Article

A COMPLEX OF GENES INVOLVED IN ADAPTATION OF *Leptinotarsa decemlineata* LARVAE TO INDUCED POTATO DEFENSE

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The Colorado potato beetle (Leptinotarsa decemlineata) is the most important pest of potato in many areas of the world. One of the main reasons for its success lies in the ability of its larvae to counteract plant defense compounds. Larvae adapt to protease inhibitors (PIs) produced in potato leaves through substitution of inhibitor-sensitive digestive cysteine proteases with inhibitor-insensitive cysteine proteases. To get a broader insight into the basis of larval adaptation to plant defenses, we created a "suppression subtractive hybridisation" library using cDNA from the gut of L. decemlineata larvae fed methyl jasmonate-induced or uninduced potato

Supporting Information is available in the online issue at wileyonlinelibrary.com.
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ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 79, No. 3, 153–181 (2012)
Published online in Wiley Online Library (wileyonlinelibrary.com).
 DOI: 10.1002/arch.21017

leaves. Four hundred clones, randomly selected from the library, were screened for their relevance to adaptation with DNA microarray hybridizations. Selected enzyme systems of beetle digestion were further inspected for changes in gene expression using quantitative PCR and enzyme activity measurements. We identified two new groups of digestive cysteine proteases, intestains D and intestains E. Intestains D represent a group of structurally distinct digestive cysteine proteases, of which the tested members are strongly upregulated in response to induced plant defenses. Moreover, we found that other digestive enzymes also participate in adaptation, namely, cellulases, serine proteases, and an endopolygalacturonase. In addition, juvenile hormone binding protein-like (JHBP-like) genes were upregulated. All studied genes were expressed specifically in larval guts. In contrast to earlier studies that reported experiments based on PI-enriched artificial diets, our results increase understanding of insect adaptation under natural conditions. © *2012 Wiley Periodicals, Inc.*

Keywords: Colorado potato beetle; insect adaptation; digestive enzymes; gene expression

INTRODUCTION

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is the most devastating pest of potato worldwide (Ferro, 1985). It can completely defoliate potato plants prior to tuber initiation, causing a total loss of crop (Hare, 1980). The pest is managed mainly with insecticides, but its ability to rapidly develop resistance to chemical insecticides requires alternative strategies to be developed (Forgash, 1985; Hare, 1990). A detailed knowledge of processes and genes involved in the adaptation of the CPB digestive system to potato defense will be needed to develop biotechnological pest control strategies such as genetically engineered plant protease inhibitors (PIs) (Haq et al., 2004; Abdeen et al., 2005) or RNAi-based insecticides (Baum et al., 2007; Mao et al., 2007).

To date, the best studied components of beetle digestion are proteases. Like most coleopterans, CPB primarily uses cysteine proteases for protein digestion (Wolfson and Murdock, 1987; Bolter and Jongsma, 1995). Aspartic proteases also contribute substantially to gut proteolytic activity, and minor activities with characteristics of serine and metalloproteases have been detected as well (Novillo et al., 1997). Larvae adapt to PIs produced in potato leaves by substituting inhibitor-sensitive digestive cysteine proteases with inhibitor–insensitive cysteine proteases. Three cysteine proteases with different substrate specificities and inhibition profiles have been isolated from midguts of CPB larvae fed on the methyl jasmonate (MeJA) induced potato leaves (Gruden et al., 2003). cDNA sequences coding for cysteine proteases have been isolated from adapted larval guts and classified into three intestain groups, A, B, and C. Expression of intestain A and C genes is induced two-fold during the adaptation of CPB larvae to potato defenses (Gruden et al., 2004).

The pool of genes involved in digestion in different leaf beetle species is reviewed in Gomez-Zurita and Galian (2005). Additionally, the entire genomic sequence of the red flour beetle *Tribolium castaneum* (Richards et al., 2008) and the recent pyrosequencing results from the gut transcriptome of several phytophagous beetles (Morris et al., 2009; Pauchet et al., 2009, 2010) have made important contributions to understanding the diversity of genes expressed in larval guts of Coleoptera. Adaptation of the gut transcriptome to dietary supplementation with the plant PI soyacystatin N was studied in the cowpea bruchid, *Callosobruchus maculatus*. These studies showed that the larval gut responds to proteinase inhibitors by upregulating a set of inhibitor-insensitive digestive cysteine proteases and inhibitor-degrading proteases (Zhu-Salzman et al., 2003; Ahn et al., 2004; Koo et al., 2008; Ahn and Zhu-Salzman, 2009). Besides upregulation of protease genes, a microarray study found differential expression of genes encoding carbohydrate digestive enzymes, antimicrobial peptides, and detoxification enzymes (Moon et al., 2004). A later, more extensive microarray study by Chi et al. (2009) confirmed the upregulation of protease and carbohydrate hydrolase genes, but also identified differentially expressed (DE) genes involved in lipid transport and juvenile hormone (JH) metabolism, structure (collagens, tubulins), defense (mucin, antibacterial peptides, serpins), and stress tolerance (cytochromes P450, glutathion S-transferases, catalases, peroxidises, chaperones). A similar study that focused on transcriptional regulation in southern corn rootworm (*Diabrotica undecimpunctata howardii*) guts in response to a diet containing soyacystatin N showed upregulation of digestive protease genes and a peritrophin-encoding gene, whereas genes involved in insect development were downregulated (Liu et al., 2004). Another microarray study of adaptation to serine- and cysteine-type PIs in *T. castaneum* (Oppert et al., 2010) focused on interpretation of DE serine and cysteine digestive proteases, although the list of DE genes (especially with a combination of inhibitors) also includes chitin and lipid metabolism genes, defense-related genes, and potential receptor and regulatory genes. However, little research has been done on the transcriptional regulation of genes in phytophagous beetles during their adaptation to host plant defenses.

Plant responses to herbivores involve complex regulatory network. Treatment of potato plants with MeJA was shown to activate herbivore damage-responsive jasmonate and ethylene signaling pathways and consequently induce transcription of defense-related genes regulated by these phytohormones (Bolter and Jongsma, 1995; Rivard et al., 2004). Larval feeding, however, in addition to activating jasmonate and ethylene signaling pathways, also induces other genes associated with mechanical damage (Bricchi et al., 2010). Additionally, elicitors in saliva or oral secretions of herbivores can also modulate plant responses (Lawrence et al., 2008; Bonaventure et al., 2011).

The aim of the present study was to identify novel genes involved in the adaptation of CPB larvae to potato defenses and to investigate their potential role in this adaptation. Contrary to previous studies, where beetle larvae were fed with a PI-supplemented diet, plant defenses against herbivores were induced using MeJA or CPB larval feeding. Our study employed an "-omics" approach. First, a library enriched for relevant genes was prepared using suppression subtractive hybridization (SSH). This was followed by cDNA microarray hybridizations, where candidate genes involved in adaptation were identified. Subsequently, the involvement of candidate components of adaptation was inspected in more detail by reverse transcription quantitative PCR (RT-qPCR) and enzyme activity measurements.

MATERIALS AND METHODS

Biological Material and Insect-Feeding Trials

Potato plants (*Solanum tuberosum* cv. Désirée and cv. Igor) were grown under conditions described in Gruden et al. (1998). Briefly, to induce plant defense, each plant was put in separate airtight glass jar (9.8 l) containing 2 ml MeJA oil (Fermenich SA, Switzerland) on filter paper for 24 h. After treatment, plants were placed in a growth chamber for a further 24 h before being used for experimentation. In parallel, control plants were left undisturbed in the growth chamber. Insect-feeding experiments were carried out in incubators at 25°C. Individual leaves or whole plantlets were cut off and placed in an Eppendorf tube containing 0.4% water–agar gel. Newly hatched CPB larvae were picked randomly from the colony and placed together with leaves into Petri dishes. Preliminary experiments have shown minimal accumulation of PIs produced in response to larval herbivory during the first 24 h (Bolter and Jongsma, 1995); therefore, fresh leaf material was provided every 24 h. To study short-term adaptation, larvae were reared in Petri dishes on control leaves until reaching their fourth instar, and then half of the population was transferred to another Petri dish containing MeJA-induced potato leaves. From both Petri dishes, larvae were sampled 4 h, 10 h, and 24 h after the start of differential feeding. The short-term adaptation feeding was repeated twice (once for microarray analysis and once for RT-qPCR analysis). For establishing long-term adaptation to plant defense, larvae were fed on control and MeJA-induced plants from the first instar till they reached fourth instar (10 days). In all experiments, 13–15 average-sized larvae were sampled per group. The long-term adaptation experiment was performed four times, once for construction of subtractive libraries, once for microarray hybridization, and twice for RT-qPCR analysis.

To study the effects of plant defense induction by CPB feeding, 20 first-instar larvae were placed on potted potato plants and reared within the plant growth chamber in a glass container open at the top. The control larval group was kept in the growth chamber in a glass Petri dish and reared on control leaves that were freshly cut every day. Larvae from both groups were sampled after 10 days of differential feeding.

Prior to dissection, the larvae were anesthetized on ice. Larval guts were gently removed, and foregut and hindgut were separated from the midgut. The midgut was briefly dried on tissue paper to remove hemolymph fluid. The midgut content, which was used to measure protease activity, was separated from the midgut wall, which was used for RNA analysis. Samples were immediately frozen in liquid nitrogen and stored at –80°C. More thorough tissue dissection was performed on five fourth-instar larvae fed 10 days on MeJA-induced potato leaves; samples of foregut, midgut, hindgut, hemolymph, leg, and the rest of beetle larvae (carcass) were collected. These samples were used to determine the gene expression tissue specificity. Special care was taken to avoid cross-contamination of tissue samples.

Isolation of RNA

For construction of SSH cDNA libraries, mRNA was isolated from 100 mg of midguts from 14 larvae fed on control plants and, separately, from 100 mg of midguts from 12 larvae fed over a long term with MeJA-induced plants. mRNA was purified in one step, using a Quickprep Micro mRNA Purification Kit (Amersham Biosciences AB, Sweden).

For gene expression analyses, samples from three or four larval specimens from the same group were pooled to obtain four biological replicates. Total RNA was isolated

using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. One microgram of purified RNA was treated with one unit of DNase I (Invitrogen) according to the manufacturer's instructions in order to degrade genomic DNA potentially present in the RNA samples. The efficiency of DNase treatment was checked by qPCR of RNA samples without prior reverse transcription. No products were observed in these reactions.

The concentration and integrity of RNA samples before and after DNase treatment was determined using the RNA 6000 Nano LabChip kit (Agilent Techologies, USA) in an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions.

Construction of SSH Libraries

For construction of SSH libraries, cDNA was synthesized using the Time Saver cDNA Synthesis kit (GE Healthcare, USA). A PCR-Select cDNA Subtraction kit (Clontech, Japan) was used to construct SSH cDNA libraries, according to the manufacturer's instructions. Two libraries of DF genes in midguts of adapted larvae (one of upregulated genes and one of downregulated genes) were prepared, each with approximately 50,000 independent transformants. Three hundred transformants from the library of upregulated genes (cpb1–cpb300) and 100 from the library of downregulated genes (cpb301–cpb400) were picked randomly and sequenced.

Microarray Production and Hybridization

Four hundred clones (cpb1–cpb400) from the up- and downregulated SSH libraries were processed and printed on the microarrays as described in Gruden et al. (2004). The microarray hybridizations were performed in two technical replicates as described in Gruden et al. (2004) for analysis of gene expression in adapted midguts after short-term adaptation (4 h and 24 h after the start of differential feeding) and after long-term adaptation (10 days after the start of differential feeding). The results were expressed as $\log_2$ of the ratio of gene expression in adapted guts to control guts (logFC), averaged over four replicate spots within the slide and over the two replicate spots of the dye-swap slide (eight independent spots). Measurement uncertainty was calculated from $cv = (cv_1^2 + cv_2^2)^{1/2}$, where cv_1 is the variability of spot intensity in adapted guts, and cv_2 the variability of spot intensity in control guts. The cutoff cv value of 0.5 was used for quality control, filtering out highly variable measurements. After calculation of the hybridization ratios, the transcripts were treated as downregulated if the logFC values were lower than –0.8, and upregulated if the logFC values were higher than 0.8.

cDNA Cloning

The previously prepared cDNA library from adapted midguts (Gruden et al., 2004) was used as a template for isolation of the full-length cDNAs of the selected partial cDNAs obtained from SSH libraries. The 5′-end of full-length cDNA was amplified using a combination of the selected cDNA-specific reverse primer and a vector-specific T7 promoter primer (Table S1). The 3′-end of full-length cDNA was amplified using a combination of the selected cDNA-specific forward primer and a vector-specific T7 terminator primer (Table S1). All primers were used at a final concentration of 0.1 μM. Amplification was carried out by touchdown PCR, with a BD Advantage 2 Polymerase Kit (BD Biosciences, USA) using various temperature settings. PCR products were ligated into the pGEM-T Easy

vector system (Promega, USA) and sequenced. Full-length cDNAs were amplified using a combination of cDNA-specific forward primers that were designed over the start codon and reverse primers that were designed over the stop codon of the partial cDNAs. Where necessary, additional primers were designed for full-length cDNA sequencing (Table S1).

Sequence Analysis

Nucleotide sequences of partial 5′-end, 3′-end, and full-length cDNAs were trimmed for low-quality sequence strings and vector/adaptor sequences using Vector NTI (Invitrogen, USA). Sequencing reads were manually inspected for read errors and low-quality sequence regions. The BLAST algorithm (Altschul et al., 1997) was used for sequence similarity search in nucleotide (NCBI-nt and dbEST) and protein databases (NCBI-nr and UniProt). CAP3 (Huang and Madan, 1999) was used to assemble nucleotide sequences obtained from SSH libraries into contigs. Contigs were annotated using Blast2GO software suite version 2.4.6 (Götz et al., 2008) under default settings, except for the high-scoring segment pairs length, which was set to 10 (rather than the default of 33) to enable annotation of shorter sequences.

The ExPASy translate tool (http://expasy.org/tools/dna.html) was used to translate cDNA sequences to amino acid sequences. Functional domain signatures of deduced amino acid sequences were queried using InterProScan (Zdobnov and Apweiler, 2001). Multiple sequence alignments were made in ClustalW version 2.0.10 (Larkin et al., 2007) except in cases where local alignment was preferred and DIALIGN-TX (Subramanian et al., 2008) was used instead. BoxShade Server version 3.21 (http://www.ch.embnet.org/software/BOX_form.html) was used for shading identical/similar amino acids in sequence alignments. Signal peptides in putative amino acid sequences were predicted using SignalP 3.0 (Bendtsen et al., 2004). The MEROPS database blast service (Rawlings et al., 2010) was used to classify deduced amino acid sequences of serine protease transcripts on the basis of sequence similarity with sequences in the database. Calculation of antimicrobial peptide properties and identification of closest homologues for deduced defensin-like amino acid sequences were performed using Antimicrobial peptide database web services (Wang and Wang, 2004).

Design and Optimization of RT-qPCR Assays

RT-qPCR assays for expression analysis of selected genes were designed using Primer-Express 2.0 software (Applied Biosystems, USA). Genes for which RT-qPCR assays were designed were selected on the basis of sequence annotations and DNA microarray expression results. Assays were designed for the following genes: intestains C, intestains D, intestains E, chymotrypsin family serine proteases, endopolygalacturonase GH28Pect-11, cellulase GH45–6, *smt3*, defensin-like genes, and juvenile hormone binding protein-like (JHBP-like) genes (Table 1). Analyses were performed using PowerSYBR Green PCR Master Mix (Applied Biosystems, USA) in 5-μl reaction volume using universal PCR conditions on a LightCycler 480 System (Roche Applied Science, USA). Melting curve analysis was applied to control for primer dimer formation and the specificity of PCR product in each reaction. In the testing phase, primer concentrations were optimized to eliminate primer dimer formation. Optimal concentrations of primers used in quantitative analysis are given in Table 1. Specificities of different intestain C and D assays (IntC and IntD, respectively) were tested on plasmids containing different intestain cDNA as a template. The linear ranges and amplification efficiencies were determined for each amplicon across four

Table 1. Assays for RT-qPCR Analysis of Selected Target Genes. Primer Sequences, Their Optimal Concentrations (c), Efficiency of Amplification (E), and Linearity of Amplification (R^2—Coefficient of Determination) Are Given for Each Assay. The 18S Primer and Probe Sequences Are Proprietary Property of Applied Biosystems. The "Ld" Prefix for Gene Names Is an Abbreviation of the Latin Species Name Leptinotarsa decemlineata

Assay	*Target genes*	*Forward primer*	*c (nM)*	*Reverse primer*	*c (nM)*	*E (%)*	R^2
18S	18S rRNA	Proprietary of Applied Biosystems	900	Proprietary of Applied Biosystems	900	88	0.9999
IntC	intestains C	AGGCCGATGGCATTATGTCA	900	TCTGCACGTGGTATTTCTTTCTTC	900	104	0.9967
IntD	intestains D	TTCAGGAACAATCAGCGGAAA	900	CAGACCACTGGGATGCCTT	900	90	0.9984
IntE	Intestains E	CATGACGAACGCTTTCAAATATATC	300	CTGCAGGAACCCACTCTTCCT	300	97	0.9960
Ld_ser_prot	Ld_ser_prot_S1A-1, Ld_GO270919	GAGGTGGACTTAATCCTGAGACTGA	300	ACTGCCCCTTTCCACCTTCT	300	95	0.9995
Ld_GH28Pect-11	endopolygalucturonase GH28Pect-11	CCAAACTGACATTCCAGGGTACA	900	TGACAAGAGGGCCTTTCCAT	900	94	0.9992
Ld_GH45–6	cellulase GH45–6	CATTCGGCAGAGGAATGTAACA	900	TGGATGCAGGTTCAGGTTTG	900	103	0.9981
Ld_JHBP-like	Ld_JHBP-like_3p1, Ld_JHBP-like_3p2, Ld_JHBP-like_5p2	CTTCACCGGTCTTGGAATTTTT	900	TGTATGCTTTAACGAGTCCACTTATTG	900	87	0.9994
Ld_smt3	ubiquitin-like Smt3	TACCGATACCCCAACCACATTAG	900	CCAGTTTGCTGTTGGTATACTTCAA	900	103	0.9999
Ld_def	defensin-like 1, defensin-like 3	GCAGGAACATGCCAAAGCTT	300	TCCACCTTTATATCCGGAATTCA	300	93	0.9977

10-fold serial dilutions of cDNA. Amplification efficiency was calculated from the slope of the log-linear regression curve using the equation $10^{(-1/\text{slope})}$. Efficiency in percentage was calculated using equation $[10^{(-1/\text{slope})} - 1] \times 100$.

RT-qPCR Analysis

For RT-qPCR gene expression analyses, cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) in a 25-μl reaction volume as instructed by the manufacturer. For the analysis of genomic DNA, qPCR was performed directly on a non-DNase treated RNA sample, which according to gel electrophoresis contained approximately 50% RNA and 50% genomic DNA.

The relative quantification was performed using the same quality control system as described in Hren et al. (2009). Briefly, the amplification efficiency and linear range of amplification were followed for each amplicon on each plate by analyzing one randomly chosen sample in five dilution steps of cDNA with two replicate wells per dilution step (range of cDNA dilutions: 10–10^5). Each sample was analyzed in two replicates of two dilutions (100× and 1,000×) to check for the presence of inhibitors in the sample. The 18S rRNA gene (Eukaryotic 18S rRNA Endogenous Control, Applied Biosystems) was used as a reference gene. Appropriateness of the 18S rRNA as a reference gene was tested using GeNorm software (Vandesompele et al., 2002). The standard curve quantification approach was applied, and the reference gene was used for normalization of gene expression in each sample. For every gene, the limit of detection (LOD) was determined from the standard curve. If the determined Cq value of a sample was below the LOD, the sample's copy number was assigned the LOD copy number. Statistical significance of differences in gene expression was calculated using Student's *t*-test.

Biochemical Characterization of Chymotrypsin Activity

Protein extracts of adapted (long-term feeding) and control midgut content were prepared using acetone precipitation. Proteins were further separated by gel filtration as described in Gruden et al. (2003). Enzyme activity in crude extracts and separated fractions was followed using Z-Phe-Arg-pNA substrate in Tris-HCl assay buffer, pH 8, with or without 5 μM PI E-64 under the conditions described in Gruden et al. (1998). Comparison of enzyme activities in control and adapted midguts was calculated as the ratio of measured activity per milligram of protein as measured by Bradford assay.

The pH optimum of enzymatic activity was determined in the presence of 5 μM E-64 using Z-Phe-Arg-MCA as a substrate in the following 0.1 M buffers: sodium acetate for pH 2.5–5.5, sodium–potassium phosphate for pH 5.5–7.5, Tris-HCl buffer for pH 7.5–9, and glycine/NaOH buffer for pH 9–10.5. After 15-min incubation at 37°C, 2 ml of 10 mM iodoacetic acid was added to stop the reaction. The product of hydrolysis was monitored on a Perkin Elmer fluorimeter LS 30 at 370 nm excitation and 460 nm emission wavelengths. The activity of the fraction with peak trypsin-like activity was tested on different 7-methylcoumarylamide substrates (Boc-Gly-Arg-Arg-MCA, Boc-Gly-Lys-Arg-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, and Suc-Ala-Ala-Ala-MCA, all from Bachem, USA), at a final concentration of 100 μM in the assay buffer (0.1 M Tris-HCl, pH 8.8). The influence of different inhibitors on enzyme activity was tested by incubating each inhibitor at an excess concentration in the assay buffer (pH 8.8) with the enzyme for 15 min prior to the addition of the substrate Z-Phe-Arg-MCA. The inhibitors used were Pefabloc SC, chymostatin, benzamidine, aprotinin, antipain (all from

Boehringer Mannheim, Germany), Bowman–Birk inhibitor (BBI), and soybean trypsin inhibitor (both from Sigma-Aldrich, USA), and a potato serine proteinase inhibitor (PSPI) isolated from potato tubers (Pouvreau et al., 2003). The reaction concentrations of inhibitors were 0.2 mM for Pefabloc, benzamidine, and antipain; 0.15 mM for aprotinin, 80 μM for chymostatin, 20 μM for BBI, 10 μM for PSPI, and 1 μM for soybean trypsin inhibitor.

RESULTS

Larval Performance on Induced Potato Plants

Previous studies have shown that larvae fed on MeJA-induced potato leaves had a reduced growth rate compared to larvae feeding on fresh leaves (Gruden et al., 1998). We have here further tested the effect of defense induction by larval feeding. We followed the growth rate and mortality of larvae fed with control leaves or leaves induced by CPB feeding. Larvae reared on potato plants that had been induced by continuous larval feeding (adapted larvae) had a significantly lower weight from the fifth day of differential feeding onward than larvae fed on fresh leaves (Fig. 1). The difference in growth rate was bigger (up to a 50% weight difference) in larvae fed on feeding-induced plants than

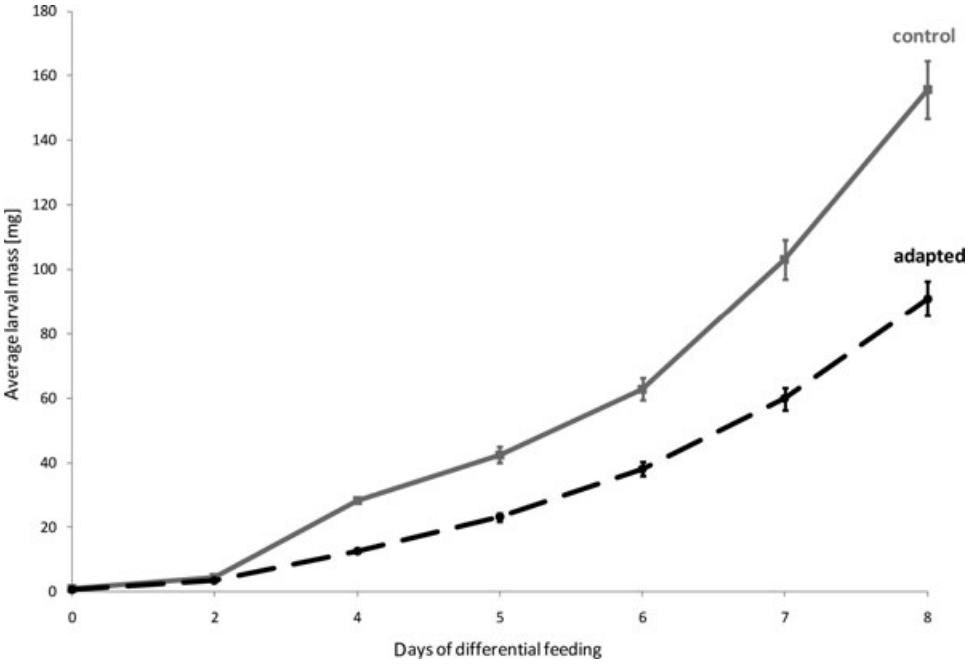

Figure 1. Larval performance on plants with defenses induced by larval feeding. The graph shows average Colorado potato beetle (CPB) larval mass in the feeding trial in which potato plant defense was induced by larval feeding. Larvae were weighed as a group through the fourth day; beginning on the fifth day, larvae were weighed individually and their masses averaged. Control larvae (solid gray line) were fed with fresh potato leaves every 24 h. Adapted larvae (dashed black line) were fed continuously on potato plants beginning at the first instar and were removed only for weighing. Error bars show standard error (SE) of the mean.

on MeJA-induced plants (30% difference in the second instar, Gruden et al. 1998). The mortality rate was negligible in all experiments.

Identification of Novel Components Involved in CPB Adaptation to Induced Potato Defenses

To identify novel components involved in adaptation of beetle larvae to induced potato defenses, SSH libraries for up- and downregulated transcripts were prepared. More clones from the upregulated library were sequenced (300) than from the downregulated (100), because it is generally known that in stress responses many genes are downregulated as a compensatory mechanism and are therefore not directly involved in the studied process. The sequences assembled into 33 contigs and 150 singletons, hereafter referred to as CPB transcripts (see Supplementary Data File 1). BLAST querying of different databases produced significant similarity hits for 54% (98) of the identified transcripts. The highest number (73) of highly similar sequences was found in dbEST, from which 27 hits represented *L. decemlineata* expressed sequence tags (ESTs). High similarities to *T. castaneum* genes were identified for only 38 transcripts. Nineteen CPB transcripts showed the highest similarity to transcripts from intracellular parasites or gut symbionts and were excluded from further analysis. Eighty-five transcripts were not significantly similar to any of the sequences in the databases and encode proteins with an unknown function (see Supplementary Data File 1).

Fifty-eight transcripts were assigned by at least one Gene Ontology (GO) term using Blast2GO algorithm. According to GO, one-third of the transcripts are annotated with hydrolase activity function and more than half participate in primary metabolic processes (Fig. 2). Besides metabolism-related transcripts, several transcripts with potential regulatory roles were identified; four transcripts were annotated to regulation of biological processes, three to stress responses, and one to signaling processes (Fig. 2).

cDNA microarrays were used as an additional screening tool for identification of genes that are differentially expressed in the process of larval adaptation to induced potato defenses. Thirty-five percent of transcripts identified through SSH were confirmed to be differentially expressed based on microarray experiments (Fig. 2, Supplementary Data File 2). Interestingly, most of the DE genes putatively encode proteins with a catalytic activity: cellulases belonging to families GH45 and GH48, polygalacturonases, alpha-amylases, or novel peptidases. Furthermore, a few genes putatively encoding proteins with a regulatory function were differentially expressed in cDNA microarray analysis, for example, a gene with a predicted receptor activity (Contig_150), a calreticulin gene (Contig_211), and a JHBP-like gene (Contig_29). On the basis of the sequence annotations and/or cDNA microarray expression results, eight genes that were upregulated in adapted midguts were selected for further analysis. These genes encode novel intestains, chymotrypsins, cellulases, a polygalacturonase, defensin-like genes, and JHBP-like genes.

Two New Groups of Intestains Identified

From the SSH library of larval midguts enriched for genes upregulated by adaptation to MeJA-induced potato defenses, seven partial cDNA clones (cpb77, cpb125, cpb155, cpb160, cpb175, cpb183, cpb262) with nucleotide sequences most similar to intestain digestive cysteine proteases were isolated. None, however, were isolated from the SSH library enriched for downregulated genes. Four new full-length cDNA sequences and 15 partial cDNA sequences were subsequently obtained by amplification of specific cDNAs from a plasmid from adapted midgut cDNA library. On the basis of sequence similarity

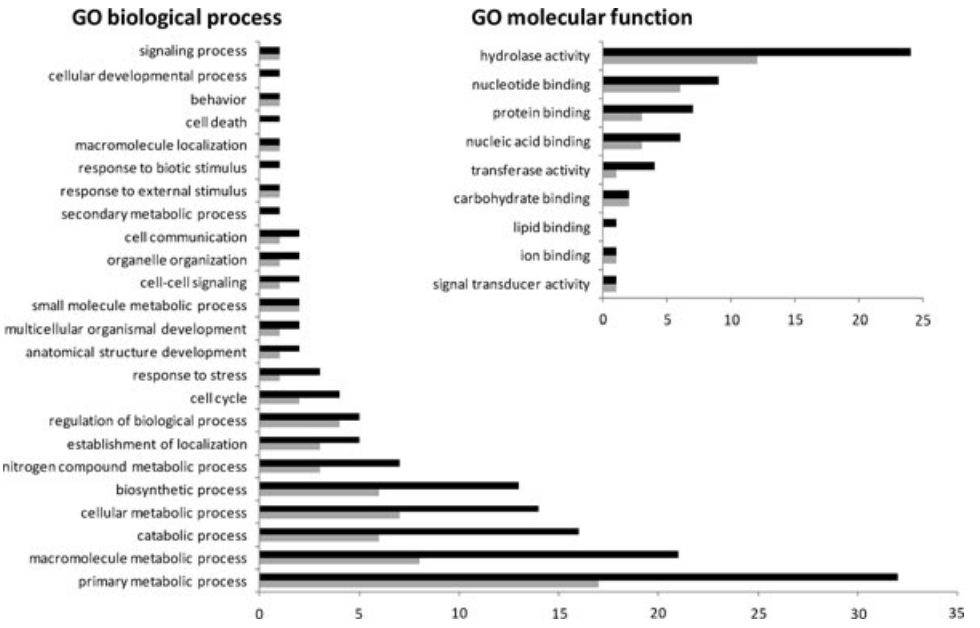

Figure 2. Representation of Gene Ontology (GO) biological process (left) and molecular function terms (top right) in the functionally annotated suppression subtractive hybridization (SSH) library assembly. The number of all sequences in the assembly assigned to a particular GO term is represented by black bars and the number of differentially expressed genes in at least one time point in microarray hybridizations is represented by gray bars. The sequence count was performed using GOSlim ontology at level 3.

to intestains A, B, and C, and other cysteine proteases, the new cDNA sequences were classified into two new intestain groups, D and E. Members of the intestain D group have 85–95% of their amino acids in common, whereas members of the intestain E group are more than 98% identical at the amino acid level. Novel intestains are most similar (61–76% identity) to previously isolated intestains A and B, and less similar (40% identity) to intestains C at the amino acid level. Both new groups of intestains are 40–52% identical to cathepsin-L-like proteases (digestive and nondigestive) from insects and other arthropods and 30–40% identical to mammalian cathepsins L, K, S, and P at the amino acid level (Fig. 3, Supplementary Data File 4).

In order to predict potential insensitivity of intestains D and E to PIs induced in a plant defense response to CPB larval feeding, amino acid regions at inhibitor binding sites were analyzed in more detail. The important structural features that may make these molecules less susceptible to potato PIs are in the S1 binding site (amino acids at positions 23 and 64 according to papain numbering, Fig. 3). Members of the intestain D and E groups have Glu inserted at position 64. Similarly, intestains A and B, but not intestains C, have an amino acid insertion at position 64 (Asp in intestains A, His in intestains B; Gruden et al., 2004). The only other insect cysteine protease with the same structural characteristics in the S1 binding site (Glu at the position 64) is DVCal1 (GenBank ID: AAG17127) from *D. virgifera* (Fig. 3). In the S2 binding site at position 205, intestains D have an Asp residue, charged similarly to Glu in cathepsin B, whereas intestains E have a hydrophobic Met residue, similar to Ala in cathepsin L.

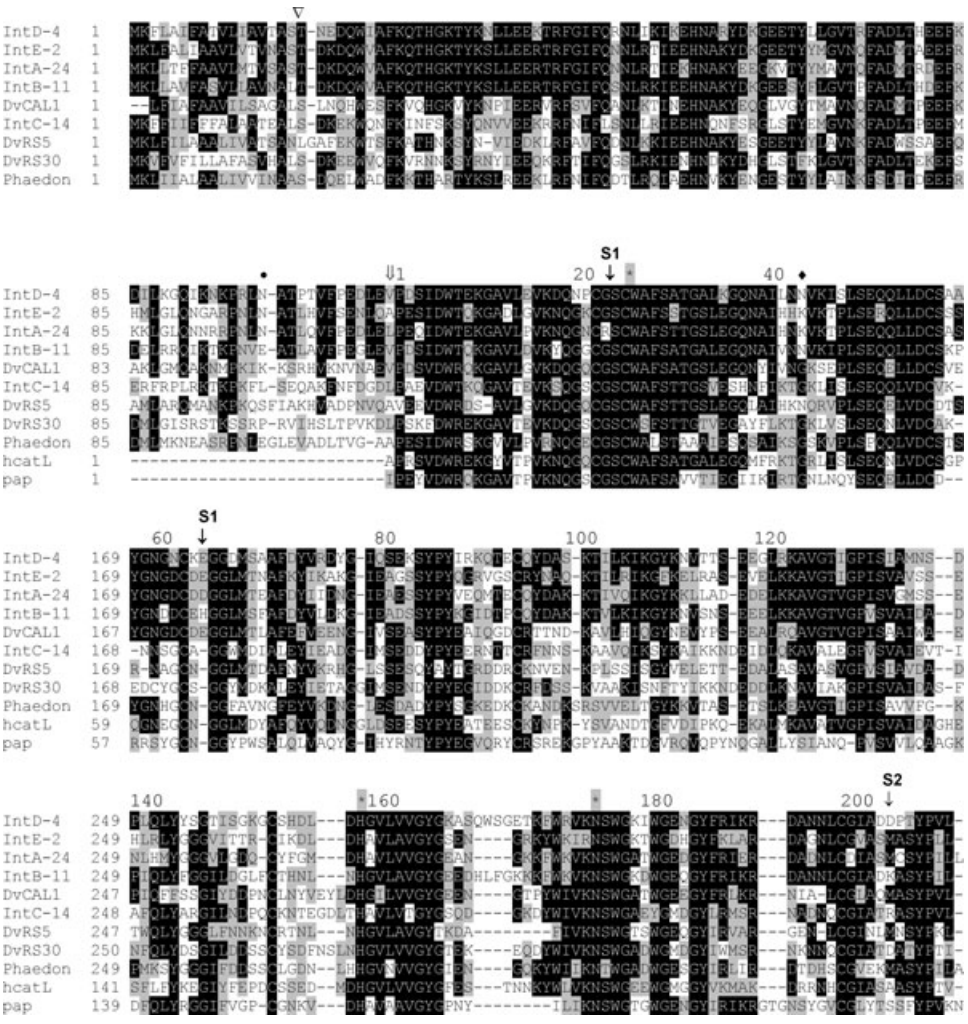

Figure 3. Alignment of deduced sequences of intestain representatives with some of the most similar cysteine proteases. Representatives of novel intestain groups IntD-4 (GenBank ID: EF154436) and IntE-2 (GenBank ID: EF154432) were compared to intestain A, B, and C groups (IntA-24 (GenBank ID: AY528226), IntB-11 (GenBank ID: AY528229), IntC-14 (GenBank ID: AY528231)), other insect cysteine proteases including a thiol protease from larval gut of *Phaedon cochleariae* (Phaedon, GenBank ID: CAA76927); digestive cysteine proteases from *Diabrotica virgifera virgifera* (DvRS5, GenBank ID: CAE47497; and DvRS30, GenBank ID: CAE47500); a digestive cathepsin L-like cysteine protease from *D. virgifera virgifera* (DvCAL1, GenBank ID: AAG17127); the mature form of human cathepsin L (hcatL, PDB ID: 1ICF); and the mature form of papain, (pap, PDB ID: 1STF). Papain numbering is shown above the alignment. The beginning/N-terminal residue of the predicted proenzyme is marked by (∇) and of the predicted mature protein by (⇓). Stars indicate active site residues. The potential Asn-glycosylation site in intestains D is marked by (♦) and in intestain E by (•). The residues that are part of the S1 binding site and important for insensitivity of enzymes to inhibitors are marked by (S1↓). The residue that is part of the S2 binding site and important for substrate specificity is marked by (S2↓).

Chymotrypsin Family Transcripts

Two SSH library clones similar to serine proteases of the chymotrypsin family were identified (cpb92 and cpb397) and another similar to serine carboxypeptidases of family S10

(cpb67). In this study, we chose to focus further on the chymotrypsins. In addition to the SSH library clones, one full-length and two partial chymotrypsin cDNAs were isolated from the cDNA library of adapted midguts. Querying of BLAST databases further identified two very similar ESTs (more than 96% identity at the nucleotide level): Ld_GO270919 (GenBank ID: GO270919) and Ld_EB756830 (GenBank ID: EB756830; Fig. 4, Supplementary Data File 4).

The amino acid sequences deduced from the new chymotrypsin-like cDNAs show highest sequence similarity (44%) to *Tenebrio molitor* putative serine protease (UniProt ID:

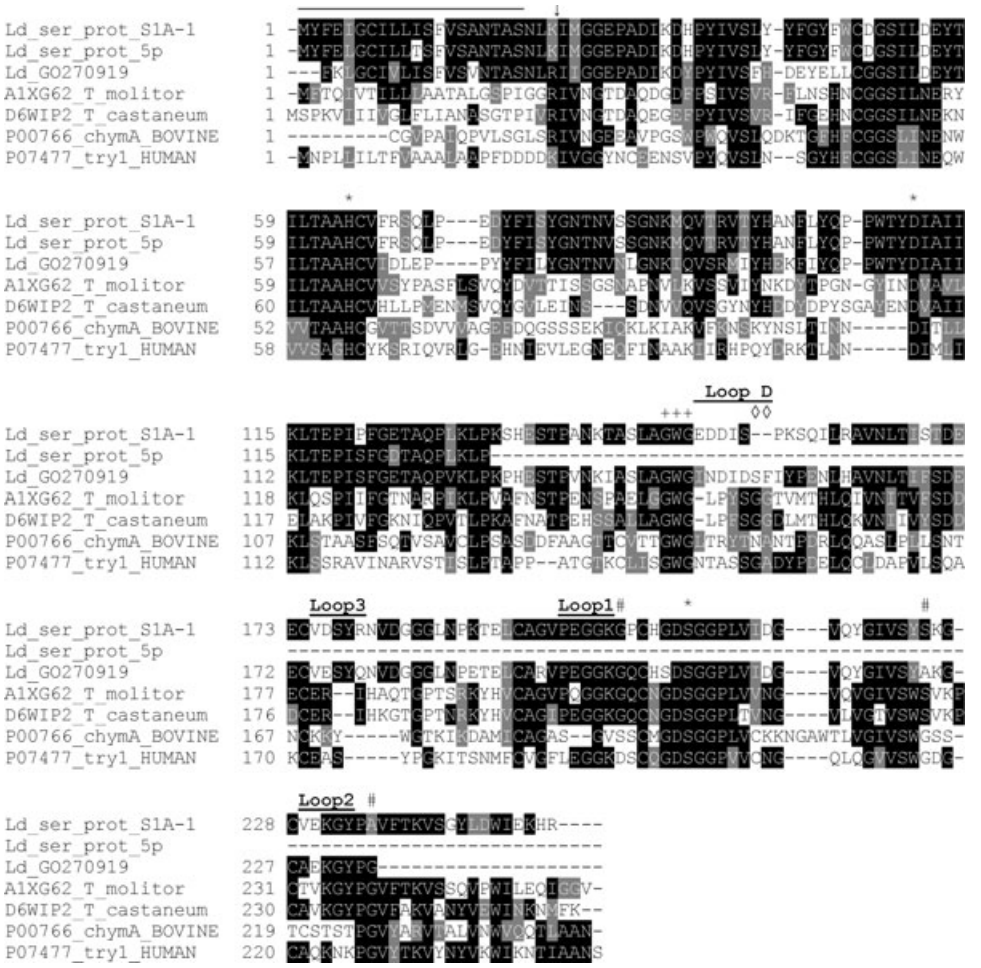

Figure 4. Sequence alignment of *Leptinotarsa decemlineata* chymotrypsin family members. Translated partial *L. decemlineata* transcripts (Ld_ser_protS1A-1, Ld_ser_prot_5p, Ld_GO270919) are aligned with the beetle serine proteases with the highest sequence similarity (*Tenebrio molitor* serine protease, Uniprot ID: A1XG62; and *T. castaneum* P40 serine protease, Uniprot ID: D6WIP2). Bovine chymotrypsinogen A (Uniprot ID: P00766) and human trypsin 1 precursor (Uniprot ID: P07477) are aligned as reference sequences. Active site residues are denoted by (*), the propeptide cleavage site by (↓), the specificity sites by (#), and the conserved GWG site by (+++). Deletion, potentially related to insect adaptation, is marked by (◊◊). The line above the alignment shows the length of signal peptide predicted for Ld_ser_protS1A-1. Loops that participate in enzyme-inhibitor interactions are marked above the alignment (Loop 1–3, Loop D).

A1XG60) and *T. castaneum* serine protease P40 (UniProt ID: D6WIP2). Analysis of the putative amino acid sequences in MEROPS revealed structural characteristics typical for chymotrypsin protease subfamily S1A, such as conserved catalytic triad residues, a conserved GWG structural motif, and a propeptide cleavage site (Fig. 4). The main structural difference between deduced amino acid sequences of the identified chymotrypsin-like cDNAs is a two amino acid deletion present in all of the CPB sequences except Ld_GO270919. This deletion is unique among chymotrypsins so far identified (Fig. 4) and is located on loop D, which was shown to be a part of the S1 substrate binding pocket (Ma et al., 2005; Fig. 4).

Cellulase and Endopolygalacturonase Transcripts

Eight clones with similarities to genes coding for cellulases were identified in the SSH libraries. Clones cpb116, cpb143, cpb303, cpb324, cpb329, and cpb337 were annotated as glycoside hydrolase 45 (GH45) family cellulases, whereas clones cpb277, cpb243, and cpb380 were annotated as glycoside hydrolase 48 (GH48) family cellulases. The deduced amino acid sequences of these clones align with more than 92% identity to previously identified CPB cellulase transcripts GH45–1 (GenBank ID: ADU33345), GH45–3 (GenBank ID: ADU33347), GH45–6 (GenBank ID: ADU33350), GH48–1 (GenBank ID: HM175847), and GH48–2 (GenBank ID: HM175848), respectively (see Supplementary Data File 4, Pauchet et al., 2010). Therefore, we did not perform additional cDNA cloning.

Four SSH clones (cpb245, cpb269, cpb292, and cpb306) were annotated as endopolygalacturonases. Nucleotide sequences of cpb292 and cpb245 are more than 90% identical to the *L. decemlineata* polygalacturonase GH28Pect-9 transcript (GenBank ID: HM175858) and GH28Pect-10 transcript (GenBank ID: HM175859), respectively. The differences in nucleotide sequences, however, do not translate into different amino acid sequences (Supplementary Data File 4).

Additionally, a full-length endopolygalacturonase cDNA was isolated from the cDNA library and was designated GH28Pect-11 (Genbank ID: JN603581). Nucleotide sequences of the remaining SSH clones with endopolygalacturonase annotation, cpb269 and cpb306, align perfectly to the GH28Pect-11 sequence. The amino acid sequence deduced from the newly identified GH28Pect-11 is 53% identical to *L. decemlineata* endopolygalacturonase GH28Pect-10 (GenBank ID: ADU33364), 59% identical to *Chrysomela tremulae* endopolygalacturonase (GenBank ID: ACP18831), 52% identical to *Phaedon cochleariae* polygalacturonase (GenBank ID: CAA76930), and 50% identical to *Sitophilus oryzae* endopolygalacturonase (GenBank ID: AAG35693) (Fig. 5).

Defensin-Like Transcripts

Four SSH clones were annotated as defensin-like transcripts (cpb36, cpb59, cpb213, and cpb220). Clone cpb36 represents a full-length transcript that was named defensin-like 1 (Ld_def1). Clone cpb213 aligns 100% to the *L. decemlineata* EST sequence FG591146 (GenBank ID: FG591146)—this transcript was named defensin-like 2 (Ld_def2). Identical clones cpb59 and cpb220, named defensin-like 3 (Ld_def3), represent a partial defensin-like transcript (Fig. 6). The amino acid sequence deduced from Ld_def1 is 71% identical to the sequence deduced from Ld_def2 and 80% identical to the sequence deduced from

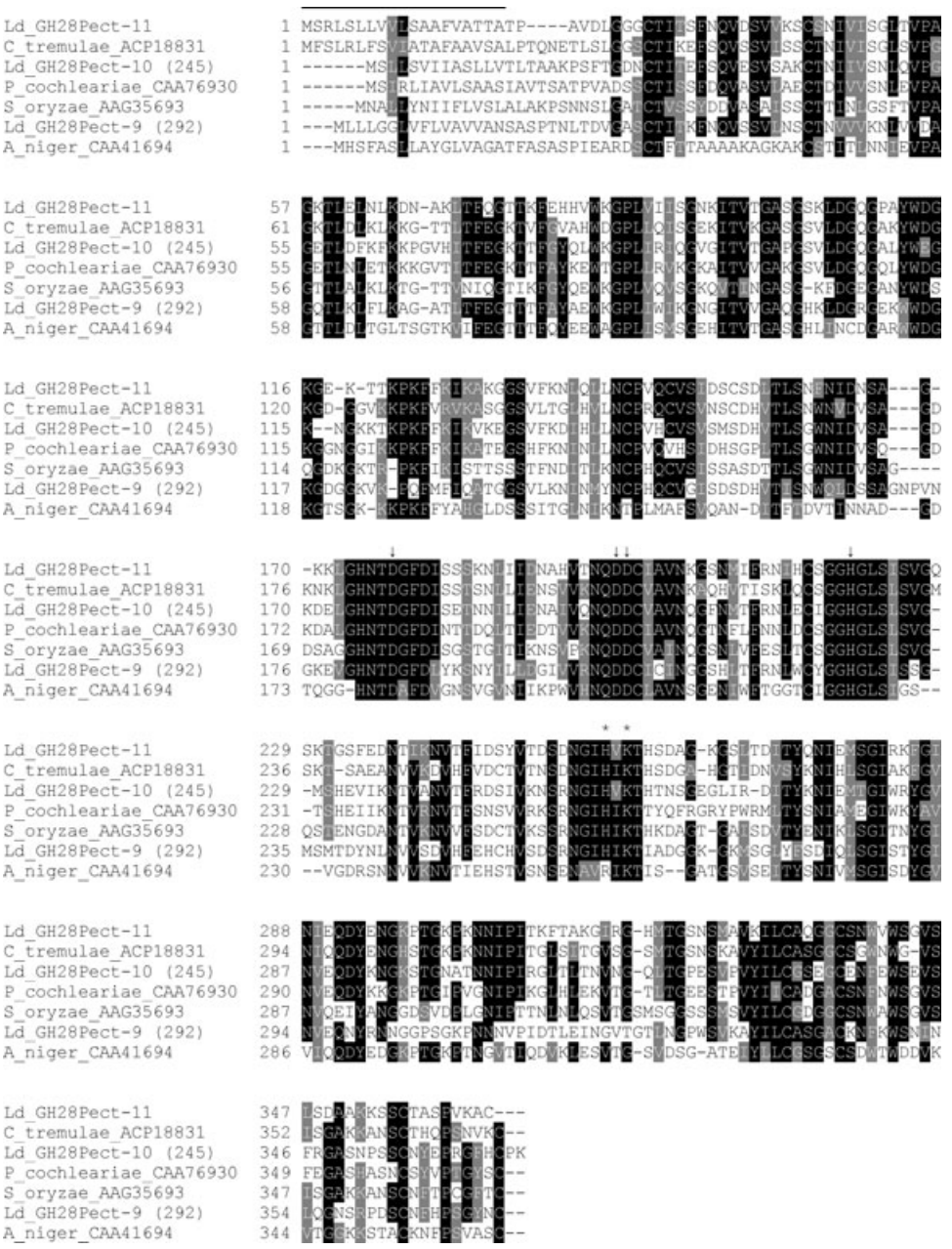

Figure 5. Alignment of the newly identified *L. decemlineata* endopolygalacturonase GH28Pect-11 (GenBank ID: JN603581) with GH28Pect-9 (GenBank ID: ADU33363), GH28Pect-10 (GenBank ID: ADU33364), other coleopteran endopolygalacturonases with highest similarity (*Chrysomela tremulae*, GenBank ID: ACP18831; *P. cochleariae*,GenBank ID: CAA76930; *Sitophilus oryzae*, GenBank ID: AAG35693), and a reference *Aspergillus niger* endopolygalacturonase (GenBank ID: CAA41694). The line above the alignment depicts the length of predicted signal peptide for GH28Pect-11. Polygalacturonase active site residues (Asp^{180}, Asp^{201}, Asp^{202}, His^{223}—numbered according to the *A. niger* sequence) are marked by arrows (↓), and residues implicated in substrate binding (Arg^{256} and Lys^{258}) are marked by asterisks (*). Residue Arg^{256} in the *A. niger* sequence (first asterisk) is replaced by His in coleopteran endopolygalacturonases. The accession numbers of SSH clones identical to the corresponding full-length endopolygalacturonases are shown in brackets.

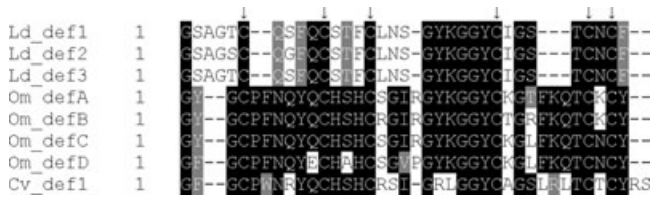

Figure 6. Sequence alignment of potential mature *L. decemlineata* defensin-like peptides (Ld_def1, Ld_def2, Ld_def3) with the most similar invertebrate defensins, *Ornithodoros moubata* defensins (Om_defA–Om_defD;UniProt ID: Q9BLJ3, UniProt ID: Q9BLJ4, UniProt ID: Q8MY08, UniProt ID: Q8MY07) and *Crassostrea virginica* mature Defensin-1 (Cv_def1; UniProt ID: P85008). Conserved defensin-characteristic cysteine residues are indicated by arrows (↓).

Ld_def3. The identity between the deduced amino acid sequence from Ld_def2 and the partial sequence deduced from Ld_def3 is 96%.

Querying InterPro with the 50 amino acid C-termini of the putative peptides deduced from Ld_def1, Ld_def2, and Ld_def3 resulted in a significant hit to the PFAM arthropod defensin domain (PFAM ID: PF01097). To explore the relevance of defensin-like annotation for these peptides, a similarity search was performed in the antimicrobial peptide database (Wang and Wang, 2004). Active defensins require a net positive charge to function as antimicrobial peptides. The longest C-terminal sequences having a positive net charge were 33 amino acids long. The antimicrobial peptide database query with these 33 amino acid sequences identified four mature tick *Ornithodoros moubata* defensins (UniProt IDs: Q9BLJ3, Q9BLJ4, Q8MY08, and Q8MY07) with more than 45% similarity in amino acid sequence and American oyster (*Crassostrea virginica*) Defensin-1 (UniProt ID: P85008) with 43% similarity (Fig. 6). In addition, all structurally important residues were conserved.

JHBP-Like Transcripts

Identical clones cpb42 and cpb178 differ in two nucleotides from *L. decemlineata* EST 525346 (GenBank ID: EB757388). We have additionally cloned three partial cDNAs with 90–95% identity to Ld_EB757388 (Supplementary Data File 4). Altogether, evidence for existence of at least four transcripts with highly similar sequences was confirmed in *L. decemlineata*.

BLAST queries of the deduced amino acid sequences against the NCBI-nr database resulted in no significant similarities. Nevertheless, InterPro query identified two motifs: a hemolymph JHBP PFAM family motif (PFAM ID: PF06585) and a PANTHER gene family motif without any functional annotation (PANTHER ID: PTHR11008; see Supplementary Data File 1). Sequences with significant similarity to our sequences were identified in the NCBI dbEST database, in gut cDNA libraries of two beetle species, *D. virgifera* and *C. maculatus*. The deduced amino acid sequence of Ld_JHBP-like_5p1 is most similar (30% identity) to a sequence deduced from a *D. virgifera* EST (GenBank ID: CN498346). The Ld_JHBP-like_3p2-deduced amino acid sequence is most similar (29% identity) to the *D. virgifera* EST (GenBank ID: CN498346). The amino acid sequence alignment of sequences deduced from the newly identified CPB cDNAs and sequences deduced from *D. virgifera* and *C. maculatus* ESTs shows that several nonpolar amino acid residues are conserved (Fig. 7).

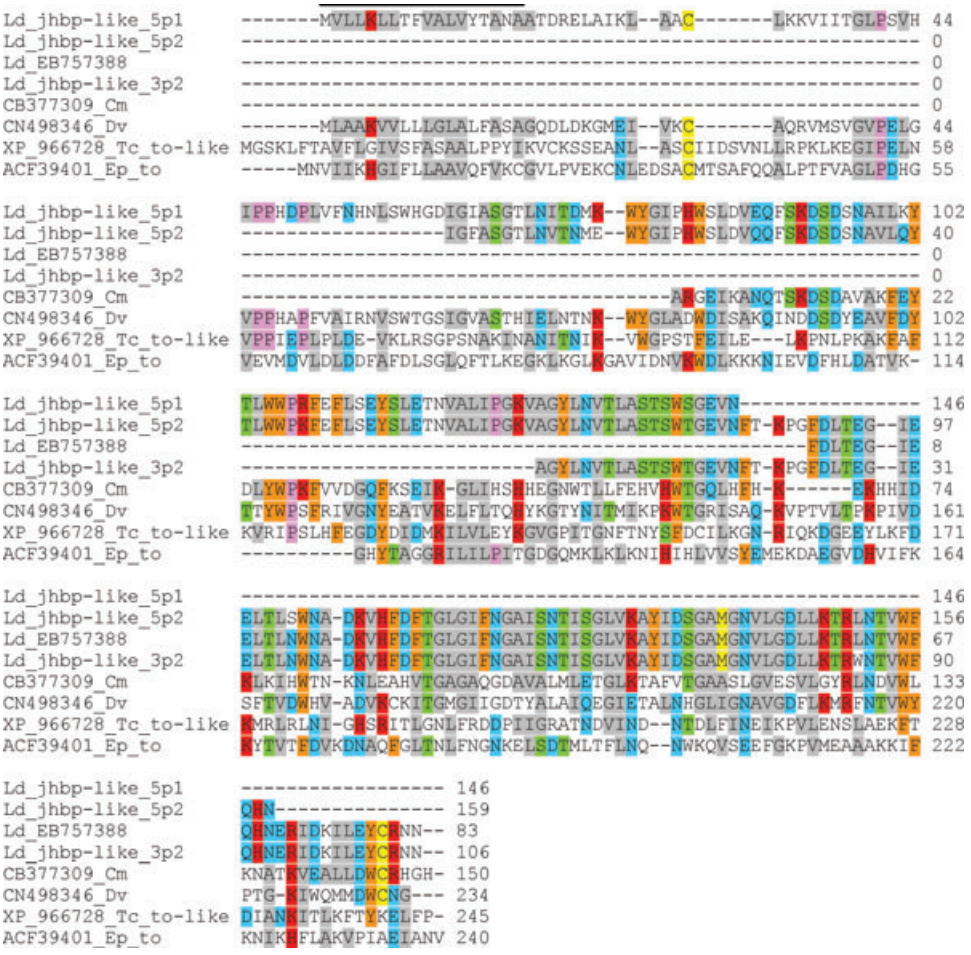

Figure 7. Sequence alignment of peptides deduced from the partial juvenile hormone binding protein-like (JHBP-like) *L. decemlineata* transcripts identified in this study with the most similar JHBP family sequences. Ld_EB757388—peptide deduced from *L. decemlineata* EST (GenBank ID: EB757388); CB377309_Cm—peptide deduced from *D. virgifera* EST (GenBank ID: CB377309); CN498346_Dv—peptide deduced from *C. maculatus* EST (GenBank ID: CN498346); ACF39401_Ep_to – *Epiphyas postvittana* takeout-like protein 1 (GenBank ID: ACF39401); XP_966728_Tc_to-like – *T. castaneum* similar to takeout-like protein (GenBank ID: XP_966728). The line above the alignment depicts the length of predicted signal peptides for Ld_ JHBP-like peptides. The conserved amino acid residues are colored according to their biochemical properties (gray—aliphatic; orange—aromatic; red—basic; blue—acid/amides; yellow—sulphur containing; green—nonaromatic with hydroxyl groups; pink—proline).

All Selected Genes Are Encoded in the Beetle Genome and Specifically Expressed in the Gut Wall

qPCR assays were designed to detect genes/transcripts of the complete gene families of intestains C, D, and E, chymotrypsins, defensin-like transcripts, and JHBP-like transcripts. Because differences between transcripts coding for glycoside hydrolases were too extensive for general qPCR primers, assays were designed to target one specific transcript. For the *smt3* (suppressor of MIF2 homolog 3) gene, only one nucleotide sequence was identified, and therefore only one set of primers was needed (Table 1). No cross-reactivity between

intestain assays exists as no false positive amplification was observed in analysis of cDNA clones belonging to different intestain groups with all assays. All qPCR assays were tested to determine efficiency (E), dynamic range, and linearity (R^2). All tests were performed within the quality control limits for quantitative analysis ($85\% < E < 105\%$, $R^2 > 0.99$; Table 1).

The qPCR assays were first used to verify the presence of the isolated genes in the genome of CPB. We successfully amplified amplicons Ld_ser_prot, Ld_GH45–6, Ld_def, Ld_GH28Pect–11, and Ld_JHBP-like in genomic DNA isolated from larval hemolymph and leg muscles, confirming that these genes originated from the beetle genome and not the metagenome of gut microflora. We further tested the tissue-specific expression of selected genes in fourth-instar larvae. Interestingly, several genes showed specific expression patterns within different gut compartments. Defensin and intestain C genes were more intensely expressed in the larval foregut; for all other genes, expression was stronger in the larval midgut (Fig. S1). Protease genes (serine peptidases, intestains C, and intestains D) were expressed in fore- and midgut, whereas the expression of cell wall degrading genes (cellulase GH45–6 and endopolygalacturonase Ld_GH28Pect-11) was limited to midgut. Low expression of all digestive enzyme genes, but not of JHBP-like or defensin-like genes, was detected in larval hindgut (Fig. S1). No expression was detected in other sampled tissues for analyzed genes, except for very weak intestain C gene expression in the carcass (1,000-fold lower than in midgut). The *smt3* gene is ubiquitously expressed in all analyzed tissues.

Dynamics of Gene Expression During Adaptation of CPB Larvae

To get an insight into dynamics of insect adaptation to plant defenses, we performed qPCR-based gene expression monitoring of larval midgut samples. We investigated both short-term (4, 10, and 24 h) and long-term (10 days) adaptation of fourth-instar larvae to the potato defense response. Expression analysis results show that none of the selected genes were significantly differentially regulated at any of the short-term adaptation time points. On the other hand, defense-induced increases in gene expression were detected in long-term feeding (Fig. 8). Genes encoding digestive proteolytic enzymes (intestains C, intestains D, and chymotrypsin) showed an increase in expression by 24 h, albeit not significant. Similarly, induction of Ld_GH28Pect-11 occurred consistently in all three long-term feeding tests, while GH45–6 cellulase was significantly upregulated in two of three long-term feeding tests. Similar patterns were measured for Ld_JHBP-like gene expression. No significant differential expression was observed for intestain E genes or the *smt3* gene in these experiments. The results for defensin-like gene expression measurements were unreliable because transcript quantities were close to or below the limit of qPCR quantification. For all the genes, differential expression was even more pronounced when larvae were fed on plants induced by CPB feeding than on plants induced by MeJA (Fig. 8).

Biochemical Characterization of Chymotrypsin Activity From Larval Midgut Contents

Although digestive cysteine proteases have been biochemically characterized extensively in CPB (Gruden et al., 1998, 2003), only one report thus far has detected serine proteases in CPB midguts (Novillo et al., 1997). The identification of differentially regulated chymotrypsin transcripts in our study led us to biochemically analyze these minor digestive proteolytic enzymes. We first inspected whether chymotrypsin proteolytic activity can be

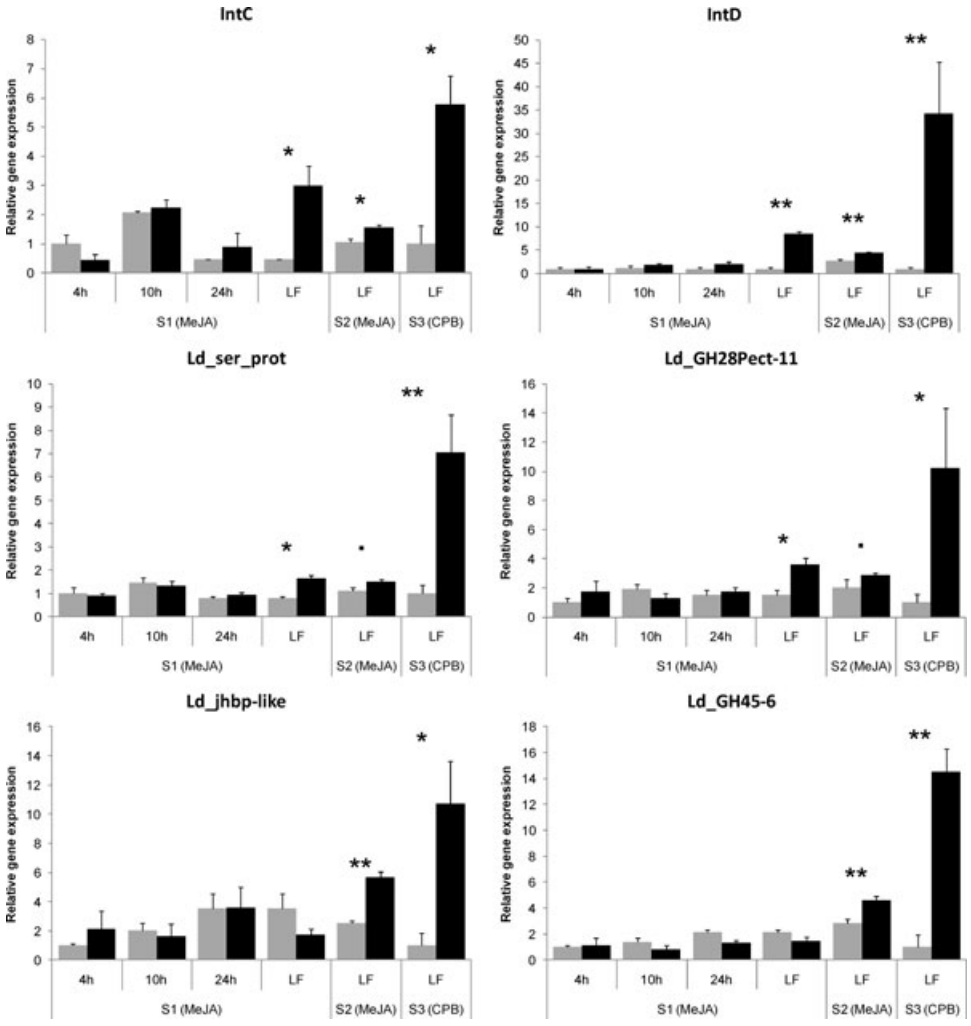

Figure 8. Dynamics in expression of selected genes. Gene expression was measured in midgut of fourth-instar larvae after 4 h, 10 h, 24 h (short-term adaptation) and 10 days (long-term adaptation, LF) of differential feeding. S1, S2, and S3 denote the three independent feeding trial series. The method of potato plant defense induction for the trials is shown in parentheses—MeJA treatment for S1 and S2 and CPB larvae feeding for S3. 18S rRNA was used as a reference gene. Expression of the tested gene in the 4-h control sample was used for normalization of gene expression in each sample. Gray bars represent control midgut samples from larvae fed with fresh potato leaves and black bars represent samples of midguts from larvae fed with defense-induced potato leaves. No significant differences were observed for intestains E or *smt3* gene expression (results not shown). Results of statistical testing are denoted with $^{**}P < 0.01$, $^{*}P < 0.05$ or $^{\cdot}P < 0.1$. Error bars show SE of the mean.

detected in the guts of larvae. Fractions were obtained by gel filtration of protein extract from adapted gut contents on standard substrate Z-Phe-Arg-pNA under conditions favorable for activity of serine proteases (pH 8). A peak of activity in the presence of the potent irreversible inhibitor of cysteine proteases E-64 was observed, consistent with residual serine protease activity (Fig. 9A). In addition, we have shown that less than 30% of total

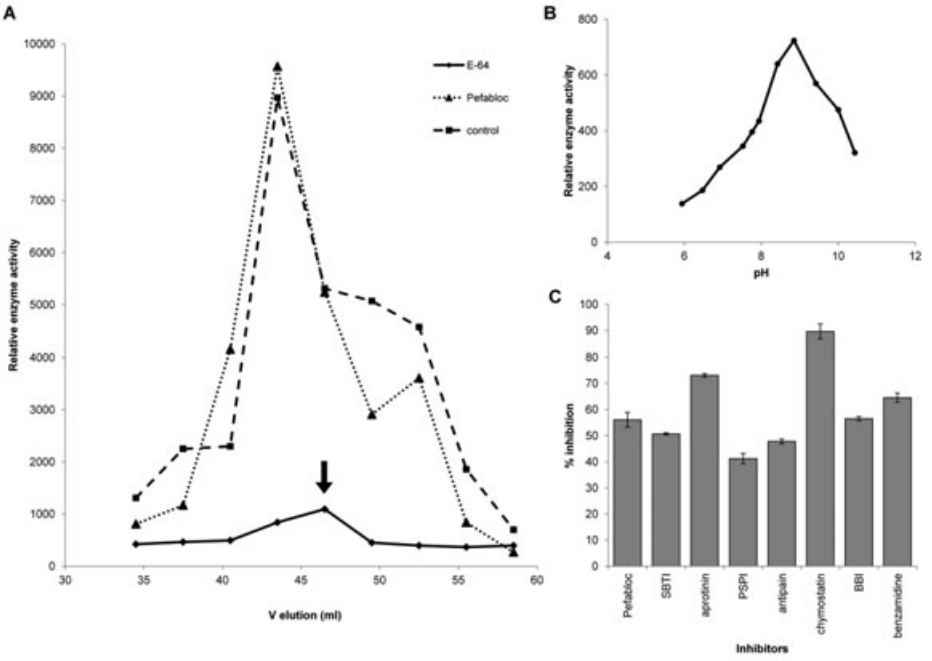

Figure 9. Biochemical characterization of chymotrypsin activity in adapted CPB midguts. (A) Partial purification allowed detection of chymotrypsin proteolytic activity in adapted CPB midguts. Gel filtration fractions of protein extracted from adapted beetle gut content were analyzed using Z-Phe-Arg-pNA substrate at pH 8 in the absence of any interfering substances (control), in the presence of cysteine protease inhibitor E-64, and in the presence of general serine proteinase inhibitor Pefabloc. The E-64-inhibited activity profile shows a peak (indicated by arrow) in residual activity attributed to serine proteases. (B) Effect of pH on the Z-Phe-Arg-pNA activity of the gel filtration fraction with the highest serine protease activity. (C) Inhibition assays performed on the gel filtration fraction with the highest serine protease activity. The following inhibitors were applied: Pefabloc, Soybean Kunitz Trypsin inhibitor (SBTI), aprotinin, potato serine Kunitz type proteinase inhibitor (PSPI), antipain, chymostatin, Bowman–Birk inhibitor (BBI), and benzamidine. Error bars correspond to SE of the mean.

proteolytic activity in any fraction can be inhibited by Pefabloc, an irreversible general serine PI.

More detailed characterization of serine protease activity revealed that the optimal pH is 8.8 (Fig. 9B), similar to the range of optima (pH 7–11) reported for chymotrypsin family members in other insects (BRENDA database, http://www.brenda-enzymes.org). Substrate specificity was tested on a number of synthetic molecules at pH_{opt} to relate the identified activity to specific members of the chymotrypsin family (Fig. 9C). The CPB chymotrypsin most efficiently degraded Boc-Gly-Arg-Arg-MCA, followed by Boc-Gly-Lys-Arg-MCA (10% less efficient degradation compared to Boc-Gly-Arg-Arg-MCA), Z-Arg-Arg-MCA (50% less efficient degradation than Boc-Gly-Arg-Arg-MCA), and Z-Phe-Arg-MCA (70% less efficient degradation than Boc-Gly-Arg-Arg-MCA), typical substrates for trypsins. The enzyme was not able to degrade Suc-Ala-Ala-Pro-Phe-MCA or Suc-Ala-Ala-Ala-MCA, which are typical substrates for chymotrypsin and elastase, respectively. Rough inhibition profiling showed that chymostatin (a strong inhibitor of mammalian chymotrypsin, but not of trypsin) and aprotinin (Ki = 0.06 pM for mammalian trypsin, and Ki = 9 nM for mammalian chymotrypsin) effectively block activity, while partial inhibition was also

detected with Pefabloc, benzamidine (a reversible general serine PI), soybean trypsin inhibitor, and BBI (both strong inhibitors of mammalian trypsins and to a lesser extent of chymotrypsins). PSPI, a Kunitz-type serine proteinase inhibitor isolated from potato tubers, was the least effective inhibitor among those tested (Fig. 9C).

Involvement of Chymotrypsin-Like Activity in CPB Adaptation

Increased cysteine protease activity in guts of CBP larvae fed with defense-induced leaves was shown in previous studies (Bolter and Jongsma, 1995; Gruden et al., 1998, 2003). In this study, we have further tested whether the induction of chymotrypsin-like gene expression is reflected in an increase of chymotrypsin-like proteolytic activity. Enzymatic activities were measured in crude extracts at the optimal pH in the presence of E-64 and EDTA. A four-fold increase in chymotrypsin-like activity per milligram of protein content (t-test, $P = 0.005$) was detected in CPB larvae fed long term with MeJA-treated leaves (1337 $\pm$ 312) compared to control larvae (283 $\pm$ 88), in agreement with the results obtained on the transcriptional level.

DISCUSSION

Several previous studies have investigated adaptation of insects to increased PI content in the diet using nontargeted transcriptomics (Liu et al., 2004; Moon et al., 2004; Chi et al., 2009; Oppert et al., 2010). Our focus, however, was on the larval response to broad spectrum of plant defense molecules in contrast to one PI alone. As pointed out by Rivard et al. (2004), the induction of PIs is only one part of the plant defense repertoire against herbivores, and components other than proteases may have an important role in beetle adaptation. In this study, we show that indeed, feeding of larvae with either MeJA- or CPB-induced plants changes the expression of several larval digestive enzyme genes in addition to cysteine proteases (Gruden et al., 2004) as well as the expression of potential regulatory genes.

A Broad Range of Digestive Proteases Contributes to CPB Larval Adaptation

At least three groups of cysteine proteases intestains exist in midguts of CPB larvae, and some of them contribute to insect adaptation to plant defenses (Gruden et al., 2003, 2004). In this study, we isolated transcripts of two new groups of cysteine proteases, intestains D and E. Altogether, 39 intestain transcripts (22 in this study) from different intestain groups have been identified in adapted CPB larvae midgut, confirming the involvement of a wide array of cysteine proteases in adaptation to potato defense proteins.

The expression of intestain C and D genes appears to be differentially regulated during larval adaptation to the potato defense response. The expression patterns of intestains D are similar to those of intestains A and C (Gruden et al., 2004), showing an increase in the amount of transcript following long-term feeding on potato PI rich diet (Fig. 8). However, expression of intestain E genes remains unchanged in short- and long-term adaptation. Similar up- and downregulation, as well as unchanged levels of the main class of digestive protease genes in response to PI ingestion, has been observed in *Helicoverpa armigera*, *H. zea*, *Agrotis ipsilon*, *C. maculatus*, and *T. castaneum* larvae (Bown et al., 1997, 2004; Mazumdar-Leighton and Broadway, 2001; Moon et al., 2004; Chi et al., 2009; Oppert et al., 2010). While inhibitor-insensitive proteases in larval guts of *H. zea* and

A. ipsilon were induced within 2 h after PI incorporation into the diet (Mazumdar-Leighton and Broadway, 2001), in our experiments and those of Gruden et al. (2004) expression of specific proteases was induced later than 24 h after feeding on MeJA-induced potato leaves (Fig. 8, Supplementary Data File 2). Thus, in different insect species, the timing of response can be different.

Cysteine proteases are the main digestive proteolytic enzymes of CPB; however, minor aspartic, serine and metallo-protease activities were detected in the gut (Novillo et al., 1997; Brunelle et al., 1999). In this study, we show for the first time that serine protease activity in CPB midgut increases four-fold in long-term adaptation to plant defenses. Additionally, we show that newly identified chymotrypsin genes are upregulated (Fig. 8, Supplementary Data File 2). Interestingly, the *L. decemlineata* transcripts identified in our study are most similar to the chymotrypsin serine protease transcript of *T. castaneum* (GenBank ID: XM_967427; see Fig. 4) that is most strongly upregulated after exposure to a PI-rich diet (Oppert et al., 2010). The substrate specificity and inhibition profile of the partially purified serine protease from CPB show some trypsin-like and some chymotrypsin-like properties, indicating that these insect enzymes most probably have unique enzymatic characteristics intermediate between the two most widespread members of the chymotrypsin family. Similarly, the deduced amino acid sequences of chymotrypsin-like transcripts identified in this study show characteristics of both mammalian trypsins and chymotrpysins. We are, however, aware that structural–functional implications are possible if the active protein is produced in heterologous systems or purified protein sequence is known.

There are several possible physiological explanations for upregulation of serine-type digestive enzymes during CPB adaptation. First, the beetle may compensate for the loss of activity of serine proteases by production of novel enzymes that are insensitive to potato serine PIs, as it does for digestive cysteine proteases. The structural basis of such potential insensitivity can only be hypothesized, but sequence alignment of the novel serine proteases shows that insensitivity might be attributed to deletion of two residues in loop D (Fig. 4). However, the increase in serine protease activity may simply compensate for loss of activity of cysteine digestive proteases. A compensatory shift from digestive cysteine to digestive serine proteases was observed in *T. castaneum* when exposed to the irreversible cysteine proteinase inhibitor E-64 (Oppert et al., 2005). In *T. castaneum*, serine proteases respond in a diverse manner to E-64, with some enzymes induced and others repressed (Oppert et al., 2010). However, one has to note that serine protease activity in *Tribolium* gut is substantially higher than in CPB, as 40% of total gut proteolytic activity can be inhibited by soybean Kunitz serine PI (Oppert et al., 2005), while in CPB the contribution of serine proteases to overall proteolytic activity is minor (Bolter and Latoszek-Green, 1997; Novillo et al., 1997).

Alternatively, serine proteases in CPB guts may serve a specific function, as was suggested for digestive aspartic proteases, which only initiate digestion of the major plant protein RUBISCO (Brunelle et al., 1999). When exposed to high levels of cathepsin D inhibitor, CPB larvae compensate for the loss of aspartic protease activity by expressing proteases of the serine/cysteine class that perform the same function (Brunelle et al., 2004). A shift from cysteine to aspartic digestive proteases was detected in another beetle, *C. maculatus*, when exposed to high levels of cysteine PIs (Zhu-Salzman et al., 2003). We isolated a clone with sequence similarity to aspartic proteases (Contig_204, Supplementary Data File 1) from the library of upregulated genes, but because microarray screening did not indicate strong changes in expression, this transcript was not investigated further. No metallopeptidase-like transcript was

identified in our study although metalloprotease activity was detected in the work of Novillo et al. (1997).

Glycoside Hydrolases Are Also Involved in L. decemlineata Adaptation to Plant Defense Compounds

The cellulases in insect guts were long thought to be encoded only in genomes of symbiotic bacteria, fungi, and protists. The first proof that they are also encoded in insect genomes came with the work of Watanabe et al. (1998) on termites. Recently, cellulase genes were also confirmed in genomic DNA of several plant-feeding beetles (reviewed in Pauchet et al., 2010; Watanabe and Tokuda, 2010). Here, we show experimental evidence that cellulases of the GH45 family are also encoded by the *L. decemlineata* genome. Moreover, they participate in CPB adaptation to potato defenses. From the SSH library, we isolated six clones that correspond to three *L. decemlineata* GH45 family cellulases (GH45–1, GH45–3, GH45–6) and two clones that correspond to *L. decemlineata* GH48–1 cellulase. All members of the GH45 family were upregulated, while GH48–1 was downregulated, according to microarray hybridizations (Supplementary Data File 1). We have further examined the expression of the GH45–6 transcript using qPCR. This cellulase was significantly upregulated in two of three long-term feeding trials analyzed; however, more experiments are needed to elucidate its role in adaptation.

Another group of enzymes that is widely distributed in bacteria, plants, and fungi but was only recently confirmed in genomes of insects is polygalacturonases (Shen et al., 2003; Pauchet et al., 2010). In insect guts, these proteins degrade pectin from plant cell walls to oligosaccharides. Several polygalacturonase transcripts have been isolated from CPB larvae gut (GH28Pect-1–10, Pauchet et al., 2010), but we have identified an additional novel transcript (GH28Pect-11). We confirmed that it is encoded in the *L. decemlineata* genome and showed that this specific transcript was induced in CPB larvae guts following exposure to potato defense compounds. Differential expression of polygalacturonases in insect adaptation could be explained, analogous to upregulation of proteases, as a response to high amounts of polygalacturonase-inhibiting proteins (PGIPs) in potato foliage (Machinandiarena et al., 2001). It is also known that inhibitors of alpha-amylase are induced in plant leaves after insect attack (Svensson et al., 2004), and we have identified an upregulated alpha-amylase gene (Contig_161) in microarray hybridizations (Supplementary Data File 2). Physiological explanation of cellulase gene involvement in insect adaptation to plant defenses is less straightforward. Plant inhibitors of cellulases are secondary (mostly phenolic) compounds (Sami and Shakoori, 2008; Kim et al., 2011) and were shown to be induced following insect infestation (Hartley and Firn, 1989). Similarly, the induction of phenylalanine ammonia lyase, polyphenol oxidase, and several other enzymes involved in biosynthesis of phenolic compounds was detected in insect infested or MeJA–treated plants (Kruzmane et al., 2002; Lawrence et al., 2008; Zhu-Salzman et al., 2008).

Gut-Specific Defensin-Like Transcripts Found in L. decemlineata

Defensins are cysteine-rich cationic peptides involved in vertebrate and invertebrate antibacterial defense, that form pores in bacterial cell membranes. They are induced as a part of insect innate immunity after bacterial infection and usually expressed at highest levels in the fat body (Hwang et al., 2009) and hemolymph (Bulet et al., 1991; Evans and Lopez, 2004). In this study, we identified three novel defensin-like transcripts (Ld_def1–3)

expressed in CPB larvae guts. The peptides show sequence similarity to defensins previously identified in tick *O. moubata* and oyster *C. virginica* (Fig. 6). We have also shown that these defensins are expressed specifically in the guts of CPB larvae. As the gut is the primary entry site for pathogenic bacteria in beetles, the presence of defensins was not unexpected. Possibly related to their function the expression was approximately nine-times higher in the foregut compared to the midgut (Fig. S1).

In *C. maculatus* larvae, antibacterial peptides were differentially expressed in short-term and long-term adaptation to a PI-supplemented diet (Chi et al., 2009). In our study, all defensin-like clones were isolated from the SSH library enriched in upregulated transcripts. The expression in midguts was nearing the lower limits of RT-qPCR detection. Analysis of foregut samples that were shown to be the primary expression site of defensin-like genes (Fig. S1) could yield better results.

Potential Role of JHBP-Like Proteins in Larval Adaptation

JHBP-like genes identified in our SSH library attracted our attention due to various functions already described for JHBPs in insects. The JHBP family (PF06585) consists of more than 500 insect proteins of which 17 were identified in beetles. Ld_JHBP-like transcripts share relatively low sequence identity to other JHBP proteins; nevertheless, the protein secondary structures and fold likely are conserved because of the functional similarity of amino acid residues (Fig. 7).

JHBPs bind JHs, unique sesquiterpenoid compounds that regulate embryonic development, repress metamorphosis, and induce vitellogenin synthesis and pheromone production in most insect species (Nijhout et al., 1998). JHBPs assist in transportation of JH and protect them from degradation in hemolymph (Zalewska et al., 2009). JHBPs were found to be downregulated by JH in *Manduca sexta* (Du et al., 2003). Additionally, JHBPs are upregulated after starvation in *Drosophila melanogaster* and *Bombyx mori*; therefore, their involvement in sensing nutritional status and regulating metabolism was proposed (Sarov-Blat et al., 2000, Saito et al., 2006). They were also upregulated in insects exposed to pathogens in *B. mori* (Liu et al., 2009). In our study, Ld_JHBP-like gene expression was induced after long-term feeding in three of four independent trials (Supplementary Data File 2, Fig. 6) implicating a role in CPB adaptation to plant defense compounds.

We have additionally shown that the Ld_JHBP-like genes identified in this study are specifically expressed only in the midgut (Fig. S1). Other known JHBP-like genes are expressed in different tissues, with the highest expression levels in the fat body. The most studied JHBPs, the *D. melanogaster* takeout family members, are expressed in the fat body, brain, antenna, and gut (Sarov-Blat et al., 2000; So et al., 2000; Saito et al., 2006). A member of *Aedes aegipti* JHBPs was found to be expressed also in antenna and its role in regulation of chemosensory responses was speculated (Bohbot and Vogt, 2005). Taken together, diverse functions for members of the JHBP family were suggested. The current study implicates another potential function of these proteins, which is regulation of responses after dietary exposure to antinutritive compounds. Downregulation of one JBHP family member was detected also in *Heliothis virescens* feeding with leaves hindered in synthesis of jasmonic acid to support this idea (Govind et al., 2010).

In summary, we have identified novel components of adaptation to induced plant defenses in CPB larvae midguts using a combination of transcriptomics, biochemical and bioinformatics techniques. Our results thus increase the understanding of the process of insect adaptation as it occurs under natural conditions. Interestingly, the changes in the gene expression largely overlap with the changes identified in larvae exposed to high

levels of dietary PIs (Moon et al., 2004; Chi et al., 2009; Govind et al., 2010; Oppert et al., 2010). We propose two possible explanations for this common pattern. Although there is an array of defense molecules produced in plants after exposure to pests (Ehlting et al., 2008; Govind et al., 2010), the induced PIs may have a dominant influence on the physiology of the pest because they target availability of amino acids essential for successful larval development. An alternative, more intriguing explanation would be that the same regulatory network is triggered whenever larvae are exposed to unfavorable diets.

ACKNOWLEDGMENTS

This work was supported financially by the Slovenian Research Agency (P4–0165). We thank Daniel J. Palmer (Department of Agriculture, State of New Jersey, USA) for providing us with Colorado potato beetle eggs and mag. Peter Dolničar, Agricultural Institute of Slovenia, for providing potato tubers and Dr. Elizabeth Covington for critical review of the manuscript.

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