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Short Communication

Characterization of four midgut aminopeptidase N isozymes from *Ostrinia furnacalis* strains with different susceptibilities to *Bacillus thuringiensis*

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ABSTRACT

The full-length cDNA of four *Ofapn* aminopeptidases were cloned and sequenced from susceptible and resistant *Ostrinia furnacalis* strains. Four sequences were identified as APN because they shared the common structural features with APN from Lepidoptera, including the signal peptide, GPI anchor signal, the zinc binding/gluzincin motif $HEX_2HX_{18}E$ and the gluzincin aminopeptidase motif GAMEN. Compared with APN sequences from the susceptible strain, there were 9, 5, 10 and 12 amino acid variations in the deduced protein sequences from the resistant strain. There were also differences in mRNA expression of the four *Ofapn* genes between resistant and susceptible *O. furnacalis* strains.

1. Introduction

The toxins produced by *Bacillus thuringiensis* (*Bt*) have been widely used as biopesticides, and *Bt* genes are now commonly used for the development of transgenic *Bt* crops to control agricultural insect pests. The planting of *Bt* crops has significantly suppressed yield losses by insect pest damage and reduced insecticide use (Romeis et al., 2008). In lepidopteran insects, the toxicity of *Bt* is correlated with binding to receptors in the epithelial brush border membrane vesicles (BBMV) of the midgut (Estada and Ferre, 1994). Aminopeptidases N (APNs) are one of the most important receptors for *Bt* toxins (Valaitis, 2008).

Since it was first shown that Cry toxins could bind to APN (Knight et al., 1994; Sangadala et al., 1994), over 140 APN cDNAs have been cloned from more than 20 lepidopteran species (http://www.ncbi.nlm.nih.gov/). Knock-down studies using siRNA suggested APN was a receptor for *Bt* toxins (Rajagopal et al., 2002; Sivakumar et al., 2007). Zhang et al. (2009) identified a deletion mutation of *apn1* in a Cry1Ac-resistant strain of *Helicoverpa armigera,* which was associated with Cry1Ac resistance. In the Cry1Ac-resistant *Trichoplusia ni*, APN1 was significantly down-regulated, whereas APN6 was significantly up-regulated. The Cry1Ac resistance was correlated with down-regulation of APN1 (Tiewsiri and Wang, 2011).

Asian corn borer (ACB), *Ostrinia funacalis*, is one of the most economically important lepidopteran pests of maize in China. Estimated average annual losses due to this insect ranged from 6 to 9 million tons per year and can be much greater in an outbreak year (Zhou et al., 1995). Available evidence demonstrates that Cry1Ab-expressing maize can offer season-long protection against the ACB (He et al., 2003; Wang et al., 2004). However, a strain of ACB selected with Cry1Ab protein has developed more than 100-fold resistance to Cry1Ab and various levels of cross-resistance to Cry1Ah, Cry1Ac, and Cry1Fa (Xu et al., 2010). To investigate the potential role of APN in this observed resistance, cDNAs coding for APN from ACB with different levels of susceptibility to Cry1Ab were cloned. The mRNA expression levels of the APN genes through larval development in *Bt*-susceptible and Cry1Ab-resistant strains were examined.

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2. Materials and methods

2.1. Insect strains and the midgut cDNA synthesis

Two strains of Asian corn borer (ACB), *O. funacalis*, a *Bt*-susceptible strain (ACB-*Bt*S) and a Cry1Ab-resistant strain (ACB-AbR), were established in the laboratory. The ACB-*Bt*S originated from a population collected from corn fields in the summer maize region of central China and reared on artificial diet. The ACB-AbR was selected from ACB-*Bt*S using trypsin-activated Cry1Ab toxin (94% pure protein, provided by Monsanto Company, St. Louis, MO, USA).

Total RNA was isolated from the midguts of fifth instar larvae of ACB using Trizol reagent (Invitrogen, USA). Total RNA concentration was determined by measuring ultraviolet absorbance at 260 nm. RNA purity was checked by determining the A260/A280 ratio and its integrity confirmed by formaldehyde agarose gel electrophoresis. The first strand cDNA used for PCR was synthesized from the total RNA using a cDNA Synthesis Module Kit (Promega, USA).

2.2. Design and synthesis of primers

To amplify the internal partial cDNA fragment, oligonucleotide primers were designed by alignment of APN sequences derived from other insect species. To obtain full-length cDNAs, gene-specific primers (GSPs) for rapid amplification of cDNA ends (RACE) were designed based on the sequences of the PCR product. According to the sequence information of RT-PCR and RACE, primers from 3′ to 5′ terminal for the full length sequence were designed. The sequences and functions of all primers used in this study are given in supplementary Table S1. All primers were synthesized by Sangon Co., Ltd. (Shanghai, China).

2.3. cDNA cloning and sequence analysis of APN genes

RT-PCR for the internal fragment of APN was conducted in 5 μl 10 × ExTaq buffer (Mg^{2+} added), 2 μl cDNA template, 2 μl primer (20 μmol/L) each, 4 μl dNTP (2.5 mmol/L), 0.25 μl ExTaq polymerase (TaKaRa, 5 U/μl), with hot start at 94 °C for 3 min, and 35 cycles of 94 °C for 1 min and 55 °C for 2 min, 72 °C for 1 min, with an additional final extension at 72 °C for 10 min. 5′-RACE (Invitrogen, USA) and 3′-RACE (Takara, Japan) reactions were conducted following the manufacture's protocol using cDNA from ACB-*Bt*S as template. Since there is a possibility that sequence errors may be generated during PCR amplification with Taq polymerase, the full length sequence for *Ofapn*s were also cloned using a DNA polymerase with a higher fidelity, the AccuPrime™ *Pfx* DNA Polymerase (Invitrogen, USA). PCR for full length sequences of APN were conducted with the same protocol for the fragments of RT-PCR described above, except the primer annealing time was extended to 3.5 min and AccuPrime™ *Pfx* DNA Polymerase (Invitrogen, USA) was used.

The PCR products amplified by ExTaq polymerase were gel purified by DNA purification kit (OMEGA, USA) and cloned into pEasy-T1 vector (TransGen, China), then transfected into DH5α *E. coli* cells. Positive recombinants were completely sequenced in both directions at Sangon Biotech (China). The products amplified by AccuPrime™ *Pfx* DNA Polymerase were sequenced directly. Sequence analysis and alignments of putative protein sequences from ACB-*Bt*S and ACB-AbR strains were performed using the computer software DNAMAN and other software available from online servers described in the results.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (q-PCR) was preformed to further compare expression levels of the APN genes in ACB-*Bt*S and ACB-AbR strains from frozen, triplicate samples of midgut from second to fifth larval instars. The sequences of all primers and probes used for q-PCR are listed in supplementary Table S2. The amplifications were conducted on an ABI7500 thermal cycler (ABI, USA) with β-actin as a reference. The probes were tagged with 6-carboxyfluorescein (FAM) as the 5′-reportor dye and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end. The mRNA levels were examined utilizing the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Data were statistically analyzed using analysis of variance (ANOVA), and the means were separated by LSD Test for significance, by using SAS program (SAS Institute Inc., 1999).

3. Results and discussion

Using RT-PCR and RACE, cDNAs coding for four *Ofapn* genes from the larval midguts of ACB-*Bt*S were obtained. MEGA (V4.0) sequence alignment showed that the four *Ofapn* genes belonged to four different classes of lepidopteran APN (Fig. 1). A recent phylogenetic study of lepidopteran aminopeptidase sequences resulted in their clustering into nine classes (Crava et al., 2010). Based on phylogenetic analysis, the four genes identified in the present study were named *Ofapn1s* (GQ927480), *Ofapn2s* (EU564811), *Ofapn3s* (EU137839) and *Ofapn4s* (EU571948); the relative ORF sequences consisted of 2985, 2823, 3045 and 2856 nucleotides, coding for the APNs with predicted molecular weights of 113.1, 106.8, 115.1 and 107.8 kDa, respectively. All four *Ofapn* protein sequences have the common features of lepidopteran APNs, including the zinc binding/gluzincin motif $HEX_2HX_{18}E$, the gluzincin aminopeptidase motif GAMEN, and a cleavable N-terminal signal peptide, predicted using SignalP V2.0 (www.cbs.dtu.dk/services/SignalP), which directs nascent polypeptides to the outer surface of the cytoplasmic membrane. Furthermore, all four APNs also contained a glycosylphosphatidylinositol anchor (GPI), predicted using the online software Big-π Predictor (http://mendel.imp.ac.at/gpi/gpi_server.html), which enables the protein to attach to the membrane. APN1, APN2, APN3 and APN4 of ACB had 4, 11, 8, and 6 predicted amino acid N-glycosylation sites, respectively (predicted using the NetNGlyc 1.0 Server; www.cbs.dtu.dk/services/NetNGlyc/). APN1 and APN3 were predicted to contain one and four O-glycosylation sites respectively, all in the region close to the C-termini of these proteins (predicted using the NetOGlyc 3.1 Server; www.cbs.dtu.dk/services/NetOGlyc/). However, no O-glycosylation sites were predicted in the entire protein of APN2 or APN4. A homology search of GenBank using BLAST revealed that the four APNs from ACB shared the greatest nucleic acid identity with the corresponding APNs from *O. nubilalis*; these were 98.49%, 99.04%, 97.04%, and 96.85%, respectively. Currently, seven cDNA sequences coding for APNs, have been cloned and sequenced from *O. nubilalis*. Linkage analysis revealed that the *onapn* genes clustered in a single linkage group (Crava et al., 2010).

The cDNAs of the four APN isoforms from ACB-AbR, referred to as *Ofapn1r* (GQ927479), *Ofapn2r* (EU826127), *Ofapn3r* (EF538427) and *Ofapn4r* (EU826126), were also obtained by amplification with specific primers. Comparison of the deduced protein sequences of the four *Ofapn* genes from the two strains revealed that there were 9, 5, 10 and 12 amino acids mutations in *Ofapn1, Ofapn2, Ofapn3* and *Ofapn4*, respectively. In addition, Thr^{225} in APN1 from ACB-AbR was a predicted O-glycosylation site, however, the same site in the sequence from the susceptible strain was not. Furthermore, at the non-translatable 3'-end region, a deletion of 11 nucleotides (positions 2828–2838) and an insertion of 9 nucleotides (position 2893–2901) occurred in *Ofapn2r* (resistant strain) relative to the Ofapn2s (susceptible strain), again demonstrating a difference in these APN genes between the resistant and susceptible ACB strains.

Specific amino acid mutations in the conserved region of lepidopteran APNs could lead to a change in affinity between the *Bt*