



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 (Cristofolletti 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 (Sayars 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:

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

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

control and each treatment dataset for each time point, and significance was determined by the Student's t-test ($p < 0.05$), corrected by Benjamini and Hochberg (1995).

3. Results

3.1. Gene expression analysis by RNA-Seq

3.1.1. Cysteine peptidases

Cysteine peptidases are the major digestive peptidases in *T. molitor* larvae and are responsible for the initial stages of dietary protein cleavage. Therefore, we examined the RNA-Seq databases from control and Cry3Aa-treated larvae to determine if ingestion of Cry3Aa affected the expression of transcripts encoding cysteine peptidases. Overall, nine distinct transcripts encoding cysteine peptidases from the C1 family were found in the 454 datasets (Supplemental Fig. 3). Three peptidase transcripts were similar to cathepsin B, one was cathepsin B-like (with differences in the structure of the additional loop), and five corresponded to cathepsin L, with ERFNIN and GNFD conserved motifs in the propeptide (Karrer et al., 1993), including a possible cathepsin K (Table 1). All sequences had conserved active site residues and presumably encoded active peptidases.

The relative expression of cysteine peptidase transcripts in control or Cry3Aa-treated *T. molitor* larvae was compared by the estimated expression values (Fig. 1). The overall importance of cathepsin L transcripts was apparent by the larger expression values, in particular Cont-08897/Bt-07583 which constituted approximately 70 and 50% of the total expression of cysteine peptidase transcripts in control and Cry3Aa-treated larvae, respectively (Table 1). Cont-08897/Bt-07583 correlated to the most highly-expressed cysteine peptidase in the anterior midgut of *T. castaneum* (Morris et al., 2009) and was repressed approximately 2-fold in intoxicated larvae. Minor changes in expression, from 1.1 to 2.5-fold, were noted with all cathepsin L and B transcripts. Overall, four cysteine peptidase transcripts were decreased and three were increased in Cry3Aa-treated compared to control larvae. One cathepsin B (Cont-00890) transcript was unique to control larvae (Table 2), and one cathepsin L (Bt-07886) transcript was unique to Cry3Aa-treated larvae (Table 3), both expressed at relatively low levels.

3.1.2. Serine peptidases

Digestive serine peptidases have been found mostly in the posterior midgut of *T. molitor* larvae and act preferentially on the partially digested products from the anterior midgut (Elpidina et al., 2005; Tsybina et al., 2005; Vinokurov et al., 2006b). In addition, serine peptidases, especially trypsin and chymotrypsin, are involved in the activation of Bt protoxins and in some cases of resistance (reviewed in

Oppert, 1999). Therefore, we also surveyed differences in expression patterns of serine peptidase transcripts in our databases. Transcripts encoding serine peptidases from the S1 family and similar sequences (analogs and homologs) were overall more numerous than cysteine peptidase transcripts, in both expression values and number of different transcripts (compare Figs. 1 and 2).

Serine peptidase transcripts were represented by 36 sequences containing conserved motifs around three residues of the active site, and 12 incomplete sequences with only two conserved motifs (Supplemental Fig. 4). We analyzed sequences for conserved catalytic residues H57, D102, and S195, found in the conserved motifs TAAHC, DIAL, and GDSGGP, and binding pocket residues in positions 189, 216 and 226 according to the numbering in bovine chymotrypsin (P00766) (Perona and Craik, 1995). Among the transcripts encoding putative serine peptidases, 19 were predicted as active peptidases with conserved amino acids in the active site, two were annotated as peptidase analogs that had synonymic substitutions in the active center (serine replaced by threonine), 19 were serine peptidase homologs (SPH) presumably lacking proteolytic function due to nonsynonymic substitutions in catalytic residues, and eight lacked sufficient sequence to be properly annotated (Table 4). Three active peptidase transcripts were related to trypsin, six to chymotrypsin, and four were predicted as elastase 1-like peptidases. We classified predicted peptidases from Cont-09283, Bt-00011, Cont-08675/Bt-00013 and Cont00845/Bt-08328, all with the substitution S189 to G189, as chymotrypsin-like. Similar substitutions have been observed in other insect peptidase transcripts (Oppert et al., 2010b), and we proposed that these substitutions would have minimal effect on structure based on modeling of loop structures (data not shown), although this functionality remains to be proven experimentally. In addition, we classified six sequences as unidentified serine peptidases from the S1 family due to unusual residues in the binding site, or lack of necessary information. Among the active serine peptidase sequences, seven were found in both control and Cry3Aa-treated larvae, but 10 were unique to control (Table 2) and four were unique to Cry3Aa-treated larvae (Table 3).

The most abundant trypsin peptidase transcript, Cont-00231/Bt-03613, biochemically characterized as the major digestive trypsin in the posterior midgut and found in a previous EST (Tsybina et al., 2005; Prabhakar et al., 2007), accounted for nearly 33% of all active serine peptidase transcripts (Fig. 2, Table 4). The major chymotrypsin transcript, Cont-09450/Bt-007640, also previously characterized as the major digestive chymotrypsin in the posterior midgut (Elpidina et al., 2005; Prabhakar et al., 2007), was responsible for approximately 16% of all serine peptidase transcripts. Both transcripts were severely repressed by Cry3Aa-intoxication. Other peptidase transcripts were of intermediate to low expression (<6.5% of the total).

Table 1

Analysis of predicted cysteine peptidase sequences and expression levels in control and Cry3Aa-treated larvae, as determined by RNA-Seq analysis.

Contig # in CONT database ^a	Expression in control larvae ^b	Contig # in BT database ^a	Expression in Cry3Aa-treated larvae ^b	Catalytic active site residues ^c	Loop active site residues ^c	Predicted classification
Cont-09310	12.1	Bt-00249	4.9	QCH-	HH	Cathepsin B
Cont-00240	9.9	Bt-01453	4.1	QCHN	HH	Cathepsin B
Cont-00890	3.7	None	-	QCHN	AH	Cathepsin B ^e
Cont-08975	9.3	Bt-08237	19.0	QCHN	- ^d	Cathepsin B-like
Cont-00009	7.3	Bt-01497	8.1	QCHN	No loop	Cathepsin L
Cont-08897	171.7	Bt-07583	77.5	QCHN	No loop	Cathepsin L
Cont-09057	10.2	Bt-00111	6.3	QCHN	No loop	Cathepsin L
None	-	Bt-07886	5.6	QCHN	No loop	Cathepsin L
Cont-01354	15.5	Bt-07528	29.7	QC-	No loop	Cathepsin L or K

^a Accession number of the contig in the corresponding database. Contigs in the same line correspond to the same predicted protein.

^b See Materials and methods for formula to estimate expression.

^c Amino acids of the catalytic center correspond to *Carica papaya* papain (P00784) Q152/C158/H292/N308. '-' indicates a truncated sequence. The loop active site residues correspond to H189/H190 in human cathepsin B (P07858).

^d No homology in loop active site residues (see Supplemental Fig. 3).

^e Annotation was based on previous research (Prabhakar et al., 2007).

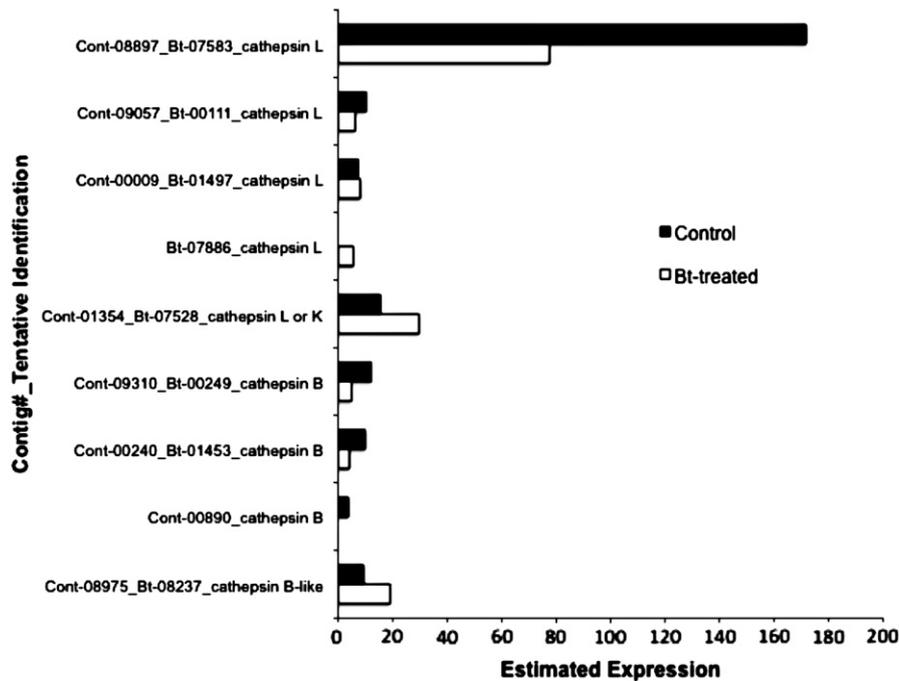


Fig. 1. Relative expression of transcripts encoding cysteine cathepsins in *T. molitor* larvae fed control or 0.1% Cry3Aa (Bt-treated) diets for 24 h, as determined by RNA-Seq analysis. Transcripts are tentatively identified as cathepsins B, K, or L, or cathepsin B-like.

Unlike the differences observed in cysteine peptidase transcripts, the expression profile of serine peptidase transcripts in Cry3Aa-intoxicated larvae changed dramatically. Five serine peptidases common to both groups were expressed 2.1 to 15.0-fold lower in Cry3Aa-

Table 2

Contigs encoding potential peptidases found only in CONT database.

Tentative identification	Contig #	Estimated expression ^a
Cathepsin B	Cont-00890	3.7
Trypsin	Cont-07065	4.6
Chymotrypsin-like	Cont-09283	5.2
Elastase 1	Cont-09389	5.8
Serine peptidase	Cont-08824	24.7
Serine peptidase	Cont-01606	16.5
Serine peptidase	Cont-01621	8.7
Serine peptidase	Cont-02277	6.9
Serine peptidase	Cont-01165	5.8
SP analog	Cont-00748	7.0
SP analog	Cont-09333	4.5
Elastase 1 homolog	Cont-09110	12.4
Elastase 1 homolog	Cont-01250	7.6
SP homolog	Cont-08723	19.8
SP homolog	Cont-02455	18.7
SP homolog	Cont-09177	12.0
SP homolog	Cont-08773	9.7
SP homolog	Cont-01452	9.1
SP homolog	Cont-09003	8.3
SP homolog	Cont-08766	7.2
SP homolog	Cont-08580	4.8
SP homolog	Cont-02809	3.8
SP homolog	Cont-00229	3.6
UTD	Cont-09471	32.7
UTD	Cont-01720	8.6
UTD	Cont-02359	7.3
UTD	Cont-03817	4.3
UTD	Cont-02507	4.0
UTD	Cont-01610	3.8
UTD ^b	Cont-02699	3.5
UTD	Cont-00142	2.3

^a See **Materials and methods** for formula to estimate expression.

^b Unable to determine specificity due to insufficient sequence data.

treated larvae, and only two were expressed 1.1 to 1.4-fold higher (Fig. 2, Table 4). Two major digestive peptidases with the highest level of expression, trypsin (Cont-00231/Bt-03613) and chymotrypsin (Cont-09450/Bt-07640), were expressed 15.0- and 6.2-fold lower, respectively, in Cry3Aa-treated larvae. Three transcripts encoding chymotrypsin-like enzymes were either increased in expression or were unique to intoxicated larvae. The remaining serine peptidase transcripts were mostly repressed in Cry3Aa-treated larvae. Overall, the expression of SPH was relatively low (3% of the expression level of active peptidases), although one (Cont-00768/Bt-00326) was moderately expressed. Only six of 18 SPH were found in the BT database, and only three had an enhanced level of expression. Therefore, exposure to Cry3Aa had a major effect on the expression of SPH in *T. molitor* larvae. It is also interesting that SPH had substitutions only in the first and/or third conserved motifs of the active site, with the most common a H57 to Q57 substitution (Supplemental Fig. 4). In addition, most unclassified peptidase transcript sequences were found only in control larvae.

Most serine peptidase transcript expression was specific to control or was decreased in intoxicated larvae (Fig. 2, Table 4). The major trypsin and chymotrypsin transcripts had higher expression values in control larvae. The highest expression values among serine peptidases in Cry3Aa-treated larvae were due to three chymotrypsin-like transcripts with the S189 to G189 substitution. Within this group, Cont-

Table 3

Contigs encoding potential peptidases found only in BT database.

Tentative identification	Contig #	Estimated expression ^a
Cathepsin L	Bt-07886	5.6
Trypsin	Bt-01424	5.4
Chymotrypsin	Bt-00011	56.5
Elastase 1-like	Bt-08266	8.7
Serine peptidase	Bt-01301	20.3
SP homolog	Bt-00581	4.4
UTD ^b	Bt-00193	38.1

^a See **Materials and methods** for formula to estimate expression.

^b Unable to determine specificity due to insufficient sequence data.

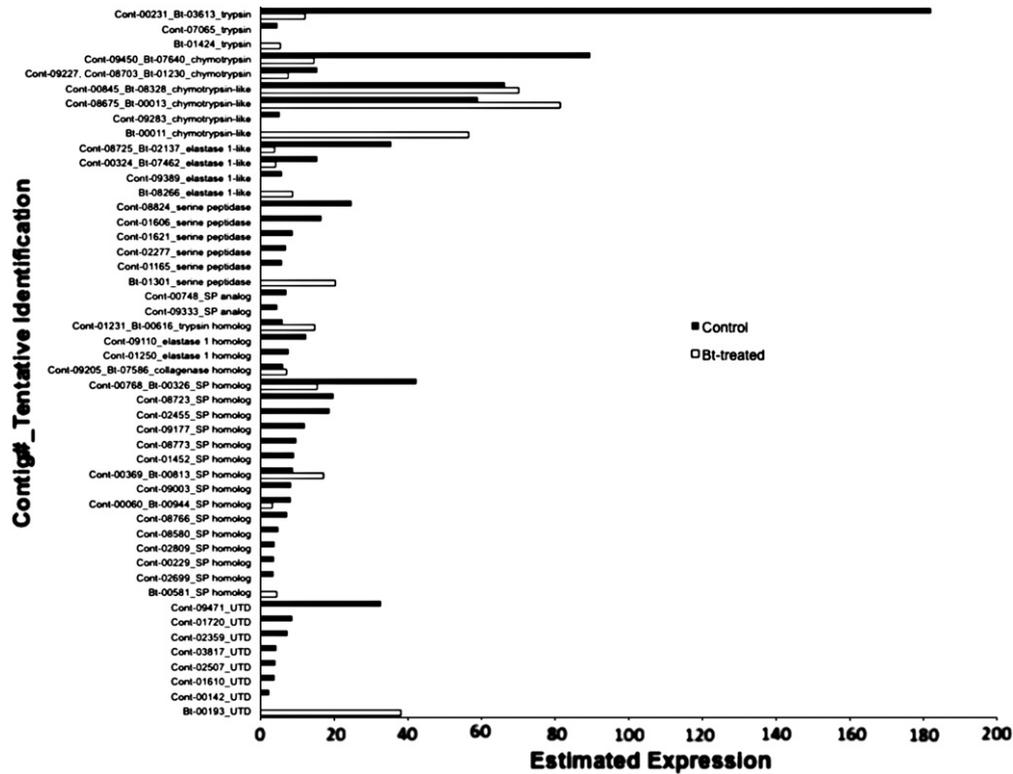


Fig. 2. Relative expression of transcripts encoding serine peptidases in *T. molitor* larvae fed control or 0.1% Cry3Aa (Bt-treated) diets for 24 h, as determined by RNA-Seq analysis. Transcripts are tentatively identified as trypsin, chymotrypsin or chymotrypsin-like, elastase 1-like, unclassified serine peptidase, analog (synonymic substitutions in conserved residues), homolog (nonsynonymic residues in the catalytic triad), or unable to determine (UTD).

08675/Bt-00013 and Cont-00845/Bt-08328 had increased expression values and were found in control and intoxicated larvae, and a contig with a similar expression value (Bt-00011) was unique to intoxicated larvae; these three accounted for 73% of all active serine peptidase transcripts in intoxicated larvae. Cont-00845/Bt-08328 was the sole RNA-Seq serine peptidase transcript located in the anterior midgut in the previous annotation of *T. molitor* larvae midgut ESTs (Prabhakar et al., 2007).

3.2. Gene expression analysis by custom microarray

Microarray analysis was used to verify expression patterns of peptidase genes in Cry3Aa-intoxicated *T. molitor* larvae, and also to determine critical time point(s) in the intoxication process for examining differential expression of peptidase transcripts. Sequences from control and Cry3Aa-treatment groups were combined in a second assembly, and oligos from these sequences were arrayed for hybridization of labeled cRNA from control larvae or larvae exposed to the LD₅₀ of Cry3Aa for 6, 12, or 24 h.

In agreement with the RNA-Seq analysis, the expression of many peptidase transcripts was repressed in Cry3Aa-treated larvae, especially at 12 and 24 h (Table 5, Supplemental Fig. 5). The expression patterns of peptidase transcripts in control and Cry3Aa-treated larvae were correlated between the microarray and RNA-Seq analyses, with the exception of one cathepsin L. In that discrepancy, microarray Contig_17683 was repressed at all time points, and RNA-Seq analysis indicated that the corresponding transcript, Cont-00009/Bt-01497, was slightly induced at 24 h, but the difference may be within experimental error. Interestingly, Contig_11744 was repressed at early intoxication timepoints but recovered at 24 h. Contig_11744 was similar to a *T. molitor* gene encoding a serine protease involved in activation of the TOLL pathway, modular serine protease zymogen, leading to the induction of antimicrobial peptides (Kim et al., 2008).

Of particular interest were the SP that were induced in both microarray and RNA-Seq analysis, especially the oligo representing Contig_19810, corresponding to chymotrypsin-like RNA-Seq Cont-08675/Bt-00013 (with a relative expression ratio of 59/81, respectively) and containing the GGS signature for binding pocket residues. Transcripts hybridizing to Contig_19810 were highly induced at all time points, although the degree of variability among biological replicates prevented the data from being included as statistically significant. Another chymotrypsin-like transcript, Contig_16973, was significantly induced at 24 h and corresponded to Cont-00845/Bt-08328 (with a relative expression ratio of 66/70, respectively) with a GAS signature. Contig_16752, with a GVS signature corresponding to an elastase 1-like enzyme, was significantly induced at all time-points of intoxication, and was found only in Cry3Aa-treated larvae in the 454 dataset (Bt-08266). Thus, the up-regulation of serine peptidase transcripts with different substrate binding site signatures was correlated to Bt intoxication: chymotrypsin-like transcripts with GGS and GAS residues, and an elastase 1-like transcript with GVS residues. These peptidases may have roles that are downstream of protoxin activation in the intoxication response and warrant further investigation. Two transcripts encoding SPH were significantly induced at all time-points: Contig_20748, corresponding to Cont_00369/Bt-00813, which was induced also in RNA-Seq (relative expression values of 8.8/17, respectively), and Contig_19676, a unique SPH obtained in the second assembly; two other SPH transcripts were induced only at 6 h. Other serine peptidase transcripts were also induced in the microarray, but we were unable correlate to RNA-Seq data or assign function.

One objective of the microarray was to determine the best time point of Cry3Aa-intoxication for future studies of peptidase gene expression. Slightly more genes were significantly differentially regulated at the 12 h intoxication time point in this study of peptidase genes (Table 5), and a corresponding analysis of all differentially-regulated genes indicated that many more genes were changing in expression at 12 h (data not shown). In addition, while many of the major serine

Table 4
Analysis of predicted serine peptidase sequences and related analogs and homologs, and expression levels in control and Cry3Aa-treated larvae, as determined by RNA-Seq analysis.

Contig # In CONT Database ^a	Expression in Control Larvae ^b	Contig # in BT Database ^a	Expression in Cry3Aa- treated Larvae ^b	Conserved Motifs around Active Site Residues ^c			Binding Pocket Residues ^d	Predicted Classification ^e
				TAAHC	DISI	GDSGGP		
Cont-07065	4.6	none	-	TAAHC	DISI	GDSGGP	DGG	trypsin
Cont-00231	182.0	Bt-03613	12.1	TAAHC	DISV	GDSGGP	DGG	trypsin
none	-	Bt-01424	5.4	TAAHC	DI AV	GDSGGP	D--	trypsin
Cont-09227, Cont-08703 ^f	15.4	Bt-01230	7.5	TAGHC	DIAL	GDSGGP	SGA	chymotrypsin
Cont-09450	89.5	Bt-07640	14.5	TAAHC	DIAL	GDSGGP	SGS	chymotrypsin ^g
Cont-09283	5.2	none	-	TAGHC	DI AV	GDSGGP	GGG	chymotrypsin-like
none	-	Bt-00011	56.5	TAGHC	DVGL	GDSGGP	GGG	chymotrypsin-like
Cont-08675	59.0	Bt-00013	81.4	TAGHC	DVGL	GDSGGP	GGG	chymotrypsin-like
Cont-00845	66.3	Bt-08328	70.1	TAGHC	DIGL	GDSGGP	GAS	chymotrypsin-like
none	-	Bt-08266	8.7	TAGHC	DIGL	GDSGGP	GVS	elastase 1-like
Cont-09389	5.8	none	-	TAGHC	DVGL	GDSGGP	GIS	elastase 1-like
Cont-08725	35.5	Bt-02137	3.8	TAAHC	DIGL	GDSGSP	GIS	elastase 1-like
Cont-00324	15.4	Bt-07462	4.1	TAAHC	DVAL	GDSGGP	SVS	elastase 1-like
Cont-02277	6.9	none	-	TAAHC	DIGI	GDSGGP	GFS	serine peptidase
Cont-01165	5.8	none	-	TAGHC	DIGL	GDSGGP	SVA	serine peptidase
none	-	Bt-01301	20.3	TAAHC	DIGL	GDSGAP	STS	serine peptidase
Cont-08824	24.7	none	-	TAAHC	DIGL	GDSGAP	STS	serine peptidase
Cont-01621	8.7	none	-	TSAHC	DIGL	GDSGSP	G--	serine peptidase
Cont-01606	16.5	none	-	TAGHC	DVGL	GDSGSP	GI-	serine peptidase
Cont-09333	4.5	none	-	TAAHC	DIGL	GDTGGP	G--	SP analog
Cont-00748	7.0	none	-	TSGHC	DIGL	GDTGSP	GLS	SP analog
Cont-01231	6.0	Bt-00616	14.7	TGAHC	DIAL	GDTGGP	DGG	trypsin homolog
Cont-09110	12.4	none	-	TAAHC	DIGL	GDTGSP	SIT	elastase 1 homolog
Cont-01250	7.6	none	-	TAGQC	DIGM	GDTVGA	GIT	elastase 1 homolog
Cont-09205	6.1	Bt-07586	7.1	TAAQC	DI AV	GDAAGP	GAD	collagenase homolog
Cont-09003	8.3	none	-	TAAHC	NIGL	GDTG--	G--	SP homolog
Cont-00369	8.8	Bt-00813	17.1	TSGSC	DVGL	GDSGDA	G--	SP homolog
Cont-08580	4.8	none	-	TAGHC	DVGL	GDLGAP	GG-	SP homolog
Cont-08766	7.2	none	-	TAGQC	DVGM	GDTVGA	G--	SP homolog
Cont-01452	9.1	none	-	TAGQC	DIGL	GDTGSG	G--	SP homolog
Cont-00060	8.2	Bt-00944	3.2	TAGQC	DIAL	GDSGSP	GFS	SP homolog
Cont-02809	3.8	none	-	TAGHC	DIGL	GDTNGSP	GIS	SP homolog
Cont-02455	18.7	none	-	TSGSC	DIGV	GDTVGGP	G--	SP homolog
Cont-09177	12.0	none	-	TAGQC	DLGL	GDTGSS	GVS	SP homolog
Cont-00768	42.3	Bt-00326	15.4	TAGQC	DIGL	GDLGSS	GIG	SP homolog

Table 4 (continued)

Contig # In CONT Database ^a	Expression in Control Larvae ^b	Contig # in BT Database ^a	Expression in Cry3Aa- treated Larvae ^b	Conserved Motifs around Active Site Residues ^c			Binding Pocket Residues ^d	Predicted Classification ^e
Cont-08723	19.8	none	-	TAG QC	DLGL	GDSGGP	GIS	SP homolog
none	-	Bt-00581	4.4	-	DIAL	GD Q GGP	DG-	SP homolog
Cont-02699	3.5	none	-	TAG QC	DVGL	G-----	G--	SP homolog
Cont-00229	3.6	none	-	TV ARC	DIGL	-	-	SP homolog
Cont-08773	9.7	none	-	TAG QC	DIGL	-	-	SP homolog
Cont-03817	4.3	none	-	TA AHC	DIAL	-	-	UTD ^d
Cont-02507	4.0	none	-	TAG HC	DVTL	-	-	UTD
Cont-02359	7.3	none	-	TA AHC	DVGL	-	G--	UTD
Cont-01720	8.6	none	-	TS AHC	DIGL	-	-	UTD
Cont-01610	3.8	none	-	-	DVGL	GDSGGP	G--	UTD
Cont-00142	2.3	none	-	-	DV AV	GDSGGP	GSG	UTD
Cont-09471	32.7	none	-	-	DIAL	GDSGGP	G--	UTD
none	-	Bt-00193	38.1	TAG HC	D VAL	-	-	UTD

^aAccession number of the contig in the corresponding database. Contigs in the same line correspond to the same predicted protein.

^bSee **Materials and methods** for formula to estimate expression.

^cConserved active site residues from *Drosophila melanogaster* trypsin alpha (P04814) were used as a model. '-' indicates lack of homologous residues due to truncated sequence. The residues of catalytic triad (H, D, S) are marked in bold. Synonymic residues of the catalytic triad are in purple; nonsynonymic changes in the residues of catalytic triad that may indicate a change in function are in blue.

^dS1 binding site residues corresponding to bovine chymotrypsin (P00766) S189, G216, and G226 (Rawlings and Barrett, 1993).

^ePredicted to function as trypsin, chymotrypsin, elastase 1-like, unclassified serine peptidase (SP), analog (with synonymic residues in the catalytic triad), or homologs (nonsynonymic residues in the catalytic triad); UTD, unable to determine due to truncated sequence data.

^fBased on alignment (see Supplemental Fig. 4) contig Cont-08703 is probably a "hybrid", as it has a fragment of sequence identical to Cont-09227 (expression was for Cont-09227).

^gAnnotation based on previous research (Elpidina et al., 2005; Prabhakar et al., 2007).

peptidase genes from RNA-Seq were slightly induced at 6 h, most were repressed by 12 h, including the major digestive trypsin (Contig_16981 and Contig_20347) and major digestive chymotrypsin (Contig_15721). The data suggest that significant events related to Bt intoxication occurred between 6 and 12 h.

4. Discussion

This study constitutes the first in-depth analysis of the expression patterns of insect gut peptidase transcripts in response to Bt intoxication through high throughput sequencing and microarrays. In general, we found that Cry3Aa-intoxication had more impact on the expression of serine than cysteine peptidase transcripts in *T. molitor* larvae. However, transcripts encoding the three major digestive enzymes, cathepsin L, trypsin, and chymotrypsin, represented by the most abundant number of reads in RNA-Seq, were repressed in Cry3Aa-intoxicated larvae. Repression of these transcripts and feeding cessation in intoxicated larvae may be connected.

Cysteine peptidases have different roles in normal and pathological metabolism across the phyla. They are involved in bulk proteolysis, like in the *T. molitor* midgut, and in specific regulation (Brix et al., 2008; Turk et al., 2012). Cysteine peptidase transcripts in our datasets included four putative cathepsin B and five cathepsin L (one of which may possibly be cathepsin K), with many more cathepsin L transcripts. Of the nine, four had increased and five decreased expression under the effect of toxin, but the changes in expression did not exceed 2.5-fold. Structural peculiarities of *T. molitor* peptidases from the papain family were noted, with the ERFNIN and GNFD motifs not found in cathepsin B-like transcripts, but present in all transcripts encoding putative cathepsin L.

The most abundant cysteine peptidase transcript in our databases, Cont-08897/Bt-07583, corresponded to what was previously described as a minor digestive peptidase in *T. molitor* larvae, ppCAL3 (Cristofolletti et al., 2005). In that study, the predicted major digestive cathepsin L in *T. molitor* larvae, ppCAL2, corresponded to the transcript in our database Cont-01354/Bt-07528, which contributed less than 6% of total cysteine peptidase expression. Therefore, our RNA-Seq data do not support the previous classification of cathepsin L transcripts in this insect. In support of our data, the major cathepsin L in this study (Cont-08897/Bt-07583) corresponds to the major digestive cysteine cathepsin in *T. castaneum* larvae, as determined by microarray analysis (Morris et al., 2009). Expression of the major digestive cathepsin L transcript in *T. castaneum*, Tc11001, was increased when larvae were exposed to a cysteine peptidase inhibitor (Oppert et al., 2010b), and yet the *T. molitor* ortholog, Cont-08897/Bt-07583, was reduced approximately 2-fold in Cry3Aa-intoxicated larvae. The differential response to peptidase inhibitors and bacterial toxins suggests that different pathways are involved in altering peptidase expression. Alternatively, the relative importance of this major cathepsin L in digestion may differ in the two tenebrionids, as has been suggested in our previous studies (Vinokurov et al., 2006a,b, 2009).

Serine peptidases are arguably among the most studied peptidase class (Polgár, 2005; Page and Di Cera, 2008). Serine peptidase transcripts were more numerous than those encoding cysteine peptidases in *T. molitor* larvae, possibly the result of adaptation to cereals with a high content of serine peptidase inhibitors. We found putative trypsin, chymotrypsin, and elastase 1-like peptidases, as well as analogs, homologs, and those in which functional prediction was problematic due to unusual residues in the binding site or insufficient sequence data.

Table 5
Results of microarray analysis of expression patterns of potential peptidase genes in *Tenebrio molitor* larvae fed 0.1% Cry3Aa or control diet for 6, 12, and 24 h intoxication (ANOVA, $p < 0.05$), with repressed expression in grey for easier pattern detection. Correspondence to RNA-Seq data and NCBI accession numbers are provided.

Microarray ID	Predicted Classification ^a	Relative Fold Change ^b			CONT Contig# ^c	BT Contig# ^c	Relative RNA-Seq Expression Change ^b	Other ^d
		6 h	12 h	24 h				
Contig_19810	chymotrypsin	7.92	7.20	4.80	08675	00013	1.38	-
Contig_16752	elastase 1-like	2.76*	2.01*	2.36*		08266	Bt-treated only	DQ356032
Contig_18081	SP	2.16	1.55	1.32	-	-	-	DQ356029
Contig_19676	SPH	1.95*	2.15*	1.84*	-	-	-	DQ356029
Contig_16973	chymotrypsin-like	1.83	1.52	2.30*	00845	08328	1.06	DQ356032/3
Contig_20748	SPH	1.80*	1.42*	2.29	00369	00813	1.94	-
Contig_16426	SP	1.55	3.41	1.79	-	-	-	-
Contig_20213	SP	1.19	1.30	1.06	-	-	-	-
Contig_19593	SPH	1.45	-3.48	-3.27	02455	-	Control only	DQ356044
Contig_18678	elastase 1-like	1.38	-1.04	-1.13	08725	02137	-9.34	DQ356045
Contig_16981	trypsin ^e	1.35	-1.54	-3.83	00231	03613	-15.0	DQ356014/5/6/7 AY845177
Contig_20232	SP	1.34	-2.58*	-2.67*	-	-	-	DQ356044
Contig_16950	SPH	1.27	-1.16	-2.12	00768	00326	-2.75	DQ356042/3
Contig_15721	chymotrypsin ^f	1.15	-1.51	-2.53	09450	07640	-6.17	DQ356031
Contig_20347	trypsin ^e	1.11	-1.60*	-1.86*	00231	03613	-15.0	DQ356014/5/6/7 AY845177
Contig_19208	UTD	-2.53	-9.09	-6.27	09471	-	Control only	-
Contig_4385	UTD	-1.98*	-7.02*	-5.22*	00142	-	Control only	-
Contig_3746	SP	-1.82	-4.00*	-2.56	-	-	-	-
Contig_7249	SP	-1.82	-3.02	-2.84	-	-	-	-
Contig_5570	SP	-1.76	-3.51	-4.80	-	-	-	-
Contig_21823	SP	-1.57	-2.94	-3.43	-	-	-	-
Contig_11744	SP	-1.5	-1.93	1.04	-	-	-	AB363982
Contig_7975	SP	-1.34	-1.93*	-1.59	-	-	-	-
Contig_18087	SPH	-1.29	-1.86	-1.32	00768	00326	-2.75	DQ356042/3
Contig_18994	SPH	-1.28*	-2.65	-2.68	02699	-	Control only	-
Contig_17683	cathepsin L	-1.28	-1.48	-1.06	00009	01497	1.11	-
Contig_20429	SPH	-1.26	-2.66*	-2.34*	08766	-	Control only	DQ356035/6/7
Contig_7784	SP	-1.26	-1.55	-1.16	-	-	-	DQ356027/8
Contig_21691	SP	-1.20*	-1.73	-1.30	-	-	-	-
Contig_18207	SP	-1.20	-1.74	-1.39	-	-	-	-
Contig_23442	SP	-1.10	-1.45	-1.03	-	-	-	-
Contig_16759	SP	-1.09	-1.41	1.94	-	-	-	-
Contig_4136	trypsin	-1.04	-1.30	-1.14	-	-	-	-

^aPredicted to function as trypsin, chymotrypsin, elastase 1-like, unclassified serine peptidase (SP), or homologs (nonsynonymic residues in the catalytic triad); UTD – predicted proteins could not be classified due to truncated sequence data.

^bRelative fold change of expression in Cry3Aa-treated larvae.

^cCorresponding contig # in CONT or BT databases from the first assembly of 454 reads.

^dNCBI ID for putative corresponding *T. molitor* sequences, as determined by Blast analysis of microarray contig sequences to *T. molitor*.

^eMajor digestive trypsin in the posterior midgut of *Tenebrio molitor* larvae (Tsybina et al., 2005; Prabhakar et al., 2007).

^fMajor digestive chymotrypsin in the posterior midgut of *Tenebrio molitor* larvae (Elpidina et al., 2005; Prabhakar et al., 2007).

*Data were statistically significant ($p < 0.05$) by t-test in pairwise analysis.

Glutamine was a common substitution for the critical histidine in the first conserved motif of peptidase homologs. Interestingly, most of the truncated sequences that we were unable to classify were found only in control larvae.

A significant reduction in serine peptidase transcripts was found in Cry3Aa-treated larvae, especially those related to analogs and homologs. Microarray analysis indicated that a reduction in classified serine peptidases occurred primarily between 6 and 12 h post intoxication, while unclassified serine peptidases were already reduced in

expression at the 6 h post intoxication treatment. Also several specific transcripts encoding serine peptidases and homologs, as well as one cathepsin B, were found only in RNA-Seq data from control larvae. We have speculated that homolog sequences may be constitutively expressed in the *T. molitor* larval gut as a protective mechanism against cereal inhibitors (Prabhakar et al., 2007; Oppert et al., 2010b), but their overall down-regulation in response to toxins was unknown prior to this study. However, two specific SPH transcripts were specifically induced at all time points of intoxication. Therefore, we speculate that

these SPH transcripts may provide a defense response to intoxication by binding and sequestering protoxin or toxin. In fact, toxin sequestration by elastase has been proposed as a potential resistance mechanism in a previous study (Milne et al., 1998).

Unlike the other serine peptidases, transcripts encoding putative chymotrypsin-like peptidases with a S189 to G189 substitution were either induced or only found in Cry3Aa-treated larvae, constituting about 73% of the total serine peptidase expression in intoxicated larvae. This result was unexpected and indicated an intricate correlation between larvae intoxication and serine peptidase expression. Transcripts encoding chymotrypsin-like peptidases also were significantly increased in *T. castaneum* larvae exposed to cysteine peptidase inhibitors (Oppert et al., 2010b). Increased expression of chymotrypsins may represent a general stress response, or may be related to dietary compensation responses when transcripts encoding major digestive enzymes are repressed. Important to Bt intoxication, proteolysis of Cry3Aa protoxin in coleopterans has been attributed to chymotrypsin (Carroll et al., 1989). We noted one chymotrypsin-like transcript that was expressed similarly in control and intoxicated larvae (Cont-00845/Bt-08328) and correlated to AM2-68 isolated from the anterior midgut (Prabhakar et al., 2007). Therefore, this transcript may function in protoxin processing. However, the relevance of increased expression of other chymotrypsin-like transcripts in Cry3Aa-intoxicated larvae is unknown and merits further study.

A recent report indicated that a trypsin-like peptidase transcript was repressed within 24 h of Cry1Ca1 intoxication of *Spodoptera frugiperda* and was predicted to play determinant role in toxicity (Rodriguez-Cabrera et al., 2010). While our study was of a different toxin and in a coleopteran, our results suggest that Cry intoxication does not affect only one critical protease, but instead induces the differential expression of a suite of peptidase genes in the midgut. Approximately half of the predicted 150 serine peptidase genes and 25 cysteine peptidase genes in the genome of *T. castaneum* are expressed in the gut under normal dietary conditions (Morris et al., 2009). Furthermore, expression patterns of midgut peptidase genes were found to be highly variable when *T. castaneum* larvae ingested peptidase inhibitors (Oppert et al., 2010b). In an in-depth study of the cysteine peptidase genes in the *Tribolium* genome, we found that many appear to be the result of gene duplication events, with as many as five in tandem (*Tribolium* Genome Sequencing Consortium et al., 2008). This phenomenon is found also with serine peptidase genes (unpublished data). Therefore, it is logical to predict that insects have retained a high degree of duplicity in peptidase genes and regulate their expression in response to external variables, including toxins and inhibitors.

Our previous studies have described peptidase transcripts in the anterior and posterior midgut of *T. molitor* (Prabhakar et al., 2007). Cry3Aa-intoxication affected the expression of serine peptidase transcripts, found mostly in the posterior midgut, more than that of anterior midgut cysteine peptidase transcripts. These data correlate with the localization of the Cry3Aa cadherin receptor in the mid to posterior *T. molitor* midgut (unpublished data). The impact of Cry3Aa intoxication on peptidase transcripts can also be observed in the value of expression change for cysteine peptidases (about 2-fold) compared to that of serine peptidases (up to 15-fold). As a result, our prediction is that the anterior midgut of *T. molitor* larvae is less affected by Cry3Aa intoxication than the posterior midgut, presumably the site of toxin binding and subsequent intoxication events. In addition, the anterior midgut is more acidic and may not be conducive to solubility or pH dependent activity of the toxin, including protoxin activation.

5. Conclusions

The data in this study represent the first application of high-throughput sequencing to the study of the effects of Bt intoxication on the expression of insect midgut peptidase transcripts. Microarray analysis was used to validate expression data from RNA-Seq. The

results indicate that Cry3Aa intoxication in *T. molitor* induces overall reduction and widespread changes in transcripts encoding serine peptidases in the larval gut, with specific induction of certain transcripts encoding predicted chymotrypsins and SPH.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbd.2012.03.005>.

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B Methodol.* 57, 289–300.
- Brix, K., Dunkhorst, A., Mayer, K., Jordans, S., 2008. Cysteine cathepsins: cellular roadmap to different functions. *Biochimie* 90, 194–207.
- Carroll, J., Li, J., Ellar, D.J., 1989. Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* 261, 99–105.
- Cristofaletti, P.T., Ribeiro, A.F., Terra, W.R., 2005. The cathepsin L-like proteinases from the midgut of *Tenebrio molitor* larvae: sequence, properties, immunocytochemical localization and function. *Insect Biochem. Mol. Biol.* 35, 883–901.
- Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797.
- Elpidina, E.N., Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P., Oppert, B., 2005. A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. *Biochimie* 87, 771–779.
- Fabrick, J., Oppert, C., Lorenzen, M.D., Morris, K., Oppert, B., Jurat-Fuentes, J.L., 2009. A novel *Tenebrio molitor* cadherin is a functional receptor for *Bacillus thuringiensis* Cry3Aa toxin. *J. Biol. Chem.* 284, 18401–18410.
- Hedstrom, L., 2002. Serine protease mechanism and specificity. *Chem. Rev.* 102, 4501–4524.
- Karrer, K.M., Peiffer, S.L., DiTomas, M.E., 1993. Two distinct gene subfamilies within the family of cysteine protease genes. *Proc. Natl. Acad. Sci. U. S. A.* 90, 3063–3067.
- Kim, C.H., Kim, S.J., Kan, H., Kwon, H.M., Roh, K.B., et al., 2008. A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. *J. Biol. Chem.* 283, 7599–7607.
- Milne, R., Wright, T., Kaplan, H., Dean, D., 1998. Spruce budworm elastase precipitates *Bacillus thuringiensis* dendotoxin by specifically recognizing the C-terminal region. *Insect Biochem. Mol. Biol.* 28, 1013–1023.
- Morris, K., Lorenzen, M.D., Hiromasa, Y., Tomich, J.M., Oppert, C., Elpidina, E.N., Vinokurov, K., Jurat-Fuentes, J.L., Fabrick, J., Oppert, B., 2009. *Tribolium castaneum* larval gut transcriptome and proteome: a resource for the study of the coleopteran gut. *J. Proteome Res.* 8, 3889–3898.
- Oppert, B., 1999. Protease interactions with *Bacillus thuringiensis* insecticidal toxins. *Arch. Insect Biochem. Physiol.* 42, 1–12.
- Oppert, B., Kramer, K.J., Johnson, D.E., MacIntosh, S.C., McGaughey, W.H., 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. *Biochem. Biophys. Res. Commun.* 198, 940–947.
- Oppert, B., Kramer, K.J., Johnson, D., Upton, S.J., McGaughey, W.H., 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryI(Ac) protoxin. *Insect Biochem. Mol. Biol.* 26, 571–583.
- Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H., 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *J. Biol. Chem.* 272, 23473–23476.
- Oppert, B., Ellis, R.T., Babcock, J., 2010a. Effects of Cry1F and Cry34Ab1/35Ab1 on storage pests. *J. Stored Prod. Pests* 46, 143–148.
- Oppert, B., Elpidina, E.N., Toutges, M., Mazumdar-Leighton, S., 2010b. Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors. *Comp. Biochem. Physiol. D* 5, 280–287.
- Oppert, B., Dowd, S.E., Bouffard, P., Li, L., Conesa, A., Lorenzen, M.D., Toutges, M., Marshall, J., Huestis, D.L., Fabrick, J., Oppert, C., Jurat-Fuentes, J.L., in press. Transcriptome

- profiling of the intoxication response of *Tenebrio molitor* larvae to *Bacillus thuringiensis* Cry3Aa protoxin. PLoS One. <http://dx.plos.org/10.1371/journal.pone.0034624>.
- Page, M.J., Di Cera, E., 2008. Serine peptidases: classification, structure and function. *Cell. Mol. Life Sci.* 65, 1220–1236.
- Perona, J.J., Craik, C.S., 1995. Structural basis of substrate specificity in the serine proteases. *Protein Sci.* 4, 337–360.
- Polgár, L., 2005. The catalytic triad of serine peptidases. *Cell. Mol. Life Sci.* 62, 2161–2172.
- Prabhakar, S., Chen, M.S., Elpidina, E.N., Vinokurov, K.S., Smith, C.M., Marshall, J., Oppert, B., 2007. Sequence analysis and molecular characterization of larval mid-gut cDNA transcripts encoding peptidases from the yellow mealworm, *Tenebrio molitor* L. *Insect Mol. Biol.* 16, 455–468.
- Rawlings, N.D., Barrett, A.J., 1993. Evolutionary families of peptidases. *Biochem. J.* 290, 205–218.
- Rawlings, N.D., Barrett, A.J., Bateman, A., 2010. MEROPS: the peptidase database. *Nucleic Acids Res.* 38, D227–D233.
- Rodriguez-Cabrera, L., Trujillo-Bacallao, D., Borrás-Hidalgo, O., Wright, D.J., Ayra-Pardo, C., 2010. RNAi-mediated knockdown of a *Spodoptera frugiperda* trypsin-like serine-protease gene reduces susceptibility to a *Bacillus thuringiensis* Cry1Ca1 protoxin. *Environ. Microbiol.* 12, 2894–2903.
- Sayers, E.W., Barrett, T., Benson, D.A., Bolton, E., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Federhen, S., Feolo, M., Fingerhann, I.M., Geer, L.Y., Helmberg, W., Kapustin, Y., Landsman, D., Lipman, D.J., Lu, Z., Madden, T.L., Madej, T., Maglott, D.R., Marchler-Bauer, A., Miller, V., Mizrahi, I., Ostell, J., Panchenko, A., Phan, L., Pruitt, K.D., Schuler, G.D., Sequeira, E., Sherry, S.T., Shumway, M., Sirotkin, K., Slotta, D., Souvorov, A., Starchenko, G., Tatusova, T.A., Wagner, L., Wang, Y., Wilbur, W.J., Yaschenko, E., Ye, J., 2011. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* 39, D38–D51.
- Stothard, P., 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* 28 (1102), 1104.
- Tribolium Genome Sequencing Consortium, Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Beeman, R.W., Brown, S.J., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J., Klingler, M., Lorenzen, M., Richards, S., Roth, S., Schroder, R., Tautz, D., Zdobnov, E.M., Muzny, D., Gibbs, R.A., Weinstock, G.M., Attaway, T., Bell, S., Buhay, C.J., Chandrabose, M.N., Chavez, D., Clerk-Blankenburg, K.P., Cree, A., Dao, M., Davis, C., Chacko, J., Dinh, H., Dugan-Rocha, S., Fowler, G., Garner, T.T., Garnes, J., Gnirke, A., Hawes, A., Hernandez, J., Hines, S., Holder, M., Hume, J., Jhangiani, S.N., Joshi, V., Khan, Z.M., Jackson, L., Kovar, C., Kowis, A., Lee, S., Lewis, L.R., Margolis, J., Morgan, M., Nazareth, L.V., Nguyen, N., Okwuonu, G., Parker, D., Richards, S., Ruiz, S.J., Santibanez, J., Savard, J., Scherer, S.E., Schneider, B., Sodergren, E., Tautz, D., Vattahil, S., Villasana, D., White, C.S., Wright, R., Park, Y., Beeman, R.W., Lord, J., Oppert, B., Lorenzen, M., Brown, S., Wang, L., Savard, J., Tautz, D., Richards, S., Weinstock, G., Gibbs, R.A., Liu, Y., Worley, K., Weinstock, G., Elsik, C.G., Reese, J.T., Elhaik, E., Landan, G., Graur, D., Arensburger, P., Atkinson, P., Beeman, R.W., Beidler, J., Brown, S.J., Demuth, J.P., Drury, D.W., Du, Y.Z., Fujiwara, H., Lorenzen, M., Maselli, V., Osanai, M., Park, Y., Robertson, H.M., Tu, Z., Wang, J.J., Wang, S., Richards, S., Song, H., Zhang, L., Sodergren, E., Werner, D., Stanke, M., Morgenstern, B., Solovyev, V., Kosarev, P., Brown, G., Chen, H.C., Ermolaeva, O., Hlavina, W., Kapustin, Y., Kiryutin, B., Kitts, P., Maglott, D., Pruitt, K., Sapojnikov, V., Souvorov, A., Mackey, A.J., Waterhouse, R.M., Wyder, S., Zdobnov, E.M., Zdobnov, E.M., Wyder, S., Kriventseva, E.V., Kadowaki, T., Bork, P., Aranda, M., Bao, R., Beermann, A., Berns, N., Bolognesi, R., Bonneton, F., Bopp, D., Brown, S.J., Bucher, G., Butts, T., Chaumot, A., Denell, R.E., Ferrier, D.E., Friedrich, M., Gordon, C.M., Jindra, M., Klingler, M., Lan, Q., Lattorff, H.M., Laudet, V., von Levetzow, C., Liu, Z., Lutz, R., Lynch, J.A., da Fonseca, R.N., Posnien, N., Reuter, R., Roth, S., Savard, J., Schinko, J.B., Schmitt, C., Schoppmeier, M., Schroder, R., Shippy, T.D., Simonnet, F., Marques-Souza, H., Tautz, D., Tomoyasu, Y., Trauner, J., Van der Zee, M., Vervoort, M., Wittkopp, N., Wimmer, E.A., Yang, X., Jones, A.K., Sattelle, D.B., Ebert, P.R., Nelson, D., Scott, J.G., Beeman, R.W., Muthukrishnan, S., Kramer, K.J., Arakane, Y., Beeman, R.W., Zhu, Q., Hogenkamp, D., Dixit, R., Oppert, B., Jiang, H., Zou, Z., Marshall, J., Elpidina, E., Vinokurov, K., Oppert, C., Zou, Z., Evans, J., Lu, Z., Zhao, P., Sumathipala, N., Altincicek, B., Vilcinskis, A., Williams, M., Hultmark, D., Hetru, C., Jiang, H., Grimmelikhuijzen, C.J., Hauser, F., Cazzamali, G., Williamson, M., Park, Y., Li, B., Tanaka, Y., Predel, R., Neupert, S., Schachtner, J., Verleyen, P., Raible, F., Bork, P., Friedrich, M., Walden, K.K., Robertson, H.M., Angeli, S., Foret, S., Bucher, G., Schuetz, S., Maleszka, R., Wimmer, E.A., Beeman, R.W., Lorenzen, M., Tomoyasu, Y., Miller, S.C., Grossmann, D., Bucher, G., 2008. The genome of the model beetle and pest *Tribolium castaneum*. *Nature* 452, 949–955.
- Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P., Oppert, B., Elpidina, E.N., 2005. Digestive proteinases of yellow mealworm (*Tenebrio molitor*) larvae: purification and characterization of a trypsin-like proteinase. *Biochemistry (Mosc)* 70, 300–305.
- Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., Turk, D., 2012. Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim. Biophys. Acta* 1824, 68–88.
- Vinokurov, K.S., Elpidina, E.N., Oppert, B., Prabhakar, S., Zhuzhikov, D.P., Dunaevsky, Y.E., Belozersky, M.A., 2006a. Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. *Comp. Biochem. Physiol. B* 145, 126–137.
- Vinokurov, K.S., Elpidina, E.N., Oppert, B., Prabhakar, S., Zhuzhikov, D.P., Dunaevsky, Y.E., Belozersky, M.A., 2006b. Fractionation of digestive proteinases from *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae and role in protein digestion. *Comp. Biochem. Physiol. B* 145, 138–146.
- Vinokurov, K.S., Elpidina, E.N., Zhuzhikov, D.P., Oppert, B., Kodrik, D., Sehnal, F., 2009. Digestive proteolysis organization in two closely related tenebrionid beetles: red flour beetle (*Tribolium castaneum*) and confused flour beetle (*Tribolium confusum*). *Arch. Insect Biochem. Physiol.* 70, 254–279.