Large-scale gene expression patterns are altered in *Tribolium castaneum* larvae to compensate for dietary cysteine and serine protease inhibitors

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ABSTRACT

Our previous studies indicate that larvae of the red flour beetle, *Tribolium castaneum*, compensate for dietary protease inhibitors by altering protease expression profiles. To evaluate the response at the transcriptome level, we used whole-genome microarrays to identify and quantify RNA transcripts from guts of larvae fed diet without inhibitor or diets containing a cysteine protease inhibitor (CPI), serine protease inhibitor (SPI), or both inhibitors. Data were analyzed by pairwise analyses of transcripts in the gut of larvae exposed to each inhibitor treatment group compared to control larvae unexposed to inhibitor, or ANOVA of all treatment and control groups. In pairwise analysis, the expression of only 253 genes was significantly altered ($p<0.05$) in response to SPI treatment, whereas CPI and combination treatments resulted in 1,574 and 1,584 differentially regulated genes. **Therefore, treatments containing CPI, either alone or in combination, significantly impacted gene expression in *T. castaneum* larvae.** ANOVA analysis revealed 2,175 genes differentially expressed in inhibitor-treated larvae compared to control ($p<0.05$). Protease-related genes that were significantly up-regulated included those encoding cathepsins B and L, chymotrypsins, and nonproteolytic cysteine cathepsin or serine protease homologs. However, inhibitor treatments also induced the differential expression of other gut-related genes, as well as genes encoding proteins of unknown function. **The data indicate that *T. castaneum* larvae compensate for dietary CPI through qualitative and quantitative changes in gene expression patterns.**

•Experimental Design

•Neonate *T. castaneum* larvae from two biological replicates were placed on 0.5 g of 85% stabilized wheat germ, 10% hard red winter wheat flour, and 5% brewer's yeast. 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 (CPI), 5% STI (SPI), 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. Guts were obtained from 6-10 larvae and placed in RNAlater (Ambion, Austin, TX). Total RNA was obtained using the Absolutely RNA Kit, (Agilent Technologies, La Jolla, CA) and was labeled with Cy dyes (Quick Amp Labeling Kit, Agilent, Santa Clara, CA).

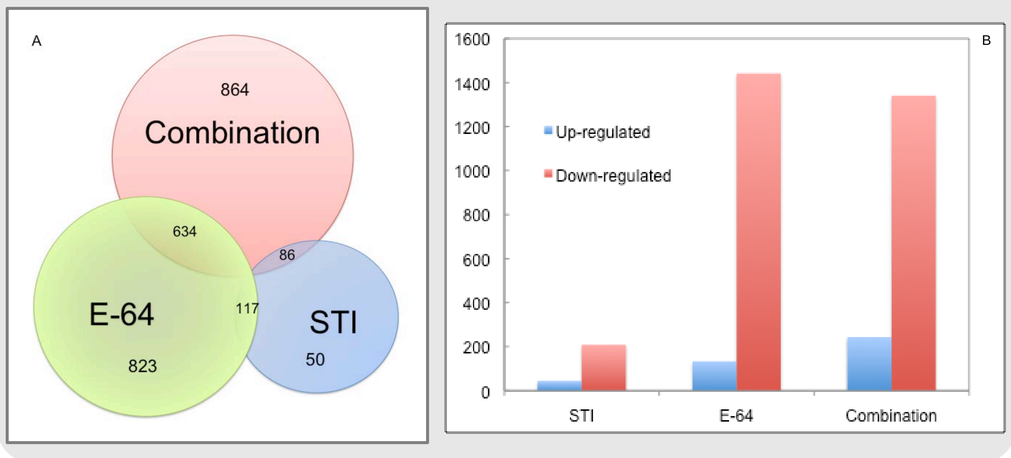
•A whole-genome microarray for *T. castaneum* was developed using all annotated and predicted gene sequences from the *T. castaneum* genome annotation project (GLEAN numbers, Tribolium Genome Sequencing Consortium, 2008). Oligos were designed and arrayed in duplicate or triplicate on a custom array chip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

•Labeled cRNA was hybridized to the Tribolium genome microarray (Gene Expression Hybridization Kit, Agilent). Microarrays were scanned with 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.

•Data was normalized by relative intensity of the means and were log transformed and analyzed for associated gene ontology (GeneSifter, Geospiza, Seattle, WA, USA). Pairwise comparisons were made between the control and each treatment dataset, and significance was determined by the Student's t-test ($p<0.05$); treatment groups also were compared by ANOVA ($p<0.05$); all were corrected by Benjamini and Hochberg (1995).

•The expression of selected genes was verified using the total RNA from the microarray analysis a template and specific primers in a qRT-PCR reaction (Superscript III First Strand Synthesis System, Invitrogen, Carlsbad, CA; Applied Biosytems 2720 thermocycler, Life Technologies, Carlsbad).

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 data indicate that the number of differentially expressed genes in E-64 and combination treatments was similar, and significantly more than with STI treatment. Furthermore, many more genes were down-regulated in response to inhibitor treatment than were up-regulated.



•More details on the study can be found in the publication: Oppert, B., Elpidina, E. N., Toutges, M. and Mazumdar-Leighton, S. 2010. Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors. Comp. Biochem. Physiol., 5D: 280-287.

Table 1. Comparison of relative-fold expression levels of genes encoding cysteine cathepsin proteases (cathepsin B, L, or O) and nonproteolytic homologs that were significantly different ($p<0.05$) in *T. castaneum* larvae fed diets containing either 0.1% E-64 or 0.1% E-64 + 5.0% STI (combination) compared to control; there were no significantly differentially expressed cysteine cathepsin genes in 5.0% STI-treated larvae. Grey squares indicate genes that were down-regulated. Larvae responded to E-64 with mostly a larger increase in cathpesin B and L.

GLEAN#	NCBI Gene	Gut Rank[1]	Predicted Function	E-64	Combination
10691 5954	XM_963596	2.36	cathepsin B	4.90 ↑	17.9 ↑
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 5956	XM_963450	0.43		-	4.24 ↑
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 9365	XM_966605	1.35 43.9		-	1.95 ↑
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)

Fig. 2. Ontology of genes that were differentially expressed in protease-inhibitor treated *T. castaneum* larvae (ANOVA, $p<0.05$), in the category Molecular Function (GO Ontology; The Gene Ontology Consortium, 2000; picture exported from GeneSifter, Geospiza). The majority of differentially expressed genes were in the categories catalytic activity (33.6%) or binding (41.6%).

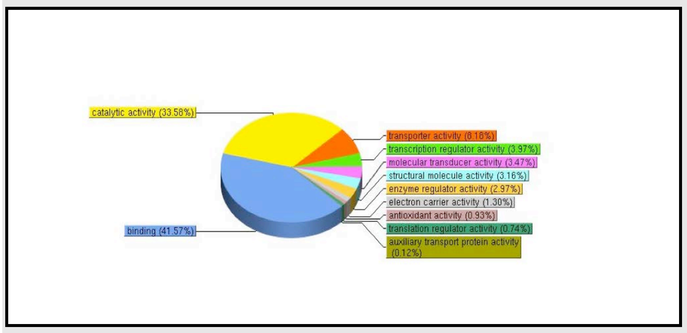


Fig. 3. Validation of relative-fold quantities of transcripts in the gut of *T. castaneum* larvae fed protease inhibitors relative to those fed control diet by qRT-PCR (using specific primers for genes designated by Glean numbers from the genome annotation project, Tc#). Data are ln transformed, average of two biological replicates. Numbers and arrows indicate the direction and magnitude of fold-change in the microarray analysis.

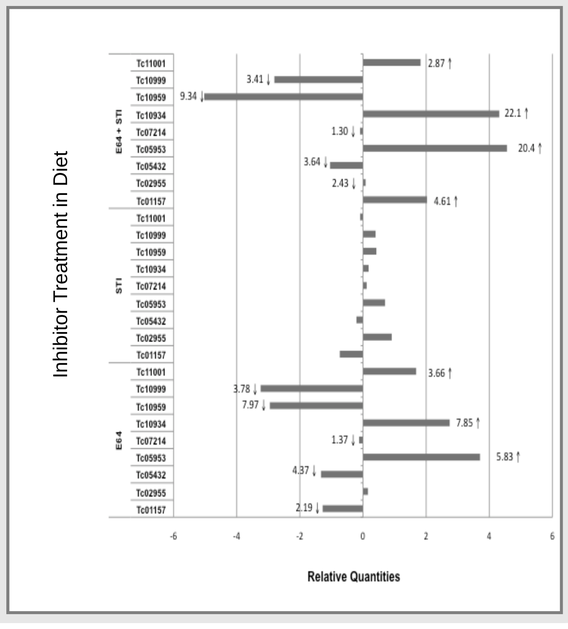


Table 2. Comparison of significantly different expression levels ($p<0.05$) of genes encoding serine proteases (chymotrypsin, elastase, and trypsin) and nonproteolytic homologs in *T. castaneum* larvae fed diets containing 0.1% E-64, 5.0% STI, or 0.1% E-64 + 5.0% STI (combination) compared to control. Grey squares are those genes that were down-regulated. Overall, there were more differentially expressed protease genes in combination-treated larvae than in E-64- or STI-treated larvae. Most differentially-expressed protease genes were up-regulated, and most were serine proteases, especially chymotrypsin-like. Many homologs also were up-regulated.

GLEAN#	NCBI Gene	Gut Rank[1]	Predicted Function	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_0011676 15	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 ↓

[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)

CONCLUSIONS

•The data support our previous bioassay and biochemical studies, in which we found that *T. castaneum* larvae respond to a cysteine protease inhibitor in their diet by increased serine protease activities, especially chymotrypsin.

•Larvae exposed to either cysteine protease inhibitor or both serine and cysteine protease inhibitors mount a complex genetic response, with many more genes down-regulated than up-regulated.

•Genes other than those encoding proteases also were affected by inhibitor-treated diets, including genes that enable larvae to shift metabolic resources to survive, or those that may repair damaged gut structures.

•Because our data has demonstrated that primary protein digestion in larvae fed control diet is by cysteine proteases, **the shift to another protease subclass, serine proteases, is a dramatic example of the plastic response of insects to toxins and inhibitors in their diet.**

•Knowledge of differentially-regulated genes, especially the identification of specific genes and the magnitude and direction of the shift in gene expression, provides information to develop new control strategies that block the compensation response.