# Chimeric *vip3Aa16$_{TC}$* Gene Encoding the Toxic Core of the Vegetative Insecticidal Protein Enhanced *Bacillus thuringiensis* Entomopathogenicity

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**ABSTRACT**

**Sellami, S., Cherif, M., Jaoua, S., and Jamoussi, K. 2015. Chimeric *vip3Aa16$_{TC}$* gene encoding the toxic core of the vegetative insecticidal protein enhanced *Bacillus thuringiensis* entomopathogenicity. Tunisian Journal of Plant Protection 10: 15-22.**

Vip3 insecticidal protein is produced by *Bacillus thuringiensis* during the vegetative stage. Its proteolysis by the midgut juice of susceptible larvae formed four major products of approximately 66, 45, 33 and 22 kDa. In this study, we cloned the *vip3Aa16$_{TC}$* DNA encoding the "Vip3Aa16 toxic core (TC)" of 33 kDa corresponding to the Vip3Aa16 region from amino acid 200 to 456. The *vip3Aa16$_{TC}$* chimeric gene carried by the pHT-*vip3Aa16$_{TC}$* plasmid was under the control of the sporulation dependent promoters (BtI-BtII) and the Shine Dalgarno sequence of *cry1Ac* gene as well as the *cry1Ia* gene terminator. Western-blot analysis of the culture supernatants of the recombinant *B. thuringiensis* strain detected Vip3Aa16$_{TC}$ after growing for 14 to 56 h proving that this protein can be produced without the Vip3 amino- and carboxy-terminal regions. Interestingly, the preservation of the Vip3Aa16$_{TC}$ toxicity against the polyphagous lepidopteran *Spodoptera littoralis* makes it a promising polypeptide for the pest biological control.

*Keywords: Bacillus thuringiensis*, *Spodoptera littoralis*, Vip3Aa16$_{TC}$

The polyphagous Egyptian cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), is one of the most devastating pests, causing damage by feeding to a wide variety of crops including cotton, tobacco, corn and several other vegetables (7). The most common control strategy against *S. littoralis* has been the application of chemical insecticides (14, 23). However, intensive use of these insecticides caused the development of *S. littoralis* resistance to 28 synthetic molecules (3,11). For this reason, various biological control agents have been used to substitute chemical control, among them the use of bioinsecticides based on microorganisms (bacteria, viruses and fungi), sex pheromones and plant extracts.

The entomopathogenic bacterium *Bacillus thuringiensis* is able to form crystal inclusions containing the Cry proteins which are toxic to a wide range of insects. Most of the *cry* genes are over-expressed during sporulation via the overlapping promoters BtI and BtII (6). The intensive application of the δ-endotoxins as pesticides increased the insect resistance, so particular attention is

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now managed to the entomopathogenic Vip3 proteins to supervise the resistance problems (27). The Vip3 toxins are produced and secreted by *Bacillus* spp. during vegetative growth stage. They displayed a broad insecticidal spectrum against a range of lepidopteron pests like *Agrotis ipsilon* which is relatively resistant to Cry1A and Cry1C (19). It has been shown that Vip3A (789 aa) protein was processed by the midgut juice of the susceptible larvae at two major sites, producing four major proteolytic products of about 22, 33, 45 and 66 kDa (12). The 66 kDa protein constitutes the activated form and the 33 kDa represents the "toxic core". The activated toxin binds to specific receptors in the midgut epithelium often described as different from those of Cry proteins, and forms pores which are able of making ion channels conducting to the larvae death (2, 17). Here, we cloned the *vip3Aa16*$_{TC}$ encoding the "toxic core (200-456 aa: 33 kDa)" of the Vip3Aa16 under the control of the sporulation dependent promoters BtI-BtII, the *cry1Ac* Shine Dalgarno-SD and the *cry1Ia* terminator, then we studied its expression in *B. thuringiensis* and evaluated its activity.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions.

*B. thuringiensis* strain BUPM106 lacking the *vip3* gene was isolated from soil samples. *Escherichia coli* strain Top10 (Invitrogen, USA) was used as the cloning host. LB medium was used for the growth of *B. thuringiensis* at 30°C and *E. coli* at 37°C. Ampicillin concentration used for recombinant *E. coli* was 60 µg/ml.

### Construction of the pHT-*vip3Aa16*$_{TC}$ shuttle plasmid.

The plasmid pHT-*spo-vip3LB* (26) carried the *vip3Aa16* gene (9) which is under the control of the strong BtI-BtII promoters and the *cry1Ac* Shine Dalgarno-SD (GenBank: U87793), as well as the *cry1Ia* terminator (GenBank: AJ315121). The *vip3Aa16*$_{TC}$DNA (0.78 kb) was obtained by PCR amplification of the (598-1368 pb) region of the *vip3Aa16* coding sequence by using the forward primer PS3 (AGTAAATCGATATGGG CTCTCCTGCAGATATTCT), bringing the *Cla*I site and the start codon (ATG), and the reverse primer PS9 (GGG AGATCTGGCGCCCTGCAGTTTTTCT TATTTAAGTCAATTTCTC) containing the *Pst*I, *Nar*I and *Bgl*II sites. PS3 and PS9 primers are designed during this study. The *vip3Aa16*$_{TC}$ DNA was digested by *Cla*I and *Bgl*II and then cloned into pHT-*spo-vip3LB* previously digested by the same restriction enzymes, to substitute the *vip3Aa16* and to obtain pHT-*vip3Aa16*$_{TC}$. Plasmid DNA was transferred into *E. coli* by the heat shock method (25). The pHT*Blue* vector and the pHT-*vip3Aa16*$_{TC}$ plasmids were electro-transferred into *B. thuringiensis* strain BUPM106 (26).

### Precipitation assay and immunoblot analysis.

The supernatant samples collected during the growth of *B. thuringiensis* recombinant strains were concentrated by precipitation with trichloroacetic acid (26). The proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with rabbit polyclonal antibody anti-Vip3Aa16. This antibody was detected with a peroxidase-labelled anti-rabbit IgG (Bio-Rad) and the signal was developed using the ECL plus Western blotting detection system (Amersham).

**Bioassays.**

The toxicity assays were performed by integrating 150 µl of the 48 h culture supernatant of BUPM106 (pHT-*vip3Aa16*$_{TC}$), BUPM106 (pHT*Blue*), or LB medium into 1.5 g of artificial semi-solid diet cubes (24) placed in a Petri dish. Afterwards, ten *S. littoralis* second-instar larvae were added to each cube and the plate was incubated at 25°C and under a photoperiod of 16 h light and 8 h dark. The experiments were repeated three times and the larval weights were recorded.

## RESULTS

**Synthesis of the Vip3Aa16$_{TC}$ protein in *B. thuringiensis*.**

Previous studies demonstrated that the 33 kDa polypeptide, artificially called Vip3Aa16$_{TC}$, remained stable when incubated with proteases (17). Hence, we constructed the pHT-*vip3Aa16*$_{TC}$ where *vip3Aa16*$_{TC}$ was controlled by the promoters BtI (active between t2 and t6) and BtII (active about t5 onwards, tn is n hours after the end of the exponential phase) causing high expression level (6). As a result of cloning strategy, the produced Vip3Aa16$_{TC}$ protein would be C-terminally supplemented with the (LQGARSPGAS) peptide. The Western blot analysis of the 50 fold concentrated culture supernatants by using the antibody anti-Vip3Aa16 revealed the presence of an approximately 33 kDa band for BUPM106 (pHT-*vip3Aa16*$_{TC}$) which was absent in the negative control BUPM106 (pHT*Blue*) (Fig. 1). These results confirmed the efficiency of the *vip3Aa16*$_{TC}$ expression under the control of the *cry1Ac* Shine Dalgarno (SD) (or ribosome binding site) together with the BtI-BtII promoters and the *cry1Ia* terminator. Vip3Aa16$_{TC}$ was detected from 14 h after the beginning of the culture until 56 h, which correspond to the sporulation phase since the BtI and BtII are sporulation dependent promoters. The persistence of the Vip3Aa16$_{TC}$ protein could reflect its stability although the absence of the other Vip3Aa16 regions, particularly the C-terminal region. Additionally, the 10 amino acids added at the C-terminal did not prevent the production of Vip3Aa16$_{TC}$ indicating its limited effect on the polypeptide stability.

**Investigation of the Vip3Aa16$_{TC}$ insecticidal toxicity towards *S. littoralis*.**

During four days, we demonstrated that the weight of the *S. littoralis* second-instar larvae decreased when the diet was supplemented with the BUPM106 (pHT-*vip3Aa16*$_{TC}$) supernatant by comparing with the negative control BUPM106 (pHT*Blue*) lacking any Vip3Aa16 derivative protein and being not toxic to *S. littoralis* larvae (Fig. 2). This finding demonstrated the ability of *B. thuringiensis* to produce a stable and activeVip3Aa16$_{TC}$, the "toxic core" corresponding to the amino acids from 200 to 456 of Vip3Aa16, without the other Vip3Aa16 regions.

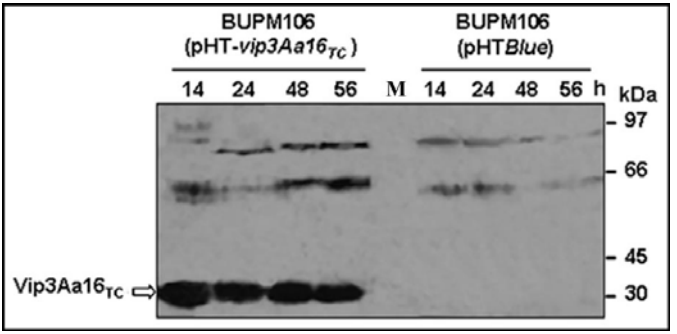


**Fig. 1.** Expression time course of the Vip3Aa16$_{TC}$ protein in the BUPM106 (pHT-*vip3Aa16*$_{TC}$). The 50 fold concentrated proteins of the culture supernatants taken during the growth were analysed by Western blot using the anti-Vip3Aa16 antibody. M: molecular weight markers (LMW-SDS, Amersham).

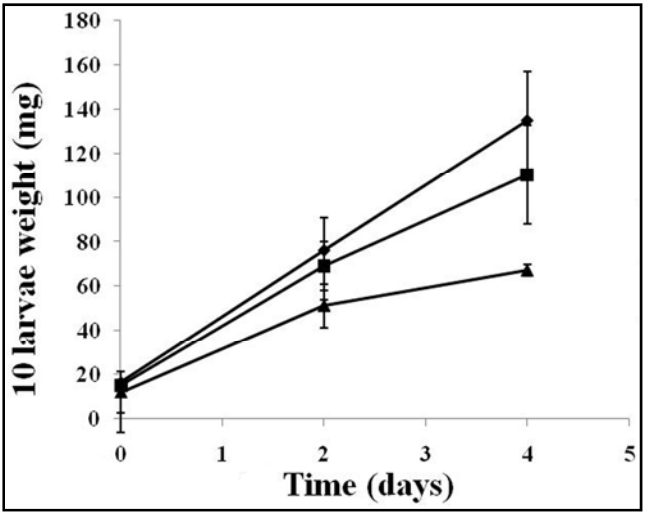


**Fig. 2.** Toxicity bioassay of the BUPM106 (pHT-*Vip3Aa16*$_{TC}$) recombinant strain on the growth of *Spodoptera littoralis* larvae.(◆) without bacteria, (■) BUPM 106 (pHT*Blue*) and (▲) BUPM 106 (pHT-*vip3Aa16*$_{TC}$).The weights of 10 larvae were recorded periodically and the experiment was repeated three times. Error bars represent a standard deviation of the mean values.

## DISCUSSION

Although *B. thuringiensis* Cry toxins are effective insecticidal proteins, several insect species larvae such as *Plutella xylostella* and *Heliothis virescens* become resistant to these toxins (13, 15). Furthermore, numerous devastating insects such as *Agrotis ipsilon* (18) and *Spodoptera exigua* (20) are less sensitive to their action. In order to increase the effectiveness of *B. thuringiensis* toxins, numerous research programs are carried out to look for other *B. thuringiensis* toxins with new insecticidal spectra. For all these reasons, attentions are now focused on the second generation of *B. thuringiensis* toxins (Vips) by screening programs, genetic manipulation, not only to improve the insecticidal activities but also to delay or prevent the development of resistance in target pest species (16, 27).

It was known that the sporulation dependent BtI and BtII are the most famous promoters during the stationary phase since they caused the obtaining of high level of expression (6). Moreover, a number of cis-elements have been identified which proceed as transcript stabilizers (4). In fact, a positive retroregulator sequence was identified in the 3' region of the *cry1Aa* gene from *B. thuringiensis* subsp. *kurstaki* HD1 gene; this retroregulator improved the expression level by the increase of the transcript half-life. Furthermore, a Shine Dalgarno (SD) sequence located in the 5' untranslated region of *cry3A* has been revealed to stabilize the corresponding transcript (4, 22). Sporulation overlapping promoters together with the cis elements stabilizers constitute a highly efficient transcription machinery, which is responsible for the accumulation of large amounts of Cry toxins in mother cell. This kind of expression system was used often by researchers to improve the expression level of *vip3A* genes (5, 8, 10, 26, 28). During this study, we had expressed the Vip3Aa16$_{TC}$protein under the control of BtI-BtII promoters, the *cry1Ac* Shine Dalgarno-SD sequence and the *cry1Ia* terminator. In fact, to be active, Vip3A proteins must be proteolysed inside the midgut of susceptible larvae into 4 major proteolytic products representing a molecular weight of approximately 66, 45, 33 and 22 kDa (1, 12, 17). The 33 kDa part, called Vip3Aa16$_{TC}$ is the main component of the Vip3A protein that remains stable after more than two hours of incubation. Western-blot analysis of the 50 fold concentrated supernatants demonstrated the presence of a 33 kDa band corresponding to the Vip3Aa16$_{TC}$ protein and its persistence during a late times of sporulation phase proving its extreme stability. To our knowledge, this is the first report describing the cloning of the *vip3Aa16* "toxic core" in *B. thuringiensis* strain.

Toxicity bioassays demonstrated that the 33 kDaVip3Aa16$_{TC}$ retains the full insecticidal activities against the second-instars of *S. littoralis* larvae, since the larvae's weights fed with the BUPM 106 (pHT-*vip3Aa16$_{TC}$*) supernatant was decreased compared to the negative control BUPM 106 (pHT*Blue*). This finding showed that the expression of Vip3Aa16$_{TC}$ was done in spite of the absence of the C-terminal region and the beginning of the N-terminal region. Previous studies confirmed this result since they demonstrated that Vip3A toxin is activated by midgut proteases to four proteolysis products where the 66 kDa protein which contains the 33 kDa part formed the active toxin (1, 17). Yu *et al*.(30) showed also that the Vip3A protoxins must be activated by a deletion of 199 amino acids in the N-terminal part.

Besides, it was demonstrated that the Vip3A protein C-terminal domain provides stability to the protein in the midgut of susceptible insects (12, 29). Furthermore, many reports were previously done to evaluate the truncated proteins and the active toxins parts. For example, in order to understand the function of the Vip3A 65 kDa core fragment, Dong *et al*. (10) substituted the three conserved cysteine residues (Cys292, Cys401, and Cys507) located within the 62 kDa core fragment in Vip3Aa7 with serines. It was demonstrated that this substitution caused a decreased resistance of 62 kDa core fragment to trypsin proteolysis, thereby resulting in the loss of the toxicity towards *Plutella xylostella*. Similar works were achieved with Cry toxins. In fact, the truncated Cry1C protein consisting on the 65 kDa active toxin was expressed using the *cyt1A* promotors, the *cry3A* STAB-SD stabilizing sequence in 5' region and the *cry3A*stem-loop transcription terminator in 3' region. Increased levels of Cry1C truncated synthesis were achieved which cause an enhancement of the toxicity towards *S. exigua* as much as fourfold (21).

In conclusion, we succeeded for the first time the homologous expression of the chimeric *vip3Aa16*$_{TC}$ gene encoding the "Vip3Aa16 toxic core" in *B. thuringiensis*. Moreover, the expression level could be increased with *cis*-acting elements proceeding as transcript stabilizers. Thus, the Shine Dalgarno (SD) sequence located in the 5' untranslated region of *cry3A* stabilized the corresponding transcript (4) and the positive retro regulator sequence localized downstream the *cry1Aa* coding sequence of *B. thuringiensis* subsp. *kurstaki* HD1 which increased the transcript half-life (21). Therefore, it will be interesting to design a chimeric protein constituted of the Vip3Aa16$_{TC}$ and the C-terminal region of a Cry1 protein to try its integration inside the crystal inclusion in order to improve the δ-endotoxins toxicity and to expand the insecticidal spectra since Vip3A and Cry1 proteins act differently against lepidopteran larvae.

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**RESUME**

**Sellami S., Cherif M., Jaoua S. et Jamoussi K. 2015. Le gène chimérique *Vip3Aa16*$_{TC}$ codant pour le core toxique de la protéine insecticide végétative améliore le pouvoir entomopathogène de *Bacillus thuringiensis.* Tunisian Journal of Plant Protection 10: 15-22.**

La protéine Vip3 est produite par la bactérie *Bacillus thuringiensis* durant le stade végétatif. Sa protéolyse par le jus larvaire des larves susceptibles forme quatre produits de protéolyse de 66, 45, 33 and 22 kDa, approximativement. Dans ce travail, nous avons cloné le gène *vip3Aa16*$_{TC}$ qui code pour le "noyau toxique de la protéine Vip3Aa16 (TC)" de 33 kDa correspondant à la région de 200 à 456 acides aminés de la protéine Vip3Aa16. Le gène chimérique *vip3Aa16*$_{TC}$ porté par le plasmide pHT-*vip3Aa16*$_{TC}$ est sous le contrôle des promoteurs de sporulation dépendants de BtI-BtII et de la région Shine Dalgarno du gène *cry1Ac* ainsi que de la région terminatrice du gène *cry1Ia*. L'analyse par western blot des surnageants de cultures de la souche recombinante *B. thuringiensis* montre la détection de Vip3Aa16$_{TC}$ après 14 à 56 h de culture prouvant que cette protéine peut être synthétisée en absence des régions amino et carboxy-terminales. La préservation de la toxicité par ce polypeptide envers le lépidoptère *Spodoptera littoralis* lui permet d'être un agent prometteur dans le contrôle biologique.

*Mots clés*: *Bacillus thuringiensis*, *Spodoptera littoralis*, Vip3Aa16$_{TC}$

**ملخص**

**السلامي، سماح ومروى الشريف وسمير الجوة وقيس الجموسي. الجين المدمج *vip3Aa16*$_{TC}$ من البروتين النباتي الحشري يحسن فاعلية البكتيريا *Bacillus thuringiensis*.**

**Tunisian Journal of Plant Protection 10: 15-22.**

تنتج البكتيريا *Bacillus thuringiensis* خلال نموها البروتين النباتيVip3. ويجزئ عبر عصير اليرقات الحساسة إلى أربعة بروتينات يبلغ حجمها حوالي 66 و45 و33 و22 كيلودالتون. قمنا خلال هذه الدراسة، باستنساخ الجين *vip3Aa16*$_{TC}$ الذي يرمز إلى القلب السام للبروتين Vip3Aa16 (TC) الذي يبلغ حجمه حوالي 33 كيلودالتون ويتطابق مع البروتين Vip3Aa16 على مستوى منطقة الأحماض الأمينية من 200 إلى 456. إن الجين *vip3Aa16*$_{TC}$ المحمول على البلازميد *pHT-vip3Aa16*$_{TC}$ يقع تحت تحكم مطورات التبويغ التابعة لـ BtI-BtII ومنطقة Dalgarno Shine للجين *cry1Ac* وكذلك للمنطقة النهائية للجين *cry1Ia*. بينت تقنية *Western-blot* لطاف مزرعات العزلة المؤتلفة *B. thuringiensis* كشف Vip3Aa16$_{TC}$ بعد 14 إلى 56 ساعة من النمو. وهذا يثبت أن هذا البروتين يمكن توليفه في غياب المناطق النهائية الأمينية والكربوكسية. تمكن المحافظة على سمية متعدد الببتيدات هذا تجاه الحشرة قشرية الأجنحة *Spodoptera littoralis* من أن يكون عاملا واعدا في المكافحة البيولوجية.

*كلمات مفتاحية*: Vip3Aa16$_{TC}$، *Spodoptera littoralis*، *Bacillus thuringiensis*

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