affected, approximately fivefold, due to purification through affinity chromatography (metal-chelate). These authors further confirmed that there was no significant difference in the activity of crude extracts as protoxin or activated toxins. Vip3A proteins that had partially lost their activity during purification showed only the growth inhibition effect on larvae. This loss of activity was described through leakage and binding of Ni^{2+} ions of the column to the 6 histidine residues present at the N-terminus of these proteins. This binding leads to permanent conformational changes in particular protein, which is not abolished even after removal of N-terminus after trypsin digestion (Hernández-Martínez et al. 2013). The Vip3A-protein aggregation was also observed during elution through imidazole (Palma et al. 2013).

Therefore, in our study, the supernatant fraction was used for toxicity analysis and filtered with a 0.45 µM and thereafter with 0.22 µM membrane filter. This fraction was not further purified because there are studies available in the literature, which do not support the purification of Vip3A-type proteins for the purpose of toxicity evaluation. In previously reported studies, crude protein extracts of Vip3-type proteins were consistently found toxic against lepidopteran pests *S. exigua*, *S. litura*, *S. littoralis*, *A. epsilon*, *H. armigera* (Sattar et al. 2008; Hernández-Martínez et al. 2013; Rangeshwaran et al. 2016). Some studies have also reported whole bacterial culture more potent (ninefold) than Vip3A protein fraction alone, against whiteflies *Bemisia tabaci* (El-Gaied et al. 2014). In a recent study, it has been demonstrated that a relatively higher LC_{50} value (389 ppm) with Vip3 expressed

protein, as compared to 89 ppm, has been observed after treatment of whiteflies with whole bacterial culture. This study suggested that expressed Vip3 proteins required 12 h more to kill 50% of insects after treatment as compared to bacterial culture (El-Gaied et al. 2020). This study favored the use of whole cell culture rather than expressed Vip3 proteins fraction. Future experiments involving further processing of lysate fraction by isoelectric precipitation and anion exchange chromatography or ammonium sulfate precipitation may yield further insight into the effect of these treatments on the observed toxicity. It will also be interesting to use S-tag monoclonal antibody to determine the level of expression of Vip3A-type protein in further experiments.

Effect of Vip3A_toxins on the growth and developmental stages of *H. armigera*

Among the 6 proteins, the effect of 3 proteins Vip3A_792, Vip3A_4K1, Vip3A_986 as compared to Vip3Aa44 used as a positive control was studied on growth and various stages during the life cycle of *H. armigera*. A significant reduction in surviving larval weight and length was found in this study after 7 d, as mean larval weight and length of individual larvae have been measured after treatment with 10 ppm concentration of toxic proteins, as: Vip3A_4K1 (2.7 mg; 4.44 mm); Vip3A_986 (3.58 mg; 4.88); Vip3A_792 (6.4 mg; 6.1 mm); Vip3A_4E3 (12.95 mg; 8.0 mm); and Vip3Aa44 (7.95 mg; 5.8 mm) in comparison with water control (85.13 mg; 17.1 mm). Maximum number of larvae treated with 50 ppm concentration remained in only the first or second instar stages and did not progress to subsequent stages of the life cycle during bioassays with each toxin in comparison with the water control. These observations were in agreement with Rang et al. (2005), as these authors have observed total weight of all larvae of *Ostrinia nubilalis* after being treated with Vip3Ba1 to be 0.0055 g, in comparison with the total weight of control larvae which was 0.1124 g. Their study also reported that larvae of *O. nubilalis* remained in the first instar stage and larvae of *P. xylostella* remained in the second instar stage. In addition to this, defective larval growth has been reported in their study.

The significant effect on percent pupation, adult emergence and different kinds of malformed stages, viz. malformed larvae, malformed pupa, miniature pupa, non-viable pupal-adult intermediates and malformed adult, has been observed in our study. There was a significant delay in the larval and pupal period, which resulted in the extended life cycle of *H. armigera*. Treatment with Vip3A_986 protein significantly reduced the percent pupation and adult emergence. The extended life cycle due to delay in larval and pupal formation and development of malformed stages will also affect the fecundity and population dynamics of insects. Thus, later on, it will drastically reduce the frequency of production of the new insect population. These sublethal effects of *Bt* Cry toxins on physiological and behavioral traits of the target insect population have been studied by various researchers (Erb et al. 2001; Biondi et al. 2013). The maximum studies relied only on an acute lethal dose of insecticidal proteins, but this might be an incomplete analysis of the harmful effect of the particular toxin on insect population, because not all insects of a target population in the field are likely be exposed to the same dose of a particular toxic protein. Hence, sublethal effects must be taken into consideration for a comprehensive analysis of the efficacy of toxic protein against target insects (Desneux et al. 2007; Esmaeily et al. 2014). The sublethal effects can be explained as the effect of toxins at the low dose, which does not cause mortality, but affects the behavioral and physiological response of surviving larvae (Desneux et al. 2007; Tan et al. 2012). In corroboration with our observations, the significant sublethal effects of Vip3Aa19 on the development stages (larval and pupal period, egg viability, fecundity, adult life span) of *P. xylostella* and *Heliothis virescens* have already been reported (Gulzar and Wright 2015). The increase in larval development time was reported for various toxins like Cry1Ab on *Helicoverpa zea* (Horner et al. 2003); Cry3Bb1 on *Diabrotica virgifera* (Meissle et al. 2009). Also, the increase in larval molting was reported after treatment with Cry1Ab (Pérez-Hedo et al. 2011) and reduction in larval weight of *Crioceris duodecimpunctata* L. after treatment with Cry3Aa (Gao et al. 2012); and Cry1Ac on *H. armigera* (Lomate and Hivrale 2013). The adverse effect of Cry1Ac on the F_1 off-springs of lepidopteran pest *Chlosyne lacinia* (Sunflower patched) like higher mortality, longer developmental period as compared to parental population, because of transfer of toxin to eggs has been reported (Paula et al. 2014). Also, reduced food intake, larval growth and percent pupation (Muñoz et al. 2014) have been reported. The higher larval mortality and reduced growth, development of smaller/miniature pupae, non-viable larval–pupal intermediates and deformed adults have been demonstrated after hemocoelic treatment of Cry toxins to the third instar of *Achaea janata* (Castor semi-looper). The possible cause of reduced growth may be explained through the lysis of the fat bodies by lysozymes in the insect hemolymph, as this study found large cytoplasmic cavities in the tissues of fat bodies (Ningshen et al. 2017). The histopathological effects, viz. cytoplasmic vacuolization, swollen cells and mitochondria, disruption of midgut epithelial cells and brush border membrane have been demonstrated through TEM images after treatment of Vip3Aa protein (AF500478) to *S. litura* larvae (Song et al. 2016).

It is an established fact that the larval stage of insects has fat bodies that metabolize primarily during the metabolic process. Thus, injury to this organ could disrupt whole metabolic processing and extensively influence the

metamorphosis, larval growth and development of any insect in a negative manner (Price 1973; Haunerland and Shirk 1995; Burmester and Scheller 1999). The disintegration of fat bodies could also affect the food accumulation and disturb the insect ingestion and digestion process of insect midgut, which in general upset the nutrient supply to larvae (Ningshen et al. 2017). Thus, sublethal toxicity even at the larval stage impacts the complete life cycle of target insects and can be useful from the perspective of insect pest control.

Molecular interactions with FGFR receptor of *H. armigera*

Since Vip3 proteins have been reported to exist as tetramer (Zack et al. 2017), we have analyzed the molecular interactions of Vip3Aa16 with *H. armigera* FGFR in both the monomeric and the tetrameric forms (Fig. 8A, B) and have observed that both, the N- and C-terminal of the protein, are involved in interaction with FGFR receptor. The central region appears to be responsible in giving proper fold orientation for maximal interaction with the receptor. A significant portion (36%) of protein consists of flexible residues. The observation that most mutations in the newly identified Vip3A proteins were mapped in the rigid portion in the three-dimensional structures of the proteins and may be thus destabilizing in nature implicates the importance of flexibility in the overall molecular function of protein. Overall protein flexibility is reduced upon increase in thermostability (Vihinen 1987). Destabilization of the rigid residues may impart higher flexibility, leading to ease in protein folding and receptor recognition. This may explain the higher toxicities observed with these new Vip3A proteins. Furthermore, Vip_986 contains amino acid substitutions in the N-terminal region, which are in proximity to the receptor in the docked ligand–receptor complex. N-terminus of Vip3A proteins is involved in receptor recognition (Gupta et al. 2021). More flexibility in N-terminus may enhance interaction with the receptor which may result in higher activity of the protein. The substitution N270K in Vip_306 protein is located in the transmembrane region, and higher flexibility in this region may allow more dynamics to the protein than otherwise. Furthermore, the Vip3A protein was found to interact computationally with *S. frugiperda* FGFR receptor, implicating its possible applicability to related receptor proteins in other pathogens as well.

Conclusion

This study identified 12 new *vip3Aa* genes and has given insight into the potential of Indian native *Bt* isolates for identification and characterization of new types of alleles of *vip3*-genes. The genes reported in the present study have new variations at amino acid positions than the genes already available in the database, except for Vip3A_4K, which had nine substitutions which were identical to those previously reported in Vip3Aa65. Therefore, these proteins may exhibit different toxicity and specificity against lepidopteran pests. The observed sequence diversity indicates both evolutionary changes and conservation in *vip3A*-type genes from isolates from diverse zones. Further investigation of toxicity of these identified genes will enrich the *vip3* database. The insecticidal activity showed by Vip3Aa67, Vip3Aa68, Vip3Aa69, Vip3Aa70 and Vip3Aa71 against *H. armigera*, a polyphagous destructive pest, which has developed resistance against Cry proteins. The toxicity of these proteins evaluated in this study is a promising finding for further applications in crop protection. Thus, the toxicity potential of these genes would encourage their use individually or in combination with Cry proteins for better management of lepidopteran pests.

Author contribution

SK and MG designed this study. MG performed the experiments. SK guided and supervised the experiments. MG and HK compiled, analyzed the data, and performed the bioinformatics analysis. MG and HK wrote the manuscript. VK provided the insect bioassay facility and guided to conduct and supervised the bioassay experiments. SS analyzed the toxicity data using Polo V 2.0, its interpretation and prepared the dose response curves. AS performed the molecular docking and in silico analysis. AD assisted in the experiments. SK contributed to the interpretation of results and critically improved the manuscript. All authors read and approved the manuscript.

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Data availability Data and material can be made available upon request as per Indian Council of Agricultural Research guidelines.

Declarations

Conflict of interest MG declares that she has no conflict of interest. SK declares that she has no conflict of interest. HK declares that he has no conflict of interest. VK declares that she has no conflict of interest. SS declares that he has no conflict of interest. AS declares that she has no conflict of interest. AD declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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