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Identification of a *Bacillus Thuringiensis* Cry8Ea3 to Xin-Binding Alkaline Phosphatase from *Holotrichiaparallela*

Wei WANG[1], Dan ZHAO[1], Wei GUO[2,a,*], Xiao-ping YAN[1], Ya-kun ZHANG[1], Kun-li ZHAO[1], Yu-jie GAO and Xiao-yun WANG[3]

[1]College of Plant Protection, Agricultural University of Hebei, Baoding, Hebei, China

[2]College of Plant Science and Technology, Beijing University of Agriculture, Beijing, China

[3]College of Agriculture, Northeast Agriculture University, Harbin, Heilongjing, China

College of Plant Science and Technology, Beijing University of Agriculture, Beijing, China;

[a]1787421502@qq.com;

*Corresponding author;

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Abstract. In order to further study the Insecticidal Mechanism of Bt protein against *Holotriciaparallela*. In the study, According to the results of midgut transcriptome sequencing and RACE-PCR, the full-length *hpalp* gene was cloned. Sequencing analysis showed that the open reading frame of *hpalp* (GenBank accession NO. KY922835) was 1605 bp long, encoding 534 amino acid residues. The predicted molecular weight and isoelectric point of HpALP were 59 kDa and 5.18, respectively. Analysis of HpALP signal peptide with 21 amino acids, GPI anchor point is located in the C-terminal D^{514}, and has two N-glycosylation sites: N^{100}, N^{296}. The results of Ligand blot showed that the binding of HpALP with Cry8Ea3 toxin. Transcriptional analysis of *hpalp* in different tissues of *H. parallela* larvae was performed by qRT-PCR, which revealed that the *hpalp* was primarly expressed higher in midgut, but lower in the foregut.

Introduction

Holotriciaparallela belongs to the order Coleoptera, family Scarabeidae, and its larvae are commonly recognized as the most significant domestic underground pest affecting peanut, sweet potato, and potato which caused substantial reductions in crop yields and huge economic losses[1,2]. *Bacillus thuringiensis* (Bt) toxin Cry8Ea had specific activity against *Holotriciaparallela* larvae[3].

Research showed that the mechanism of insecticidal effect of Bt has been demonstrated to be related to brush border membrane vesicles (BBMV) receptor protein in the insect midgut epithelial cells[4,5]. There were two potential modes to explain the underlying mechanism of toxicity of Cry proteins. In the pore formation[6,7], the toxin-receptor interaction would promote toxin oligomerization and membrane-pore formation, resulting in midgut cell lysis and the eventual death of the target larvae. In the signal transduction model[8], the Cry toxins binding cadherin receptors caused a intracellular signal transduction, G protein and adenosine cyclase are activated, elevated cyclic adenosine monophosphate (cAMP), and protease A is activated, eventually leading to cells death. A number of receptors have been described for Cry toxins, including cadherin-like[9,10], aminopeptidase N (APN)[11,12],

alkaline phosphatase (ALP)[13,14], ABC transporter (ABCC2) [15].

In this work, the cDNA of the full-length *H. parallela* alp (*hpalp*) gene was cloned from larvae midguts for the first time, and the HpALP protein was separated from *Escherichia coli*. To test the binding ability of the protein with the coleopteran-specific toxin Cry8Ea3, ligand blot experiments were carried out. Furthermore, we used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine the transcriptional level of the *hpalp* gene in different larval tissues of *H. parallela*.

Materials and Methods

Insects

H. parallela were collected from the field in Baoding, China, and the adults were reared with fresh *Ulmus pumila* elm tree leaves in the rearing chamber. The larvae were reared with artificial feed.

Preparation of Cry Toxin

BTGWL, Cry8Ea3 (GenBank No. KC855216.1) engineering strains were cultured in 1/2LB medium supplemented with ampicillin and erythromycin at 30±1℃ until complete autolysis and spore crystal mixtures were prepared. The cry8Ea3 crystal proteins were prepared by differential centrifugation. Cry8Ea3 protoxin was activated by trpsin and analyed by SDS-PAGE.

Cloning of the *Hpalp* Gene

Total RNA was extracted 15 mg of fresh midguts of third-instar *H. parallela* larvae using the RNAprep pure Tissue Kit (Tiangen, Beijing, China). The first-strand cDNA was synthesized according to the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). Simultaneously, 5′-RACE primer was designed according to the results of *H. parallela* midgut transcriptome sequencing and amplified by SMARTER RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The PCR program was as follows: 5 cycles of 94°C for 30 s; 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; and 27 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. Next, using Primer 5.0 designerate full length primers (alp-F/alp/R). The PCR program was as follows: one cycle of 94°C for 4 min; 30 cycles of 94°C for 45 s, 67°C for 45 s, and 72°C for 1 min and 40 s; and a final cycle at 72°C for 10 min.

Expression of HpVAA Protein in *E. Coli* BL21

The open reading frame of *hpalp* was amplified, purified with Universal DNA Purification Kit (TIANGEN, Beijing, China), and cloned into pET21b vector via restriction enzymes digestion (*Sal* I and *Not* I).

The strain BL21 (pET21b-*hpalp*) was cultured in Luria-Bertani medium at 37°C, and induced by isopropyl-β-D-1-thiogalactopyranoside for 4 h and 8 h; BL21(pET21b) served as the negative control. HpALP protein was loaded onto 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes to determine its expression. In brief, the membrane with the transferred proteins was blocked for 1 h with 1% bovine serum albumin at room temperature. After blocking the membrane was incubated with anti-His (1:5000 in Tris-buffered saline [TBS]) mouse antibodies (Boster, Wuhan, China) for 1 h, followed by washing with TBS four times for 5 min , and then with

Tween (TBS-T) washing once for 5 min. The membrane was subsequently incubated with second antibodies (goat anti-mouse antibodies, 1:10000 in TBS) for 1 h, and the same washing steps were applied as described above. Detection was performed with Nitrotetrazolium Blue chloride (NBT) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP).

Ligand Blot Analysis of HpVAA Protein

After separating with 12% SDS-PAGE, HpALP protein was transferred onto a polyvinylidene fluoride membrane, and incubated in blocking buffer, 1% bovine serum albumin in TBS, for 1 h. The blocked membrane was then incubated with 20 μg/mL Cry8Ea3 toxin proteins, activated by trypsin in blocking buffer, and washed with TBS and TBS-T. The blocked membrane was incubated with Cry8Ea3 primary antibodies (rabbit polyclonal antibody, 1:5000 in TBS) for 1 h and then conjuncted with anti-rabbit secondary antibody for 1 h. The immunoblots were developed with NBT and BCIP.

qRT-PCR

Total RNA was traditionally separated from the midgut, foregut, hindgut, Malpighian tubes, fat body, peritrophic membrane, and egg of *H. parallela*. cDNA was synthesized and primers were designed as described above. The cDNA was amplified by qRT-PCR with specific primers (CFX96 Touch^{TM} Real-Time PCR System, BIO-RAD, Germany). PCR amplification conditions were as follows: one cycle of 95°C for 30 s, 39 cycles of 95°C for 5 s, 56°C for 30 s, and 72°C for 30 s, one cycle of 95°C for 1 min, one cycle of 50°C for 1 min, one cycle of 65°C for 5 s. The relative quantities of *hpalp* transcripts were assessed using the $2^{-\Delta\Delta Ct}$ method and normalized with β-actin.

Results

Cloning and Analysis of the *Hpalp* Gene of *H. Parallela*

The full-length sequence of *hpvaa* cDNA was successfully obtained by RACE-PCR (Table 1A). The full-length *hpalp* gene was cloned. Sequencing analysis showed that the open reading frame of *hpalp* (GenBank accession NO. KY922835) was 1605 bp long encoding 534 amino acids with a predicted molecular weight and isoelectric point of HpALP were 59 kDa and 5.18, respectively. The SignaIP 4.1 Server on-line analysis of HpALP signal peptide with 21 amino acids; Big-PI Predictor analysis of GPI anchor point is located in the C-terminal D^{514}; NetOGlyc 3.1 Server online analysis found no O-glycosylation site; Server NetNGlyc1.0 online prediction has two N-glycosylation sites: N^{100}, N^{296} (Figure 1A). Additionally, using MEGA 6.0 and the neighbor-joining (NJ) construct phylogenetic tree, HpALP was closely related to *Nicrophorus vespilloide*se ALP (GenBank accession NO. XP008200193.1) (Figure 1B). Ag, *Anoplophora glabripennis*; Tc, *Tribolium castaneum*; Ap, *Agrilus planipennis*; At, *Aethina tumida*; Dp, *Dendroctonus ponderosae*; Nv, *Nicrophorus vespilloides*. SWISS-MODLE on line was used to 3D-modeled structure of HpALP (Figure 1C).

Table 1. Summary of primers used in the study

Primer	Orientation	Primer DNA sequence
5-RACE	5'RACE	CTTCCGAATAGACGAGTTGATGGGCG
Full-F	Forward	gcgtcgacATGAACGCGAAGTGTGTG
Full-R	Reverse	ttgcggccgccGTTTCTTCCGTCATTGGA
qPCR/F	Forward	CCATCTAGACGCTGAAGATAAC
qPCR/R	Reverse	TGTGCTCTAGTTTCGTGATG
Actin/F	Forward	ATGTTGCCATCCAAGCTGTA
Actin/R	Reverse	CCAAACGCAAAATAGCATGA

A

1 MNAKCVLVLI SVIGLVVTIQ ARPENFANSY DDGHMHPNPV KRSINTKTSK INAEEYNSKF WIDQAQKVLN

71 AKLLEKPNTN IAKNVIMFLG DGLSIPTIKA SRLYLGQLHN ESGEETILSF EKFPYVGLSK TYCVDTQVPD

141 SACTATAYLS GVKNNYHTIG VTAAVQYQDC DASLNEENHV YSIAQWSQLK NKKTGIVTTT RITHASPAGA

211 YANTADRDFE SDADTPDSKC KDIAHQLVYS EVGKNLNVVL GGGRREFLPN TVTDEGGQRG RRLDGENLIE

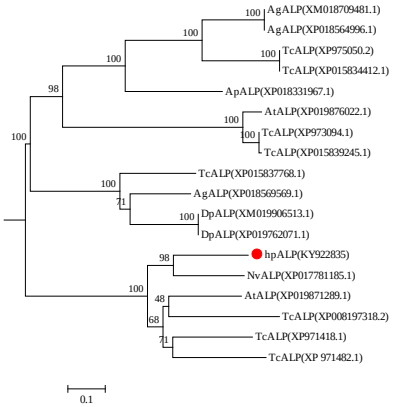
281 AWLKSKQGHK AQYVWNRTEL ENVAGDTEYL LGLFEDNHCK YHLDAEDNDP SLTEMTEKAI ELLASAENGY

351 FLFVEGGRID HAHHETRAQK ALDETVEFHK AIERAVELTN KEDTLIVVTA DHAHTMSVSG YAERGNDILG

421 ICDKADDELP YMTLSYANGP GYRASVGGRR TDVTNDNTHD KNYRFPVIAP LEDETHGGDD VSIYSQGPWS

491 HLLTGVIEQN VIPHIMAYAS CVG**D**GLKICD QELERIKKSN DGRN*

B



C

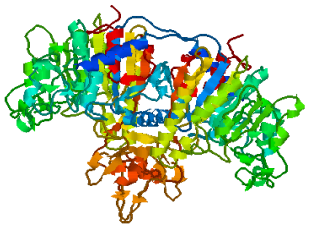


Figure 1. Gene sequence analysis of ALP from *H. parallela*. Panel A, The Amino acid sequence of ALP was deduced from *H. parallela* cloned of *alp* gene (GenBank accession NO. KY922835). Signal peptide is underlined with a double line. N-linked glycosylation sites by a solid triangle. GPI-anchor site by font-bold. Panel B, Phylogentic tree based on amino acid sequences of HpALP from *H. parallela* and other known insects. HpALP was closely related to *Nicrophorus vespilloide*se ALP (GenBank accession NO. XP008200193.1). Ag, *Anoplophora glabripennis*; Tc, *Tribolium castaneum*; Ap, *Agrilus planipennis*; At, *Aethina tumida*; Dp, *Dendroctonus ponderosae*; Nv, *Nicrophorus vespilloides*. Panel C, 3D-modeled structures of HpALP.

Expression of pET21b-*Hpalp* in *E. coli*

About 69 kDa band was visible on the gel of SDS-PAGE (Figure 2A), which was consistent with the predicted molecular weight. The recombinant protein existed in the form of an inclusion body. Western blot analysis demonstrated a clear signal with a band appearing at 69 kDa (Figure 2B).

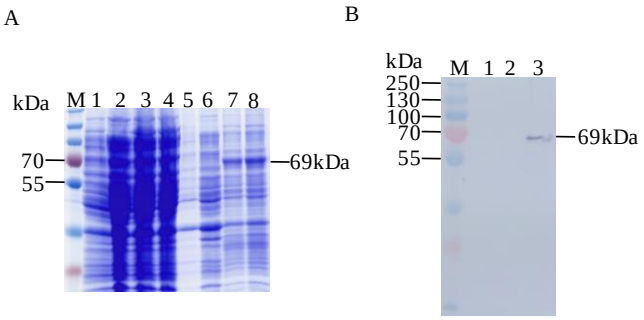


Figure 2. Identification and analysis of a recombinant HpALP protein. Panel A, Expression of the recombinant HpALP protein analyzed by SDS-PAGE in *E. coli.* M: Protein Marker; lanes 1-4: supernatant; lanes 5-8: precipitate. Lanes 1, 5: BL21(pET21b); 2, 6: BL21(pET21b-*hpalp*), not induced; 3, 4, 7, and 8: BL21(pET21b-*hpalp*) induced for 4 h and 8 h, respectively. Panel B, Western bot analysis of the recombinant expression of HpALP in *E. coli.* M: Protein Marker; lane 1: BL21(pET21b); 2: BL21(pET21b-*hpalp*), not induced; 3: BL21(pET21b-*hpalp*), induced.

Ligand Blot of HpALP Protein in *E. coli* BL21

Ligand blot of Cry8Ea3 toxins, as well as immune-blot for HpALP protein are shown in. The Cry8Ea3 ligand blot revealed strong binding to a protein of 69 kDa (Figure 3).

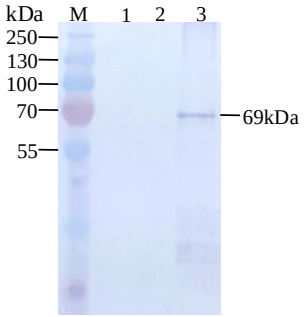


Figure 3. Ligand blot analysis of recombinant expression of HpALP in E. coli M: Protein Marker; lane 1: BL21(Pet21b); 2: BL21(pET21b-hpalp), not induced; 3: BL21(pET21b-hpalp), induced.

Transcriptional Analysis of *Hpalp* in Different Larval Tissues of *H. Parallela*

Transcriptional analysis of *hpalp* in different tissues of *H. parallela* larvae was performed by qRT-PCR, which revealed that the *hpalp* was primarly expressed higher in midgut, but lower in the foregut (Figure 4).

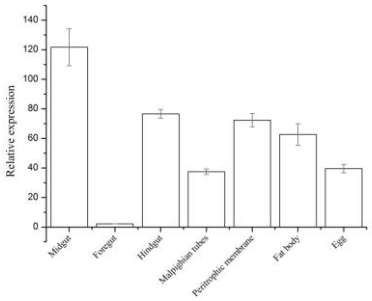


Figure 4. Relative expression level of hpalp in different tissue in the third larvae of H. parallela

Discussion

In the *S. exigua* midgut, ALP acts as Cry1Ac binding protein[16]. ALP1 silenced *Aedes aegypti* larvae showed tolerance to both Cry4Ba and Cry11Aa, but not tolerance to Cry4Aa. The result shows that ALP1 was a functional receptor that plays an important role in the toxicity of the Cry4Ba and Cry11Aa proteins[17]. Immunolocalization results showed that Cry4Ba is able to bind to only Sf9 cells-expressing AaALP. Moreover, these cells were shown to undergo cell lysis in the presence of trypsin-treated toxin. Finally, trypan blue exclusion assay also demonstrated an increase in cell death in recombinant cells treated with Cry4Ba. Overall results indicated that AaALP protein was responsible for mediating Cry4Ba toxicity against Sf9 cells, suggesting its role as a receptor for Cry4Ba toxin in *A. aegypti* larvae[18]. Further studied have found that the binding site is located on the domain II loop 2 of the Cry4Ba protein[17]. In the present study, ligand blot assay showed binding of the HpALP with Cry8Ea3 toxin.

In the *S. exigua*, ALP2 was expressed in the whole larval period, the lowest expression levels in the 1st instar larvae, and the highest expression in the 4th instar larvae[16]. However, The ALP could be expressed in the 1st, 2nd, 3rd larval period, and there were significant differences among different instars. The lowest and highest expression levels appeared in the 1st and 3rd instar larvae, and no expression in 4th and 5th instar larvae[19], in the *Manduca sexta*. In the work, transcriptional analysis of *hpalp* in different tissues of *H. parallela* larvae, which revealed that the *hpalp* was primarly expressed higher in midgut, but lower in the foregut.

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