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RNAi in the striped stem borer, *Chilo suppressalis*, establishes a functional role for aminopeptidase N in Cry1Ab intoxication

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ABSTRACT

The striped stem borer, *Chilo suppressalis*, is a major target pest of transgenic rice expressing the Cry1Ab protein from the bacterium *Bacillus thuringiensis* (Bt) in China. Evolution of resistance in this pest is a major threat to the durability of Bt rice. Since Bt exerts its activity through binding to specific receptors in the midgut of target insects, identification of functional Cry1Ab receptors in the midgut of *C. suppressalis* larvae is crucial to evaluate potential resistance mechanisms and develop effective strategies for delaying insect resistance. In this work, we identified the putative Cry1Ab toxin-binding protein, aminopeptidase-N (APN), in the midgut of *C. suppressalis* by ligand blot and mass spectrometry. After cloning the full-length cDNAs encoding APN isoforms from the *C. suppressalis* larval midgut, we studied their spatiotemporal expression in different gut tissues and developmental stages. Furthermore, RNA interference (RNAi) against *C. suppressalis* aminopeptidases (CsAPNs) was employed to illustrate a functional role for CsAPNs in Cry1Ab toxicity to *C. suppressalis* larvae using injection and oral delivery of Stealth™ siRNA. Down-regulating the expression of CsAPNs by RNAi was closely associated with reduced susceptibility of *C. suppressalis* to Cry1Ab. These data provide the first direct evidence that CsAPNs participate in the mode of Cry1Ab action and may act as the functional receptor of Cry1A in *C. suppressalis* larvae.

1. Introduction

Crystal (Cry) proteins produced by the bacterium *Bacillus thuringiensis* (Bt) are pore-forming toxins used worldwide in spray formulations or produced by transgenic crops (Bt crops) to control relevant insect pests (Bravo et al., 2011). These Cry toxins target the midgut epithelial cells of susceptible insects to disrupt the epithelial barrier and allow the onset of septicemia, which ultimately kills the insect (Raymond et al., 2010; Caccia et al., 2016). Although the specific mechanism responsible for enterocyte death by Cry toxins is still a matter of debate (Vachon et al., 2012), binding of activated Cry toxins to receptors on the surface of midgut cells is recognized as a necessary step for toxicity (Pigott and Ellar, 2007). The importance of toxin binding to receptors for Cry toxicity is further highlighted by reduced toxin binding being the most common mechanism resulting in high levels of resistance to Cry toxins (Ferré and Van Rie, 2002). Current models of Cry intoxication support binding of the toxins to midgut cadherins as necessary to initiate further toxin proteolytic cleavage, resulting in toxin oligomerization (Gómez et al., 2002). Toxin oligomers then bind with high affinity to aminopeptidase-N (APN) or alkaline phosphatase (ALP) proteins (Jurat-Fuentes and Adang, 2006), which facilitates insertion of the oligomeric structure into the membrane to form ionic pores and cause osmotic cell lysis (Bravo et al., 2004). Recently, the ATP-binding cassette transporter protein ABCC2 was reported to be a functional receptor for Bt Cry toxins in *Bombyx mori* (Tanaka et al., 2013), and mutations of ABCC genes is associated with Cry1A resistance in many insects (Atsumi et al., 2012; Baxter et al., 2011; Gahan et al., 2010; Guo et al., 2015; Xiao et al., 2014).

The striped stem borer, *Chilo suppressalis*, is a major rice pest widely distributed in all rice-growing areas of China. While chemical control has been the most common method for control of this pest, efficacy of pesticides has declined rapidly due to the evolution of resistance (Li et al., 2007). Transgenic rice expressing the *cry1Ab* toxin gene was introduced as an environmentally sound alternative to control *C. suppressalis* in China (Shu et al., 2000; Tu et al., 2000; Ye et al., 2001), yet the development of resistance to Bt rice in *C. suppressalis* is considered the most important threat to the future of this technology (Cohen et al., 2000). Due to the crucial role of toxin receptors in susceptibility (Ferré and Van Rie, 2002), the identification of relevant Cry1Ab receptors is needed to

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develop effective strategies for delaying *C. suppressalis* resistance and to design alternative Bt products targeting this pest. Previous competition binding studies demonstrated that Cry1A toxins shared binding sites with Cry1Ba in midgut brush border membrane vesicles (BBMV) in *C. suppressalis* larvae (Fiuza et al., 1996). In addition, binding sites shared by Cry1A toxins are not recognized by Cry2A, Cry9C, or Cry1C toxins (Lee et al., 1997; Alcantara et al., 2004). Binding of the Cry1Ab toxin to a *C. suppressalis* aminopeptidase (CsAPN) and a cadherin (CsCad) protein expressed in insect cell cultures suggests a role for these proteins in Cry intoxication (Yu et al., 2010). Proteomic analyses revealed binding of the Cry1Ac toxin to two putative APNs in the midgut brush border membrane of *C. suppressalis*, although transcripts for these proteins were not detected in the corresponding midgut transcriptome (Ma et al., 2012). Despite available toxin binding evidence, the functional role of *C. suppressalis* APNs in Cry1A toxicity has not been demonstrated. To address this knowledge gap, in this study, we identified four APN isoforms potentially interacting with the Cry1Ab toxin in *C. suppressalis* BBMV and tested their spatiotemporal expression and role in Cry1Ab toxicity in *C. suppressalis* larvae.

2. Materials and methods

2.1. Insects and Cry1Ab toxin

A laboratory colony of *C. suppressalis* was initiated from larvae collected at paddy fields in Beijing (China) in 2009. Insects were reared on an artificial diet as previously described (Han et al., 2012) without exposure to any Bt toxins for 10 generations before use. All cultures were kept under constant conditions (27 ± 1 °C, 70–80% RH, and a photoperiod of 16:8 h light: dark).

Trypsin-activated Cry1Ab toxin purified from recombinant *Escherichia coli* cultures containing the *cry1Ab* toxin gene was provided as lyophilized powder by Marianne Pusztai-Carey (Case Western Reserve University, Cleveland, OH, USA).

2.2. BBMV preparation

BBMVs were isolated by a differential centrifugation method (Wolfersberger et al., 1987) from dissected midguts of *C. suppressalis* 3rd instar larvae. Specific activity of APN, using leucine-ρ-nitroanilide as the substrate (Jurat-Fuentes et al., 2011), was used as a marker for brush border enzyme enrichment in the BBMV preparations. Activities in the final BBMV preparations were 5–7-fold higher when compared to initial midgut homogenates. Final BBMV sample proteins were quantified by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard, and kept at −80 °C until used.

2.3. Ligand blot and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

BBMV proteins (20 μg) were separated by 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) filters. After blocking with dry skim milk (5%) in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST), the filters were probed with 10 nM Cry1Ab toxin for 2 h. Bound toxin was detected using polyclonal anti-Cry1Ab antibody (Youlong Biotech, Shanghai, China) (1:10,000, 1 h) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-) (1:20,000, 1 h). The blots were developed using the enhanced chemiluminescence (ECL) Western Blot Kit (CWBIO, China) and imaged on the ImageQuant LAS 4000 digital imaging system (GE Healthcare).

To detect CsAPN proteins potentially binding to Cry1Ab in the ligand blots, the corresponding bands in a Coomassie-stained gel of *C. suppressalis* BBMV (SDS-PAGE) were excised, trypsinized, and analyzed by LC-MS/MS. Peptide spectra were searched against the predicted CsAPN protein sequences with one missed trypsin cleavage allowed, 15 ppm peptide mass tolerance, fixed carbamidomethylation of cysteines, methionine oxidation, and N-terminal glutamate to pyroglutamate conversion as variable modifications. All matches were significant identity as determined by their ion scores ($P < 0.05$). The relative abundance of CsAPN proteins in the excised gel band was calculated using the exponentially modified protein abundance index (emPAI) method based on protein coverage by the peptide matches in the database search results (Ishihama et al., 2005). While the relative emPAI values are dependent on the sizes of the proteins, the similar predicted sizes of CsAPNs validates direct comparisons.

2.4. Sequencing of aminopeptidase cDNAs

Total RNA was extracted from midguts of 3rd instar *C. suppressalis* larvae using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNA was treated with DNase I (Invitrogen) to eliminate genomic DNA, and reverse transcription PCR (RT-PCR) was performed using SuperScript III RNaseH$^-$ reverse transcriptase (Invitrogen). The first strand cDNA was used as a template for PCR. Degenerate primers used for PCR amplification (Table S1) were designed to amplify a conserved amino acid region among APNs from *C. suppressalis* (GenBank accession no DQ342305), *Diatraea saccharalis* (GenBank accession no HM231317 and HM231318), *Helicoverpa punctigera* (GenBank accession No. AF217249), *Helicoverpa armigera* (GenBank accession No. AAN04899), *Manduca sexta* (GenBank accession No. X97877), *B. mori* (GenBank accession No. BAA33715), *Plutella xylostella* (GenBank accession No. ADD39718), and *Spodoptera exigua* (GenBank accession No. AAP44967). Amplicons were subcloned into the pGEM-T Easy vector (Promega), transformed into *E. coli* TOP10 cells (Invitrogen), and sequenced. Four different APNs were detected among the sequenced clones and selected for further cloning efforts. Specific primers were designed (Table S1) based on the four different APN clones obtained from degenerate PCR and used in rapid amplification of cDNA ends (RACE) with the Gene Race Kit (Invitrogen) following the manufacturer's instructions to obtain full-length cDNAs.

The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and two glycosylphosphatidylinositol (GPI) modification site prediction servers (http://mendel.imp.ac.at/sat/gpi/gpi_server.html and http://gpi.unibe.ch/) were used to determine the presence of a signal peptide and a GPI-anchor signal sequence (Nielsen et al., 1997; Eisenhaber et al., 2001), respectively. The molecular weight and isoelectric points of the predicted proteins was determined using the ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). The ExPASy NetOGlyc 3.1 and NetNGlyc 1.0 servers (http://www.cbs.dtu.dk/services) were used to predict potential O- or N-glycosylation sites, respectively, on the predicted APN protein sequences (Gasteiger et al., 2003). Sequence homology analyses and multiple sequence alignments were performed using BLASTp and ClustalX 2.0.9, respectively. A phylogenetic tree including lepidopteran APN protein sequences available in GenBank was constructed using the neighbor-joining method with 1000 bootstrap resamplings with Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 6.06).

2.5. Quantitative real-time PCR

Total RNA was isolated from different developmental stages of *C. suppressalis* and tissues of 3rd instar *C. suppressalis* larvae using