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Gene cloning and sequencing of aminopeptidase N3, a putative receptor for *Bacillus thuringiensis* insecticidal Cry1Ac toxin in *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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Abstract. A cDNA encoding aminopeptidase N3 was cloned by degenerated PCR and RACE techniques. The full-length of *APN3harm* is 3486bp. Open reading frame is 3042bp in length, encoding 1014 amino acid residues. Its predicted molecular weight and isoelectric point are 117.04 kDa and 5.14, respectively. This deduced amino acid sequence shares some common structural features with aminopeptidase N from Lepidoptera, including the consensus zinc-binding motif $HEXXHX_{18}E$ and the GAMEN motif common to gluzincin aminopeptidases. The full-length of the *APN3harm* gene from three susceptible and three resistant strains were cloned and sequenced. Comparison analysis revealed fourteen amino acid differences in the *APN3harm* gene from resistant and susceptible strains and six mutations of amino acids exist in all resistant strains. It is possible that these mutations are related to the resistance of *Helicoverpa armigera* to Cry1Ac toxin. The results of semi-quantitative RT-PCR showed that the resistance of *H. armigera* to Cry1Ac is unrelated to the amount of *APN3harm* mRNA in midgut tissue. In susceptible strains, *APN3harm* is highly expressed in mid-gut, foregut and hindgut but not in other tissues. To determine if the APN3harm is the receptor of Cry1Ac, recombinant APN3harm protein was successfully expressed in *E. coli*. A ligand binding experiment showed purified product could bind Cry1Ac toxin. So it is proposed that APN3harm is a putative receptor of Cry1Ac in *H. armigera*. The sequence of *APN3harm* was deposited in GenBank with the accession number AY052651.

INTRODUCTION

Bacillus thuringiensis, a Gram-positive bacterium, produces inclusion bodies of insecticidal proteinaceous crystals during sporulation. These inclusion bodies consist of one or several proteins called δ-endotoxin. When digested by susceptible insect larvae the inclusion bodies are solubilized and the protoxins are converted to toxins. The activated toxins bind to receptors on the surface of midgut epithelial cells of susceptible insects, cause this lysis and the death of the insect (Höfte & Whiteley, 1989; Gill et al., 1992; Knowles, 1994).

Cotton bollworm, *Helicoverpa armigera* (Hübner), is a serious pest of cotton, maize and many other crops in Asia and parts of Australia (Guo, 1997; Gujar et al., 2000). Because the growers have used large quantity of chemical insecticides to control cotton bollworm in the past it became resistant to insecticides and since the 1990s has became a major threat to cotton production (Wu et al., 1997). After 1997, Cry1Ac-transgenic cotton was planted in China, the area increased rapidly to more than 200 million ha in 2002. The Bt cotton is not only a major factor in the integrated management of *H. armigera*, it has also delayed the development of *H. armigera* resistant to chemical insecticides. However, selection experiments in laboratory indicating that *H. armigera*, just like other Lepidoptera, is potentially capable of developing resistance to Bt or Cry1Ac-transgenic cotton (Liang et al., 2000a, b). It is important to clarify how *H.armigera* develops resistance to Cry1Ac in order to delay the development of resistance in *H. armigera* to Bt cotton.

A change in the ability of Bt toxin to bind to insect midgut receptors is the most important reason why insect pests develop resistance to Bt (Heckel, 1994). Specific Cry 1 toxin-binding proteins present on the brush border membrane of midgut cells have been reported for many Lepidoptera. Aminopeptidase N (APN) has been identified as the putative receptor for Cry1Ac toxin in *Helicoverpa armigera* (Rajagopal et al., 2003), *Manduca sexta* (Knight et al., 1994; Sangadala et al., 1994), *Heliothis virescens* (Gill et al., 1995; Lee et al., 1996), *Lymantria dispar* (Valaitis et al., 1995) and *Plutella xylostella* (Luo et al., 1997a). Other, distinct APNs also interact with Cry1Ab/Cry1Aa toxin in *M. sexta* (Masson et al., 1995; Denolf et al., 1997), *H. virescens* (Luo et al., 1997b), *Bombyx mori* (Yaoi et al., 1997), Cry1C in *M. sexta* (Luo et al., 1996) etc. cDNAs for APN genes from many Lepidoptera have been cloned and sequenced (Gill et al., 1995; Knight et al., 1995; Hua et al., 1998; Chang et al., 1999; Garner et al., 1999; Yaoi et al., 1999; Zhu et al., 2000; Emmerling et al., 2001; Rajagopal et al., 2003; Wang et al., 2003a) but only from Bt-susceptible strains. Zhu et al. (2000) report that mutation of the APN gene is associated with Bt-resistance in *Plodia interpunctella* and that the APN mRNA expression levels in a Bt-resistant strain are slightly higher than those in a Bt-susceptible strain. Liang et al. (2004) cloned two APN genes from the

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midgut of *H. armigera* and found mutations of the APN1 gene in a Cry1Ac-resistant strain. In the present study, we clone and express a new APN gene named as *APN3harm*. The expressed products of *APN3harm* gene can bind to Cry1Ac. To determine whether this gene is responsible for Cry1Ac resistance in *H. armigera*, the mutations and mRNA expression level of the *APN3harm* gene from both Bt-susceptible and Bt-resistant strains of *H. armigera* was examined.

MATERIALS AND METHODS

Insects

Individuals of the Bt-susceptible strain SS1 of *H. armigera* were collected at Xinxiang, Henan Province in 1996. Bt-susceptible strain SS2 and SS3 were collected at Langfang, Henbei Province in 2001 and 2002, respectively. The three strains were maintained on an artificial diet without Bt toxin as described by Liang et al. (1999). Three Bt-resistant strains (Rdu, Rsh, Rm) were selected from the SS1 strain by incorporating Cry1Ac toxin, Bt insecticides and Cry1Ac transgenic cotton into the diet separately for 46 generations. The relative resistance of the Rdu, Rsh and Rm strains was 278, 1083.3 and 48.7. The resistance ratios of the three strains were determined according to Liang et al. (2000a, b).

Fifth instar larvae of *H. armigera* were chilled on ice for 15 min. The midgut was removed and washed with ice-cold phosphate buffer saline (0.7% NaCl/ 10 mmol L^{-1} sodium phosphate pH 7.0), immediately frozen in liquid nitrogen and then stored at –70°C until used.

Chemicals

T-easy vector, cDNA synthesis kit and DNA purification system were purchased from Promega (USA). Restriction enzymes, *Taq* DNA polymerase, 3′-RACE kit, dNTP, DEPC, IPTG, X-gal were purchased from TaKaRa Biotech. Co., Ltd (Japan). 5′-RACE kit and RNA isolated reagent (Trizol) from Invitrogen Life Technologies (USA).

Total RNA isolation and cDNA synthesis

The midgut tissue was ground into a fine powder in liquid nitrogen and transferred into a centrifuge tube. 1 ml Trizol reagent and 200 ml chloroform were added and the mixture centrifuged at 12000 g for 15 min, after which the supernatant was transferred into a new centrifuge tube, 500 µl isopropanol was added and then centrifuged at 12000 g for 10 min, after which the sediment was washed with 70% ethanol. Finally, dried RNA was dissolved in DEPC-treated ddH_2O and the concentration of RNA measured using a spectrophotometer.

A cDNA synthesis kit was purchased from Promega and used as outlined in the instructions. In a microcentrifuge tube, 2 µg RNA and 1 µl OligdT(50 µmol L^{-1}) were added a volume of < 15 µl of water. The tube was heated to 70°C for 10 min and then cooled immediately on ice, after which it was spun briefly to collect the solution at the bottom of the tube. 5 µl dNTP(10 mmol L^{-1}), 0.75 µl RNase inhibitor(40 U μl^{-1}), 5 µl M-MLV 5× reaction buffer and 1 µl M-MLV RT(200 U μl^{-1}) were added, mixed gently and incubated for 30 min at 42°C. The product was used as a PCR template.

PCR and RACE Primers

Five APN amino acid sequences of several insects were compared with DNAMAN software (Los Angeles, USA). A pair of degenerate primers (APN3S1 and APN3A2) was designed according to the characteristics of the conservative region. These APN sequences were obtained from NCBI. The accession numbers are as follows: *Heliothis virescens* APN AAK58066, *Helicoverpa punctigera* APN3 AAF37559, *Lymantria dispar* APN3 AAL26894, *Spodoptera litura* APN AAK69605, *Manduca sexta* APN AAM18718, *Bombyx mori* APN AA33715. In addition, another three degenerate primers (APN3S3, APN3A4 and APN3A5) were designed according to the characteristics of the conservative regions and three specific primers (APN3A3, APN3S4 and APN3S5) were designed from the above PCR sequencing. These primers were used to amplify another three fragments of the APN3 gene. Specific primers for RACE were derived from the sequence of the PCR product. Specific primers (APN3FF and APN3RR) to amplify full-length ORF of APN were designed based on RACE products. In order to facilitate cloning two restriction enzyme sites (*Bam*HI and *Sal*I) were introduced. The sequences of all primers used in this study are given in Table 1 and the relative positions of PCR and RACE primers are shown in Fig. 1. All primers were synthesized by BioAsia Biotechnology Co., Ltd (Shanghai, China).

PCR and RACE reactions

PCR was carried out in a GeneAmp 480 thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The reaction mixture contained 4 µl 2.5 mmol L^{-1} dNTPs (N = C,G,A,T), 0.25 µl *ExTaq* polymerase (5 U μl^{-1}), 5 µl of 10× *ExTaq* buffer (added Mg^{2+}), 2 µl degenerate primer (or 1 µl specific primer), 1 µl cDNA template and 35.75 µl (37.75 µl) of distilled water. The reaction mixture was initially heated for 3 min at 94°C, then incubated for 32 cycles (94°C 1 min, 50–55°C for 1 min, and 72°C for 1–3 min) and finally for 10 min at 72°C.

5′-RACE and 3′-RACE kits were purchased from TaKaRa Biotechnology Co. Ltd. The reaction was conducted following the manufacture's protocol.

Cloning and identification of PCR and RACE products

PCR and RACE products were separated by electrophoresis on 1% agarose gels in TAE buffer. The resulting band was visualized by ethidium bromide staining and the target fragment was cut and purified by DNA purification kit (Promega) following the manufacture's protocol. The purified target fragment was ligated with T-easy vector using T4 DNA ligase (4°C, 18 h), and competent DH5α *E. coli* cells were transformed by 90 s heat shock at 42°C followed by growing for 45 min at 37°C in 800 ml of LB medium. LB agar plates containing ampicillin were inoculated and incubated overnight at 37°C, and the posi-

TABLE 1. Primers used in PCR amplification and RACE reaction.

Primer name	Sequence
APNS1	5′-T(C/T)CTCITG(C/T)TA(C/T)GA(C/T)GAGCC-3′
APNA2	5′-GCGAAICC(C/T)TCGTT I AGCCA-3′
APNS3	5′-TACMGHYTVCCBAAYACTAC-3′
APNA3	5′-AAGCCAGAAGGTAGGTAGAC-3′
APNS4	5′-GAGCAGCAACTTCAAGCAAATC-3′
APNA4	5′-TGRGCHCKRTTRTAYTCATG-3′
APNS5	5′-CTATGATGATTACACCTGGG-3′
APNA5	5′-(A/G)(A/G)G(A/T)TGGAGTGGAGGGTTCC-3′
APNS6	5′-CCGCTTACAACAGCATCC-3′
APNrt	3′-CCTACCGTTGCTGA-5′ (5′-end phosphorylation)
5APNa2	5′-TACTTTGTAGTGGGTAGGGA -3′
5APNa1	5′-TCAAGTGGTCGCAGTGGATG -3′
5APNs1	5′-CACCCCTATACCTCAATCTC-3′
5APNs2	5′-CCGTGCAATATACCCTTTCC-3′
APN3FF	5′-GTGGATCCATGGCGGCGATAAAACTCTTAG-3′
APN3RR	5′-GTGTCGACTAAAATAAGGTGCAAAATGGC-3′
APN3F	5′-TTCGACATCGACACTGGCTAC-3′
18SS	5′-TTAGTGAGGTCTTCGGACCG-3′
18SR	5′-CAGTTCACACTATGACGCGC-3′

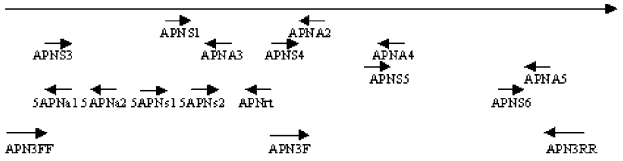

Fig. 1. Relative positions of primers in mRNA.

tive clones on the LB agar plates were identified by PCR amplification and restriction analysis.

DNA sequencing and analysis

Positive recombinants were selected and inoculated into liquid LB medium containing 100 μg μl^{-1} ampicillin. After an overnight incubation at 37°C, plasmid DNA was extracted (Sambrook et al., 1989) and sequenced using an Applied Biosystem Model 377 DNA sequencer (USA).

Six *APN3* genes from three susceptible strains and three Cry1Ac-resistant strains were cloned and sequenced. All cDNA sequences and inferred amino acid sequences were analyzed and compared by DNAMAN and DNASIS software (Hitachi, Japan).

Semi-quantitative RT-PCR of APN3 gene

Specific primers (APN3F and APN3RR) were designed and used to amplify APN3 fragments. Specific primers (18SS and 18SR) were designed and used to amplify 18S RNA fragments as a control (Table 1). The reaction mixture contained 1 μl cDNA template, 4 μl 2.5 mmol L^{-1} dNTPs (N = C,G,A,T), 0.25 μl *ExTaq* polymerase (5 U μl^{-1}), 5 μl of 10× *ExTaq* buffer (added Mg^{2+}), 1 μl each primer (APN3F and APN3RR, 18SS and 18SR) and appropriate distilled water. The reaction mixture was initially heated for 3 min at 94°C, then incubated for 28 cycles (94°C 1 min, 55°C for 1 min and 72°C for 1–3 min) and finally for 10 min at 72°C.

Recombinant protein expression and purification

The entire APN3 coding region from a susceptible strain was subcloned into the *Bam*H I / *Sal* I sites of pGEX-4T-1 (Pharmacia, USA). Competent *E. coli* DH5α cells were transformed by 90 s heat shock and colonies were grown on LB ampicillin agar plates. Positive colonies were identified by PCR and restriction analysis. The plasmid was then extracted and inserted into competent *E. coli* BL21(DE3) cells. A selected positive clone was grown in 5 ml LB ampicillin medium for 12 h and the culture inoculated into 5 ml of liquid medium. Cells were induced with IPTG (0.8 mM) at early log phase (OD = 0.5) and isolated by centrifugation after 8 h. The soluble fraction and the whole pellet of the induced and non-induced cells were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

For large-scale protein production, 2 L of LB ampicillin medium was inoculated with 20 ml of overnight culture and induced by 0.8 mM IPTG. After 8 h, cells were isolated by centrifugation (5000 g), dispersed in lysis buffer (80 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 4% glycerol, pH 7.2, 1 mM PMSF) and burst by sonication (10 s, 5 pulses). Soluble proteins were isolated by ultracentrifugation (30,000 g). Insoluble inclusion bodies from 1 L of culture were washed with 0.2% Triton X-100 in 50 Mm Tris-HCl (pH 6.8) and then dissolved in 10 ml 6 N guanidinium hydrochloride.

Soluble rAPN3 was primarily purified by Glutathione Sepharose 4B affinity chromatography (Pharmacia) and desalted by dialysis in Tris buffer (50 mM Tris-HCl, pH 7.5) overnight. Desalted protein (100 ml) was digested by thrombin in lysis buffer, including 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 2.5 mM $CaCl_2$. Finally the product of this lysis was purified by Sephadex-G50 gel filtration (Wang et al., 2003b).

Preparation of antisera (anti-Cry1Ac)

Antisera were obtained by injecting an adult rabbit subcutaneously and intramuscularly with 150 μg of purified Cry1Ac toxin, followed by two additional injections of 100 μg after 18 and 30 days. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for subsequent injections. The animal was bled 10 days after the last injection and the serum was used without further purification.

Protein blotting and ligand binding experiment

A twenty μl aliquot of protein was separated on 1.0% SDS-PAGE and electroblotted onto a nitrocellulose membrane for 4 h at 40 V. Membranes were stained with Ponceau S to visualize molecular weight markers, washed with water, blocked with 5% dry milk in PBST and incubated with activated Cry1Ac toxin overnight. After washing the unbound Cry1Ac with PBST it was incubated with rabbit anti-Cry1Ac antiserum in PBST with 3% BSA for 2 h and washed three times with PBST (10 min each time). Protein-antibody complexes were visualized by secondary antibody-alkaline phosphate conjugate and chromogenic substrate (BCIP, NBT) as described in the instructions (Promega).

RESULTS AND DISCUSSION

Cloning and identification of PCR and RACE products

Using cDNA from the midgut of *H. armigera* as a template, APNS1 and APNA2 as primers, the expected ca. 550 bp PCR product was obtained, isolated, purified and ligated with T-easy vector. Positive recombinant pGEM/APN was identified by PCR amplification and restriction analysis. In the same way, three cDNA fragments of the *APN* gene of different lengths (ca. 600 bp, 900 bp and 1000 bp, respectively) were also amplified. In addition, the ca. 650 bp 3′-RACE and ca. 350 bp 5′RACE products were obtained. All these fragments were also ligated with T-easy vector and identified by restriction analysis. These primers in PCR and RACE reactions are shown in Table1 and their relative positions in mRNA in Fig 1.

Sequencing and analysis

PCR products and the 5′ and 3′ RACE products were aligned to form a contig of 3486 bp in length. The cDNA encoding APN of *H. armigera* was named as *APN3harm*. *APN3harm* contains 53.72% A-T, 46.26% C-G. This sequence was deposited in GenBank with the accession number AY052651. It contains an open reading frame (ORF) of 3042 base pairs. The start codon (ATG) and terminal codon (TAA) are in bold. The 3′ end of *APN3harm* contains polyadenylation signals typical of eukaryotes. Therefore, *APN3harm* appears to contain the complete 3′ and 5′ ends. The predicted amino acid sequence of *APN3harm* is given in Fig. 2. The cDNA encoded 1014 amino acid residues. The molecular mass and isoelectric point were calculated to be 117.04 kDa and 5.14, respectively. There are 4 N-glycosylation sites in the referred amino acid sequence (http://www.cbs.dtu.dk/services). The first 18 amino acid residues have the characteristic

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MAAIKLLVLS LACACVIAHS PIPPVSRTIF LDERLEGGAF ENIDAFKNIE LSNAAASPYR   60
LPNTTFPTHY KVLWVINLSE NVQSYSGTVD ITLQATQPNV NEIVIHCGHL TVTSVVLRQG  120
TATEGTLIPT TPTPQSQYHF LRVALNDGVL LYNENVPVQY TLSIAFNADM RDDMYGIYRS  180
WYRNLPSDNN IKWMATTQFQ ATAARYALPC YDEPGYKAKF DVTIRRPLDY ISWFCTRQRI  240
TRPSTTPGYA EDEYHTTPEM STYLLALIVA EYDSLATLDA DNRVLHEVIA RPGAIINGQA  300
AYAQRAGQDL LAEMSDHTDF DFYKQDENLK MTQAAIPDFG AGAMENWGLL TYREAYILYD  360
EQHTSSNFKQ IIAYILSHEI AHMWFGNLVT NAWWDVLWLN EGFARYYQYF LTAWVEDMGL  420
ATRFINEQVH ASLLSDSSID AHPLTNPGVG SPAAVSAMFS TITYNKGASV IRMTEHLLGF  480
EVHRAGLRKY LEDKKFKTVQ PIDLFTALET AGNDAGALDA YGDHFDFVKY YESWTEQPGH  540
PVLNVHINHQ TGHMTIYQRR FDIDTGYSVQ NRNYIVPITF TTGADPDFDN TKPSHVISKA  600
VTVIDRGVVG DVWTIFNIQQ TGFYRVNYDD YTWDLIILAL RGADREKIHE YNRAQIVNDV  660
FQFARSGLMT YERALNILSY LENETDYAPW VAAITGFNWL RNRLVGKPQL AELNAKIVQW  720
SSKVMSELTY MPIEGESFMR SYLRWQLAPV MCNLNVPACR AGARVIFENL RLYQHEVPVD  780
SRSWVYCNAL RDGGADEFNH LYNRFKGHNV YTEKILILQT LGCTSHAASL TTLLNDIVTP  840
NNIIGPQDYT TAFSTAVSGN EENTLFVLNY IQNNLETVLK AFSSPRTPLS YIAARLRTVE  900
DVTAYQTWLN LTTTREVLGT SYNNIYGDSV AAYNSILWVA TIEDSLSAYL TNGDNVIQST  960
TSTTTTTVAP TTVTQPPITE PSTPTLPVPV TDGAMTSFAS LFIISLGAIL HLIL      1020
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Fig. 2. Inferred amino acid sequences from *APNharm* gene. Signal peptide at N-terminal is underlined. Dashed underlined are GPI-anchored sequence at C-terminal. Potential N-glycosylation site is double underlined. The zinc-binding motif $HEXXHX_{18}E$ is in bold. The consensus GAMEN motif, indicative of gluzincin aminopeptidase, is boxed. Italics at N- and C-terminals are hydrophobic and transmembrane helices.

features of a signal peptide common for secretory proteins. The cleavage site of the signal peptide is between the 18th and 19th amino acid residues (Nielsen et al., 1997; Signal P 3.0 Server, Technical University of Denmark).

Searching the PROSITE database revealed that there are conservative motifs of insects' aminopeptidase N in the amino acid sequence. Amino acid residues between 342 and 346 are the zinc-binding region signature, GAMEN, a conservative structure of gluzincin aminopeptidase, and $HEXXHX_{18}E$, a zinc binding site, is present in the amino acid sequence (Haeggstrom et al., 1990; Rawlings & Barrett, 1995). Analysis of the potential transmembrane domain showed that except for the signal peptide on N-termini, there was a hydrophobic and transmembrane helix on C-termini, by which APN3harm anchored to the brush border membrane.

There are more than 10 kinds of insect APN genes cloned and deposited in GeneBank. Homology analysis showed that all the aminopeptidase Ns of insects had a GAMEM structure and zinc binding site $HEXXHX_{18}E$, which indicates that these proteins might have originated from the same ancestor. Insect aminopeptidase Ns are classified into 3 groups. Four *APN* genes of *H. armigera* cloned in our laboratory belong to the 3 groups (Wang et al., 2003; Liang et al., 2004) (Fig. 3). Every APN group is very similar (>50%). The homology of the 3 kinds of APN from *H. armigera* is not high, but they have the same conservative structure and characteristic of peptidase M1. So the APN gene duplicated at least three times in the ancestor of *H. armigera* and in other Lepidoptera (Chang et al., 1999; Emmerling et al., 2001).

The full-length *APN3harm* gene from three susceptible and three resistant strains were cloned and sequenced. Comparison analysis revealed several amino acid differences among the three susceptible strains, which could be a polymorphism. Those from the resistant strains differed by fourteen amino acids from those of the susceptible strains. In particular, there are six mutations in all resistant strains that are absent in the susceptible strains (Table 2). PCR amplification of the *APN3harm* gene from every strain was repeated three times and the PCR products

TABLE 2. Summary of amino acid mutations of APN3harm in Cry1Ac-resistant and susceptible strains.

Location of amino acids	Three susceptible strain SS1-3	Resistant strain Rdu	Resistant strain Rm	Resistant strain Rsh
65	F	F	F	I
107	G	**D***	**D**	**D**
132	T	I	I	T
168	D	E	E	D
186	S	T	T	S
230	I	R	R	I
280	D	N	N	D
307	Q	H	Q	Q
343	M	M	M	T
736	S	**P**	**P**	**P**
763	R	**K**	**K**	**K**
767	E	**D**	**D**	**D**
802	N	**E**	**E**	**E**
826	A	**S**	**S**	**S**

* Amino acids of mutation in all resistant strains are in bold.

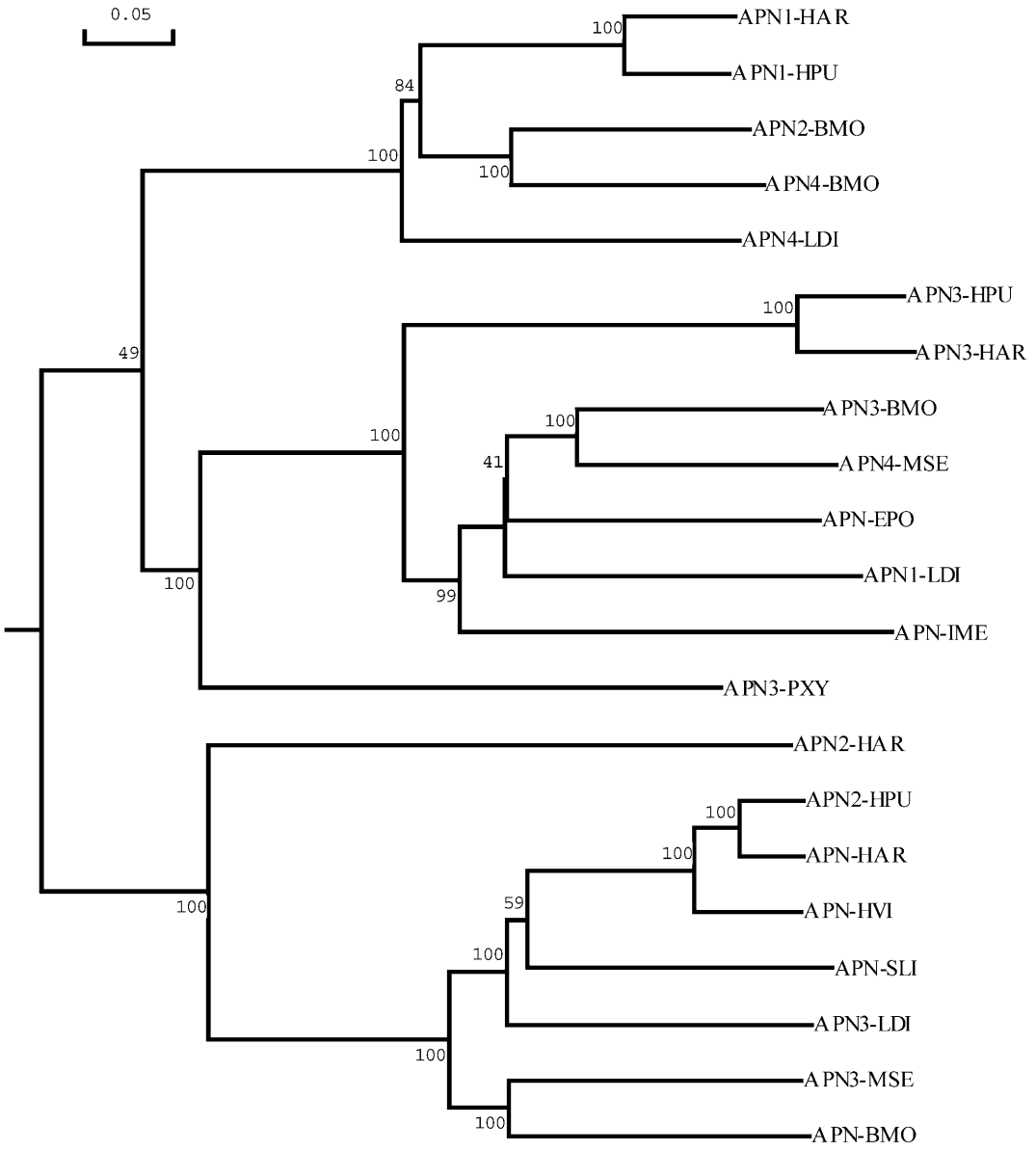

Fig. 3. Neighbour joining phylogeny relating APN-Harm in lepidopterous insects. The scale of the distance is indicated. Bootstrap support values (%) based on 1000 replicates are indicated. APN sequences were obtained from NCBI using ENTREZ search engine. The accession numbers are as follows: *Helicoverpa punctigera* APN1-HPU (AAF37558), APN2-HPU (AAF37559) and APN3-HPU(AAF37560); *Lymantria dispar* APN1-LDI (AAD31183), APN3-LDI (AAL26894) and APN4-LDI (AAL26895); *Heliothis virescens* APN-HVI (AAK58066); *Spodoptera litura* APN-SLI (AAK69605); *Manduca sexta* APN3-MSE (AAM18718) and APN4-MSE(AAM13691); *Bombyx mori* APN-BMO(BAA33715), APN2-BMO(AAF07223), APN3-BMO(AAL83943) and APN4-BMO (BAA32475); *Plutella xylostella* APN3-PXY(AAF01259); *Helicoverpa armigera* APN-HAR(AY181026), APN1-HAR (AAL34109), APN2-HAR (unpublished) and APN3-HAR (AY052651); *Plodia interpunctella* APN-IME(T30942); *Epiphyas postvittana* APN-EPO (AAF99701).

sequenced in both directions, which excluded the error in PCR. Three resistant strains were derived from the susceptible strain SS1. So it is possible that these mutations lead to different levels of resistance. But it is not possible to exclude a polymorphism unrelated to resistance. So further study is necessary to confirm the roles of these mutations using site-directed mutagenesis of the *APN3harm* gene.

Expression of *APN3harm* gene in different strains and tissues

Semi-quantititative RT-PCR was used to determine the expressed quantity of *APN3harm* at the transcriptional level in three Cry1Ac-resistant strains and one susceptible strain, and in different tissues of the susceptible strain. The results showed that the expressed quantity of *APN3harm* is not obviously different in resistant strains. So the resistance of *H. armigera* to Cry1Ac is unrelated to the amount of *APN3harm* mRNA in midgut tissue. In

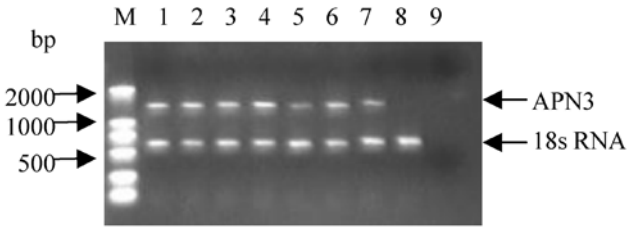

Fig. 4. Semi-quantitative RT-PCR of *APN3* gene in different strains and tissues. M: marker (DL2000); Lane 1: cDNA template from the midgut of a susceptible strain of *H. armigera*; Lane 2–4: cDNA templates from the midgut of resistant strains of *H. armigera* (Rdu, Rsh and Rm); Lane 5–8: cDNA templates from the foregut, midgut, hindgut and the rest of the larval body after removing the gut; Lane 9: template control with water.

the susceptible strain, *APN3harm* is highly expressed in midgut, foregut and hindgut, but not in other tissues (Fig. 4).

Recombinant protein expression and ligand binding experiment

PCR amplification of protein expression cassettes was carried out using primer pairs (APN3FF and APN3RR), and a ca. 3000 bp product was obtained. After extraction, precipitation, and digestion, the *APN3harm* fragment was ligated into a pGEX-4T-1 expression vector. A positive clone was selected for sequencing to affirm the linked-domain. Competent *E. coli* BL21(DE3) cells were transformed, and more than 200 colonies were obtained. Three colonies were randomly selected from the plate and grown in 5 ml LB ampicillin culture. Plasmids were isolated, purified and digested. All three colonies showed the correct insert size. Another 5 ml of culture was induced with 0.8 mM IPTG, resulting in the expression of a ca. 146 kDa protein revealed by SDS-PAGE of cell pellet lysates. Uninduced cells did not contain the new protein (Fig. 5). The pGEX-4T-1 expression vector has a strong *Ptac* promoter and a gene of glutathione S-transferase upstream of the multiple cloning sites. So an expressed

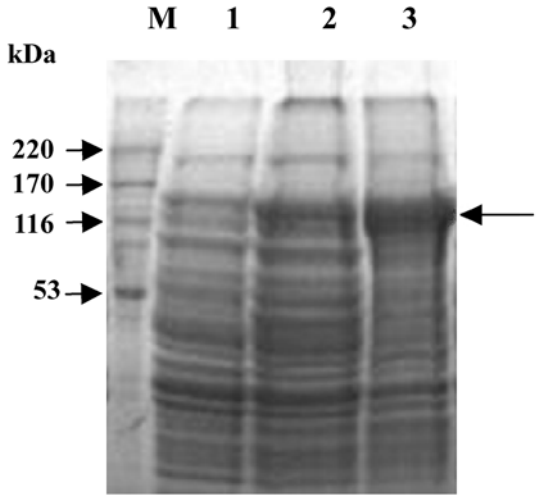

Fig. 5. SDS-PAGE of recombinant APN3harm protein. M: Marker, Lane 1: Uninduced cells, Lane 2: Supernatant fluid of induced cells, Lane 3: Sediment of induced cells.

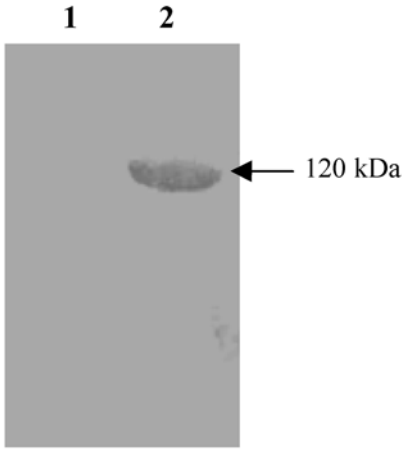

Fig. 6. Ligand blotting of recombinant *H. armigera* APN. The expressed and purified protein was separated by SDS-PAGE and transferred from gel onto a PVDF membrane filter. The membrane filter was incubated with Cry1Ac (1 nM). Binding Cry1Ac was detected with alkaline phosphate conjugate rabbit anti-Cry1Ac (lane 2). Lane 1: Control.

protein resulted from the fusion of the target protein and glutathione S-transferase (the molecular weight of GST was ca. 26 kDa). The majority of rAPN3 was in the form of insoluble inclusion bodies, so after dissolving and refolding of the inclusion bodies, the renatured recombinant protein was purified using affinity chromatography. In order to remove the glutathione S-transferase from rAPN3, the partially purified protein was digested with thrombin and further purified by affinity chromatography and gel filtration after desalting in Tris-HCl buffer by dialysis. The final purified product (120 kDa) bound Cry1Ac toxin in the ligand binding experiment (Fig. 6). So it is proposed that APN3harm is a putative receptor of Cry1Ac in *H. armigera*.

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