Saudi Journal of Biological Sciences 27 (2020) 1363–1367

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Characterization, cloning, expression and bioassay of *vip3* gene isolated from an Egyptian *Bacillus thuringiensis* against whiteflies

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ARTICLE INFO

Article history:
Received 15 September 2019
Revised 24 November 2019
Accepted 8 December 2019
Available online 17 December 2019

Keywords:
Cloning
Expression *vip3* gene
Bacillus thuringiensis
Whiteflies

ABSTRACT

Throughout the vegetative life of *Bacillus thuringiensis*, vegetative insecticidal proteins (Vip) are produced and secreted. In the present study, the vip3 gene isolated from Bacillus thuringiensis, an Egyptian isolate, was successfully amplified (2.4 kbp) and expressed using bacterial expression system. The molecular mass of the expressed protein was verified using SDS-PAGE and western blot analysis. Whiteflies were also screened for susceptibility to the expressed Vip3 protein (LC50). In addition, ST_{50} was determined to assess the kill speed of the expressed Vip3 protein against whiteflies compared to the whole vegetative proteins. The results showed that the potency of whole *B. thuringiensis* vegetative proteins against whiteflies was slightly higher than the expressed Vip3 protein with 4.7-fold based on LC50 value. However, the ST50 parameter showed no significant difference between both the *B. thuringiensis* vegetative proteins and the expressed Vip3 alone. The results showed that the vip3 gene was successfully expressed in an active form which showed high susceptibility to whiteflies based on the virulence parameters LC_{50} and ST_{50}. To our knowledge, this study showed for the first time the high toxicity of the expressed Vip3 proteins of *B. thuringiensis* toward whiteflies as a hopeful and promising bio-control agent.

1. Introduction

Bacillus thuringiensis (Bt) is a natural happening bacterium originate in soil. Due to its importance as a bio-pesticides, Bt widely used nowadays for the effective control of different insect pests mainly belonging to order Lepidoptera (Balaraman, 2005). The death effect of the Bt on susceptible insect host is occurred due to the inclusions bodies formed from spores produced during Bt sporulation phase. These inclusions bodies are activated from pro-toxins into toxins (δ-endotoxins) inside the insect midgut leading to its death (Rangeshwaran et al., 2016; Osman et al., 2018,2019).

Cry proteins are the most widely produced insecticidal protein by Bt. These are active agents for controlling some Coleopteran pests as well as a wide range of Lepidopteran pests, mosquito species, and black fly (Sanchis, 2011; Sanahuja et al., 2011; Atia et al., 2020). B. thurenginsis produces another form another form of insecticide called vegetative proteins (Vips). During the Bt vegetative development period, this form is created. Vips demonstrate insecticide activity against a broad spectrum of insects and do not have a DNA homology with the renowned Cry (Estruch et al., 1996; Osman, 2012). Among the four families recognized for Vips are Vip1 and Vip2 that form a binary toxin that affects various Hemipterans and Coleopterans. While Vip3 has its effect to a broad range of lepidopteras and has no sequence similarities to Vips 1 and 2. However, its mode of action is mimic that of Cry.Vip4 family has recently reported to have no target insects (Abulreesh et al., 2012). Whiteflies are vectors for several genera of plant viruses such as; Begomovirus, Crinivirus, Carlavirus, Ipomovirus and Torradovirus which dramatically affect the production and yields of

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2019.12.013
1319-562X/

important food crops (Lapidot and Polston, 2010). Most viruses require whitefly feeding for acquisition and inoculation, while others require only probing for transmission (Navas-Castillo et al., 2011). Some factors affecting the widespread distribution of whiteflies such as; broad host range, the capability of virus transmission, and their high reproductive capacity (Schuster et al., 2010). Several reports demonstrated that whitefly transmits Geminiviruses that cause significant loss in vegetable as well as many crops in tropical and sub-tropical regions, which are regarded as restricting factors in the production of open field crops. Geminiviruses can cause different plant diseases such as tomato yellow leaf curl disease (causing severe damage to tomatoes crop), brown streak disease and mosaic disease in cassava, and golden mosaic disease in beans (Thiago et al., 2012; Elgaied et al., 2017; Ahmed et al., 2019). Due to the dramatic increase in whitefly transmitting geminivirus (WTGs), the control of such insects requires precise and quick techniques. This research seeks to specifically characterize Vip3′s potential toxicity to whiteflies as a new strategy for the control of whiteflies in Egypt, as well as to provide a helpful vision into the sustainable longstanding whiteflies control. δ-endotoxins

2. Materials and methods

2.1. PCR amplification and sequencing of vip genes

Bt subsp. *aegypti* BtaC18 isolate (BtaC18 USA patent no. 5986177) was grown on LB medium for overnight at 30 °C to reach the vegetative phase. About 100 mg of grown cells were pelleted through centrifugation. Pellet was disolved in 100 µL of resolution I containing lysozyme (20 mg/mL), sucrose (1 M), Tris-HCl (25 mM), and EDTA (25 mM). After keeping it for 30 min at 37 °C, cells were lysed by adding 200 µL of Solution II containing EDTA (10 mM), Tris-HCl (200 mM), NaCl (1 M), SDS (2%), phenol (tenth volume). After incubation for 1 h at 37 °C, 400 µL of chloroform were added. After centrifugation for 10 min at 12,000 × g, genomic DNA was precipitated by adding isopropanol to the supernatant. Precipitated pellet was washed 2 times with ethanol (70%). Pellet containing DNA was resuspended in sdH_2O to be handled as a template for *vip3* PCR isolation. A set of specific primers were designed and used to amplify *vip3* gene in BtaC18 isolate based on the sequences in GenBank under accession numbers; JF811911. The sequence of the designed primers used for amplification of *vip3* Table 1 and To facilitate subsequent cloning, BamHI and KpnI recognition sites were added to the forward and the reverse primer, respectively as in Table 1. PCR was performed using 10 pmol/µL of each forward and reverse primer, 1 µL of the reverse-primer (10 pmol/µL), 12.5 µL of master mix (Takara, Japan) and 0.5 µg of DNA template. The following program was used for PCR: 94 °C for 3 min as an initial temperature followed by 30 cycles of 94 °C for 45sec, 61 °C for 1 min, and 72 °C for 1 min. PCR-amplified DNA bands were analyzed by electrophoresis on 1.2% agarose gel.

Table 1
Showing the designed primer for amplification of *Vip 3* gene.

Primer name	Primer sequence
Vip-3-ORF_forward	5′-GGATCCATGAACAAGAATAATACTAAATT-3′
Vip-3-ORF_revers	5′-GGTACCTAGAGACATCGTAAAAATGTAC-3′
Vip–3-ORF_F:	5′-GGATCCATGAACAAGAATAATACTAAATT-3′ (29 mer) BamHI
Vip–3-ORF_R:	5′-GGTACCTAGAGACATCGTAAAAATGTAC-3′ (30) KpnI
Vip–3-ORF_F:	5′-GGATCCATGAACAAGAATAATACTAAATT-3′ (29 mer) BamHI
Vip–3-ORF_R:	GGTACCTAGAGACATCGTAAAAATGTAC-3′ (30) KpnI

2.2. Cloning and expression of vip3 gene

The PCR amplicon was gel purified using the GeneJET Gel Extraction kit, Thermo Scientific, USA, and subsequently cloned into pGEM-T Easy vector using a TA cloning kit (Promega, USA). Probable colonies were screened and positive colonies were confirmed by plasmid DNA double restriction digestion using BamHI and KpnI endonucleases. The *vip3* gene fragment was released from the verified plasmid followed by subcloning in the P^{QE-30} expression vector (Qiagen) as previous protocolled (Salem et al., 2018). The clones harboring the *vip3* gene were verified and transformed into Top 10 competent cells, and subsequently to an expression host *E. coli*M15. A bacterial colony of *E. coli*M15 containing the recombinant vector was cultured overnight and further inoculated in LB-broth (10 mL) and shacked at 37 °C (~3h) until OD reached 0.6 at 600 nm. At this point (zero time), the expression of Vip3 protein induction was done by addition 1 mM IPTG, and the bacterial growth was grown for 16 h (overnight). At each time point, 1 mL culture was collected, pelleted, and kept in −20 °C until processing for protein extraction and analysis.

2.3. Protein analysis by polyacrylamide gel electrophoresis

Whole protein extracts from bacteria were separated based on their molecular mass using SDS-PAGE as described (Laemmli, 1970), with slight modification (Salem et al., 2019a). The mini-protein II dual slab gel (Bio-Rad Laboratories, USA) was used with 10 mL of 12% separating gel composed of 3.3 mL double distilled water, 2.5 mL of 1.5 M Tris-HCL (pH 8.8), 4 mL of acrylamide solution, 0.1 mL of 10% SDS, 0.004 mL of TEMED, and 0.1 mL of 10% ammonium persulfate (APS). The solution was gently poured among two glass plates and covered with 1–2 mL of butanol-saturated double-distilled water. After complete polymerization of the gel (20 min), the overlay was detached, and the top of gel was rinsed with 1X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Almost 2 mL of 5% stacking gel containing 0.33 mL of acrylamide, 1.4 mL double distilled water, 0.25 mL 1 M Tris-HCL (pH 6.8), 0.02 mL of 10% APS, 0.02 mL of 10% SDS and 20 µL of TEMED was added onto the separating gel and then, the combs were sited. Electrophoresis was done in 1X SDS-PAGE running buffer. Protein samples were prepared in SDS sample buffer that was added before boiling for 5 min. The samples were loaded against the negative control (*E. coli*M15 transformed with P^{QE-30} empty vector). After running, the gels were stained in one percent of Coomassie brilliant blue solution with calm shaky all overnight.

2.4. Western blotting

Proteins extracted from induced and non-induced transformed BL21 as previously described (Elgaied et al., 2017; Salem et al., 2019b) were transferred onto an Immobilon® PVDF membrane (Millipore) using a trans-blot apparatus (Bio-Rad, USA). The membrane was blocked with 5% BSA in TBS and then washed with TBS-Tween (TBS-T) thrice and soaked in TBS containing anti-His-Tag antibody (1:1000) for 2 h at 25 °C. After keeping the membrane was again eroded with TBS-T thrice (5 min each) and keep it for 1 h in TBS supplemented with anti-mouse universal antibodies (1:10,000). Chemo-fluorescence detection of His-tag protein was performed using NBT/BCIP reagent in alkaline phosphatase buffer.

2.5. LC_{50} and ST_{50}

Whiteflies have been transmitted from tomato plants to cotton (Gossypium hirsutum L.), cotton has been grown for one month in an insect-proof wooden cage at controlled room temperature with

16 h illumination/day to avoid virus contamination. A group of 25 freshly hatched whiteflies from cotton crops were gathered, starved and left in a conical flask covered with a parafilm membrane for 4 h. Further, the insect was fed on 3% sucrose solution mixed with bacterial culture added over the parafilm film. Serial concentrations (25, 50, 250, 500, and 1000 ppm) of bacterial culture were utilized and kept for 12 h each. Mortality of these whiteflies was recorded at every 12 h post insect (hpi) and compared to the control insects fed only on sucrose solution (3%). The test was repeated thrice and the virulence parameters LC_{50} and ST_{50} were determined**.** The mean survival time (ST_{50}) was established by inoculating 30 newly hatched whiteflies in three independent replicates with the calculated LC_{80}, as well as three control experiments. Mortality of the whiteflies was monitored every 3 h post-infection until 24 h or flies death.

3. Results

3.1. PCR amplification of vip3

The *vip3* encoding sequence was amplified from the genomic DNA of Bt subsp. *aegypti* (BtC18) isolate. Two specific primers were designed based on the published sequence of *vip3* coding sequence (GenBank Acc. No. JF811911). As shown in Fig. 1, the PCR amplicon gave the expected fragment size of *vip3* (~2.4 Kb) and its identity was confirmed, by gel purification of the amplicon and nucleotide sequencing (data not shown).

3.2. SDS-PAGE and western blot analysis

The amplified *vip3* gene (2.4 kb) was cloned into a TA cloning vector and subsequently into the $P^{QE\text{-}30}$ vector for protein expression using *E. coli*M15 as a bacterial expression host. The T5 promoter was induced for translation of *vip3* gene using 1 mM IPTG. Induction was carried out for 16 h (overnight) and seven different positive colonies were processed for protein extraction and electrophoresis using SDS-PAGE compared to $P^{QE\text{-}30}$ (empty vector) as a negative control. As shown in Fig. 2, a clear band of ~88 KDa was detected in all examined colonies corresponding to Vip3 protein. These results suggested the successful induction of *vip3* coding sequence with adequate amounts available for western blotting and its bioassay analysis.

In order to confirm the identity of the Vip3 and its molecular mass, western blotting analysis using mono-specific anti-His antibodies was applied to total protein extracted from the same colonies as mentioned above. As illustrated in Fig. 3, Mono-specific antibodies strongly responded to the Vip3 protein with a protein band of ~88 kDa. The absence of background signals, was confirmed the specificity of the reaction.

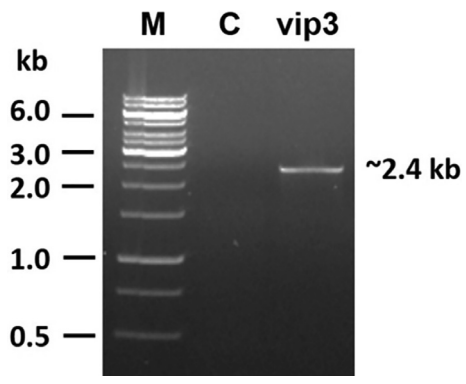

Fig. 1. Agarose gel showing PCR amplification of *vip3* gene using DGA3 genomic DNA and *vip3* ORF specific primers. M represents 1 Kb DNA ladder. C: PCR negative control.

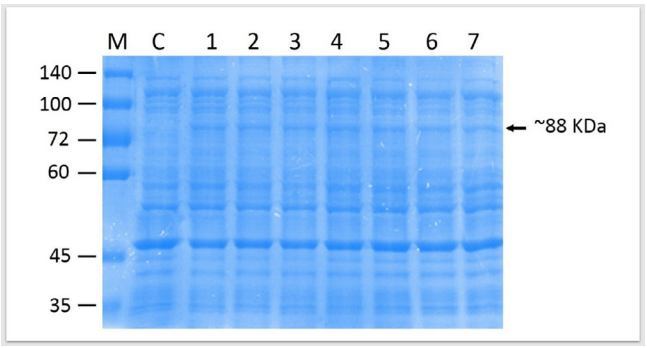

Fig. 2. SDS-PAGE gel showing protein expression of *Vip3*. Lane 1–7: Total protein extracted from induced colonies at 16 h post-inoculation. C: Negative control of Bl21 (DE3) transformed using $P^{QE\text{-}30}$ alone. M: Page Ruler Protein ladder. The arrow shows the putative vegetative insecticidal protein (VIP3) of ~88 kDa.

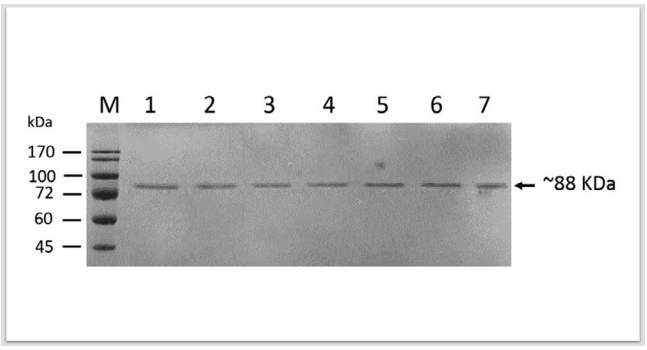

Fig. 3. Western blot to detect expression of VIP3 protein. Lanes 1–7 represent the interaction of overexpressed Vip3 protein (16 h) with mono-specific anti-His antibodies. M: Page Ruler broad-range protein ladder.

3.3. LC_{50} and ST_{50}

The biological activity of vip3 was evaluated against the whitefly population using LC_{50} and ST_{50} as virulence parameters. The bacterial culture expressing the vegetative insecticidal protein (Vip3) was used as inoculum for whiteflies at different concentrations ranging from 25, 50, 250, 500, and 1000 ppm as well as the total vegetative proteins of Bt as a whole culture. Bioassay findings for vip3 expressed protein disclosed a value of LC50 of 389 ppm relative to the entire Bt vegetative protein of 82 ppm (Table 2). On the other hand, the calculated ST_{50} for the expressed vip3 vegetative insecticidal protein was 36 h compared to the vegetative proteins of Bt, which was 24 h (Table 3). As noticed in Fig. 4, the vip3 culture survival plots is close to the survival curve of the whole vegetative proteins of Bt (BtC18), suggesting no significant difference between both cultures.

Table 2
Median lethal concentration (LC50) of whiteflies exposed to the extracts of total bacterial culture of Vip3 expressed protein at 12 h post-inoculation. Nr. Is the tested larvae number; LC50 values of the probit lines slopes; CI is the 95% confidence interval; SE: standard error. The value of LC50 and the confidence limits in ppm. According to Robertson and Preisler (1992), at $P < 0.0001$ all Wald $\chi 2$ tests were significant (DF = 1).

Type	Nr.	LC50 (CI)	Slop (SE)	X2 (calculated)
BtC18	150	82 (51–119)	2.11 (0.42)	2.14
Vip3	270	389 (335–452)	3.95 (0.79)	1.98

Table 3
Kaplan-Meier estimation of ST50 for whiteflies inoculated with VIP3 expressed protein.

Type	No. larvae	ST80 (ppm)	ST50 (h)	95% Confidence intervals (h)
BtC18	70	117	24	20–28
VIP3	73	135	36	28–42

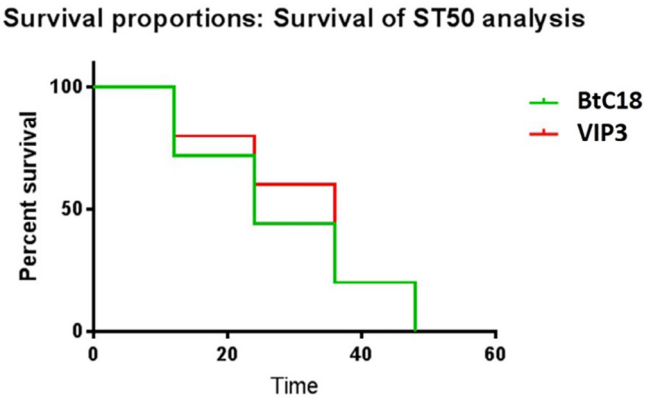

Fig. 4. Survival plots constructed by NCSS statistical analysis software on the basis of Kaplan-Meier estimator, showing the assessment of survival time of BtC18 vegetative proteins and VIP3 protein as observed on infected whiteflies.

However, during the bioassay, no distinction was observed on the whiteflies infected with either vip3 or the whole Bt bacterial culture.

4. Discussion

Whiteflies infestation causes serious crop devastation globally. Biopesticides are known to be among the most successful pest control strategies. *Bacillus thuringiensis* can develop crystal and vegetative insecticidal proteins throughout the development stage (Abouseadaa et al., 2015). From the vegetative proteins, the Vip1 and Vip2 known to be a binary toxin with considerable insecticidal activity against Aphis gossypii (Hemiptera) sap-sucking insect pest and some coleopteran pests (Sattar and Maiti, 2011; Osman et al., 2015). Vip3 proteins, on the other hand, are single-chain (not binary) toxins with insecticide activity against a wide range of lepidopteran species (Estruch, et al., 1996; El-Menofy et al., 2014; Osman et al., 2016). During the vegetative growth phase, *Bacillus thuringiensis* Vip3 proteins are synthesized and secreted. The insect gut proteases activates Vip3 proteins subsequently recognizes and binds to receptors of midgut, form pores and causes cells lysis. The current research was conducted to evaluate the likelihood of using Vip3 against whiteflies as a vegetative insecticide protein. The Vip3 was discovered being more prominent in numerous strains of *B. thuringiensis* than vip2 and vip1. Just about 10% of the isolates had a gene amplification allocation for vip1 or vip2; however, almost half of the strains contained vip3 gene (Estruch et al., 1996). This incidence of the vip3 was closer to that earlier observed by Espinasse et al. (2003). This finding promotes the assumption that vip3 may have distinct insecticide activity against insects. The wide variety of protein toxins that *B. thuringiensis* produces indicate that their encoding genes are influenced by strong selective evolutionary pressures resulting in a wide host range and making *B. thuringiensis* a rich source of protein with insecticidal activity against insects pests. Therefore, in this study, the vip3 gene of a BtC18, a local Egyptian isolate, was further expressed in bacterial expression system and applied to examine its activity against whiteflies as a promising bio-pesticide. The genome of *B. thuringiensis* subsp. aegypti (BtC18) was used as a template for isolation of vip3 coding sequence (2.4 Kb). The full-length gene of vip3 was subsequently expressed effectively in *E. coli* at 88 kDa of obvious molecular weight corresponding to Vip's expected molecular weight. Western blotting using anti-His protein as a further confirmation was performed, where its protein was particularly linked to the Vip; signals were detected at about 88 kDa, consistent with previous research (Rangeshwaran et al., 2016). Results also agreed with other research conducted by Osman et al., 2013, who verified the existence of vip3A in isolate DGA genome by western blotting for the expressed Vip3 protein. The biological activity of Vip3 protein was also successfully used as bio-insecticide against different insect species, including black cutworm (Sattar et al., 2011; Osman et al., 2013). The current investigation, was assessed the biological activity of Vip3 protein against whiteflies. Bioassay results showed that the LC50 value for the Vip3 expressed protein was 389 ppm. The results showed that the vip3 gene was expressed effectively in an active form that was biologically effective against whiteflies. On the other hand, the expressed vip3 showed considerable capacity against whiteflies population as well as the *B. thuringiensis* total vegetative proteins, which was 82 ppm, based on the LC50 values. On the other side, the calculated ST50 for BtC18 total vegetative proteins, was 24 h compared to the expressed vip3 protein alone which was 36 h. The ST50 results suggested that the vip3 protein needs more time (12 h) to reach 50% mortality of the treated insects compared to the whole bacterial culture. Thus, from the LC50 and ST50 analysis, it is concluded that insects fed on the expressed vip3 vegetative bacterial culture alone was showed a comparatively lower mortality relative to the whole culture. Although different studies have proved the pore formation by Vip3 proteins (EL-Ghareeb et al., 2012), it remains poorly understood how Vip3 proteins function. Further studies concerning the mode of action of *B. thuringiensis* vip3 protein toxicity against whiteflies are still needed in order to investigate the control mechanism of the whiteflies population.

Author contributions

Lamiaa El-Gaied: Conceptualization, Data curation. **Alshimaa Mahmoud**: Formal analysis, Funding acquisition. **Reda Salem**: Investigation, Methodology. **Wael Elmenofy**: Project administration, Resources. **Ibrahim Saleh**: Software, Supervision, Validation. **Hussein H. Abulreesh**: Visualization, Writing - original draft. **Ibrahim A. Arif**: Writing - review & editing. **Gamal Osman**: Writing - review & editing.

Funding

The author(s) received no specific funding for this work.

Declaration of Competing Interest

None declared.

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