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Bacillus thuringiensis Cry3Aa protoxin intoxication of *Tenebrio molitor* induces widespread changes in the expression of serine peptidase transcripts

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ABSTRACT

The yellow mealworm, *Tenebrio molitor*, is a pest of stored grain products and is sensitive to the *Bacillus thuringiensis* (Bt) Cry3Aa toxin. As digestive peptidases are a determining factor in Cry toxicity and resistance, we evaluated the expression of peptidase transcripts in the midgut of *T. molitor* larvae fed either a control or Cry3Aa protoxin diet for 24 h (RNA-Seq), or in larvae exposed to the protoxin for 6, 12, or 24 h (microarrays). Cysteine peptidase transcripts (9) were similar to cathepsins B, L, and K, and their expression did not vary more than 2.5-fold in control and Cry3Aa-treated larvae. Serine peptidase transcripts (48) included trypsin, chymotrypsin and chymotrypsin-like, elastase 1-like, and unclassified serine peptidases, as well as homologs lacking functional amino acids. Highly expressed trypsin and chymotrypsin transcripts were severely repressed, and most serine peptidase transcripts were expressed 2- to 15-fold lower in Cry3Aa-treated larvae. Many serine peptidase and homolog transcripts were found only in control larvae. However, expression of a few serine peptidase transcripts was increased or found only in Cry3Aa-treated larvae. Therefore, Bt intoxication significantly impacted the expression of serine peptidases, potentially important in protoxin processing, while the insect maintained the production of critical digestive cysteine peptidases.

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1. Introduction

The toxins of *Bacillus thuringiensis* (Bt) are the most successful microbial pesticides, used in insecticide spray formulations or incorporated into transgenic crops for safe and effective insect control. Bt crystal (Cry) proteins are expressed as insoluble protoxins that are solubilized and hydrolyzed to active toxins by insect midgut peptidases (reviewed in Oppert, 1999). Therefore, the complement and relative activity of peptidases in the insect gut can be a determining factor for toxicity. In addition, changes in the expression of insect peptidases can contribute to insect resistance to Bt toxins, as was first described in *Plodia interpunctella* (Hübner) (Oppert et al., 1994, 1996, 1997).

Cry toxins target a specific group of insects. For example, Cry1A toxins are toxic primarily to lepidopterans, whereas Cry3A toxins have activity against some coleopterans. The yellow mealworm, *Tenebrio molitor* Linnaeus, is a pest of stored grain products and is sensitive to Cry3Aa (Oppert et al., 2010a). Far less is known about the mode of action of Cry3A toxins in coleopterans than of Cry1A toxins in lepidopterans, but our data from *T. molitor* suggest that gut-specific cadherins, important to Cry toxicity in lepidopterans, are also conserved toxin receptors in beetles (Fabrick et al., 2009). However, unlike trypsin hydrolysis of protoxins in Lepidoptera, chymotrypsin is important in obtaining a soluble and processed Cry3Aa protoxin in a more acidic coleopteran gut (Carroll et al., 1989).

We have demonstrated through biochemical and genetic studies that tenebrionid larvae digest protein through the sequential action of primarily cysteine peptidases in the anterior and serine peptidases in the posterior midgut (Vinokurov et al., 2006a,b; Prabhakar et al., 2007; Vinokurov et al., 2009). The primary digestive peptidases in tenebrionid larvae are cathepsin L cysteine peptidases (Cristofoletti et al., 2005; Morris et al., 2009) and the serine peptidases trypsin and chymotrypsin (Elpidina et al., 2005; Tsybina et al., 2005; Vinokurov et al., 2006b). The relative expression of peptidase transcripts in the tenebrionid larval gut may be altered by dietary elements, such as peptidase inhibitors (Oppert et al., 2010b).

Studies of Cry3A intoxication in *T. molitor* have been limited by the availability of genetic data. The only coleopteran with a sequenced genome is the tenebrionid *Tribolium castaneum*, far less sensitive to Cry3Aa than *T. molitor* (Oppert et al., 2010a). Therefore, we used high-throughput sequencing to obtain EST sequences from the gut of *T. molitor* larvae, while also examining the effect of Cry3Aa intoxication on the expression of peptidase transcripts in the gut transcriptome. In

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addition, a custom microarray was used to study the temporal expression of peptidase genes in Bt intoxicated larvae of *T. molitor*.

2. Materials and methods

2.1. Insects

T. molitor were reared on 50% rolled oats, 2.5% brewers yeast, and 47.5% flour at 28 °C, ~75% R.H., in darkness.

For sequencing, approximately 1 mo old larvae (5-6th instar) were starved for 24 h and placed on control diet (85% wheat germ, 10% wheat flour, 5% brewers yeast) or the same diet containing 0.1% Cry3Aa protoxin (Bt subsp. *tenebrionis*). After 24 h, 23–26 larvae were submersed in RNAlater (Ambion, Austin, TX, USA), and guts were removed and placed in vials containing RNAlater.

For the microarray, recently molted larvae (approximately 1 mo old and mean mass of 5–6 mg) were selected from a standard laboratory colony and were starved overnight. Larvae were placed on a diet of 85% stabilized wheat germ, 10% flour, and 5% brewers yeast with a 3% dilution of a concentrated solution of FD&C Blue #1 and 97% of a mixture of 85% stabilized wheat germ, 10% flour, 5% brewers yeast with or without 0.1% Cry3Aa (pre-equilibrated at 28 °C, 75% R.H. over saturated sodium chloride). Larvae were monitored for blue color in the gut, indicating that they had ingested either control or treated diet, and allowed to continue feeding on their respective diets for 6, 12, or 24 h. For each treatment, midguts were dissected from four larvae in RNAlater and were placed into vials of RNAlater. A second biological replicate was obtained from larvae in a different oviposition tray (i.e., different generation).

2.2. 454 pyrosequencing

High-throughput sequencing was used to obtain EST databases from the midguts of *T. molitor* larvae fed either control diet or diet containing 0.1% Cry3Aa for 24 h. Briefly, total RNA was obtained from gut tissues using an RNeasy kit (Invitrogen, Carlsbad, CA USA), and samples were shipped to 454 Life Sciences for processing. PolyA$^+$RNA was purified and fragmented, first strand cDNA was prepared with Superscript II (Invitrogen), and directional adaptors were ligated for clonal amplification and sequencing on the Genome Sequencer FLX pyrosequencing system (454 Life Sciences, Branford, CT USA). Specific details of the sequencing procedure are in Oppert et al. (in press). Initial contig assembly was from 258,377 total reads, performed with the Roche GS De novo Assembler (v1.1.03) with default parameters (total nucleotides = 1,026,138 and 898,431; N50 contig size = 764 and 788; Q40 + Bases = 88.5 and 88.23%, respectively). Filtered read datasets from control and Cry3Aa-treated larvae were assembled separately, resulting in two RNA-Seq databases: 1318 large contigs from sequences obtained from control larvae (labeled Cont#, database CONT) and 1140 large contigs from sequences obtained from Cry3Aa-treated larvae (labeled Bt#, database BT).

2.3. Bioinformatics

Sequences similar to annotated peptidases (*Drosophila melanogaster* trypsin alpha, P04814; *D. melanogaster* trypsin beta, P35004; *Homo sapiens* trypsin II, P07478; *H. sapiens* trypsin I, P07477; *D. melanogaster* cathepsin L Q95029; *Carica papaya* papain, P00784) were identified in RNA-Seq databases using tBLASTn (default parameters, Altschul et al., 1990). Sequences with similarity in at least two motifs around the active site residues (serine peptidases), or conservation in a GSCWAF motif of the active site (cysteine peptidases), were selected as homologous. Open reading frames in sequences were selected using NCBI ORFfinder (Sayers et al., 2011) and ORFfinder Sequence Manipulation Suite (Stothard, 2000). Multiple alignment of all homologs and query proteins was used to identify active sites and substrate binding centers (MUSCLE, default parameters; Edgar, 2004). Serine peptidase annotation was based on previous research on associations between amino acids in the S1 binding subsite and classification of peptidases (Perona and Craik, 1995; Hedstrom, 2002) and MEROPS database (Rawlings et al., 2010). Cysteine peptidase annotation was based on previous data (Prabhakar et al., 2007), structural specificity, and searching for orthologs in the NCBI database. Manual editing of errors in the assembly was made using original unassembled reads.

2.4. Expression analysis

Average read length was estimated as:

$$\frac{\sum_{database}\text{Read length}}{\text{Number of reads in the database}}.$$

Expression (average contig coverage) was estimated as the number of expressed contigs normalized to the length of contigs:

$$\text{Number of reads} * \frac{\text{Average read length}}{\text{Contig length}}.$$

Expression change for contigs appearing in both databases was estimated as:

$$\frac{\text{Estimated expression in database CONT}}{\text{Estimated expression in database BT}}.$$

2.5. Development of the microarray, hybridization, and analysis

We used microarray analysis to validate the differential expression of peptidase transcripts from RNA-Seq. For the microarray, a second assembly was made of all transcripts combined from control and Cry3Aa-treated sequences using Seqman NGEN (DNAstar, Madison, WI USA). Contigs from this assembly were denoted "Contig_#". Assembly parameters were optimized over time to obtain a final configuration and assembly (limiting false joins) with default parameters (match size = 15, match percentage = 97, match spacing = 25, maximum coverage = 10,000 and minimum coverage = 2). With this assembly, uniESTs were manually evaluated and edited using Seqman. Of the 25,201 uniESTs, 23,671 unambiguous oligos were arrayed in duplicate or triplicate on a custom array chip (4×44K, Agilent Technologies, Santa Clara, CA USA), incorporating standards supplied by eArray.

For RNA extraction, excess RNAlater was blotted and guts were ground with a plastic pestle in a 1.5 ml microcentrifuge tube containing liquid nitrogen. Total RNA was isolated using the Absolutely RNA Kit with on-column DNase treatment (Agilent Technologies, La Jolla, CA USA). mRNA was reverse transcribed from total RNA using oligo-dT with T7 promoter and amplified with T7 polymerase to obtain Cy3 or Cy5-labelled antisense cRNA (Quick Amp Labeling Kit, Agilent; dyes were swapped in the biological replicate). Hybridization of the microarray was for 18 h at 45 °C with approximately 500 ng of labeled cRNA (Gene Expression Hybridization Kit, Agilent). Microarrays were scanned at 532 and 635 nm using a GenePix 4000B scanner and GenePix Pro 6.1 (Molecular Devices, Sunnyvale, CA, USA) at the Gene Expression Facility at Kansas State University (Manhattan, KS, USA).

For data analysis, the relative intensity for each spot (raw data) was imported into GeneSifter (Geospiza, Seattle, WA, USA) for statistical analysis. Data was normalized by relative intensity means and was log transformed. To verify potential peptidase transcripts, oligo sequences were submitted to publicly-available databases for similarity searches, as well as custom databases containing previously annotated *T. molitor* and *T. castaneum* peptidase genes or fragments. Pairwise comparisons were made between oligos representing peptidase transcripts in the