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Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors

Brenda Oppert [a,*], Elena N. Elpidina [b], Michelle Toutges [a], Sue Mazumdar-Leighton [c]

[a] USDA ARS Center for Grain and Animal Health Research, 1515 College Ave., Manhattan, KS 66502, USA
[b] A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119991, Russia
[c] Department of Botany, Delhi University, Delhi 110007 India

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ABSTRACT

The transcriptome response of *Tribolium castaneum* larvae to dietary protease inhibitors was evaluated by whole-genome microarray analysis. RNA was isolated from guts of larvae fed control diet (no inhibitor), or diets containing 0.1% E-64 (cysteine protease inhibitor), 5.0% soybean trypsin inhibitor (STI, serine protease inhibitor), or a combination of 0.1% E-64 and 5.0% STI. Data were analyzed by pairwise analysis, in which each inhibitor treatment group was compared to control, or ANOVA of all treatment groups. In pairwise analysis, the expression of only 253 genes was significantly altered ($p<0.05$) in response to STI treatment, whereas E-64 and combination treatments resulted in 1574 and 1584 differentially regulated genes. The data indicate that treatments containing E-64, whether alone or in combination, significantly impacts gene expression in *T. castaneum* larvae. ANOVA analysis revealed 2175 genes differentially expressed in inhibitor-treated larvae compared to control ($p<0.05$), including genes related to proteases that were mostly up-regulated, namely cathepsins B and L, chymotrypsins, and nonproteolytic cysteine cathepsin or serine protease homologs. Inhibitor treatments induced the differential expression of other gut-related genes, as well as genes encoding proteins of unknown function. These data suggest that *T. castaneum* larvae compensate for dietary cysteine protease inhibitors by altering large-scale gene expression patterns.

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1. Introduction

The tenebrionid, *Tribolium castaneum*, is a major pest of grains and stored products, inflicting considerable economic damage worldwide. Biochemical and genetic approaches have been used to identify crucial proteases and delineate their spatial organization in the gut of *T. castaneum* larvae. Protein digestion in *T. castaneum* is largely due to cysteine proteases in the acidic anterior midgut, with minor contributions by serine proteases, chymotryspins and trypsins, in an alkaline posterior midgut (Vinokurov et al., 2009). The importance of cysteine proteases in protein digestion, as well as the compartmentalization of these enzymes in the acidic anterior midgut, appears to be an adaptation in tenebrionid larvae to cereals containing abundant serine protease inhibitors. Insects adapt to plant and cereal protease inhibitors as part of an evolutionary struggle to overcome host-plant responses to insect attack (reviewed in Oppert, 2000; Agrawal, 2001; Dunaevsky et al., 2005). In another tenebrionid, *Tenebrio molitor*, our studies and others have found that protein digestion in larvae is also compartmentalized (Terra et al., 1985; Thie and Houseman, 1990; Terra and Ferreira, 1994; Vinokurov et al., 2006; Prabhakar et al., 2007). However, serine proteases in the posterior midgut of *T. molitor* apparently are more important for protein digestion than those in *T. castaneum* larvae (Vinokurov et al., 2009).

Despite the reliance on cysteine proteases for protein digestion, *T. castaneum* larvae survive diets containing relatively high levels of cysteine protease inhibitors (Chen et al., 1992; Oppert et al., 1993, 2003). This paradox was explained by examining the proteolytic activity in the guts of surviving larvae (Oppert et al., 2004, 2005). As larvae were fed increasing doses of a cysteine protease inhibitor, in vitro protease activity was less responsive to the inhibitor, and a concomitant increase in serine protease activity was observed, particularly chymotryptic activity. These data suggested that *T. castaneum* larvae were compensating for dietary cysteine protease inhibitors through the expression of up-regulated and/or novel cysteine and serine proteases.

A significant breakthrough in probing coleopteran responses to dietary toxins and inhibitors came with the sequencing of the genome of *T. castaneum* (*Tribolium* Genome Sequencing Consortium, 2008). Through a *Tribolium* whole-genome microarray analysis, our research has demonstrated that the cysteine proteases cathepsin L and B, and the serine proteases chymotrypsin and trypsin are among the most highly expressed genes in the gut of *T. castaneum* fed a wheat flour/brewer's yeast diet (Morris et al., 2009). To further examine the

Abbreviations: E-64, L-trans-epoxysuccinylleucylamide [4-guanidino] butane; STI, soybean trypsin inhibitor.

* Corresponding author. Tel.: +1 785 776 2780; fax: +1 785 537 5584.
E-mail address: bso@ksu.edu (B. Oppert).

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response of *T. castaneum* to cereal protease inhibitors, custom microarrays containing oligonucleotides representative of all genetic sequences from the *Tribolium* genome were used to examine gene expression in larvae fed a cysteine or serine protease inhibitor, or a combination thereof. The data support our observations from previous biochemical studies and emphasize the role of adaptative responses in overcoming the effect of protease inhibitors in *T. castaneum*. These data further provide evidence that the use of combinations of protease inhibitors can be an effective method to inhibit the insect compensative response.

2. Materials and methods

Inhibitors used in this study included L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64, Sigma-Aldrich Chemical Co., St Louis, MO, USA) and soybean trypsin inhibitor (STI, Kunitz, Worthington Biochemical Corp., Lakewood, NJ, USA).

2.1. Insect bioassay and dissection

T. castaneum were reared in our laboratory on a diet of 95% wheat flour and 5% brewer's yeast at 28 °C, 75% R.H., 0L:24D. Neonate larvae from two biological replicates of *T. castaneum* larvae were placed on 0.5 g of 85% stabilized wheat germ, 10% hard red winter wheat flour, and 5% brewer's yeast and reared under normal rearing conditions. At 10 days of age, groups of 11–13 larvae were transferred to a plastic cup with 120 mg of diet and either no inhibitor (control), 0.1% E-64, 5% STI, or a combination of 0.1% E-64 and 5% STI. After 3 days, larvae were sacrificed for the collection of gut tissue. Mean larval masses (±S.E.) in control, E-64, STI, and combination treatments were 1.4 (0.3), 1.3 (0.2), 1.5 (0.3), and 0.6 (0.2) mg, respectively.

2.2. Development of the microarray

A whole-genome microarray for *T. castaneum* was developed using all annotated and predicted gene sequences from the *T. castaneum* genome annotation project, designated as GLEAN numbers (*Tribolium* Genome Sequencing Consortium, 2008). Oligos were designed and arrayed in duplicate or triplicate on a custom array chip (4x44K, Agilent Technologies, Santa Clara, CA, USA).

2.3. Isolation of RNA and cRNA hybrydization

For dissection, 6–10 larvae with food in the gut were chosen from each dietary treatment and were ice-anaesthetized. The anterior and posterior ends were removed with sterile scissors, and guts were removed by pulling gently from one end and placed in RNAlater (Ambion, Austin, TX) at room temperature. Prior to RNA extraction, excess RNA later was removed 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, which includes on-column DNase treatment as described by manufacturer's protocol (Agilent Technologies, La Jolla, CA). Gut mRNA was reverse transcribed from total RNA using an oligo-dT with T7 promoter and amplified with T7 polymerase to obtain Cy3 or Cy5-labeled antisense cRNA (Quick Amp Labeling Kit, Agilent), with dye swapping among the biological replicates. Hybridization of the *Tribolium* genome microarray was performed for 18 h at 45 °C with approximately 825 ng of the modified cRNA (Gene Expression Hybridization Kit, Agilent). Microarrays were scanned at 532 and 635 nm using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) at the Gene Expression Facility at Kansas State University. Images were analyzed by Genepix Pro 6.1 software.

2.4. Reverse transcription and quantitative PCR (qPCR)

Aliquots of total RNA used for microarray analysis were used as the template for reverse transcription reactions. All reverse transcription reactions were with Superscript in the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and approximately 500 ng of total RNA, following the manufacturer's protocol for RT-PCR on an Applied Biosytems 2720 thermocycler (Life Technologies, Carlsbad, CA, USA). Primer sets (Supplemental Table 6) were designed (PrimerQuest; www.idtdna.com) and obtained from Integrated DNA Technologies (Coralville, IA, USA). Amplicons were sequenced and verified against the *T. castaneum* genome. Quantitative PCR was performed on the MX3000P (Stratagene, Santa Clara, CA, USA) using 3 μL of diluted cDNA (1:20 for all primer sets except Tc11001 which was diluted 1:2500) and Takara SYBR Premix Ex Taq II (Takara Bio, Madison WI, USA). Cycling conditions were 30 s at 95 °C; 40 cycles of 5 s at 95 °C, 20 s at 55 °C, and 15 s at 72 °C; followed by a standard dissociation curve. Data are the average of two biological replicates (standard deviation ≤ 0.2, triplicate technical replicates).

2.5. Statistical and bioinformatic analysis

The relative intensity of each array (raw data) was imported into GeneSifter (Geospiza, Seattle, WA, USA) for statistical analysis. Data was normalized by relative intensity means and was log transformed. Pairwise comparisons were made between the control and each treatment dataset, and significance was determined by the Student's *t*-test ($p<0.05$), corrected by Benjamini and Hochberg (1995); all treatment groups also were compared by ANOVA ($p<0.05$) and corrected by Benjamini and Hochberg. Principle Component Analysis (PCA; Pearson, 1901) also was performed with GeneSifter.

Alignments of predicted *T. castaneum* protease and homolog protein sequences were made with ClustalW2 (Larkin et al., 2007).

3. Results

To determine the effect of protease inhibitors on the *T. castaneum* larval transcriptome, microarrays containing oligos representative of the predicted gene sequences from *T. castaneum* were used to estimate the relative expression of mRNA from the gut of larvae fed diets containing 0.1% E-64, 5.0% STI, a combination of E-64 and STI, or inhibitor-free diet (control). Data were compared in pairwise analyses between control and each treatment group, or an ANOVA analysis of differences among control and all treatments ($p<0.05$).

3.1. Pairwise analysis of treatment effects

In pairwise analyses, there were 1574, 253, and 1584 genes that were differentially expressed in E-64, STI, and combination treatments, respectively (Fig. 1A). Therefore, the number of differentially expressed genes in E-64 and combination treatments was similar, and significantly more than that of STI treatment. Specifically, the number of genes that were up- or down-regulated in each treatment group was 133 and 1441 in E-64-treated larvae; 44 and 209 in STI-treated larvae; and 243 and 1341 in combination-treated larvae, respectively (Fig. 1B). Therefore, there were many more genes that were down-regulated in response to inhibitor treatment than were up-regulated.

Genes expressed at more than 5-fold difference in E-64 and combination treatments compared to control are summarized in Tables 1 and 3, respectively; those with more than 2-fold difference in STI treatment compared to control are found in Table 2. Included in the comparisons are “gut rankings”, a prediction of the expression level of the gene in the gut based on a previous microarray analysis of RNA from *T. castaneum* larval gut tissue (Morris et al., 2009).

In larvae fed 0.1% E-64, there were 38 genes that were more than 5-fold differentially expressed compared to control larvae (Table 1).

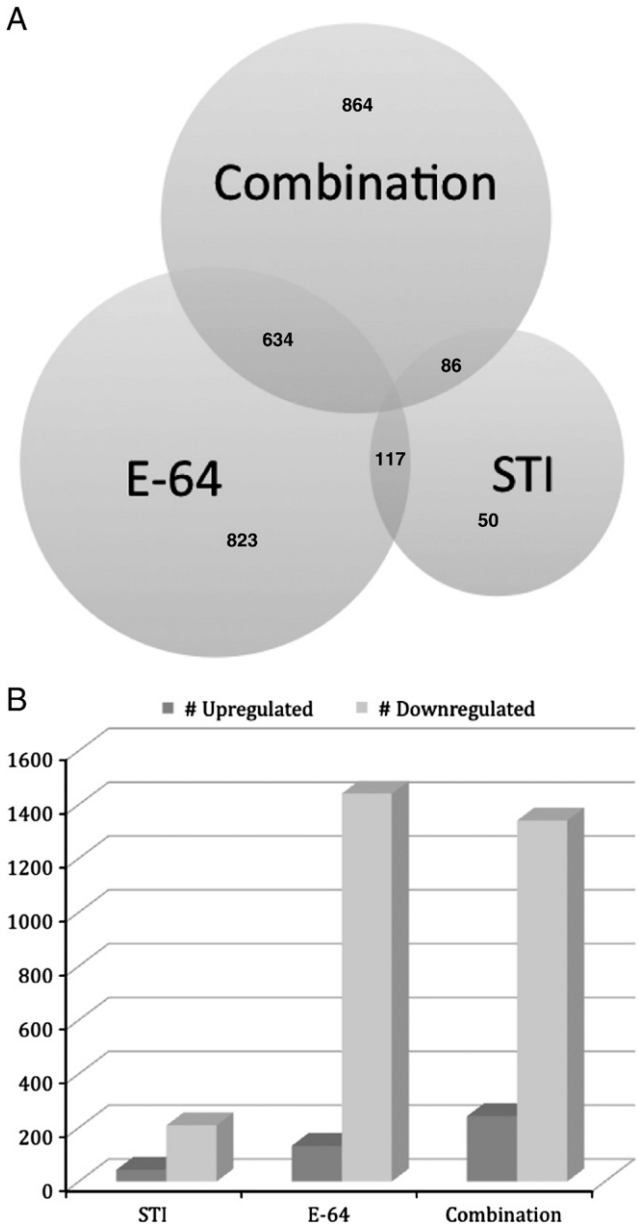

Fig. 1. Comparison of the total number of differentially expressed genes (A) or the number of significantly ($p<0.05$) up-regulated or down-regulated genes (B) in the gut of *T. castaneum* larvae fed 0.1% E-64, 5.0% STI, or a combination of inhibitors.

The most striking differentially expressed gene was 30.6-fold down-regulated and was related to the mosquito transcript AGAPO12043-PA of unknown function. A gene encoding RNA polymerase II was down-regulated 17.6-fold and may indicate that either a shut-down or redirection of transcription is occurring; RNA pol II is involved in transcription of all protein coding genes but also transcribes small nuclear RNAs which are involved in splicing. Many of the remainder encode chymotrypsins, or serine protease homologs (SPH) from the S1 protease family according to the MEROPS classification (Rawlings et al., 2010) which lack sequence conservation in catalytic residues of typical serine proteases and therefore are predicted to be nonproteolytic; most were up-regulated.

Only 10 genes were differentially expressed more than 2-fold in STI-treated larvae, and none more than 5-fold (Table 2). Most significantly down-regulated was a chymotrypsin encoded by Glean 16121, otherwise predicted to be relatively highly expressed in the larval gut under normal dietary conditions according to a previous microarray (Morris et al., 2009).

Table 1
Genes differentially regulated more than 5-fold (significant at $p<0.05$, pairwise analysis) in *T. castaneum* larvae fed a diet containing 0.1% E-64. Grey squares are those genes that were down-regulated. Whenever possible, the most descriptive term associated with each sequence was provided under "Description".

Glean#	NCBI#	Ratio	Gut Rank[1]	Description
5225	XM_001808333	30.6 ↓	NOC	AGAP012043-PA
7328	XM_968377	17.6 ↓	0.73	RNA polymerase II
9628	NM_001042569	9.14 ↓	1.91	Chitinase 13
3383	XM_962430	8.45 ↓	2.10	Phosphatidylinositol kinase
3275	XM_968716	8.08 ↓	46.6	Peritrophin-1
10959	XM_966152	7.97 ↓	2.23	Elastase 1
14501	XM_965570	7.78 ↓	0.91	Protein lethal(3)malignant blood Neoplasm 1 (Mbn(83))
2989	XM_966418	7.66 ↓	2.10	α-L-fucosidase precursor
7306	XM_970496	7.23 ↓	0.63	CG32603-PA
11602	XM_968802	7.22 ↓	1.40	CG17127-PA
15670	XM_961468	7.03 ↓	0.72	Serine protease homolog
16058	XM_970389	6.90 ↓	0.62	CG7709-PA
14497	XM_965288	6.76 ↓	0.55	Chitin binding protein
13059	XM_961720	6.69 ↓	1.49	Reeler domain
9178	XM_967709	6.58 ↓	3.09	Glycosyl hydrolase
13911	XM_970972	5.94 ↓	2.53	C-type lectin
13562	XM_961635	5.84 ↓	2.10	CG14661-PA
11616	XM_001812148	5.73 ↓	NOC	Inward rectifier potassium channel
5238	XM_964575	5.59 ↓	0.70	Golden goal CG32227-PA
13128	XM_968953	5.40 ↓	0.91	Cuticular protein 78, RR-1 family
13139	XM_968570	5.30 ↓	0.75	Chitin binding protein
15580	XM_967979	10.1 ↑	9.31	Elastase 1
2786	XM_967427	9.67 ↑	2.53	Chymotrypsin 1
10934	XM_963610	7.85 ↑	2.46	Serine protease homolog
8504	XM_962160	7.68 ↑	46.8	Serine protease homolog
13661	NM_001110542	7.17 ↑	NOC	Chitin deacetylase 7
10052	XM_970573	6.72 ↑	54.6	Glycosyl hydrolase
3274	NM_001168458	6.68 ↑	NOC	Peritrophic matrix protein 2-A
16349	XM_963397	6.53 ↑	23.8	Chitin binding protein
10908	XM_963904	5.91 ↑	12.2	Serine protease homolog
2952	XM_969205	5.89 ↑	43.4	Cathepsin B
5953	XM_963674	5.83 ↑	2.71	Cathepsin B homolog
10927	XM_963074	5.82 ↑	44.5	Serine protease homolog
5124	XM_971250	5.78 ↑	62.6	Hypothetical protein LOC660391
6990	XM_971269	5.64 ↑	25.4	Hypothetical protein LOC660857
2767	XM_962314	5.42 ↑	1.63	Chymotrypsin BII
11510	XM_970264	5.18 ↑	0.49	Short-chain dehydrogenase/ reductase
292	XM_971310	5.08 ↑	3.63	Hypothetical protein LOC662001

[1]Gut ranks were determined through microarray analysis (Morris et al., 2009), and they range from 0 (low or no expression in the gut) to 100 (highly expressed in the gut); NOC – not on chip in the Morris et al., 2009 array.

Table 2
Genes differentially regulated more than 2-fold (significant at $p<0.05$, pairwise analysis) in *T. castaneum* larvae fed a diet containing 5% STI. Grey squares are those genes that were down-regulated.

Glean#	NCBI#	Ratio	p-value	Gut Rank[1]	Description
2465	XM_963424	3.83 ↓	0.04	5.33	Lysosomal α-mannosidase
1160	XM_968915	2.07 ↓	0.02	2.93	Histidine phosphatase
7187	XM_967864	2.25 ↓	0.05	8.27	Lysosomal acid lipase
16121	XM_962371	8.80 ↓	0.03	8.80	Chymotrypsin
0204				0.61	
2146	XM_962798	2.40 ↑	0.02	0.79	Phosphatidylinositol kinase
2829				0.92	
11253				0.38	
15792	XM_971273	2.24 ↑	0.03	1.86	Hypothetical protein LOC660996
3274	NM_001168458	2.16 ↑	0.04	NOC	Peritrophic matrix protein 2-A

[1]Gut ranks were determined through microarray analysis (Morris et al., 2009), and they range from 0 (low or no expression in the gut) to 100 (highly expressed in the gut); NOC – not on chip in the Morris et al., 2009 array.

Table 3
Genes differentially regulated more than 5-fold (significant at $p<0.05$, pairwise analysis) in *T. castaneum* larvae fed a diet containing a combination of 0.1% E-64 and 5% STI. Grey squares are those genes that were down-regulated.

Glean#	NCBI#	Ratio	Gut Rank[1]	Gene Name
9465	XM_971307	60.2 ↓	8.85	Hypothetical protein LOC661850
7959	XM_971378	35.5 ↓	0.64	Hypothetical LOC664046
5225	XM_001808333	35.5 ↓	NOC	AGAP012043-PA
7328	XM_968377	31.7 ↓	0.73	RNA polymerase II
11616	XM_001812148	23.7 ↓	0.90	Inward rectifier potassium channel
16123	XM_971273	20.8 ↓	0.51	Hypothetical protein LOC660996
10419	XM_966795	17.3 ↓	0.93	C-type lectin
9626	NM_001039427	15.0 ↓	1.23	Chitinase 2
291	XM_001812363	15.0 ↓	23.1	Hypothetical protein LOC100142396
3708	XM_966359	14.5 ↓	0.91	C-type lectin
14069	XM_969497	13.4 ↓	5.05	ML superfamily
16345	XM_963699	11.8 ↓	1.43	AGAP001819-PA
9628	NM_001042569	11.8 ↓	1.91	Chitinase 13
5375	XM_967951	11.3 ↓	1.76	Arylphorin-like hexamerin
13137	XM_968636	11.3 ↓	3.22	Cuticular protein 78, RR-1 family
1770	NM_001039435	11.3 ↓	1.15	Chitinase 1
15238	XM_966713	10.2 ↓	0.82	CG17600-PA, isoform A
8680	NM_001045814	9.80 ↓	2.38	Chemosensory protein 11
11602	XM_968802	9.37 ↓	1.40	CG17127-PA
600	XM_962127	9.34 ↓	0.41	Venom allergen 3-like
10959	XM_966152	9.34 ↓	2.23	Elastase 1
9176	NM_001039426	9.04 ↓	37.3	Chitinase 3
9627	NM_001044630	8.59 ↓	49.2	Chitinase 11
9178	XM_967709	7.99 ↓	3.09	Glycosyl hydrolase
11140	XM_966352	7.77 ↓	1.63	Cuticular protein analogous to peritrophins
10813	XM_964920	7.57 ↓	0.30	Cytosolic fatty-acid binding protein family
7945	XM_962383	7.42 ↓	10.8	Scavenger receptor cysteine-rich protein
9681	XM_961730	7.37 ↓	2.10	Major Facilitator Superfamily
15460	XM_963805	7.35 ↓	NOC	CG2150-PA
14184	XM_962888	7.29 ↓	NOC	Lectin-binding protein
14497	XM_965288	7.01 ↓	0.55	Chitin binding protein
10350	XM_964986	6.86 ↓	0.33	Lysozyme
552	XM_968714	6.83 ↓	NOC	Hypothetical protein DM4/DM12 family
639	XM_970389	6.58 ↓	1.25	CG7709-PA
2989	XM_966418	6.11 ↓	2.10	α-L-fucosidase precursor
518	XM_970063	6.10 ↓	12.5	PR-5-like protein
7063	XM_971284	5.95 ↓	0.31	Hypothetical protein LOC661206
15151	XM_965879	5.86 ↓	0.37	Sulfatase
15583	XM_968139	5.85 ↓	0.60	Secretory/lipid-binding
6471	XM_965368	5.59 ↓	3.67	Long-chain acyl-CoA synthetases
10067	XM_970595	5.34 ↓	0.95	Odorant binding protein C02
6093	XM_964248	5.32 ↓	0.68	Translation initiation factor IF-2
6498	XM_968031	5.27 ↓	0.53	Membrane trafficking
5238	XM_964575	5.22 ↓	0.70	Golden goal CG32227-PA
12134	XM_962591	5.20 ↓	1.58	Glycerolipid metabolism
13304	XM_965700	5.13 ↓	1.69	GA22158-PA
13184	XM_965767	5.02 ↓	1.46	Phosphorylase
14914	XM_001812265	5.02 ↓	1.13	Hypothetical protein LOC100142600
2786	XM_967427	38.4 ↑	2.53	Chymotrypsin 1
8927	XM_968410	38.0 ↑	2.20	CG11127-PA
13661	NM_001110542	36.0 ↑	NOC	Chitin deacetylase 7
2681	XM_962716	35.4 ↑	0.83	Cytochrome b5-like Heme/Steroid binding
10934	XM_963610	22.1 ↑	2.46	Serine protease homolog
5953	XM_963674	20.4 ↑	2.71	Cathepsin B homolog
10691	XM_963596	17.9 ↑	2.36	Cathepsin B
11825	XM_967202	14.5 ↑	1.72	Chymotrypsin
15099	XM_967979	14.3 ↑	2.48	Serine protease homolog
291	XM_971313	12.4 ↑	23.1	Hypothetical protein LOC662037
12187	XM_966267	10.0 ↑	3.34	Anterior fat body protein
10050	XM_970571	10.0 ↑	3.09	Zinc finger
2767	XM_962314	9.95 ↑	1.63	Chymotrypsin BII
8504	XM_962160	9.73 ↑	46.8	Serine protease homolog
3274	NM_001168458	9.55 ↑	NOC	Peritrophic matrix protein 2-A
11432	XM_971367	9.44 ↑	2.49	Hypothetical protein LOC663642
15617	XM_965228	9.26 ↑	27.3	Collagenase
326	XM_964871	8.95 ↑	0.59	Zn-finger
10052	XM_970573	8.73 ↑	54.6	Glycosyl hydrolase
10927	XM_963074	8.03 ↑	44.5	Serine protease homolog
15618	XM_965293	8.02 ↑	25.4	Collagenase
5925	XM_967043	7.87 ↑	6.29	Serine protease homolog
10908	XM_963904	7.57 ↑	12.2	Serine protease homolog
15822	XM_963666	7.51 ↑	0.86	Ecdysone inducible protein L2
13662	NM_001110435	7.13 ↑	14.5	Chitin deacetylase 6
2952	XM_969205	7.00 ↑	43.4	Cathepsin B
3905	NM_001110434	6.99 ↑	36.9	Chitin deacetylase 9
9233	XM_971347	6.85 ↑	27.2	Hypothetical LOC662942
5124	XM_971250	6.74 ↑	62.6	Hypothetical protein LOC660391
11000	XM_965804	6.72 ↑	14.1	Cathepsin L
3450	XM_966968	6.55 ↑	1.68	Esterase/lipase
15445	XM_961810	6.51 ↑	2.80	Protein kinase
7157	XM_969038	6.50 ↑	1.07	Peritrophic matrix protein 3
10940	XM_964413	6.50 ↑	17.3	Elastase 1
7183	XM_968094	6.22 ↑	1.59	Esterase/lipase
15237	XM_966767	5.97 ↑	39.3	Chymotrypsin
4502	XM_965401	5.95 ↑	NOC	DNA/RNA non-specific endonuclease
8883	XM_969919	5.93 ↑	1.26	Protein kinase
15153	XM_965770	5.65 ↑	0.84	F-box and leucine-rich repeat protein 11
6284	XM_965442	5.62 ↑	3.43	γ-glutamyltranspeptidase
292	XM_971310	5.46 ↑	3.63	Hypothetical protein LOC662001
10929	XM_963220	5.40 ↑	10.9	Serine protease homolog
12574	XM_968153	5.38 ↑	19.8	Serine protease homolog
10905	XM_964051	5.37 ↑	4.70	Serine protease homolog
14147	NM_001110436	5.28 ↑	14.6	Chitin deacetylase 8
15725	XM_967704	5.24 ↑	0.37	Oxidoreductase
3179	XM_968559	5.22 ↑	3.21	Microsomal prostaglandin E synthase

[1]Gut ranks were determined through microarray analysis (Morris et al., 2009), and they range from 0 (low or no expression in the gut) to 100 (highly expressed in the gut); NOC – not on chip in the Morris et al., 2009 array.

The impact of the E-64 and STI combination on gene expression of *T. castaneum* larvae was evident, as 95 genes were differentially expressed more than 5-fold in larvae fed the combination treatment (Table 3). Notably, the most highly down-regulated gene encoded a protein with unknown function (Glean 9465), and there were eight other genes encoding hypothetical proteins that were down-regulated in this dataset. As with E-64 treatment alone, there were a number of chymotrypsin and SPH genes that were up-regulated. Notably, there also were genes encoding lectins, chitin-modifying enzymes, and peritrophins that were mostly down-regulated.

3.2. Comparison of control and treatments by ANOVA

As was observed in the pairwise analysis, comparisons of differences in protease gene expression among control and treated larvae indicated that E-64 and combination treatments resulted in significantly more differential gene expression (Supplemental Fig. 3). Classifications by gene ontology revealed categories for differentially expressed genes. In the classification Biological Processes, the largest categories were either related to cellular or metabolic processes (25.8%; Supplemental Fig. 3A). In the classification of genes by Cellular Component, the largest category was related to "cell" (25.6%, Supplemental Fig. 3B). More revealing were the classifications in Molecular Functions, in which most genes were grouped into catalytic activity (33.6%) or binding (41.6%, Supplemental Fig. 3C).

PCA was used to compare the variance in gene expression between the four different treatments (Supplemental Fig. 4). In PCA, potentially correlated variables (in this case, gene expression levels as determined by signal intensities) are grouped into uncorrelated variables, called principle components, by a mathematical transformation. The first component (Comp. 1) accounts for most of the variations, and the second component (Comp. 2) accounts for the remainder of the variability. Using PCA on the 2175 differentially expressed genes among control and treatments (significant at $p<0.05$ by ANOVA), gene

expression in STI-treated larvae were more similar to the control, while those in E-64 or combination-treated larvae were more different.

3.3. Verification of gene expression by quantitative PCR

To verify gene expression patterns from the micorarray, we designed primers for selected protease transcripts (Supplemental Table 6). In qPCR, the expression patterns of all selected transcripts were similar to the microarray except for Tc02955 (Fig. 2). With Tc02955 cathepsin B, qPCR estimated an increase of 0.08, whereas microarray analysis indicated a decrease of 2.43-fold expression in the combination-treated larvae. However, all other genes in the combination-treated group were down-regulated in both microarray and qPCR, and all up-regulated transcripts in the microarray were up-regulated in qPCR. In the case of the predicted trypsin Tc01157, both microarray and qPCR indicated that the transcript was down-regulated when larvae were fed E-64 but up-regulated when larvae were fed both inhibitors. The magnitude of expression was often higher in the microarray, and this may reflect the hybridization of highly-related transcripts and/or the more precise experimental data from the qPCR analysis due to primer specificity.

3.4. Trends in protease genes

Since we have previous data that indicates that protease activity is altered in inhibitor-fed larvae, the expression of genes encoding proteases were evaluated separately (Tables 4 and 5). Overall, there were more differentially expressed protease genes in the combination-treated larvae than in E-64- or STI-treated larvae. Only two serine protease genes were down-regulated in response to STI in the diet. The majority of differentially regulated genes in larvae fed E-64 and combination treatments were up-regulated. In most cases, genes that were up-regulated in E-64-treated larvae (1.32 to 10.1-fold) also were up-regulated in larvae fed the inhibitor combination (1.30 to 38.4-fold). The majority of differentially regulated genes were serine proteases or homologs.

In the *Tribolium* genome, there are 26 genes encoding presumably active cysteine cathepsin proteases and homologs (*Tribolium* Genome Sequencing Consortium, 2008). Cathepsin L and B enzymes are found mostly in the anterior midgut of tenebrionid larvae and are critical to the early stages of protein digestion (Vinokurov et al., 2006; 2009). The primary cysteine cathepsins in the *T. castaneum* larval gut according to the previous microarray analysis (Morris et al., 2009) are Gleans 11000 and 11001 which encode a cathepsin L-like protein, and both were up-regulated in larvae fed diets containing 0.1% E-64 (Table 4). Major cathepsin B enzymes (Gleans 2952 and 10691) also were up-regulated. There was no clear pattern in the expression of cysteine protease homolog genes.

There are more than 150 genes encoding serine proteases and homologs in the *Tribolium* genome, and approximately half of those were predicted to be highly expressed in the gut by previous microarray analysis (Morris et al., 2009). The trends in gene expression patterns of serine protease genes in inhibitor-fed larvae provided critical insights into the complex genetic response to seed protease inhibitors (Table 5). Serine protease transcripts from the chymotrypsin family S1 (Rawlings et al., 2010) differentially expressed in this study included eight putative chymotrypsins, seven trypsins, four elastases (elastase 1), and two

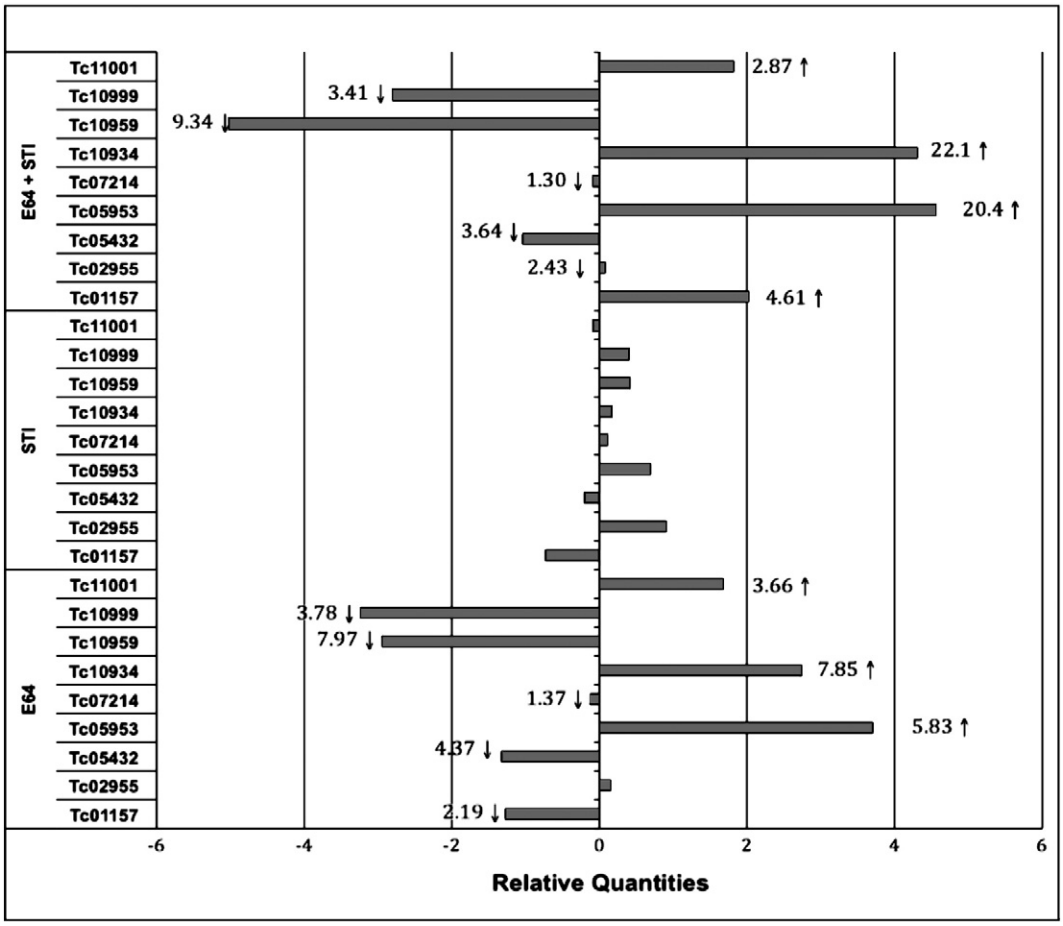

Fig. 2. Comparison of the relative quantities of transcripts in the gut of *T. molitor* larvae fed protease inhibitors or control diet by qRT-PCR (data are ln transformed, average of two biological replicates), using primers specific for selected transcripts indicated on the left (Supplemental Files Table 6). Numbers and arrows associated with each relative quantity are the direction and magnitude of significant fold-change obtained from the microarray analysis.

Table 4
Comparison of significantly different expression levels ($p<0.05$) of genes encoding cysteine cathepsin proteases and nonproteolytic homologs in *T. castaneum* larvae fed diets containing either 0.1% E-64 or 0.1% E-64 + 5.0% STI (combination); there were no significantly differentially expressed cysteine cathepsin genes in the 5.0% STI-treated larvae. Grey squares are those genes that were down-regulated.

Glean#	NCBI gene	Gut rank[1]	Predicted function	E-64	Combination
10691	XM_963596	2.36	Cathepsin B	4.90 ↑	17.9 ↑
5954					
2952	XM_969205	43.4		5.89 ↑	7.00 ↑
2955	XM_969127	2.24		–	2.43 ↓
5953	XM_963674	2.71	Cathepsin B homolog	5.83 ↑	20.4 ↑
5955	XM_963450	0.43		–	4.24 ↑
5956					
5431	XM_961657	2.66		–	4.62 ↓
5432	XM_961570	2.55		4.37 ↓	3.64 ↓
2954	XM_969151	9.22		–	2.21 ↓
11000	XM_965804	14.1	Cathepsin L	3.73 ↑	6.72 ↑
11001	XM_965745	82.0		3.66 ↑	2.87 ↑
4102	XM_966605	1.35		–	1.95 ↑
9365		43.9			
10999	XM_965858	1.62		3.78 ↓	3.41 ↓
9363	XM_966719	3.98	Cathepsin L homolog	–	3.57 ↑
7214	XM_965419	0.57	Cathepsin O	1.37 ↓	1.30 ↓

[1]Gut ranks were determined through microarray analysis (Morris et al., 2009), and they range from 0 (low or no expression in the gut) to 100 (highly expressed in the gut).

collagenases. Predictions of the protease type are based on residues in the S1 binding pocket corresponding to S189, G216, and G226 in bovine chymotrypsin, which determine substrate specificity (Perona and Craik, 1995; Hedstrom, 2002, Supplemental Fig. 5). In predicted chymotrypsins from *T. castaneum* genetic sequences, often a glycine residue is found in position S189, and we postulate that the structure of the loops would be similar to that of bovine chymotrypsin. Overall, genes encoding chymotrypsins, elastases, and collagenases were up-regulated in E-64 and combined treatments with few exceptions. Additionally in larvae fed both inhibitors, genes encoding trypsins were down-regulated. There were more SPH genes differentially expressed in larvae fed both inhibitors (24) than in those fed only E-64 (18), and mostly they were up-regulated. Interestingly, most of the up-regulated homologs were predicted to be similar to chymotrypsin, and the few down-regulated homologs were similar to trypsin. The data support the hypothesis that *T. castaneum* larvae compensate for the presence of the cysteine protease inhibitor E-64 through an overall up-regulation of genes encoding serine proteases and homologs, but addition of STI to E-64 treatment leads to down-regulation of trypsin genes.

4. Discussion

Our previous biochemical data indicated that *T. castaneum* larvae fed increasing doses of E-64 responded by an increase in overall protease activity that was less responsive to the inhibitor, either by an increase in existing proteases and/or synthesis of novel inhibitor-insensitive enzymes, particularly chymotrypsins (Oppert et al., 2004, 2005). To further explore this compensation, this study was designed to determine the effects of inhibitors on gene expression in *T. castaneum* larvae fed 0.1% E-64, 5.0% STI, or a combination of 0.1% E-64 and 5.0% STI in the diet. Dosages were chosen because they were previously demonstrated to induce changes in proteolytic activity without mortality (Oppert et al., 2005). However, in the previous study, larvae were reared continuously on inhibitor-treated diets. In the present study, larvae were reared on normal rearing diet for 10 days, at which time larvae were transferred to inhibitor treatments, either singly or in combination, for three days prior to RNA extraction of pooled larval guts from each treatment. Changing the protocol resulted in less damage to the gut and larger larvae for dissection. In previous experiments, the relative mass of larvae fed either E-64 or a combination of E-64 and STI was significantly less than controls (Oppert et al., 1993, 2003, 2005). The change in protocol also was designed to shift observations to changes at the gene rather than the protein level, which is presumably more optimal at earlier inhibitor-exposure timepoints.

The findings of this study suggest that when *T. castaneum* larvae ingest protease inhibitors, they respond through differential gene expression that enhances their chances to both survive and in some cases exploit the inhibitor. In fact, the dramatic expansion of protease genes in the *Tribolium* genome, in some cases up to six genes in tandem, are evidence of a successful survival strategy for *T. castaneum* to respond to expansions of inhibitors in cereals, both quantitatively and qualitatively (*Tribolium* Genome Sequencing Consortium, 2008).

When RNA transcripts were compared in larvae fed control diets and a diet containing inhibitor(s), more significant differences were found in larvae that were fed E-64 either singly or in an inhibitor combination. Because STI alone elicited mostly the down-regulation of a few genes, it is expected that *T. castaneum* larvae have better survival strategies to deal with proteinaceous serine protease inhibitors. This hypothesis is further supported by the ability of *T. castaneum* larvae to survive relatively larger quantities of serine protease inhibitor with less impact on larval weight and no mortality when compared to larvae fed cysteine protease inhibitors (Oppert et al., 1993, 2003, 2005). We and others have proposed that cysteine proteases are compartmentalized to the anterior midgut to degrade serine protease inhibitors in the seed and protect serine proteases in the posterior midgut (Terra et al., 1985; Thie and Houseman, 1990; Terra and Ferreira, 1994; Vinokurov et al., 2006; Prabhakar et al., 2007).

In addition to protease genes, there were a variety of differentially regulated genes that suggest that larvae are shifting metabolic resources to survive, or that significant changes are occurring in gut structures. The latter is intriguing in that digestion is compartmentalized in tenebrionid larvae, presumably as part of the regulation mechanism for proteases. In fact, 80% of the total proteolytic activity is due to cysteine proteases that are found in the acidic anterior midgut of *T. castaneum* larvae (Vinokurov et al., 2009). The remainder of proteolytic activity, mostly serine proteases, is relegated to the neutral to slightly basic posterior midgut. If *T. castaneum* larvae shift from cysteine to serine proteases as a response to cysteine protease inhibitors, significant changes may be occurring in the gut pH to allow for optimal activity of serine proteases in the gut, or new serine proteases may be able to operate in more acidic pH.

Table 5
Comparison of significantly different expression levels ($p<0.05$) of genes encoding serine proteases and homologs in *T. castaneum* larvae fed diets containing 0.1% E-64, 5.0% STI, or 0.1% E-64 + 5.0% STI (combination). Grey squares are those genes that were down-regulated.

Glean#	NCBI gene	Gut rank[1]	Predicted function	Predicted identification[2]	E-64	STI	Combination
2785 2786	XM_967427	4.86 2.53	Serine protease	Chymotrypsin	9.67 ↑	–	14.5 ↑
11825	XM_967202	1.72		Chymotrypsin	4.57 ↑	–	14.3 ↑
15580	XM_967979	9.31		Elastase 1	10.1 ↑	–	
2767	XM_962314	1.63		Chymotrypsin	5.42 ↑	–	9.95 ↑
15617	XM_965228	27.3		Collagenase	4.40 ↑	–	9.26 ↑
15618	XM_965293	25.4		Collagenase	3.47 ↑	–	8.02 ↑
10940	XM_964413	17.3		Elastase 1	3.88 ↑	–	6.50 ↑
15237	XM_966767	39.3		Chymotrypsin	4.44 ↑	–	5.97 ↑
2766	XM_962403	15.2		Chymotrypsin	3.51 ↑	–	4.73 ↑
15579	XM_967941	43.0		Chymotrypsin	4.34 ↑	–	3.24 ↑
15780	XM_965510	46.6		Chymotrypsin	3.00 ↑	–	–
15130	XM_967907	11.4		Elastase 1	–	–	1.55 ↑
10959	XM_966152	2.23		Elastase 1	7.97 ↓	–	9.34 ↓
1157	XM_968836	0.51		Trypsin	2.19 ↓	–	4.61 ↑
547	XM_963012	1.40		Trypsin	–	–	3.39 ↓
4635	XM_967270	0.62		Trypsin	–	–	3.17 ↓
15110	XM_961910	1.08		Trypsin	–	–	2.24 ↓
13042	XM_963197	0.79		Trypsin	–	–	1.73 ↓
16121	XM_962371	8.80		Chymotrypsin	–	4.43 ↓	1.62 ↑
8657	XM_968902	0.61		Trypsin	1.54 ↓	1.20 ↓	1.47 ↓
8653	XM_969036	1.18		Trypsin	–	–	1.18 ↓
10934	XM_963610	2.46	Serine protease homolog		7.85 ↑	–	22.1 ↑
5908 6246 15099	XM_967979	5.29 65.8 2.48			10.1 ↑	–	14.3 ↑
8504	XM_962160	46.8			7.68 ↑	–	9.73 ↑
10927	XM_963074	44.5			5.82 ↑	–	8.03 ↑
5925	XM_967043	6.29			–	–	7.87 ↑
10908	XM_963904	12.2			5.91 ↑	–	7.57 ↑
10929	XM_963220	10.9			4.33 ↑	–	5.40 ↑
12574	XM_968153	19.8			4.86 ↑	–	5.38 ↑
10937	XM_964051	33.3			4.47 ↑	–	5.37 ↑
10904 10905	XM_964569	5.81			–	–	4.32 ↑
10932	XM_963467	3.56			2.84 ↑	–	3.26 ↑
10938	XM_964127	14.0			3.29 ↑	–	2.83 ↑
10910 13421	XM_965876	4.64 3.02			–	–	2.64 ↑
12573	XM_968119	2.07			–	–	2.18 ↑
12575	XM_968186	5.44			2.73 ↑	–	2.12 ↑
10941	XM_964641	29.9			2.15 ↑	–	1.36 ↑
10905 10909	XM_963761	11.3 19.5			–	–	1.78 ↑
14375	XM_965934	10.6			3.27 ↑	–	–
13709	NM_001167615	39.0			2.71 ↑	–	–
10930	XM_963301	43.1			2.37 ↑	–	–
12390	XM_966947	0.93			–	–	3.97 ↓
4622	XM_963323	0.70			1.32 ↓	–	1.86 ↓

Previously, we determined that *T. castaneum* larvae respond to E-64 in their diet by decreasing cysteine and increasing serine protease activities (Oppert et al., 2005). In the present study, most of the highly expressed cysteine protease genes were up-regulated, except for two that were somewhat decreased in expression. We propose that E-64 irreversibly inhibited cysteine proteases from the papain

family C1 (Rawlings et al., 2010) in the anterior midgut of *T. molitor* larvae. An up-regulation of other cysteine protease genes occurred to compensate for the lost activity, at least partially. Although this was not apparent at the activity level, it was evident at the transcriptome level. Almost all of the serine proteases that were up-regulated in larvae fed inhibitors should be insensitive to E-64, including chymotrypsins, elastases, and collagenases, and these data support our biochemical studies that demonstrated an increase in chymotryptic activity in E-64-fed larvae (Oppert et al., 2005) to compensate for total proteolytic activity. Most of the trypsin genes were down-regulated in combination-treated larvae presumably due to E-64 inhibition, as has been reported previously with insect trypsins (Novillo et al., 1997). Therefore, the transcriptome study was much more definitive in understanding the complex response of *T. castaneum* larvae to protease inhibitors than the previous biochemical studies.

Previously, we noted an abundance of protease homolog genes in tenebrionid insects (Prabhakar et al., 2007; *Tribolium* Genome Sequencing Consortium, 2008). We speculated that protease homologs may be involved in compensation to cereal inhibitors, perhaps acting as a "sink" to sequester inhibitors. However, the observation that chymotrypsin-like homolog genes are up-regulated overall in response to diets containing E-64 does not support this hypothesis. From this study, we can conclude that apparently the insect uses several modes of compensation to dietary inhibitors: 1. Up-regulation of inhibitor-sensitive proteases (cysteine cathepsins); 2. Up-regulation of inhibitor-insensitive proteases (chymotrypsins); 3. Down-regulation of inhibitor-sensitive proteases (trypsins).

In addition to changes in protease gene expression, there were other genes differentially expressed when larvae were fed inhibitors. These diverse genes have been associated with the larval gut, such as chitin, peritrophic matrix, carbohydrases, and ion channels. However, there were a number of differentially regulated genes encoding hypothetical proteins, many that have been predicted in other insect genomes and for which there in no defined function. We are actively pursuing the identity and function of these proteins.

In trying to develop an inhibitor-based approach to combat coleopteran insect damage to cereals, our goal is to disrupt protein digestion in tenebrionid larvae through the manipulation of protease inhibitor genes in cereals. We have made significant progress in understanding the digestive physiology, transcriptome, and proteome of these insects. This study provides specific targets to develop inhibitors with enhanced efficacy for coleopteran pest control. In addition, compensation responses to inhibitors can now be addressed through deployment of more refined and deliberate strategies.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.cbd.2010.08.001.

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