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Relocating expression of vegetative insecticidal protein into mother cell of *Bacillus thuringiensis*

Naresh Arora, A. Selvapandiyan,[1] Neema Agrawal, and R.K. Bhatnagar*

International Centre for Genetic Engineering and Biotechnology, P.O. Box 10504, Aruna Asaf Ali Marg, New Delhi 110067, India

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Abstract

Vegetative insecticidal protein (VIP) is a class of insecticidal proteins produced by some strains of *Bacillus thuringiensis* during the vegetative stage of their growth. Unlike δ-endotoxins which are produced as parasporal inclusion bodies within the cell during sporulation, VIP is secreted into the culture medium. Here we report the relocation of the expression of VIP into the mother cell compartment in a manner similar to well-characterized Cry proteins. Relocation of VIP is directed to mother cell by placing its synthesis under sporulation-dependent promoters, BtI and BtII. The insertion of *cry* preferred transcription termination sequence at the 3′ region and a STAB-SD sequence at the 5′ region of the gene provided stability to the *vip* transcript and enhanced its yield. The demonstrated expression of VIP within the cells in the form of inclusion bodies would facilitate development of a suitable formulation for the application of this class of insecticidal proteins in the field.

Keywords: Bt-toxin relocation; Vegetative insecticidal protein; Sporulation-linked expression

Strains of Gram-positive, spore-forming soil bacterium, *Bacillus thuringiensis* (Bt) produce parasporal crystal in the cell during the stationary phase of growth [1]. These crystals also called as δ-endotoxins consist of several polypeptides (Cry proteins), some of which display insecticidal activity [1]. The expression of most of the *cry* genes is sporulation-dependent, *cry1Aa* being a typical example. Transcriptionally, the development of sporulation is controlled by an array of sigma factors, which bind the core RNA polymerase and direct the transcription from sporulation-specific promoters [2,3]. *Cry1Aa* and other sporulation-dependent *cry* genes are expressed only in the mother cell compartment of *B. thuringiensis* where sigma E and K are active [4]. *Cry1Aa* is under the control of sporulation-linked promoters, BtI and BtII [5]. Sporulation-linked dual promoters together with compartment-specific sigma factors constitute a highly efficient transcription apparatus, which is responsible for the accumulation of Cry protein in mother cell that can account for 20–30% of the dry weight of the sporulated cells [2,3]. In addition to sporulation-linked expression of insecticidal toxins, some strains of *B. thuringiensis* produce insecticidal proteins, unrelated to the Cry proteins, in the vegetative phase of growth, which are termed as vegetative insecticidal proteins, VIP [6]. These VIPs do not form parasporal crystal proteins and are secreted from the cell into the growth medium.

Bt has evolved a number of transcriptional and posttranscriptional mechanisms to overproduce δ-endotoxins in the mother cell compartment during the stationary phase. These mechanisms have been studied in detail and exploited to overproduce agriculturally important proteins. A number of *cis*-elements have been identified in the 3′ region and 5′ region of *cry* gene mRNAs that act as transcript stabilizers [2]. A positive retroregulator sequence was identified in the 3′ terminal fragment of the *cry1Aa* gene from *B. thuringiensis* subsp. *kurstaki* HD1 [7]. It was demonstrated that the cotranscription of the *cry* retroregulator with the upstream target gene increases the half-life of the transcript and consequently its expression level. Similarly, a Shine–Dalgarno (SD) sequence located in the 5′ untranslated

* Corresponding author. Fax: +91-11-2616-2316.
E-mail address: raj@icgeb.res.in (R.K. Bhatnagar).
[1] Present address: Laboratory of Bacterial, Parasitic and Unconventional Agents, Division of Emerging and Transfusion Transmitted Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20814, USA.

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region of *cryIIIA* (129 bp upstream of the translational start site) has been shown to stabilize the corresponding transcript. This sequence is designated as STAB-SD. When SD sequence was fused to the 5′ region of the *lacZ* gene, it resulted in a 10-fold increase in the levels of *lacZ* fusion mRNA [8]. Several studies have shown increase in the synthesis of Cry proteins by fusing these genetic elements to the corresponding *cry* mRNAs. For example, the yields of Cry3A toxin were significantly increased by using dual promoter/STAB-SD (Shine–Dalgarno sequence) system [9] and inclusions of truncated Cry1C were obtained by using *cyt1A* promoters, 5′ STAB-SD mRNA stabilizer and 3′ stem–loop transcription terminator [10].

Many *cry* genes have been expressed in large amounts in *Escherichia coli* in the form of inclusion bodies and used for binding studies [11]. Similarly, VIP has also been expressed in *E. coli* in the form of inclusion bodies but since formulations containing *E. coli* cannot be sprayed in the fields, it was imperative to redirect the expression of VIP in the mother cell compartment of Bt. The amount of insecticidal protein produced and their site of synthesis and accumulation are of commercial interest. Vegetative insecticidal proteins are active against several agronomically important insects, which are less sensitive to δ-endotoxins [6]. Unlike Cry proteins, which are accumulated in the cell, VIP is secreted from the cell into the growth medium thus limiting its field application. In the present study, the *vip* was cloned under the control of two strong sporulation-dependent BtI and BtII promoters of the *cry1Aa*. Inclusion of the STAB-SD sequence at the 5′ region and *cry* terminator sequence at 3′ region of the construct significantly increased the amount of expressed VIP. The expression of VIP in the form of inclusion in the mother cell compartment of *B. thuringiensis* would permit its application in the fields.

Materials and methods

Cloning of vip by polymerase chain reaction. The gene encoding VIP was cloned in our laboratory and expressed in *E. coli* (VIP, EMBL: Y17158) [12].

The VIP protein has a putative N-terminal signal peptide, which is believed to direct its secretion into the culture medium. To redirect the VIP toxin synthesis to mother cell, the N-terminal sequence comprising of 39 amino acids was deleted by PCR amplification of *vip*. In our previous communication we have reported differential toxicity against susceptible larvae upon removal of N-terminal residues. While deleted VIP was considerably less active against *Spodoptera litura*, its activity against *Chilo partellus* remained unchanged [12]. The *vip* with 117 bp truncated from 5′ region was PCR amplified from the parent clone (pBVIP) with primers VIP-SSD (5′ GGGATCCCCGGGATAAT CTTGAAAGGAGGGATGAAGAATCAGCAGTTACTA 3′) spanning amino acid residues 39–44 and containing STAB-SD sequence suitable for high level expression of insecticidal proteins [8]. The primer VIP-SSD contained *Bam*HI recognition site. The reverse primer VIP 4M (5′ GGAGCTCTTACTTAATAGAGACATC 3′) was designed from the 3′ region of the gene and contained *Sac*I site. The amplified fragment, SD-VIP, was cloned in pGEM-Te vector (Promega) and the construct was termed as pSD-VIP. The SD-VIP fragment was excised out from pGEM-Te by double digestion (*Bam*HI/*Sac*I) and cloned in *E. coli*/*B. thuringiensis* shuttle vector pHT3101 at the corresponding sites. This clone was termed as pHT3101-SD-VIP. In a separate PCR, the promoters BtI and BtII were amplified using forward primer CAAP1, 5′ AGATCTATGCATTGGTTAAAC 3′ and reverse primer CAAP2, 5′ AGATCTCCTCCATCTCTTTTAT 3′ both having *Bgl*II sites and *cry1Aa* DNA as template [5]. The 225 bp amplified product was cloned into pGEM-Te and then digested with *Bgl*II. The fragment was then cloned upstream of SD-VIP fragment in *Bam*HI digested pHT3101-SD-VIP construct to yield a new vector, pHT3101-Bt1-2-SD-VIP, containing *vip* under the control of BtI and BtII promoters with a STAB-SD sequence at its 5′ region. A 110 bp stem–loop structure, crySt-L, was amplified by PCR using forward primer CryTctrF, 5′ GAGCTCAAGTAATTTGTTGTAATG 3′ and reverse primer CryTctrR, 5′ GAGCTCCATATGTATTACTAGA AAATAA CA 3′ both having *Sac*I sites and *cry1Aa* DNA as template. This fragment was cloned into pGEM-Te and excised out by *Sac*I digestion. The excised fragment was further cloned at the corresponding *Sac*I site of vector, pHT3101-Bt1-2-SD-VIP, and the clone was termed as pHT3101-Bt1-2-SD-VIP-crySt-L. Both recombinants, pHT3101-Bt1-2-SD-VIP and pHT3101-Bt1-2-SD-VIP-crySt-L, were separately transformed into *E. coli* DH5α. Plasmid DNA isolated from transformed *E. coli* was used to electroporate *B. thuringiensis* 4D7.

Electroporation of B. thuringiensis strain. Host cells (*B. thuringiensis* 4D7) were grown in 50 ml brain heart infusion broth (BHIB, Difco) to an absorbance of 2.0 at 600 nm at 37 °C as a shake culture. Cells were harvested by centrifugation at 5000 rpm for 10 min, washed with cold sterile water, and resuspended in 5 ml cold electrolyte solution (0.625 M sucrose containing 1 mM $MgCl_2$). About 200 μl cells were electroporated in cuvette with 5 μg plasmid, pHT3101-Bt1-2-SD-VIP-crySt-L. The physical parameters used for electroporation were optimized at 2.0 kV, resistance 200 Ω, and 25 μF [13].

After electroporation transformed culture was transferred to 1 ml brain heart infusion (BHIB) medium and incubated at 30 °C at 200 rpm for 1 h. At the end of recovery period cells were harvested by centrifugation, resuspended in BHIB, and plated on BHIB agar medium containing erythromycin (10 μg/ml). Plates were incubated at 30 °C overnight and transformants screened by PCR using primers specific to *vip*. Single colonies were grown for further analysis.

Analysis of vegetative insecticidal protein expressed in spore mother cell. Transformed colonies of *B. thuringiensis* 4D7 were grown in G medium for sporulation [14]. Sporulated cultures were pelleted by centrifugation, washed twice with 0.9% NaCl, and finally washed with water. The Bt culture was grown for 20, 30, 40, 50, and 60 h and a time course analysis of VIP expression was carried out. The pellet of 50 ml culture was resuspended in 300 μl of 50 mM Tris–HCl buffer, pH 7.5, and 100 μl of 4× PLB (protein loading buffer) (250 mM Tris–HCl, pH 6.8, 40% glycerol, 4% SDS, 4% β-mercaptoethanol, and 0.005% bromophenol blue) was added to solubilize the proteins. The solubilized proteins were resolved on 10% SDS–PAGE, stained with Coomassie blue and destained with 10% acetic acid. The protein after resolving on the gel was also electrotransferred to nitrocellulose membrane in Tris–glycine buffer for Western blot analysis. Immunoblotting was performed using 1:20,000 diluted anti-VIP sera (polyclonal antibodies) raised in rabbits. Alkaline phosphatase-conjugated anti-rabbit antiserum was used as secondary antibody and the antigen–antibody complex was visualized by incubating the membrane in alkaline phosphatase buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) with 40 μl NBT (4-ntriblue tetrazolium chloride, 50 mg/ml in dimethylformamide) and 50 μl BCIP (5-bromo-4-chloro-3-indolyl phosphate; 50 mg/ml in dimethylformamide).

Isolation of total RNA. *Bacillus thuringiensis* 4D7 transformed with the construct, pHT3101-Bt1-2-SD-VIP-crySt-L, was grown for 10, 20, 30, 40, 50, and 60 h in 50 ml G medium at 30 °C at 200 rpm. The cells were pelleted, washed with water, and stored at −70 °C until the pellet

from last time point was harvested. Each cell pellet was resuspended in 1 ml of Trizol (Life Technologies), incubated at room temperature for 5–10 min, mixed with 200 μl chloroform, and centrifuged at 11,000*g* for 10 min. The aqueous phase was collected in a fresh Eppendorf tube and total RNA was precipitated using 0.7 volumes of isopropanol. Total RNA was pelleted, washed with ethanol, dried, and dissolved in 50 μl DEPC-treated water.

Northern blotting. The concentration of total RNA was estimated spectrophotometrically and 15 μg of total RNA from each time point was blotted onto a nitrocellulose membrane using slot-blot apparatus (Bio-Rad). RNA was crosslinked to the membrane by baking under vacuum at 80 °C for 1 h. A non-radioactive approach was followed for hybridization and signal detection using Gene Images Random Primer Labeling and Detection Modules (RPN 3540 and RPN 3510) from Amersham Pharmacia Biotech. A 1.5 kb DNA fragment was amplified using 2.4 kb *vip* cloned in pGEM-T as template and forward primer Vip3, 5′ ATGTACTTATTAACTCTACAC 3′ and reverse primer Vip4S, 5′AGTTATCTCCCCAAGCTTCATC 3′. This fragment was used as the probe. Briefly, 50–100 ng of the probe was fluorescein labeled using the contents provided in the kit. Prehybridization and hybridization were carried out at 50 °C. Washes were also carried out at 50 °C. Signal generation and detection were carried out as per the protocols given in the Gene Images Signal Detection Module.

Insect rearing and bioassay. Rearing of spotted maize stalk borer (*C. partellus*) and bioassay were performed as described previously [12]. Cells harvested from sporulated culture of Bt 4D7 transformed with pHT3101-Bt1-2-SD-VIP-crySt-L were coated on maize leaves and then fed to the second instar larvae. Sporulated culture of Bt 4D7 transformed with pHT3101 was used as control.

Results and discussion

The development of resistance against Cry proteins in susceptible larvae as well as the insensitivity of several

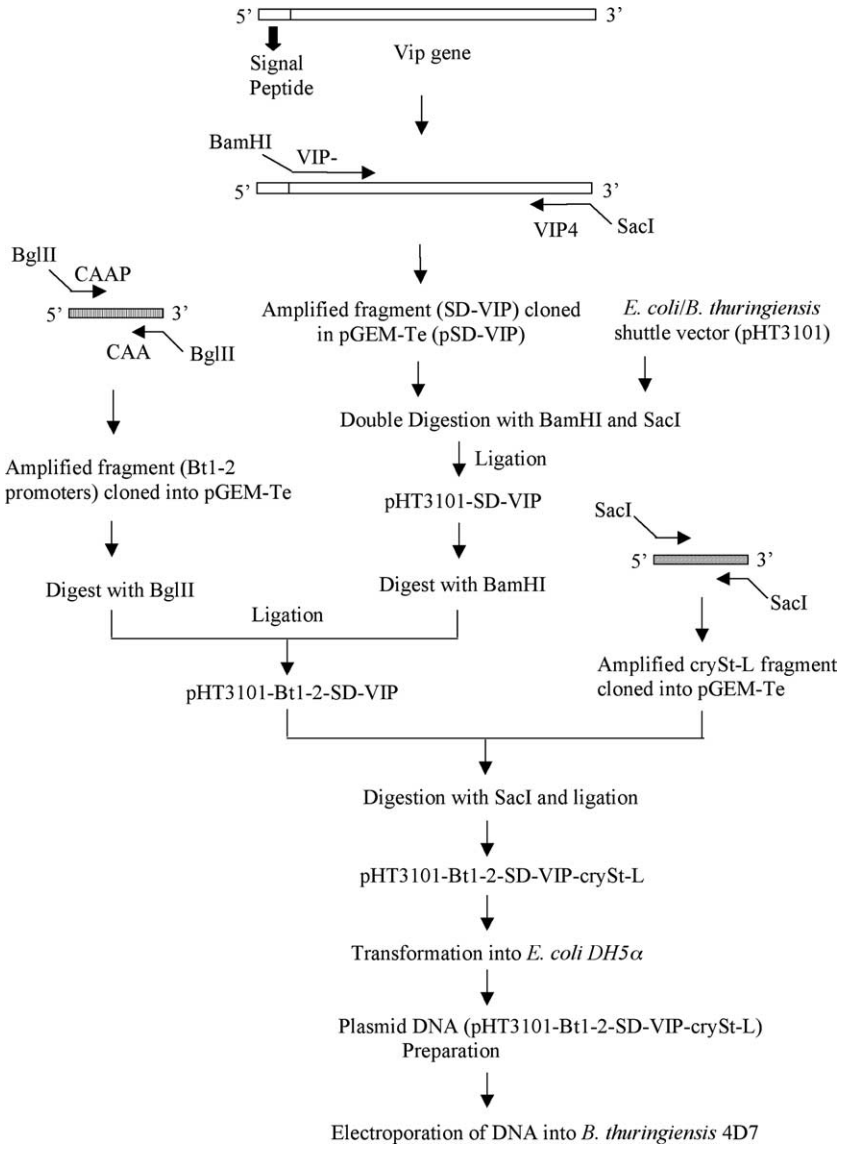

Fig. 1. Schematic representation of the strategy used for cloning modified *vip* in *E. coli*/*B. thuringiensis* shuttle vector, pHT3101. Putative signal sequence from *vip* was removed by PCR and simultaneously STAB-SD sequence was added to the 5′ of deleted *vip*. In another PCR, sporulation-specific promoters (Bt1-2) were amplified and cloned upstream of SD-VIP construct. The *cry* terminator (crySt-L) was amplified separately and then cloned at the 3′ region of SD-VIP construct. The final construct, pHT3101-Bt1-2-SD-VIP-crySt-L, was transformed into *B. thuringiensis* 4D7 strain.

agronomically important insects towards crystal proteins renders it imperative to search for Bt strains producing novel insecticidal proteins. In one such screening program, a vegetatively expressed, non-δ-endotoxin with insecticidal activity was identified and termed as VIP [6]. Unlike δ-endotoxins, VIP is secreted into the culture medium thus limiting its field applications. Most of the insecticidal Cry proteins are deposited as crystals in the sporulating *B. thuringiensis* cells [2]. The amount of toxin polypeptides synthesized could be as high as 25% of the dry wt. of sporulated cells. Molecular bases of such high expression of proteins are the coupled unit of efficient promoters BtI and BtII together with sigma factors K and E [2].

The presence of a Shine–Dalgarno sequence (STAB-SD) in the 5′ untranslated region upstream from the toxin gene is known to stabilize transcript [2,8]. The synthesis of Cry3A toxin was significantly increased when STAB-SD sequence was cloned at the 5′ region of the gene [9]. The 3′ terminal fragment of the *cry1Aa* harbors a stem–loop structure which protects the *cry* mRNA from exonucleolytic degradation thus increasing the half-life of transcript and eventually its expression level [7].

We have utilized the sporulation-dependent promoters to redirect the VIP synthesis in mother cell compartment and *cis*-elements at 5′ and 3′ regions to provide stability to *vip* mRNA to increase the yields of VIP. The construct consisting of *vip* under the control of sporulation-linked promoters, STAB-SD sequence at 5′ region of gene and *cry* terminator at 3′ region of the gene, was cloned by PCR in *E. coli–B. thuringiensis* shuttle vector pHT3101 and transformed into *B. thuringiensis* 4D7. The complete cloning strategy has been illustrated schematically in Fig. 1. Transformants were grown in sporulation inducing medium and cells were collected. In preliminary experiments wherein the gene construct was without crySt-L structure at 3′ region, the amount of VIP accumulated was only 1–2% of the total cell protein, suggesting that a further optimization was required to enhance the yield (data not shown). The fusion of the *cry* terminator sequence at the 3′ region of the gene resulted in increase in expression levels of VIP, which probably is a direct consequence of increased mRNA stability.

Resolution of the total cell protein of the sporulated Bt culture transformed with pHT3101-Bt1-2-SD-VIP-crySt-L on SDS–PAGE resulted in the appearance of a polypeptide at 84 kDa which corresponds to the size of VIP (Fig. 2, lane 2). Total cell protein of the sporulated Bt culture transformed with the vector pHT3101 alone was used as control. A threefold excess of protein loaded in the control lane (Fig. 2, lane 1) did not reveal any polypeptide at the size corresponding to VIP. The expression of VIP was confirmed by Western blot analysis using anti-VIP antiserum. To optimize the yield of VIP,

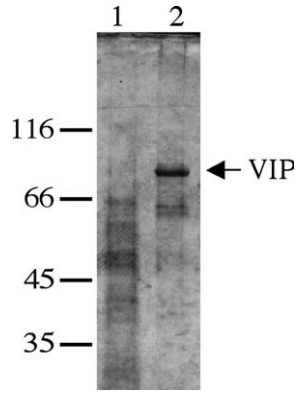

Fig. 2. Coomassie blue-stained gel of VIP expressed in *B. thuringiensis* 4D7. The total cell protein of the sporulated cultures was resolved on 10% SDS–PAGE. Lane 1, wild type Bt 4D7 strain (three times excess protein was loaded); lane 2, pHT3101-Bt1-2-SD-VIP-crySt-L transformed Bt 4D7 strain.

transformed culture was grown for different growth periods and the accumulation of VIP in sporulating culture was monitored by western blot analysis. Upon initiation of sporulation an increase in the expression of VIP was observed which reached the maximum in 40 h and declined subsequently (Fig. 3). In addition to the 84 kDa band corresponding to VIP, two more polypeptides were highlighted in the Western blot which probably are the consequence of degradation of VIP since these polypeptides are not observed in *B. thuringiensis* 4D7 transformed with vector pHT3101 alone (Fig. 3, lane C). The relative abundance of *vip*-specific transcript was examined in transformed Bt culture grown for different hours. Maximum transcript was observed in culture grown for 40 h (Fig. 4). Thus taken together the results of Western for VIP and transcript accumulation were in complete agreement with the sporulation-linked expression of VIP.

The toxicity of relocated truncated VIP was evaluated against second instar larvae of *C. partellus* and it was

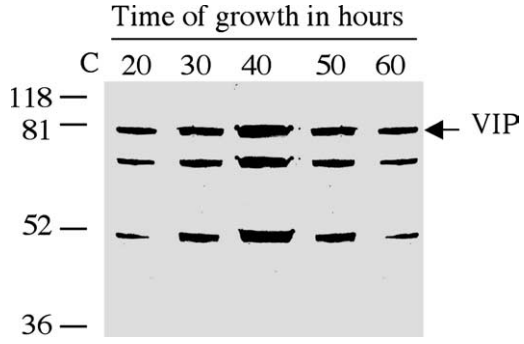

Fig. 3. Time course of VIP expression in Bt. The sporulating culture of *B. thuringiensis* 4D7 (pHT3101-Bt1-2-SD-VIP-crySt-L) was harvested at the indicated time points. Total cell protein was resolved on 10% SDS–PAGE and electrotransferred to nitrocellulose membrane. Total cell protein of Bt 4D7 transformed with pHT3101 was loaded in lane C. Western blot analysis was carried out using anti-VIP antibodies.

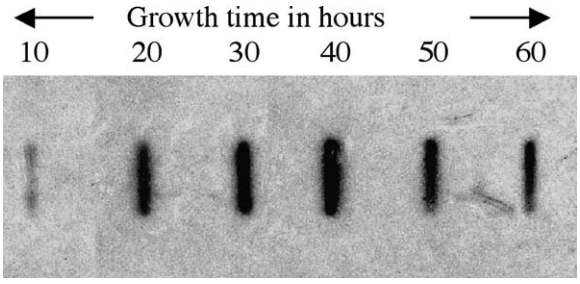

Fig. 4. Northern blot showing the levels of *vip* transcript expressed in sporulating Bt 4D7 (pHT3101-Bt1-2-SD-VIP-crySt-L) at different time points. The blot was probed with fluorescein labeled vip fragment.

found to be active against the pest. When the leaves coated with 40 h sporulated culture of Bt transformed with pHT3101-Bt1-2-SD-VIP-crySt-L were fed to the larvae, mortality was observed within 48 h. No mortality was observed in the control leaves.

The data presented here clearly demonstrate that the fusion of the sporulation-specific promoters with *vip* redirected its expression to sporulating cells. Accumulation of VIP in mother cell offers advantage in its field application as it permits formulation development strategy routinely adapted for various strains of *B. thuringiensis*.

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