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Screening of *vip1/vip2* binary toxin gene and its isolation and cloning from local *Bacillus thuringiensis* isolates

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ABSTRACT: Efforts were carried out to isolate vegetative insecticidal protein genes from local isolates of *Bacillus thuringiensis*. The study focused on *vip1/vip2* binary toxin, considering its insecticidal potential against coleopteran and hemipteran pests. Thirty nine *B. thuringiensis* local strains and one standard reference strain (HD 1) were screened for the presence of *vip1/vip2* gene by using a PCR approach. Among 39 isolates only four isolates (PDKV-08, PDKV-27, PDKV-28 and NCIM-5112) showed the presence of the desired gene. SDS-PAGE screened profiling of the isolates showed the presence of 95 kDa and 50 kDa protein which confirmed our PCR study. For further characterization, the *vip1/vip2* gene was cloned from the PDKV-08 isolate by using the pJET1 cloning vector. Sequence homologous analysis confirms the presence of the *vip1/vip2* gene. A further BLAST analysis also revealed that the isolated *vip1/vip2* gene is highly conserved and showed a maximum of 88% sequence homology with existing *vip*1/vip2 genes. Insect toxicological potential was also elucidated by performing bioassays of PDKV-08 supernatant proteins against the coleopteran store grain pest, *Sitophilus zeamais*. The results from a bioassay revealed 60% mortality.

KEYWORDS: PCR, insect bioassay, protein profiling, gene isolation, nucleotide BLAST

INTRODUCTION

B. thuringiensis, a Gram-positive soil bacterium, can produce insecticidal crystal (Cry) proteins or δ-endotoxins during the sporulation stage. *B. thuringiensis* has been successfully used in biological control for the last several decades[1]. Apart from being used as biopesticides, the utility of *cry* genes in developing the insect resistant transgenic plants is also well demonstrated and successful[2,3]. During the past decades, hundreds of *B. thuringiensis* endotoxin genes have been identified, sequenced, and classified according to their sequence homology[4]. However, ever increasing insect resistance and narrow insecticidal spectrums imposes threat on long-term sustainability of *Bt* technology[1,5]. Identification of novel *B. thuringiensis* toxin genes, having wide host range and new target sites, will mitigate the severity of this challenge.

In addition to δ-endotoxins, *B. thuringiensis* produces a novel family of insecticidal proteins named vegetative insecticidal proteins (Vip) during its vegetative stage[6]. These Vip proteins are produced throughout the growth phase of some *B. thuringiensis* and are secreted into the extracellular medium.

The *B. thuringiensis* nomenclature committee (BGSC) classified these vegetative insecticidal protein genes into 4 groups, 8 subgroups, 29 classes and 103 subclasses according to the encoded amino acid sequence similarity (see www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html). These types of proteins include Vip1, Vip2, Vip3, and Vip4. The Vip1 and Vip2 proteins are the components of the binary toxin that exhibits toxicity to the coleopterans[7]. Vip1Aa1 and Vip2Aa1 are very active against corn rootworms, particularly *Diabrotica virgifera* and *D. longicornis*[8], whereas Vip3 toxins are specific to Lepidopteran[6,7]. In addition, *vip* genes have also been explored to develop insect resistance transgenic crops. The transgenic cotton (VipCot), based on *vip3* gene developed by Syngenta[9] provides cotton growers a means to control bollworms, armyworm, and loopers.

The Vip1 and Vip2 insecticidal proteins were originally isolated and purified from a strain of *B. cereus* AB78, and then from *B. thuringiensis* var. *tenebrionis*[7]. Each polypeptide in the Vip1/Vip2 class of binary toxin evidently functions separately. The membrane-binding 95 kDa vip1 multimer provides a pathway for the 52 kDa vip2 ADP-ribosylase to enter the cytoplasm of target WCR cells[7]. The

NAD-dependent ADP-ribosyltransferase Vip2 likely modifies monomeric actin at Arg177 to block polymerization, leading to loss of the actin cytoskeleton and eventual cell death due to the rapid subunit exchange within actin filaments in vivo[10]. Recently, Vip1/Vip2 binary protein was identified from an isolate of *B. thuringiensis* showing toxicity towards cotton aphid (*Aphis gossypii*, Glover)[11,12]. Cotton aphids are polyphagous pests, which cause devastating damages in various economically important crops including cotton, melon, tomato, and a variety of garden ornamentals etc[13].

Considering the menace of hemipteran and coleopteran pest in crops, the present investigation was planned to see the abundance of *vip1/vip2* gene from local *B. thuringiensis* isolates by PCR approach. A new type of *vip1/vip2* obtained during the investigation was cloned and sequenced. In supporting to this, the toxicity potential of Vip1/Vip2 containing *B. thuringiensis isolates* was carried out against coleopteran insects.

MATERIALS AND METHODS

B. thuringiensis strains Used

Twenty eight local *B. thuringiensis* strains (*B. thuringiensis* PDKV-01–28), isolated from the premises of Dr Panjabrao Deshmukh Krishi Vidyapeeth, Akola and Nagpur campus of Dr PDKV Akola were used in the present investigation. These local *B. thuringiensis* isolates were subjected for PCR screening for surveying the presence of *vip1/vip2* gene. Reference strain HD1 was kindly obtained from Bacillus Genetic Stock Centre, Columbus, Ohio. Similarly eleven different subspecies of *B. thuringiensis* were obtained from NCIM (National Centre for Industrially useful Microbes), NCL, Pune.

Total genomic DNA isolation

Genomic DNA was isolated from the *B. thuringiensis* isolates as per the method given by Ausubel et al[14]. All PCR amplifications were performed using the Eppendorffs PCR thermal cycler system. Isolates were tested for the presence of *vip* genes with the designed primers.

PCR screening of *B. thuringiensis* isolates

The *vip1/vip2* genes specific primers were designed based on two known sequences of genes coding for *vip1Aa/vip2Aa* and *vip1Ab/vip2Ab*[7]. Forward primer (AAATTAGTGATCCGTTACCTTCTT) corresponds to positions 1076–1099 and the reverse primer (CAACTTGCTTTTCTTTCCCTTTAT) corresponds to positions 1794–1817 of *vip1Ab/vip2Ab* sequence. The amplified 742 bp fragment contained the 3-terminus of the *vip2* gene and the 5-terminus of the *vip1* gene. PCR conditions were 5 min initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and final extension for 15 min at 72 °C[15].

Characterization of vegetative insecticidal proteins

For insect bioassay, vegetative insecticidal proteins were obtained according to the method given by Sattar et al[11]. The protein was estimated by the Bradford method[16] and used for insect toxicity assay.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) profiling of partially purified Vip proteins was carried out by using 10% SDS PAGE, according to standard protocol[17].

Biological activity of Vip1/Vip2 binary toxins was confirmed using standard bioassay procedures. Coleoptera bioassay against *Sitophilus zeamais* adults was conducted with surface application of sorghum[7]. *B. thuringiensis* subsp. *kurstaki* was used as a positive control. In each experimental set, 10 adults were released at 28 °C. A control set containing sorghum seeds treated with only buffer solution was also maintained. The mortality observations were recorded after each 12 h.

Gene cloning and sequencing

Gene libraries of *vip1/vip2* fragments of PCR products amplified from *B. thuringiensis* strain PDKV-08 were constructed in pJET1 vector (CloneJET Kit, MBI Fermentas make). These chimeric colonies were confirmed by the formation of white colony and confirmed by relative migration analysis and PCR screening. Recombinant plasmids obtained from *E. coli* transformed with *vip1/vip2* gene were sent for the sequencing to the GeneOmbio Technologies Pvt. Ltd., Pune. Sequences obtained were converted in FASTA format and were subjected to BLAST by using the nucleotide BLAST programme available on NCBI web portal. BLAST was carried out against the non redundant (nr) nucleotide database. Highly similar sequences with lowest expect value were considered for assigning the putative class to the new sequence.

RESULTS

PCR screening of 39 *B. thuringiensis* isolates showed successful amplification of *vip1/vip2* genes in the 4 isolates (11% abundance) PDKV-08, PDKV-27, PDKV-28, and NCIM-5112. The Vip1/Vip2 protein

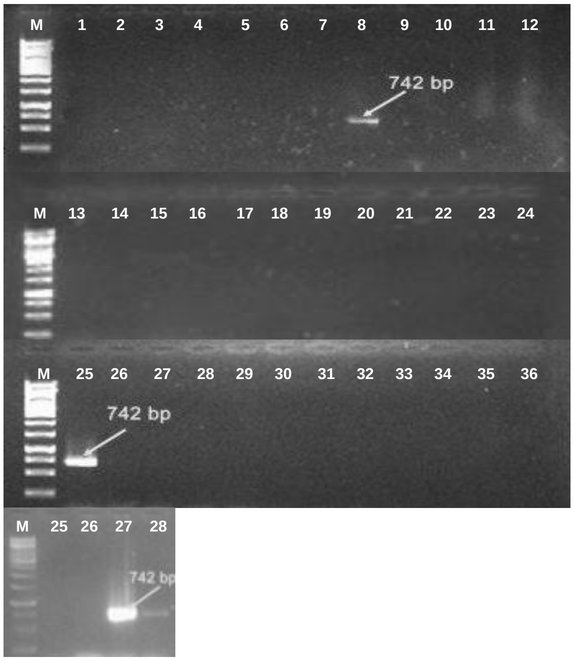

Fig. 1 Screening of *B. thuringiensis* isolates for the presence of *vip1/vip2* binary toxin gene.

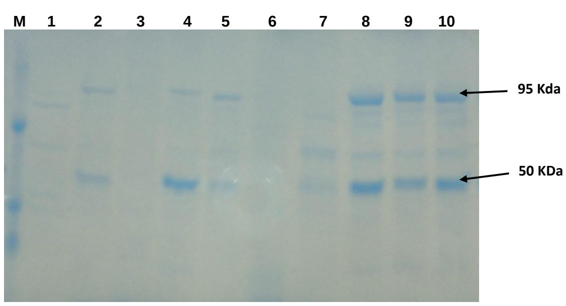

Fig. 2 SDS PAGE of selected isolates for the presence of vip1/vip2 proteins M: marker, 2: PDKV-08, 8: PDKV-27, 9: PDKV-28, 10: NCIM-5112.

will have priority in future as it possesses insecticidal activity against coleopteran and hemipteran insect pest[7].

SDS PAGE profiling of different *vip* proteins

SDS PAGE was carried out to study the presence of different *vip* proteins harboured by *B. thuringiensis* isolates under study. Earlier reports suggest that molecular weight of Vip1 protein is 47 kDa and that of Vip2 protein is around 95 kDa, respectively[11,18].

SDS-PAGE was performed by using partially purified protein, isolated from *B. thuringiensis* isolate under study. Although several common protein bands were detected among some strains, overall results clearly demonstrated a strain-specific pattern of polypeptide secretion in the culture medium, which reflect the final insecticidal potential results of the respective isolate. The isolate PDKV-08 displayed significant high larval mortality against lepidopteran pest. It is particularly relevant that a band in the position of the putative Vip1/Vip2-like polypeptide (95 kDa) appeared much more intense than in all others lanes. Also, some isolates (NCIM-5112, PDKV-27, and PDKV-28) showed the two different bands of size 50 kDa and 95 kDa which were relevant with the presence of gene *vip1/vip2* binary toxins in that isolate as indicated by arrow in Fig. 2.

Insect bioassay

The insecticidal potential of Vip1/Vip2 binary toxin was tested against coleopteran store grain pest *S. zeamais*. About 60% mortality (data not given) was obtained after 72 h. This confirms the coleopteran toxic nature of Vip1/Vip2 binary toxin. The detailed study pertaining to determination of LC_{50} is currently under way. Similar results were observed by Warren et al[7] in 1996, who identified the toxic potential of binary toxin against western corn rootworm.

S. zeamais is a major pest of store grains in tropics. Pesticides for controlling weevils are available, but the resource poor farmers cannot afford them. Also the increase in occurrence of insecticide resistance and increasing environmental concerns about the use of chemical insecticide means that alternative control methods are required[19].

Cloning of *vip* genes

The amplicon of *vip1/vip2* (742 bp) from *B. thuringiensis* PDKV-8 was amplified using gene specific primers, cloned into pJET1 vector, and transformants harbouring inserts were isolated through white colony and confirmed with gene specific amplification and relative migration analysis of plasmids.

Single pass sequencing of pJETPDKV-8 *vip1/vip*2 gene by using forward primer yielded 239 bp sequences, while the sequence obtained from using the reverse primer yielded 470 bp. The comparative analysis showed that the clones had 88% similarity comparing to the previously reported vip2Ac and vip1Ac genes (AY245547.1)

DISCUSSION

The study of distribution of the insecticidal gene among local *B. thuringiensis* isolates is helpful in understanding diversity of gene content, race evaluation and lastly to discover any new or potent insecticidal gene. Several reports demonstrated the abundance of various *cry*[20–22] and *vip*[23,24] genes, which proved effective in identification of potent *B. thuringiensis* strain and insecticidal genes. The present investigation showed that there was an 11% *vip1/vip2* gene abundance. Warren et al[7] studied the distribution of *vip1/vip2* gene in 463 *Bacillus* strains belonging to *B. thuringiensis* and *B. Cereus*, they reported 12% distribution of *vip1/vip2* genes. However, Shi et al[15]. showed only a 2% existence of *vip1/vip2* genes in *B. thuringiensis* isolates studied by them. This difference of gene distribution in our local ecological niche might be because of geographic distribution.

There were some isolates, viz., PDKV-08, PDKV-27, PDKV-28 and NCIM-5112 that showed two different bands of size 50 kDa and 95 kDa which were relevant with the presence of gene *vip1/vip2* binary toxins in that isolate. Thus SDS-PAGE profiling is useful for confirmation and characterization of Vip1/Vip2 proteins. Selective profiling of the individual Vip proteins after cloning will be more helpful in characterization of these important insecticidal proteins which will be carried out in near future. However, this protein profiling study is supportive to PCR analysis and insect bioassay study.

The binary toxin Vip1Aa and Vip2Aa isolated from *B. cereus* AB78 is active at 20–40 ng/g against WCR and NCR belonging to coleopteran insects[7,8]. However, Vip1/Vip2 proteins from *B. thuringiensis* appeared to have no activity against lepidopteran pests[15]. In our study, Vip1/Vip2 from *B. thuringiensis* strain PDKV-08 represented 60% mortality to *S. zeamais* a coleopteran pest. However, HD-1 a standard reference strains protein showed no activity against Coleopteran insects. This was probably owing to a proper target insect, such as *S. zeamais*, being used for our bioassays which indicated the toxicity of Vip1/Vip2 proteins against coleopteran pests.

The *vip1/vip2* binary toxin gene was successfully cloned and sequenced. The nBLAST result clearly indicates that the new *vip* amplicon belongs to the *vip1/vip2* genes. On the other hand, it showed significant difference than the all existing *vip* genes. This indicates the obtained *vip1/vip2* gene having different motifs than the available Vip toxins.

The study led to the identification of a novel *vip1/vip2* gene, which is known to effective, against coleopterans[7]. It is necessary to study the effect against insect pests, clone these genes into a plant expression vector, transform crop plants, and study their effectiveness against different insect pests.

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REFERENCES

1. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**, 775–806.
2. Kaur S, Singh A (2000) Natural occurrence of *Bacillus thuringiensis* in leguminous phylloplanes in the New Delhi region of India. *World J Microbiol Biotechnol* **16**, 679–82.
3. Sanahuja G, Banakar R, Twyman R, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol J* **9**, 283–300.
4. Romeis J, Meissle M, Bigler F (2006) Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nat Biotechnol* **24**, 63–71.
5. McGaughey WH (1985) Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science* **229**, 193–5.
6. Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc Natl Acad Sci USA* **93**, 5389–94.
7. Warren GW (1997) Vegetative insecticidal proteins: novel proteins for control of corn pests. In: Carozzi NB, Koziel M (eds) Advances in insect control, the role of transgenic plants, Taylor & Francis Ltd, London, pp 109–21.
8. Han S, Craig J, Putnam C, Carozzi N, Tainer J (1999) Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nat Struct Mol Biol* **6**, 932–6.
9. Shotkoshi F, Chen F (2003) Vip: A novel insecticidal protein with broad spectrum Lepidopteran activity. In: *Proceedings of the Belwide Cotton Conference*, Nashville TN, pp 89–93.
10. Carlier M-F (1990) Actin polymerization and ATP hydrolysis. *Adv Biophys* **26**, 51–73.
11. Sattar S, Pradip K, Munshi B, Hossain A, Maiti M, Soumitra Sen K, Basu A (2008) Search for vegetative insecticidal proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against lepidopteran and homopteran insect pests. *J Biopesticides* **1**, 216–22.
12. Sattar S, Maiti MK (2011) Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking. *J Microbiol Biotechnol* **21**, 937–46.
13. Blackman RL, Eastop VF (2000) *Aphids on the World's Crops: An Identification and Information Guide*, 2nd edn, Wiley, New York.

14. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Strihl K (1995) *Short Protocolsin Molecular Biology*, John Wiley & Sons.
15. Shi Y, Ma W, Yuan M, Sun F, Pang Y (2007) Cloning of *vip1/vip2* genes and expression of Vip1Ca/Vip2Ac proteins in *Bacillus thuringiensis*. *World J Microbiol Biotechnol* **23**, 501–7.
16. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **17**, 248–54.
17. Sambrook J, Russell D (2007) *Molecular Cloning: A Laboratory Manual*, 3rd edn, Cold Spring Harbor Laboratory Press, New York, pp 31–162.
18. Hernández-Rodríguez CS, Boets A, Van-Rie J, Ferré J (2009) Screening and identification of vip genes in *Bacillus thuringiensis* strains. *J Appl Microbiol* **107**, 219–25.
19. Dhliwayo T, Pixley K (2001) Breeding for resistance to the maize weevil (*Setophilus zeamais* Motsch). In: *Proceedings of the Seventh Eastern and Southern Africa Maize Conference*, pp 134–8.
20. Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos F, Peña G, Nuñez-Valdez M, Soberon M, Quintero R (1998) Characterization of cry genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol* **64**, 4965–72.
21. Alvarez A, Licia MP, Flavia L, Virla EG, Baigori MD (2009) Insecticidal crystal proteins from native *Bacillus thuringiensis:* numerical analysis and biological activity against *Spodoptera frugiperda*. *Biotechnol Lett* **31**, 77–82.
22. Patel KD, Bhanshali FC, Ingle SS (2011) Diversity and Characterization of *Bacillus thuringiensis* isolates from alluvial soil of Mahi river basin, India. *J Adv Dev Res* **2**, 14–20.
23. Beard CE, Court L, Boets A, Mourant R, Van Rie J, Akhurst RJ (2008) Unusually high frequency of genes encoding vegetative insecticidal proteins in an Australian *Bacillus thuringiensis* collection. *Curr Microbiol* **57**, 195–9.
24. Yu X, Zheng A, Zhu J, Wang S, Wang L, Deng Q, Li S, Liu H, Li P (2010) Characterization of vegetative insecticidal protein *vip* genes of *Bacillus thuringiensis* from Sichuan Basin in China. *Curr Microbiol* **62**, 752–7.