Genomics 99 (2012) 256–264

Contents lists available at SciVerse ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

De novo intestine-specific transcriptome of the brown planthopper *Nilaparvata lugens* revealed potential functions in digestion, detoxification and immune response

Yan-Yuan Bao, Ying Wang, Wen-Juan Wu, Dong Zhao, Jian Xue, Bao-Qin Zhang, Zhi-Cheng Shen, Chuan-Xi Zhang *

State Key Laboratory of Rice Biology and Ministry of Agriculture Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Institute of Insect Sciences, Zhejiang University, Hangzhou 310028, China

ARTICLE INFO

Article history:
Received 8 October 2011
Accepted 6 February 2012
Available online 15 February 2012

Keywords:
Nilaparvata lugens
Intestine transcriptome
Illumina sequencing
Digestion
Immunity
Detoxification

ABSTRACT

The brown planthopper (*Nilaparvata lugens*, BPH) is the most serious rice plant pests in Asia. In this study, we performed transcriptome-wide analysis on BPH intestine. We obtained more than 26 million sequencing reads that were then assembled into 53,553 unigenes with a mean size of 388 bp. Based on similarity search with the nucleotide sequences available at NCBI, BPH intestine-specific transcriptome analysis identified 21,405 sequences. Assembled sequences were annotated with gene description, gene ontology and clusters of orthologous group terms. The digestion-, defense- and xenobiotic metabolism-related genes were abundantly detected in the transcripts from BPH intestine. Many novel genes including 33 digestion-related genes, 25 immune responsive genes and 27 detoxification-related genes are first reported here. We investigated the gene expression patterns at the transcript levels in different tissues by quantitative real-time PCR analysis, which revealed that some genes had intestine-specific expression, implicating their potential significance for BPH management.

1. Introduction

The brown planthopper (*Nilaparvata lugens* Stål, BPH) is a typical vascular feeder that is considered to be one of the most destructive pests for rice throughout Asia. This insect sucks sap from rice phloem, which can lead to stunted plant growth, and it transmits 'grassy stunt' and 'ragged stunt' virus diseases [1]. BPH control by chemical insecticides is still the first choice for BPH management; however, the widespread use of insecticides has caused planthopper resurgence and environmental risks. It is therefore urgently important to develop novel strategies for BPH management. In recent years, RNAi-based technology has been demonstrated for its feasibility and potential in protecting crops against agriculturally important insect pests [2–5]. The RNAi approach is very promising because it allows a wide range of potential targets for the suppression of gene expression [6].

At present, though the entire BPH genome is not available, an expressed sequence tags (EST) database (http://bphest.dna.affrc.go.jp/) offers 10,200 clusters that were generated from 18 BPH tissues and stages [7]. Furthermore, a transcriptomic dataset gives more comprehensive gene expression information regarding development, wing dimorphism and sex differences in BPH [8]. These data are useful for the understanding of BPH biochemical and physiological mechanisms, but they are not fully analyzed.

The BPH intestine mainly functions in nutrient ingestion and utilization, and it plays an important role in xenobiotic metabolism (i.e., insecticide metabolism). Several detoxification enzymes have been identified from the BPH gut, which were associated with resistance to insecticides, including carboxylesterase [9] and glutathione S-transferase [10]. In addition to digestive and detoxification functions, the intestinal epithelium serves as a barrier between the internal and external environment [11]. This barrier protects the host against invasion and systemic dissemination of pathogens and maintains intestinal immune homeostasis. Despite the importance of this tissue, little is known about the intestine-specific responses underlying the physiological and immunological processes in BPH intestine. Therefore, a full transcriptome analysis of the BPH intestine would provide valuable information for identifying potential gene targets for BPH management.

Next-generation high-throughput DNA sequencing platforms, such as 454-FLX, SOLID and Illumina systems, have emerged as powerful tools for studying genome-wide expression and profiling of mRNAs, small RNAs and methylated DNAs [12]. These novel techniques offer rapid methods for gaining huge sums of data in a short amount of time. For example, the Illumina–Solexa technique is able to generate over 1 billion bases of high-quality DNA sequence in a single run at less than 1% of the cost of capillary sequencing [13]. Owing to the massive amount of sequences analyzed simultaneously, RNAs of low abundance are able to be detected. Thus, this approach dramatically

* Corresponding author. Fax: +86 571 88982991.
E-mail address: chxzhang@zju.edu.cn (C.-X. Zhang).

0888-7543/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2012.02.002

improves the efficiency and speed of gene discovery in the absence of available genome information. The Illumina sequencing approach has been successfully used to study development-specific gene expression in BPH and Whitefly (*Bemisia tabaci*) [8,14].

In order to fully understand BPH physiological and immunological mechanisms in the intestine, we performed a transcriptome analysis using the Illumina approach. We obtained 53,553 unigenes, including abundant digestion-, immune- and detoxification-related genes. Some genes exhibited gut-specific expression in BPH. These data would be useful for the identification of the potential target genes involved in the immune response, insecticide resistance and macromolecule cabalism.

2. Results and discussion

2.1. N. lugens digestive system

We dissected the intestinal tracts from *N. lugens* adults for *de novo* transcriptome analysis. The structure of the intestinal tract was observed under a Leica S8AP0 stereomicroscope. The observations showed that a complete BPH intestinal tract begins with the esophagus neighboring the fore-diverticulum, followed by the midgut and ending with the hindgut. The two Malpighian tubes were located between the midgut and hindgut (Fig. 1). To obtain the gene expression profile of the entire *N. lugens* intestinal tract, we isolated the whole intestines, including the esophagus, fore-diverticulum, midgut and hindgut from approximately 3,000 nymph and adult individuals and used this tissue to prepare pooled RNA samples.

2.2. Illumina sequencing and reads assembly

N. lugens intestine transcriptome assembly by Illumina sequencing generated a total of 26,261,054 reads with the length of 2,363,494,860 nucleotides (nt). These raw reads were assembled into 317,347 contigs by SOAP *de novo* software. Using paired-end joining and gap-filling, the contigs were further assembled into 76,749 scaffolds with a mean size of 308 bp. After clustering the scaffolds together with nucleotide sequences available at NCBI, we obtained 53,553 unigenes with a mean size of 388 bp.

To annotate these unigenes, we searched reference sequences using BLASTX within the non-redundant (nr) NCBI nucleotide database using a cut-off *E*-value of 10^{-5}. A total of 32,147 unigenes (60% of all distinct gene sequences) did not have an annotation with matches in the NCBI database due to both a short nucleotide length and a lack of *N. lugens* genome availability; however, 21,405 unigenes (40% of all distinct gene sequences) had BLAST results with annotation. The size distribution of these annotated unigenes showed that the lengths of 2,714 unigenes (12.7%) were more than 1000 bp, 5,572 unigenes (26%) were between 500 and 1000 bp and 13,119 unigenes (61.3%) ranged from 150 to 500 bp (Fig. 2).

2.3. Sequence homology distribution

As shown in Fig. 3, the *E*-value distribution of the 21,405 annotated unigenes showed that 24% of the sequences have significant homology (smaller than $1.0E10^{-50}$) matching in the NCBI database, while 76% of the sequences ranged between $1.0E10^{-5}$ to $1.0E10^{-50}$. The similarity distribution showed that 14.2% of the sequences had significant homology higher than 90%, followed by 78% of the sequences with homology between 50 and 90%; only 7.6% of the sequences had homology lower than 50%. Species-specific distribution revealed that 17.1% of the sequences most closely resembled *Apis mellifera* and *Tribolium castaneum*, respectively, followed by *Acyrthosiphon pisum* (14.4%) and *Nasonia vitripennis* (11.3%). Approximately 14.1% of unigene sequences showed top matches with diptera, including *Drosophila*, *Culex quinquefasciatus* and *Aedes aegypti*. There were 229 hits (1.1%) matched with *N. lugens* sequences in the NCBI database (Additional file 1). Our intestine-specific transcriptome analysis identified a large number of high-quality sequences, suggesting that the corresponding proteins may have special functions in the BPH ingestion system.

2.4. Classification of gene ontology (GO) and clusters of orthologous groups (COG)

To classify the functions of *N. lugens* unigenes, we searched the GO database by using the WEGO tool (Web Gene Ontology Annotation Plotting) for plotting GO annotation results (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). A total of 6,220 *N. lugens* sequences (29.1% of the annotated unigenes) were categorized into 47 functional groups (Fig. 4). In each of the three main categories (biological process, cellular component and molecular function) of the GO classification, the 'Cellular and Metabolic process', 'Cell or cell part' and 'Binding and Catalytic activity' terms were the most dominant. In contrast, the 'Cell killing' and 'Viral reproduction' terms under the Biological process category were least abundant, and these terms contained only one and five unigenes, respectively. The unigenes associated with the 'Auxiliary transport protein activity', 'protein tag' and 'metallochaperone activity' terms under the Molecular function category also showed the

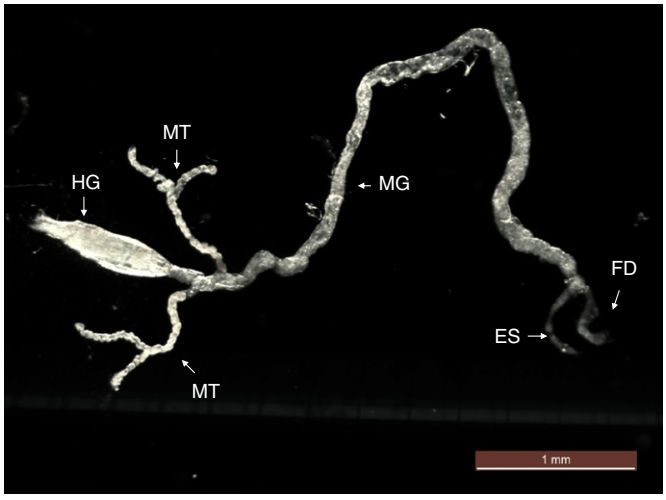

Fig. 1. Structure of an adult BPH intestine. The structure of an intestine was observed under a Leica S8AP0 stereomicroscope. ES, esophagus; FD, fore-diverticulum; MG, midgut; HG, hindgut; MT, Malpighian tube.

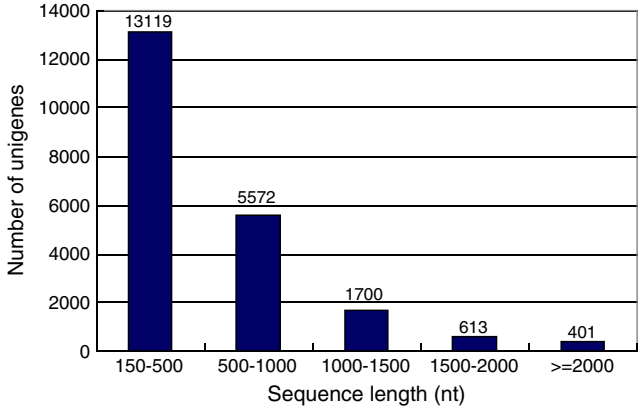

Fig. 2. Size distribution of the intestine-specific BPH unigenes. The sizes of 21,405 unigenes that have a BLAST annotation in the NCBI database were calculated.

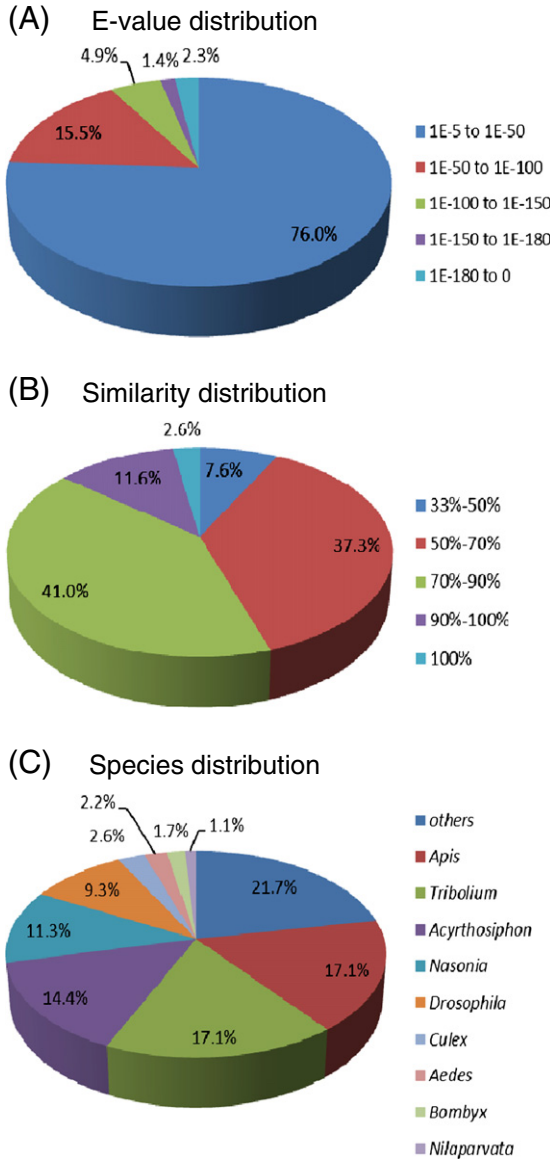

Fig. 3. Homology analysis of the intestine-specific BPH unigenes. All distinct gene sequences (21,405) that had BLAST annotations within the nr database with a cut-off *E*-value $\leq 10^{-5}$ were analyzed for (A) *E*-value distribution, (B) similarity distribution, and (C) species distribution. In each analysis, proportions greater than 1% are shown.

lowest abundance, as there were no more than three unigenes in each term.

To further evaluate the effectiveness of the annotation process, we searched the annotated unigenes involved in COG classifications. Out of 21,405 unigenes, 11,961 sequences had a COG annotation (Fig. 5). Among the COG categories, the cluster for 'General function prediction' represented the largest group (2103, 17.6%), followed by 'Translation, ribosomal structure and biogenesis' (1061, 8.8%), 'Replication, recombination and repair' (985, 8.2%) and 'Transcription' (962, 8.0%). The clusters for 'RNA processing and modification', 'defense mechanisms', 'Nucleotide transport and metabolism' and 'Cell motility' shared similar abundance (83–147, 0.7–1.2%). In contrast, 'Extracellular structures' and 'Nuclear structure' were the smallest clusters, which contained only 6 and 4 unigenes, respectively. The annotations of unigenes against the GO and COG databases provide useful information for confirming the gene function and specific processes occurring in BPH intestine.

2.5. Digestion-related genes

It has been assumed that sap-sucking BPH did not carry out digestive proteolysis, but instead relied on free amino acids in the phloem and xylem saps for their nutritional requirements. Besides many inorganic ions, organic acids, sugars and amino acids, more than 150 characteristic proteins were detected in the phloem sap of rice plants through cut BPH stylets [15]. These proteins are synthesized continuously, and their composition in the phloem sap is rather stable. This finding suggested that rice phloem sap proteins are capable of entering BPH intestinal tracts and probably serve as a source of nitrogen that could be utilized by the sap-suckers. Despite the fact that the molecular biology of digestion in BPH remains poorly understood, a study has demonstrated serine and cysteine protease activities in BPH gut [16]. Thus, it would be expected that BPH possesses these potential digestive functions. Understanding BPH digestive mechanisms will be useful for elucidating the ecological and economic significance of this species.

In this study, BPH intestine transcriptome-wide analysis revealed 120 hits encoding various proteinases, which included 56 hits for all types of serine proteases (i.e., trypsin-like proteases), 14 cysteine proteases (i.e., cathepsins), 24 aminopeptidases, 23 carboxypeptidases and 3 dipeptidyl peptidases. In addition, 15 hits encoding lipases and 20 hits encoding glucosidases were identified in the intestine. Among these sequences, 33 hits are newly identified in BPH (Additional file 2). We compared BPH intestine transcriptome dataset with several other insect species including the sand fly *Lutzomyia longipalpis*, the tobacco hornworm *Manduca sexta* and the poplar leaf beetle *Chrysomela tremulae*. The transcriptome dataset of *L. longipalpis* contained 9,601 ESTs, which were sequenced from the midgut cDNA libraries [17]. Proteases were among the most abundant transcripts captured in the midgut cDNA libraries that included trypsin-like serine proteases, chymotrypsins and carboxypeptidases. Pauchet et al. identified over 387,000 ESTs and 23,238 contigs in *M. sexta* and *C. tremulae* midguts using 454 sequencing, respectively [18,19]. The most dominant enzymes in these midguts are serine and cysteine proteases followed by carboxypeptidases, lipases and aminopeptidases. Dipeptidyl peptidases, α- and β- glucosidases and α-amylases were also found in the midgut transcriptome datasets. The comparison of the gut transcriptomes from different insect species showed that the distribution of the BPH digestion-related enzymes are similar to those described in *M. sexta*, *C. tremulae* and *L. longipalpis*, implying the predicted role of intestine enzymes in BPH digestion, possibly like herbivorous and hematophagus insect species.

In order to understand if these genes are involved in digestion mechanisms, we randomly selected 41 genes to analyze their expression specificity. As a result, nine genes showed intestine-specific expression, including the following products: two serine proteases, trypsin-like protease (ID50510) and serine proteinase-like protein 1 (ID39856); two cysteine proteases, cathepsin B-like protease (ID45985) and cathepsin L proteases (ID50175); two lipases, lipase 1 precursor (ID42634) and lipase 3 (ID14017); one beta-glucosidase (ID51029); one alpha-amylase (132); one sugar transporter 15 (ID49203). These genes displayed similar tissue-specific expression patterns. Their transcripts were detected at significantly higher expression levels in intestine, but expression of these genes was extremely low or almost non-detectable in carcass and fat body tissues (Fig. 6). The intestine-specific expression strongly implied that protein, lipid and glucose cabalisms occur in BPH intestine. The exclusive expression of several genes in the intestine (i.e., *trypsin-like protease*, *cathepsin B-like protease*, *cathepsin L proteases*, *lipase 1 precursor* and *alpha-amylase*) suggests that these digestive enzymes probably function in the digestion of macromolecules in BPH intestine and may play important roles in absorption and utilization of nutrients from fluid food sources. The detailed molecular mechanisms need to be elucidated to understand whether these digestive enzymes are indispensable for BPH physiological processes.

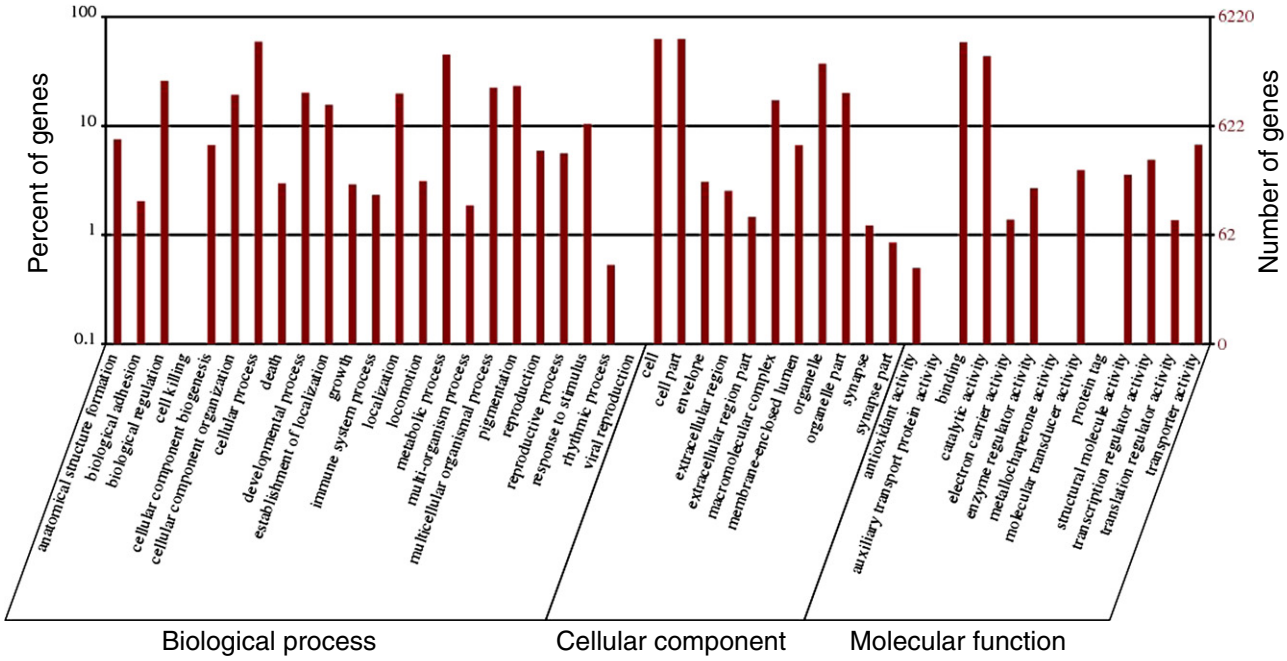

Fig. 4. Gene ontology classification (GO) of the intestine-specific BPH unigenes. Out of 21,405 unigenes, 6,220 sequences are annotated within the GO database into three main categories: biological process, cellular component and molecular function. The left and right Y-axes indicate the percentage and number of genes in a category, respectively.

2.6. Immune responsive genes

Insects rely on innate immune reactions for their defense against microbial infection. Bacterial and fungal cell wall components, such as peptidoglycan, lipopolysaccharide and beta-1,3-glucan, are strong elicitors of immune responses in insects. Accordingly, a variety of pattern recognition proteins mediate immune responses and induce the expression of immune responsive genes and activation of the prophenoloxidase activating cascade [20]. However, as one of the most serious agricultural pests, little is known about BPH immune mechanisms and molecular pathways that recognize and eliminate invaders. The understanding of immune mechanisms is meaningful for identifying potential target molecules in the BPH immune system.

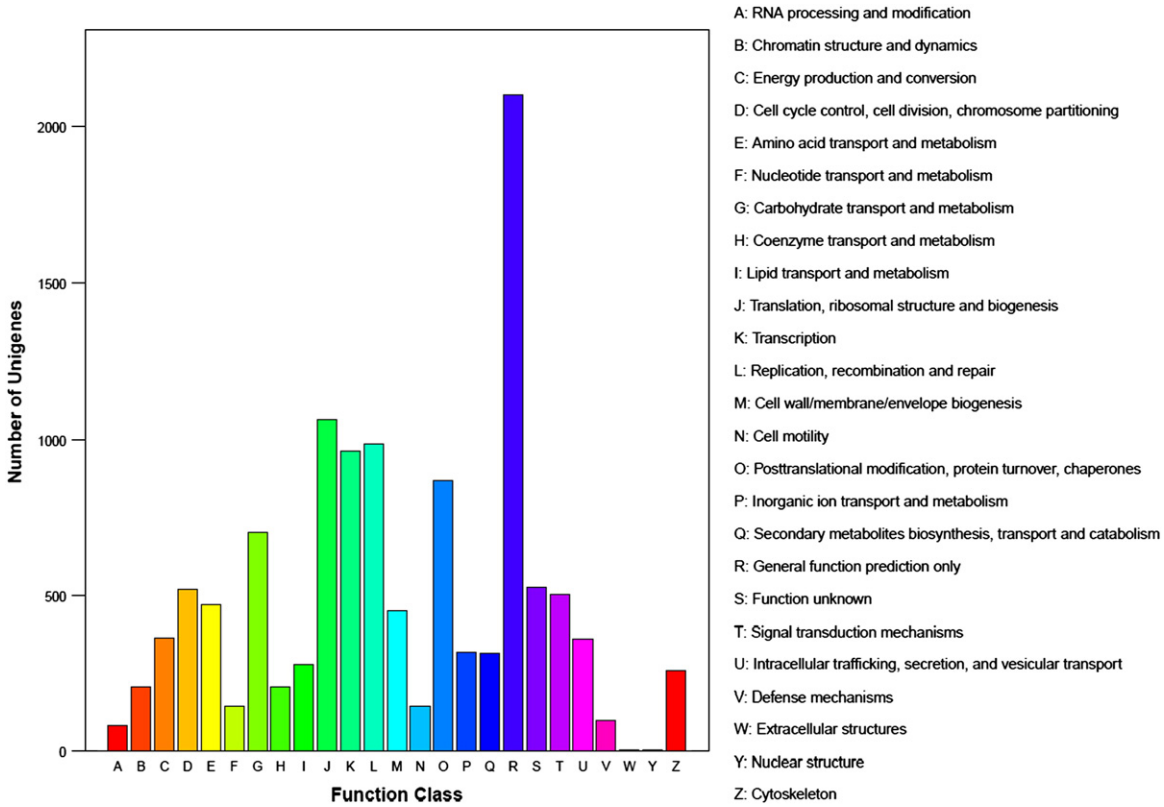

Fig. 5. Clusters of orthologous groups (COG) classification of the intestine-specific BPH unigenes. More than half of the unigenes (11,961 sequences) have a COG annotation among the 25 categories.

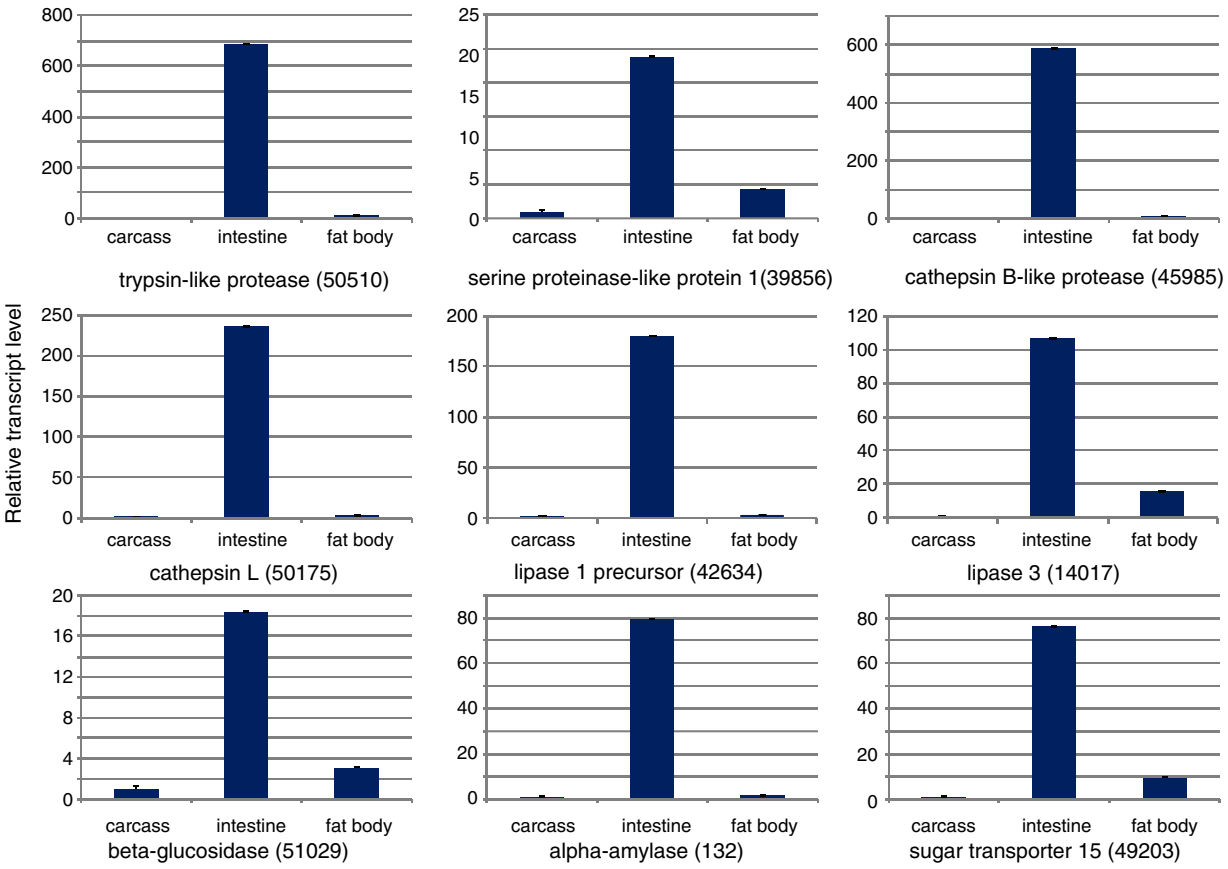

Fig. 6. Tissue specificity of digestion-related gene expression. Total RNA was extracted from the intestines, fat bodies and carcasses of 30 BPH nymphs, respectively. First-strand cDNA (20 ng) was analyzed in each qRT-PCR reaction. The reactions were performed with specific primers for amplifying the digestion-related genes. The relative expression levels of each gene in each tissue were normalized using the BPH *18s rRNA* threshold cycle (C_t) values that were obtained from reactions run on the same plate. In each assay, the expression level was normalized to the lowest expression level, which was arbitrarily set at one. Three technical replication was conducted and the mean ± SD ($n = 3$) was calculated to measure relative transcript levels using the $\Delta\Delta C_t$ method. The number in parentheses represents the ID number of unigenes in the BPH intestine-specific transcriptome data.

The insect midgut has classically been viewed as a tissue primarily involved in digestion and detoxification [19]. In this study, intestine-specific transcriptome analysis revealed a wealth of hits corresponding to genes implicated in immune response (Additional file 3). All of these hits are distinct sequences that correspond to 7 peptidoglycan recognition proteins (PRGP), 5 beta-1,3-glucan recognition proteins (beta-GRP), 4 gram-negative bacteria-binding proteins (GNBP), 19 serine proteinase inhibitors or serpins, 11 C-type lectins, 11 lysozymes, 8 transferrins and 2 immune-induced Hdd11 homologues. Out of these hits, 25 sequences had not been identified yet in BPH, based on searching NCBI and UNKA (BPH) EST databases.

Comparison of the BPH intestine transcriptome with other insect species showed that the diversity of predicted immune responsive genes expressed in the BPH intestine is very similar to that in *M. sexta* and *L. longipalpis* midguts, which had a wide range encoding the recognition proteins, lectins, lysozymes and defensins and the most abundant transcripts representing serine protease inhibitors or serpins [17,19]. Two transcripts for the immune responsive *Hdd11* genes were identified only in BPH transcriptome datasets.

PGRPs are conserved in insect and mammalian innate immunity [21,22]. *Drosophila melanogaster* PGRPs are the most abundant immune proteins, and they constitute a family of highly diverse 16 PGRP homologues [23]. PGRPs are not only the initial sensors of bacteria, but also serve as an off switch for innate immunity with their amidase activity [24]. In this study, seven distinct sequences encoding PGRPs, which included two novel *PGRP* genes, were identified in BPH intestine (Additional file 3). We investigated their expression specificity in BPH tissues. As shown in Fig. 7, BPH *PGRP* genes exhibited different tissue-specific expression patterns. Two *PGRP* transcripts (ID21730 and 44613) were detected at extremely low levels in carcasses and fat bodies, but were at very high levels in intestines, whereas three *PGRPs* (ID33525, 38087 and 28448) had the highest expression levels in intestines, followed by lower expression levels in fat bodies and carcasses. Two other *PGRP* (ID38993 and 37496) genes were detected at very low levels in carcasses, fat bodies and intestines (data not shown). In insect immune system, GNBP and beta-GRP are important pattern recognition molecules. In this study, we identified two novel *GNBP* genes in the BPH intestine transcriptome dataset. We analyzed *GNBP* (ID37537) and *beta-GRP* (ID13924) gene expression in these tissues. Significantly higher expression levels of these two genes were observed in fat bodies, while much lower levels were seen in carcass and intestine tissues. These results suggested the possibility that the BPH intestine is able to respond to bacterial infections, i.e., gram-positive and some negative bacteria strains, whereas the immune responses against gram-negative bacteria and fungal infections may be translocated across the intestine to the fat body as suggested in *Drosophila* [25].

Of particular note in BPH intestine transcriptome dataset is the immune responsive gene, *Hdd11*. *Hdd11* homologues have been identified from several insect species and their expression was strongly induced by bacteria infection [26–30]. The recent studies reported that Hdd11 protein is involved in melanization cascade in *Antheraea mylitta* and *Bombyx mori* [29,31]. In this study, we investigated *Hdd11* gene expression specificity. Two *Hdd11* genes displayed completely different expression patterns. One gene (ID43241) had very high transcript levels in the intestine, but very low expression levels in carcasses and fat bodies; the other gene (ID45889) had extremely low transcript levels in the intestine and

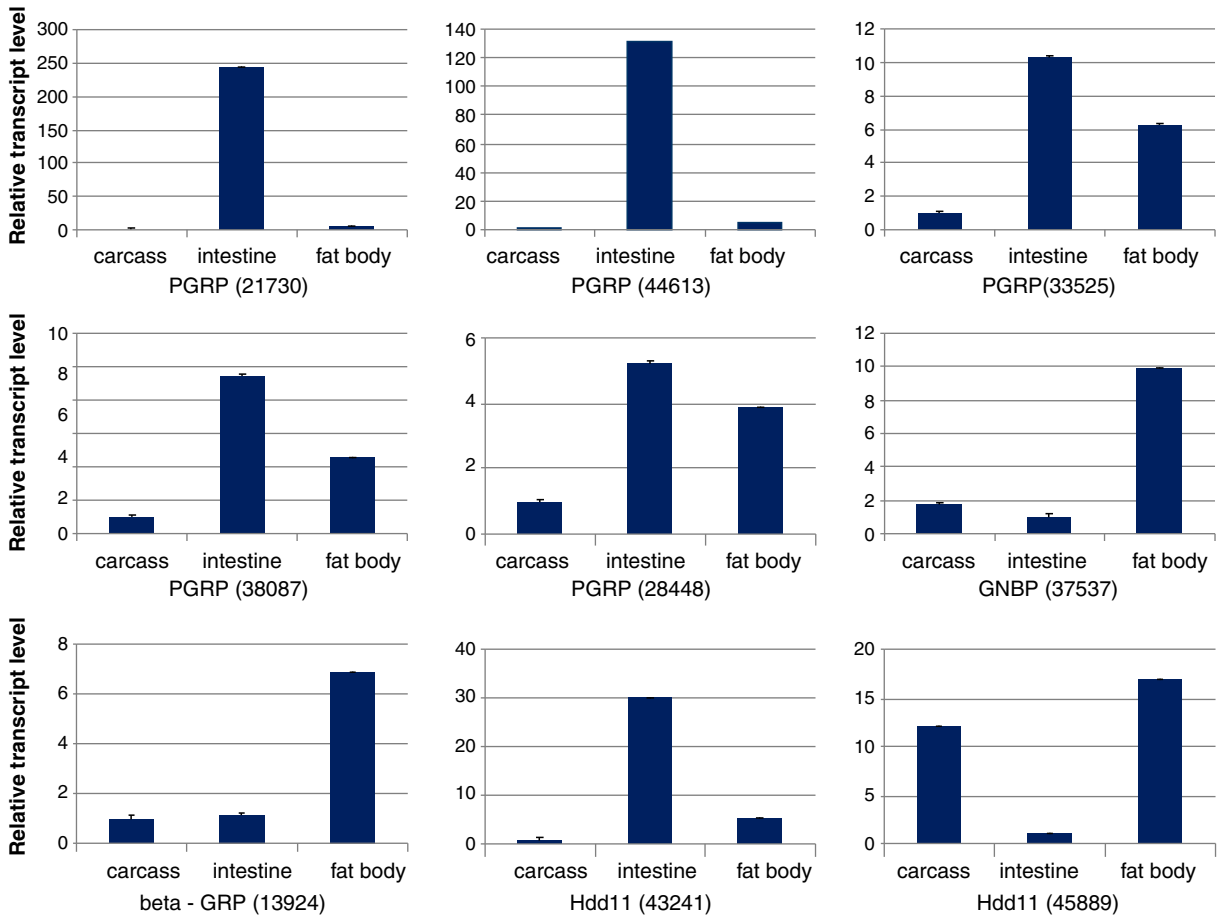

Fig. 7. Tissue specificity of immune responsive-related gene expression. The genes involved in immune responses were analyzed for their tissue-specific expression by qRT-PCR. The experimental methods were performed as described in Fig. 6.

high expression levels in carcasses and fat bodies, implying that these genes might have the different functional purposes in the BPH immune system.

2.7. Detoxification-related genes

We obtained abundant transcripts that are related to detoxification mechanisms (Additional file 4). Cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs) and carboxylesterases are members of the three major multigene enzyme families that are primarily responsible for xenobiotic metabolism (i.e., insecticides and plant toxic allelochemicals) [32]. Some individual enzymes from several insect species including BPH have been clearly implicated in insecticide resistance [9,10,33,34], but more enzymes remain with unknown functions. In this study, our transcriptome data identified 102 hits encoding P450s, which were the most abundant sequences among the detoxification-related genes. Twenty-two out of these hits predicted novel P450 genes in BPH. Besides, the abundant sequences encoding carboxylesterases (37) and GSTs (28) were identified in BPH intestine. Of these, three *carboxylesterases* and two *GST* sequences had not been previously identified in BPH.

Most insect species metabolise xenobiotics such as insecticides and secondary plant chemicals using a suite of detoxification enzymes, i.e., P450s, GSTs and carboxylesterases [35]. Analysis of fully sequenced insect genomes have identified 160 P450s in *Aedes aegypti* [36], 115 in the green peach aphid *Myzus persicae* [37], 105 in *Anopheles gambiae* [38], 85 in *Drosophila melanogaster* [39], 83 in the green pea aphid *Acyrthosiphon pisum* [37], and 46 in the western honey bee *Apis mellifera* [40]. Besides, 76 and 123 P450s were identified in *M. sexta* and the greenhouse whitefly *Trialeurodes vaporariorum* transcriptomes [18,35]. The current number of P450s in the BPH transcriptome (102) is within the range of P450s identified in these insect species (46–160); additional P450 genes may await discovery due to the BPH genome information not available.

In insects, known carboxylesterases can be divided into 13 clades, seven of which are represented in the BPH intestine transcriptome dataset. The transcripts for carboxylesterases (clade E) and juvenile hormone esterases (clade F) are abundantly indentified in BPH intestine. The number of BPH carboxylesterases are more than that in *A. pisum*, *M. persicae*, *A. mellifera*, and *D. melanogaster* where 22–35 sequences were identified [37], and less than the number (40–78) found in *T. vaporariorum*, *M. sexta*, *A. aegypti* and *A. gambiae* [18,35,36]. The GSTs indentified in BPH intestine were assigned Delta, Epsilon, Omega, Sigma, Theta and microsomal classes. Most of the BPH GSTs (11) belong to the Delta class, members of which are known to play a role in insecticide detoxification in other insect species [40]. The number of GSTs (28) in BPH intestine transcriptome is within the range identified in other insect species (10–44) [18,35–37] (Additional file 5).

We randomly selected several detoxification-related genes to analyze their expression specificity in BPH tissues. As shown in Fig. 8, two *P450* genes, *CYP4CE1* (ID15215) and *CYP6AY1* (ID7650), had significantly high transcript levels in fat body, but very low expression levels in intestine and carcass tissues. A similar expression pattern was seen in *juvenile hormone esterase* (ID16400) gene. In contrast, the *GST* gene (ID27765) showed higher expression levels in intestine than fat body, suggesting the importance in BPH intestine. The tissue-specific gene expression may reflect the functional diversification of these

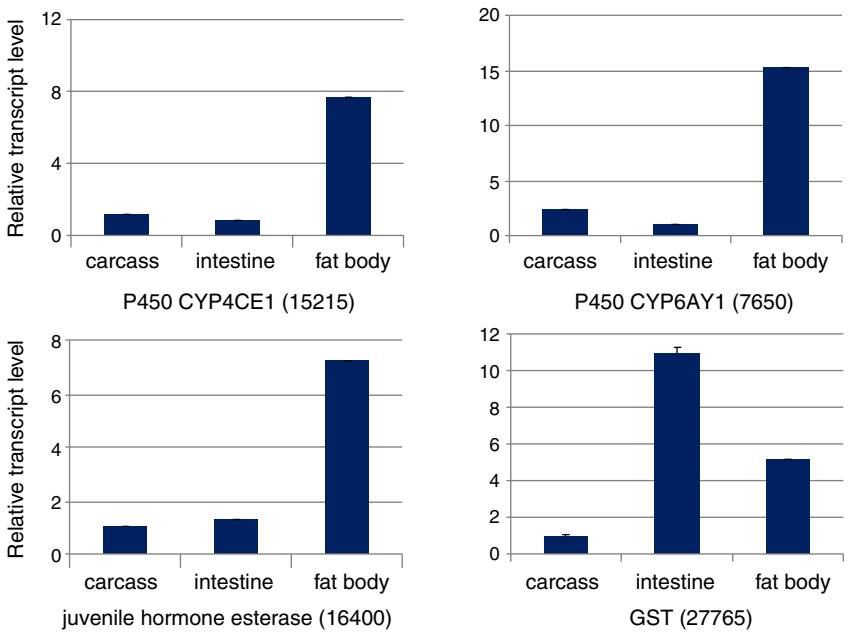

Fig. 8. Tissue specificity of detoxification-related BPH gene expression. Genes that were potentially associated with xenobiotic metabolism were analyzed for tissue-specific expression. The qRT-PCR procedure was performed as described in Fig. 6.

detoxification-related genes, and thus provides potential resistance-associated target candidates that will enable the resistance status of BPH to be monitored.

3. Conclusion

In this study, following the application of Illumina sequencing technology to genes that are important in BPH development, wing dimorphism and sex differences [8], the tissue-specific transcriptome revealed an enormous amount of genes that are potentially relevant to digestion, detoxification and immune defense in the BPH intestinal tract. Though BPH genome data were not available, this intestine transcriptome analysis provided a total of 53,553 unigenes, and half of these genes had an annotation with matches in the NCBI database. The digestion-, immune- and detoxification-related transcripts were abundantly identified in this dataset. Several genes exhibited exclusive expression in intestine, including two immune-related *PGRP*s and an *Hdd11* homologue, and digestion-related *trypsin-like protease*, *cathepsin B-like protease*, *cathepsin L proteases*, *lipase 1 precursor* and *alpha-amylase.* These genes could be potential targets for BPH management. In addition, the intestine-specific transcriptome analysis generated a large number of genes newly identified in BPH, which provided a substantial contribution to existing sequence resources. The expression specificity and function of more genes included in these data will need to be elucidated, which would be useful for understanding the physiological and immunological mechanisms occurring in BPH intestinal tract and could provide valuable strategies for BPH management in the future.

4. Materials and methods

4.1. Insects

The BPH strain was originally collected from a rice field located in the Huajiachi Campus of Zhejiang University, Hangzhou, China. The insects used in this experiment were the offspring of a single female and were reared at 28 ± 0.5 °C on rice seedlings (Xiushui 128) under a 12:12 h light:dark photoperiod.

4.2. Collection of intestines

BPH nymphs and adults were anesthetized on ice for 20 min and dissected under a Leica S8AP0 stereomicroscope. The whole intestines including the esophagus, fore-diverticulum, midgut and hindgut were isolated and quickly washed in a diethylpyrocarbonate (DEPC)-treated PBS solution (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and were immediately frozen at −80 °C.

4.3. RNA isolation and cDNA library preparation for transcriptome analysis

Total RNA was isolated from BPH intestines using the SV Total RNA Isolation System (Promega). To obtain complete gene expression information, a pooled RNA sample isolated from BPH at different developmental stages, sexes and wing forms (macropterous and brachypterous) was used for transcriptome analysis. According to the Illumina manufacturer's instructions, poly(A)$^+$ RNA was purified from 20 μg of pooled total RNA using oligo(dT) magnetic beads and fragmented into short sequences in the presence of divalent cations at 94 °C for 5 min. The cleaved poly(A)$^+$ RNA was transcribed and second-strand cDNA synthesis was performed. After the end-repair and ligation of adaptors, cDNA products were amplified by PCR and purified using the QIAquick PCR Purification Kit to create an intestine-specific cDNA library.

4.4. Illumina sequencing analysis

The cDNA library was sequenced on the Illumina sequencing platform (GAII). The raw reads were generated using Solexa GA pipeline 1.6 and were then cleaned by removal of adaptor sequences, empty reads and low quality reads. The cleaned reads with an identity value of 95% and a coverage length of 100 bp were assembled using SOAP *de novo* software [41]. The resultant contigs were joined into scaffolds using paired-end joining and gap-filling. The scaffolds were clustered to generate distinct gene sequences (unigenes) using TGI Clustering tools [42].

The obtained unigenes larger than 350 bp were annotated by searching the GenBank database with the BLASTX algorithm (http://

www.ncbi.nlm.nih.gov/), using an *E*-value cut-off of 10^{-5}. Functional annotation by gene ontology terms (GO) was performed using Blast2go software (http://www.blast2go.org/). COG annotations of unigenes were performed using Blastall software against Cluster of Orthologous Groups database.

4.5. Quantitative real-time PCR (qRT-PCR) analysis

Fat bodies and intestinal tracts were dissected from *N. lugens* 5th instar nymphs. As the quantity of an individual nymph is extremely low, the fat bodies, intestinal tracts and the remaining carcasses from 30 nymphs were pooled into each tissue sample, respectively. Total RNA was isolated from each tissue sample using the SV Total RNA Isolation System (Promega). RNA was treated with 10 U of DNase I (TaKaRa) following the manufacturer's instructions. The concentration of DNase I-treated RNA was adjusted with DEPC-treated H2O to 1 μg/μl, and 1 μg of RNA was reverse-transcribed in a 10 μl reaction using the AMV RNA PCR Kit (TaKaRa). qRT-PCR was performed on an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR *Premix Ex Taq* Kit (TaKaRa), according to the manufacturers' instructions. The first-strand cDNA (2 μl) and the no-template control (NTC, 2 μl) were used as templates for three technical replication assays in each 20 μl reaction mixture under the following conditions: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Fluorescence of PCR products was detected by adding a heat-dissociation protocol (temperature range, 60–95 °C) during the last step of each cycle. Following amplification, melting curves were constructed and data analysis was performed on an ABI 7500 system equipped with SDS software. Specific primer sets were designed for genes (Additional file 6). As an internal control, expression of the BPH *18s rRNA* gene (GenBank accession no. JN662398) was analyzed using the following primers: 5′-CGCTACTACCGATTGAA-3′ (sense primer) and 5′-GGAAACCTTGTTACGACTT-3′ (antisense primer). The specificity of the primers was confirmed using NCBI BLAST algorithms (http://www.ncbi.nlm.nih.gov/). The results were standardized to the expression level of BPH *18s rRNA*. An NTC sample was run to detect any contamination and to determine the degree of dimer formation. The $\Delta\Delta C_t$ method was used to analyze the relative differences in the transcript levels.

Supplementary materials related to this article can be found online at doi:10.1016/j.ygeno.2012.02.002.

Acknowledgments

This work was supported by National Basic Research Program of China (973 Program, No. 2010CB126205) and Grants from the Department of Agriculture of China (2008zx08010-003).

References

[1] H. Hibino, Biology and epidemiology of rice viruses, Annu. Rev. Phytopathol. 34 (1996) 249–274.
[2] J.A. Baum, T. Bogaert, W. Clinton, G.R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, Control of coleopteran insect pests through RNA interference, Nat. Biotechnol. 25 (2007) 1322–1326.
[3] Y.B. Mao, W.J. Cai, J.W. Wang, G.J. Hong, X.Y. Tao, L.J. Wang, Y.P. Huang, X.Y. Chen, Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol, Nat. Biotechnol. 25 (2007) 1307–1313.
[4] X. Zhang, J. Zhang, K. Zhu, Chitosan double stranded RNA nanoparticle mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (*Anopheles gambiae*), Insect Mol. Biol. (2010) 683–693.
[5] W. Zha, X. Peng, R. Chen, B. Du, L. Zhu, G. He, Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*, PLoS One 6 (2011) e20504.
[6] D.R.G. Price, J.A. Gatehouse, RNAi-mediated crop protection against insects, Trends Biotechnol. 26 (2008) 393–400.
[7] H. Noda, S. Kawai, Y. Koizumi, K. Matsui, Q. Zhang, S. Furukawa, M. Shimomura, K. Mita, Annotated ESTs from various tissues of the brown planthopper *Nilaparvata lugens*: a genomic resource for studying agricultural pests, BMC Genomics 9 (2008) 117.
[8] J. Xue, Y.Y. Bao, B. Li, Y.B. Cheng, Z.Y. Peng, H. Liu, H.J. Xu, Z.R. Zhu, Y.G. Lou, J.A. Cheng, Transcriptome analysis of the brown planthopper *Nilaparvata lugens*, PLoS One 5 (2010) e14233.
[9] G.J. Small, J. Hemingway, Molecular characterization of the amplified carboxylesterase gene associated with organophosphorus insecticide resistance in the brown planthopper, *Nilaparvata lugens*, Insect Mol. Biol. 9 (2000) 647–653.
[10] J.G. Vontas, G.J. Small, D.C. Nikou, H. Ranson, J. Hemingway, Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens*, Biochem. J. 362 (2002) 329–337.
[11] N. Buchon, N.A. Broderick, M. Poidevin, S. Pradervand, B. Lemaitre, *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation, Cell Host Microbe 5 (2009) 200–211.
[12] W.J. Ansorge, Next-generation DNA sequencing techniques, N. Biotechnol. 25 (2009) 195–203.
[13] W. Huang, G. Marth, EagleView: a genome assembly viewer for next-generation sequencing technologies, Genome Res. 18 (2008) 1538–1543.
[14] X.F. Wang, B.Q. Zhang, H.J. Xu, Y.J. Cui, Y.P. Xu, M.J. Zhang, Y.S. Han, Y.S. Lee, Y.Y. Bao, C.X. Zhang, ODV-associated proteins of the *Pieris rapae* granulovirus, J. Proteome Res. (2010) 2817–2827.
[15] H. Hayashi, A. Fukuda, N. Suzui, S. Fujimaki, Proteins in the sieve element-companion cell complexes: their detection, localization and possible functions, Funct. Plant Biol. 27 (2000) 489–496.
[16] X. Foissac, M. Edwards, J. Du, A. Gatehouse, J. Gatehouse, Putative protein digestion in a sap-sucking homopteran plant pest (rice brown plant hopper; *Nilaparvata lugens*: Delphacidae)—identification of trypsin-like and cathepsin B-like proteases, Insect Biochem. Mol. Biol. 32 (2002) 967–978.
[17] R. Jochim, C. Teixeira, A. Laughinghouse, J. Mu, F. Oliveira, R. Gomes, D.E. Elnaiem, J. Valenzuela, The midgut transcriptome of *Lutzomyia longipalpis*: comparative analysis of cDNA libraries from sugar-fed, blood-fed, post-digested and *Leishmania infantum chagasi*-infected sand flies, BMC Genomics 9 (2008) 15.
[18] Y. Pauchet, P. Wilkinson, M. van Munster, S. Augustin, D. Pauron, Pyrosequencing of the midgut transcriptome of the poplar leaf beetle *Chrysomela tremulae* reveals new gene families in Coleoptera, Insect Biochem. Mol. Biol. 39 (2009) 403–413.
[19] Y. Pauchet, P. Wilkinson, H. Vogel, D. Nelson, S. Reynolds, D. Heckel, R. Ffrench-Constant, Pyrosequencing the *Manduca sexta* larval midgut transcriptome: messages for digestion, detoxification and defence, Insect Mol. Biol. 19 (2010) 61–75.
[20] L. Cerenius, S. Kawabata, B.L. Lee, M. Nonaka, K. Soderhall, Proteolytic cascades and their involvement in invertebrate immunity, Trends Biochem. Sci. 35 (2010) 575–583.
[21] D. Kang, G. Liu, A. Lundstr m, E. Gelius, H. Steiner, A peptidoglycan recognition protein in innate immunity conserved from insects to humans, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 10078–10082.
[22] C. Liu, Z. Xu, D. Gupta, R. Dziarski, Peptidoglycan recognition proteins, J. Biol. Chem. 276 (2001) 34686–34694.
[23] T. Werner, G. Liu, D. Kang, S. Ekengren, H. Steiner, D. Hultmark, A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13772–13777.
[24] H. Steiner, Peptidoglycan recognition proteins: on and off switches for innate immunity, Immunol. Rev. 198 (2004) 83–96.
[25] A. Zaidman-Rémy, M. Hervé, M. Poidevin, S. Pili-Floury, M.S. Kim, D. Blanot, B.H. Oh, R. Ueda, D. Mengin-Lecreulx, B. Lemaitre, The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection, Immunity 24 (2006) 463–473.
[26] Y. Zhu, T. Johnson, A. Myers, M. Kanost, Identification by subtractive suppression hybridization of bacteria-induced genes expressed in *Manduca sexta* fat body, Insect Biochem. Mol. Biol. 33 (2003) 541–559.
[27] Y. Bao, K. Mega, Y. Yamano, I. Morishima, cDNA cloning and expression of bacteria-induced Hdd11 gene from eri-silkworm, *Samia cynthia ricini*, Comp. Biochem. Physiol. C 136 (2003) 337–342.
[28] A.B.G. Veiga, J. Ribeiro, J.A. Guimar es, I. Francischetti, A catalog for the transcripts from the venomous structures of the caterpillar *Lonomia obliqua*: identification of the proteins potentially involved in the coagulation disorder and hemorrhagic syndrome, Gene 355 (2005) 11–27.
[29] A.S. Gandhe, S.H. John, J. Nagaraju, Noduler, a novel immune up-regulated protein mediates nodulation response in insects, J. Immunol. 179 (2007) 6943–6951.
[30] S. Woon Shin, S.S. Park, D.S. Park, M. Gwang Kim, Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and subtractive cloning, Insect Biochem. Mol. Biol. 28 (1998) 827–837.
[31] Y. Bao, J. Xue, W. Wu, Y. Wang, Z. Lv, C. Zhang, An immune-induced Reeler protein is involved in the *Bombyx mori* melanization cascade, Insect Biochem. Mol. Biol. 41 (2011) 696–706.
[32] H. Ranson, C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M.V. Sharakhova, M.F. Unger, F.H. Collins, R. Feyereisen, Evolution of supergene families associated with insecticide resistance, Science 298 (2002) 179–181.
[33] G. Le Goff, S. Boundy, P. Daborn, J. Yen, L. Sofer, R. Lind, C. Sabourault, L. Madi-Ravazzi, Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*, Insect Biochem. Mol. Biol. 33 (2003) 701–708.
[34] N. Lumjuan, S. Rajatileka, D. Changsom, J. Wicheer, P. Leelapat, L. Prapanthadara, P. Somboon, G. Lycett, H. Ranson, The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides, Insect Biochem. Mol. Biol. 41 (2011) 203–209.
[35] N. Karatolos, Y. Pauchet, P. Wikinson, R. Chauhan, I. Denholm, K. Gorman, D.R. Nelson, C. Bass, R.H. Ffrench-Constant, M.S. Williamson, Pyrosequencing the

transcriptome of the greenhouse whitefly, *Trialeurodes vaporariorum* reveals multiple transcripts encoding insecticide targets and detoxifying enzymes, BMC Genomics 12 (2011) 56.
[36] C. Strode, C.S. Wondji, J.P. David, N.J. Hawkes, N. Lumjuan, D.R. Nelson, D.R. Drane, S. Karunaratne, J. Hemingway, W.C. Black IV, Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*, Insect Biochem. Mol. Biol. 38 (2008) 113–123.
[37] J.S. Ramsey, D.S. Rider, T.K. Walsh, M. De Vos, K. Gordon, L. Ponnala, S. Macmil, B. Roe, G. Jander, Comparative analysis of detoxification enzymes in *Acyrthosiphon pisum* and *Myzus persicae*, Insect Mol. Biol. 19 (2010) 155–164.
[38] R.A. Holt, G.M. Subramanian, A. Halpern, G.G. Sutton, R. Charlab, D.R. Nusskern, P. Wincker, A.G. Clark, J.M.C. Ribeiro, R. Wides, The genome sequence of the malaria mosquito *Anopheles gambiae*, Science 298 (2002) 129–149.
[39] M.D. Adams, S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, S.E. Scherer, P.W. Li, R.A. Hoskins, R.F. Galle, The genome sequence of *Drosophila melanogaster*, Science 287 (2000) 2185–2195.
[40] C. Claudianos, H. Ranson, R. Johnson, S. Biswas, M. Schuler, M. Berenbaum, R. Feyereisen, J. Oakeshott, A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee, Insect Mol. Biol. 15 (2006) 615–636.
[41] R. Li, C. Yu, Y. Li, T.W. Lam, S.M. Yiu, K. Kristiansen, J. Wang, SOAP2: an improved ultrafast tool for short read alignment, Bioinformatics 25 (2009) 1966–1967.
[42] G. Pertea, X. Huang, F. Liang, V. Antonescu, R. Sultana, S. Karamycheva, Y. Lee, J. White, F. Cheung, B. Parvizi, TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets, Bioinformatics 19 (2003) 651–652.