

Cloning and Expression of *cry9Ea10* Gene from *Bacillus Thuringiensis* Strain GZ2 Isolated from Infected *Hyphantria Cunea* Larvae

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Abstract. A novel *Bacillus thuringiensis* (Bt) strain GZ2, was isolated from infected *Hyphantria cunea* larvae. The bioassay results showed that GZ2 had insecticidal activity to *H. cunea* larvae. The genotypes of GZ2 were identified by PCR-RFLP method. GZ2 was identified to harbor *cry9Ea10* gene and the full length of *cry9Ea10* was cloned and sequenced. The engineering strain of Bt carrying *cry9Ea10* gene was constructed successfully. SDS-PAGE analysis confirmed that the expression of *cry9Ea10* as ~130kDa protein. And Cry9Ea10 protein produced by Bt engineering strain exhibited a high toxicity to *H. cunea* larvae.

Introduction

Hyphantria cunea Drury was native to North America then spread to Europe and Asia due to human activities [1]. This international quarantine pest has caused tremendous damages to forest in China since 1979 because of its high reproductive capability, good adaptability and wide reach [2,3]. The chemical control against *H. cunea* are deleterious to the environment and the health of humans [4,5]. So alternative methods for pest control that are less hazardous are highly valued.

Bacillus thuringiensis (Bt) bacteria are one of the best substitutes, which produce insecticidal crystalline proteins (ICPs) during the sporulation phase of growth. Different ICPs are highly specific to target insects [6]. After larvae ingestion, the crystalline inclusions are solubilized in midgut lumen and activated by alkaline proteases releasing 65-kDa toxic fragment that binds to several midgut receptors. The interactions facilitate toxin insertion into the membrane, forming pores that result in the death of midgut cells [7,8].

Soil is the most common source to sift Bt strain on account of carrying with vast microorganism. However, it's a long period to get a strain that has specific and high toxicity to a target pest. In the present study, We put attention to the naturally infected *H. cunea* larvae collected from the wild which show classic symptom of infection by bacteria. A novel *cry9Ea* gene was cloned and expressed from one of the isolates detected from infected *H. cunea* larvae, the crystal protein purified from engineering Bt strain have high toxicity against *H. cunea* larvae.

Materials and Methods

Hyphantria Cunea Larvae

The infected larvae were collected from *Platanus orientalis* wood out of pesticides in

Zhengding(Hebei, China) and fed by *Ailanthus altissima* leaves in lab. The healthy ones were provided by Chinese Academy of Forestry and fed by artificial diet.

Isolation and Identification of Bt strains

After 15 minutes surface sterilization by 75% alcohol, the infected *H. cunea* larvae were put in the Milli-Q water and vortexed to get the tissue homogenate. Shake for 20 minutes at room temperature (at 200 rpm). Then incubate in 75°C water bath for 17 minutes. After cold to room temperature, dilute the supernate to 10^{-2} , 10^{-3} , 10^{-4} three gradients and pipette 100 μ l to spread on 1/2 LB solid medium separately. Incubate at 30°C for three days and pick up the white irregular colony with waxy rough surface. The microorganism was identified under microscope after carbolfuchsin staining.

The colonies were cultivated in 1/2 LB liquid medium for 72 hours. After centrifugation, the supernate was removed and the sediment was resuspended in 10mM Tris-HCl (pH 8.0). The samples were added protein sample buffers boiling for 10 minutes. Proteins produced by the wild Bt strains were analyzed by SDS-PAGE.

Identification of cry Gene Types

27 pairs of universal oligonucleotide primers were used to detect the gene types by PCR [9]. The amplified PCR fragments were purified using Universal DNA Purification Kit (Tiangen, China) and the restriction fragment length polymorphism (RFLP) patterns were performed.

Cloning of the Full Length of cry9Ea10

Specific primers were then designed depending on the blast in NCBI. The full length gene was cloned and sequenced. The result was then submitted to *Bacillus thuringiensis* Toxin Nomenclature Committee and NCBI to acquire a name and GenBank accession number respectively.

Expression of Cry9Ea10 in Escherichia coli

The purified fragments of *cry9Ea10* with restrict enzyme sites were cloned into pET21b vector [10]. The recombined plasmid was transformed into *E. coli* BL21(DE3) competent cells. Protein expression was induced by the addition of 1 mM IPTG for 6hours. After centrifugation, the precipitates were resuspended with 10mM Tris-HCl buffer (pH=8.0) and sonicated to release proteins.

The expressed proteins were separated by 8% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane were blocked for 1 h with 1% BSA, incubated with mouse anti-His (1:2000) for 1h, washed four times with TBS-T and two times with TBS. The protein was detected using anti-mouse conjugated with Phosphatase alkaline (1:10000). Finally, NBT and BCIP (Sigma) was used as substrate for detection.

Expression and Purification of Cry9Ea10 in HD73⁻

The full-length fragments of *cry9Ea10* with the restrict enzyme sites were cloned into pSXY422b vector. The recombined plasmid were transformed into acrystalliferous mutant HD73⁻ competent cells by electric shock at 2500 V, 5.0ms.

The expressed Cry9Ea10 protein after culturing for 72 hours in 1/2 LB was purified with isoelectric precipitation. The concentration of the purified Cry9Ea10 was measured by BCA method according to the manufacturer's instructions (TAKARA).

The purified Cry9Ea10 was analyzed by Western Blot (as in the last section). The

membrane was revealed using rabbit anti-cry9Ea (1:20000) and secondary anti-rabbit conjugated with Phosphatase alkaline(1:10000).

Insect Bioassays

Bioassays were performed with *H. cunea* larvae by the diet surface contamination method [6]. 20 primary hatching larvae/box were fed with different doses of GZ2 spores or Cry9Ea10 proteins and 3 repeats were conducted. The boxes were incubated at 28 °C with 65±5% relative humidity. Mortality was recorded after 96 hours and the 50% lethal concentration (LC₅₀) was analyzed with SPSS software.

Results

Isolation and Identification of Bt

Two Bt isolates were gained. SDS-PAGE shows they both produce about 130kDa bands(Fig. 1, A and B).

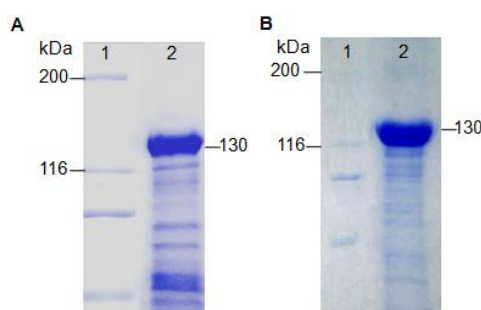


Fig. 1 SDS-PAGE analysis of proteins produced by wild Bt isolates. A, isolate GZ1(lane 2), Lane 1, molecular weight marker. B, isolate GZ2(lane 2), Lane 1, molecular weight marker.

Cloning and Analysis of *cry9Ea10* Gene

The conservative regions of several *cry* genes were obtained by universal primers and the full-length sequence of *cry9Ea10* was cloned successfully by specific primers. The gene was named as *cry9Ea10* by *Bacillus thuringiensis* Toxin Nomenclature Committee. The GenBank accession number is KT692743.

The ORF of *cry9Ea10* is 3453bp, encoding 1150 amino acids, with a calculated molecular mass of 130.0kDa, and isoelectric point (pI) of 4.68. Amino acid sequence analysis using the NCBI Conserved Domain Database, showed that the Cry9Ea10 protein includes three putative conserved Domains(Fig. 2A). The 3D-modeled structures of Cry9Ea10 were predicted by SWISS-MODEL on line (Fig. 2B).

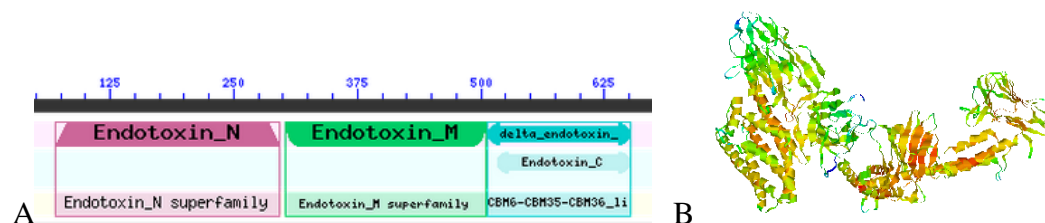


Fig. 2 The binary and ternary structure of Cry9Ea10. A, three putative Domains of Cry9Ea10. B, 3D-modeled structures of Cry9Ea10.

Expression of pET21b-9Ea10 in BL21

The pET21b-9Ea10 was constructed and transferred into BL21 successfully (Fig. 3A). Western Blot analysis showed that the molecular weight of expressed Cry9Ea10 protein was about 130kDa (Fig. 3B), which was coherent with the predicted

molecular weight.

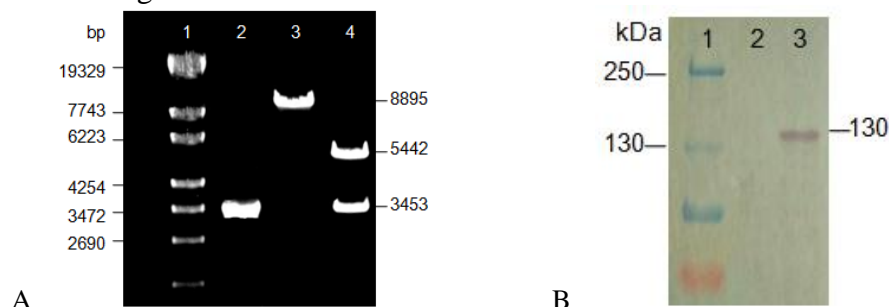


Fig. 3 The pET21b-9Ea10 was successfully constructed and expressed in BL21. A, identification of the recombinant plasmid. *Lane 1*, marker. *Lane 2*, PCR product. *Lane 3*, single endonuclease digestion. *Lane 4*, double endonuclease digestion. B, Western Blot analysis of the recombinant protein. *Lane 1*, prestained marker. *Lane 2*, the expression of pET21b. *Lane 3*, the expression of pET21b-9Ea10.

Expression and Purification of Cry9Ea10 in HD73⁻

The pSXY422b-9Ea10 was successfully constructed (Fig. 4A). The heterogenous expressed protein Cry9Ea10 was purified and Western Blot analysis showed a 130kDa band (Fig. 4, B and C). The concentration of the purified Cry9Ea10 was measured as 3.5mg/ml by BCA method.

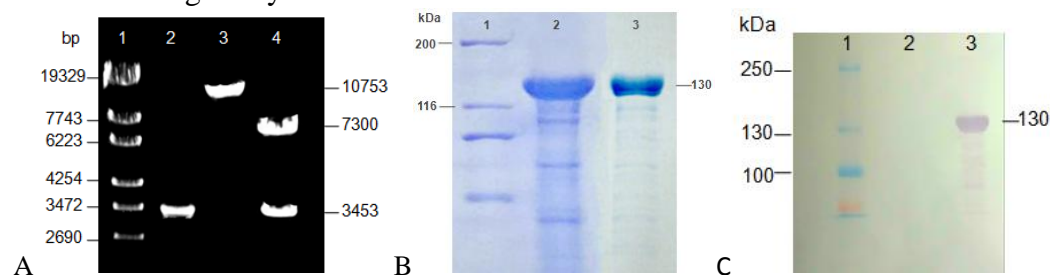


Fig. 4 The pSXY422b-9Ea10 was successfully constructed and expressed in HD73⁻. A, identification of the recombinant plas mid. *Lane 1*, marker. *Lane 2*, PCR product. *Lane 3*, single endonuclease digestion. *Lane 4*, double endonuclease digestion. B, coomassie blue staining of the heterogenous expressed protein Cry9Ea10. *Lane 1*, molecular weight marker. *Lane 2*, the expression of HD73 (pSXY422b-9Ea10). *Lane 3*, the purified protein Cry9Ea10. C, Western Blot analysis of the purified protein Cry9Ea10. *Lane 1*, prestained marker. *Lane 2*, the expression of HD73. *Lane 3*, the purified protein Cry9Ea10.

Insect Bioassays

The mortality of GZ2 strain and Cry9Ea10 protein to *H.cunea* was calculated and analyzed respectively (Fig. 5). The LC₅₀ lethal values of isolate GZ2 were 1921 pfu/ml. The LC₅₀ lethal values of Cry9Ea10 protein were 4.14 µg/ml.

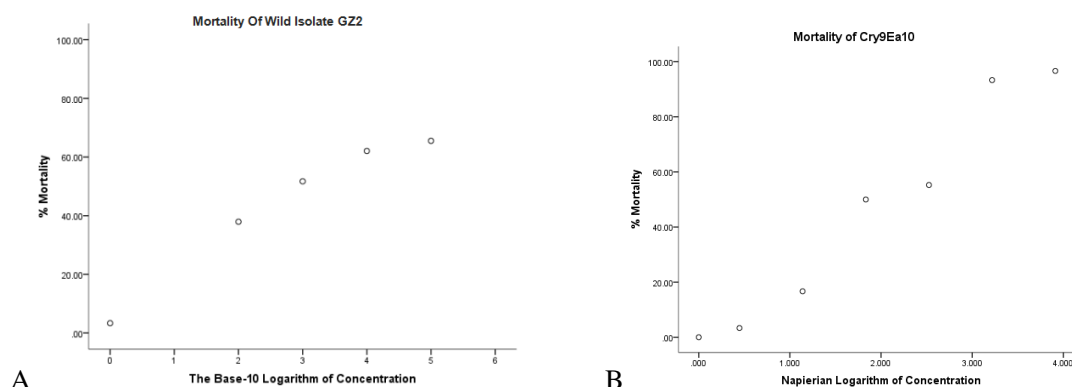


Fig. 5 Toxicity effects to *H. cunea* larvae. A, bioassay of Bt isolate GZ2 to *H. cunea* larvae. Horizontal ordinate, the base-10 logarithm of concentration. Vertical coordinates, mortality. B, bioassay of Cry9Ea10 protein to *H. cunea* larvae. Horizontal ordinate, the natural logarithm of concentration. Vertical coordinates, mortality.

Discussion

In this study, a novel Bt strain isolated from the naturally infected *H. cunea* does have high toxicity to healthy *H. cunea* larvae. It provides an effective insecticidal crystal protein to control *H. cunea* and study the interactions between Cry proteins and receptors on BBMV of insect midgut.

Previous research had indicated that Cry9Ea is highly toxic to *Trichoplusia ni* neonates ($LC_{50}=0.0639\mu\text{g/mL}$) and *Plutella xylostella* neonates ($LC_{50}=4.574\mu\text{g/mL}$) [11], but not to *Helicoverpa armigera*. According to the Ping Pong binding model, the activated cry monomer first binds to APN [12]. The 3D structures of APN from *T. ni*, *H. cunea*, *P. xylostella* and *H. armigera* predicted by SWISS MODEL were compared. The results showed that there is a deep crack between Domain Gluzincin and Domain ERAP1_C in *T. ni* APN. In *H. cunea* and *P. xylostella* APN, Domain ERAP1_C inserts into Domain Gluzincin further and two Domains joint more closely, which may block the bind between Cry9Ea and APN to some extent. In *H. armigera*, two Domains are bounded tightly together and the crack is totally blocked. These difference may indicate how Cry9Ea bind to APN on the configuration.

However, the intermolecular mechanism between Cry9Ea and several receptors is still remained to be understood. The future research will help to understand the key site of interactions and obtain insecticides of higher toxicity, which would be of great value for the biological control of *H. cunea*.

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