Transcriptomic and Expression Analysis of the Salivary Glands in White-Backed Planthoppers, *Sogatella furcifera*

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Abstract

The white-backed planthopper (WBPH), *Sogatella furcifera* (Horváth), is one of the serious rice pests because of its destructive feeding. The salivary glands of the WBPH play an important role in the feeding behaviour. Currently, however, very little is known about the salivary glands at the molecular level. We sequenced the salivary gland transcriptome (sialotranscriptome) of adult WBPHs using the Illumina sequencing. A total of 65,595 transcripts and 51,842 unigenes were obtained from salivary glands. According to annotations against the Nr database, many of the unigenes identified were associated with the most studied enzymes in hemipteran saliva. In the present study, we identified 32 salivary protein genes from the WBPH sialotranscriptome, which were categorized as those involved in sugar metabolism, detoxification, suppression of plant defense responses, immunity-related responses, general digestion, and other phytophagy processes. Tissue expression profiles analysis revealed that four of 32 salivary protein genes (multicopper oxidase 4, multicopper oxidase 6, carboxylesterase and uridine phosphorylase 1 isform X2) were primarily expressed in the salivary gland, suggesting that they played putative role in insect-rice interactions. 13 of 32 salivary protein genes were primarily expressed in gut, which might play putative role in digestive and detoxify mechanism. Development expression profiles analysis revealed that the expression level of 26 of 32 salivary protein genes had no significant difference, suggesting that they may play roles in every developmental stages of salivary gland of WBPH. The other six genes have a high expression level in the salivary gland of adult. 31 of 32 genes (except putative acetylcholinesterase 1) have no significant difference in male and female adult, suggesting that their expression level have no difference between sexes. This report analysis of the sialotranscriptome for the WBPH, and the transcriptome provides a foundational list of the genes involved in feeding. Our data will be useful to investigate the mechanisms of interaction between the WBPH and the host plant.
Introduction

The saliva of insect herbivores contains a diversity of digestive enzymes and components, which either induce or inhibit plant defence [1]. Therefore, as the first substance to contact the plant, herbivore saliva plays an important role in the ingestion of food and in the interaction between plant and herbivore [2]. Hemipterans are phloem feeders with piercing-sucking mouthparts. The salivary organs of hemipterans are a pair of primary and accessory salivary glands, which produce two primary types of saliva: coagulable and watery [3, 4]. During feeding, they discharge the gelling and watery saliva into the rice plant tissues. Furthermore, most hemipteran vectors secrete and inoculate pathogens into healthy plants through the proteins of saliva [5, 6]. Thus, the saliva of phloem feeders is a mediator of plant-(pathogen)-insect interactions [5, 6].

The white-backed plant hoppers (WBPH; Sogatella furcifera; Hemiptera: Delphacidae), which is one of the most destructive insect pests of rice (Oryza sativa L.) in Asia. Adults and nymphs cause rice physiological abnormalities through the mouth to suck rice phloem sap, seriously lead to the plants die. WBPH is also the vector of southern rice black-streaked dwarf virus (SRBSDV) [7, 8], which outbreaks in 2009 not only in the southern Chinese province but also in northern Vietnam, and Japan [9].

Despite their importance as pests, little is currently known about the secretion and composition of WBPH saliva. Because of the important functions of herbivore saliva in plant-insect interactions, the saliva of WBPHs is likely to play a central role in the interaction between insect and rice. Therefore, information on salivary secretions is crucial for understanding the interactions between WBPHs and host plants, and the characterisation of WBPH saliva will provide new insights into WBPH-rice interactions, including induced defences in rice and WBPHs. Additionally, the results will facilitate the development of better strategies for pest control.

The transcriptomes of the salivary glands of phloem-sap feeders were determined for several hemipterans, including the rice brown plant hopper (Nilaparvata lugens (Stål); Hemiptera: Delphacidae) [2], the small brown planthopper (Laodelphax striatellus (Fallén); Hemiptera: Delphacidae) [10], the whitefly (Bemisia tabaci (Gennadius); Hemiptera: Aleyrodidae) [5], the potato leafhopper (Empoasca fabae (Harris); Hemiptera: Cicadellidae) and the green rice leafhopper (Nephotettix cincticeps (Uhler); Hemiptera: Cicadellidae) [11, 12]. Many proteins in the saliva (including secretory proteins) were found. Sharma et al. (2014) [4] divided the salivary proteins of plant-feeding hemipteroids into three categories according to the function: ‘detoxifying plant allelochemicals and altering plant-defence mechanism’ (e.g., glucose dehydrogenase, glucose oxidase, phenol oxidases, laccase, peroxidases, catalase, trehalases); ‘plant-cell degrading’ (e.g., α-glucosidases, amylase, sucrase, acid, alkaline phosphatases and peptidases); ‘other proteins’ (e.g., calcium-binding proteins, effector proteins and other non-enzymatic proteins).

Glucose dehydrogenase was identified to detoxify plant defensive compounds in Russian Wheat Aphid (Diuraphis noxia) [13]. The phylogenetic relationships and expression patterns of multicopper oxidase gene family was first analysed to elucidate their functions in the context of N. lugens growth and development [14]. The tissue-, development-, and sex-specific expressions of serine protease gene family in N. lugens were clarified the potentially functional roles in the biological process [15]. Angiotensin converting enzyme was considered as a potential target for development of insect growth regulators [16]. Soluble and membrane-alkaline phosphatases were identified in N. lugens, and soluble-alkaline phosphatases may play a role in development and insecticide tolerance [17]. Levels of soluble and membrane-bound trehalases expression in various stages and tissues of B. tabaci suggested that soluble trehalases may prevent trehalose in salivary gland from leaking and entering into plants along with saliva, while membrane-bound trehalases may play role in trehalose catabolism during development [18].
Although many salivary proteins were identified in the hemipterods, the salivary components of WBPH and the functions of these different components are unknown. Because the complete genome of the WBPH is not available yet, a more effective method is required for transcriptomic analysis of the salivary glands of the WBPH. The sialotranscripome of WBPH was sequenced on Illumina sequencing platform with depth of 14.58 G, which can dig into more genes and assemble more long and accurate sequences.

In this study, the WBPH sialotranscripome was sequenced and assembled, and analysis of sialotranscripome could get accurate sequences of annotated genes and reveal gene pathways. We identified the tissue and development expression of 32 salivary protein genes in WBPH. Based on this study, a rich molecular resource for future functional studies on primary salivary glands can be provided, which will contribute to a better understanding of the insect-rice interactions.

**Methods**

**Insect rearing and salivary glands collection**

The original WBPH adults used in the experiment were collected separately from Xing'an County, Guilin City, Guangxi Zhuang Autonomous Region, China, in 2013, which is the Scientific Observing and Experimental Station of Crop Pests of Guilin/Guilin Branch, Institute of Plant Protection, Chinese Academy of Agricultural Sciences. The field studies did not involve endangered or protected species, and no specific permissions were required for these activities in this station. After collection, the planthoppers were then maintained artificially in our laboratory. The WBPHs cultured on TN-1 rice plants were reared in an artificial climatic chamber at 26 ± 1°C and 70 ± 10% relative humidity under a 16/8 h light/dark photoperiod.

Salivary glands (approximately 600 of each sex) were collected from 1- to 3-day-old adults for transcriptome sequencing. The adult WBPHs were anesthetised with ice and placed in a petri dish that was also on ice. The salivary glands of WBPHs were dissected in a drop of sterilised 1× Phosphate Buffered Saline (1× PBS) solution with microforceps (Shanghai Medical Instruments Ltd., Corp.) under an anatomical lens (Leica Microsystems GmbH, Wetzlar, Germany). The head was first pulled from the pronotum, and then the pair of salivary glands that emerged at the distal end of the severed head was carefully removed with microforceps. Different tissues including salivary gland (SG), head (without salivary gland), gut, malpighian tubule (MT), and remaining body (RB) of adult WBPHs and salivary gland at different developmental stages (2nd-3rd instar, 4th-5th instar, female and male adult) of WBPHs were collected for real-time quantitative PCR (qPCR) test. All collected tissues were immediately frozen and stored at -80°C until use.

**Library construction of the salivary glands and Illumina sequencing**

Approximately 300 female and 300 male salivary glands were pooled together for one sample. Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The degradation and contamination of RNA sample was monitored on 1% agarose gels, and the RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and the RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Messenger RNAs were purified from total RNA using poly-T oligos attached to magnetic beads. The mRNAs were then shared into short fragments using fragmentation buffer. First strand cDNA were synthesized using random hexamer primer and M-MuLV Reverse
Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBotCluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

Bioinformatics data analysis

Raw reads of fastq format were firstly processed through in-house perl scripts. In this step, clean reads were obtained by removing low quality reads and reads containing adapter, reads containing ploy-N. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. Transcriptome assembly based on clean reads with high quality was accomplished using Trinity [19]. Following the assembly, homology searches of all unigenes were conducted using the BLASTx and BLASTn programs against Nr (non-redundant protein database) and Nt (nucleotide sequence databases) in NCBI with an E-value less than 1.0E-5. Unigenes were also aligned by BLASTx with other protein databases, including Gene Ontology (GO), Swiss-Prot, Protein Family (PFAM), Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology (KO), and euKaryotic Ortholog Groups (KOG), to identify protein with high sequence similarity and assign putative functional annotations. By using Blast2GO program [20], GO terms were extracted from the best hits obtained from the BLASTx against the Nr and PFAM.

Identification of putative WBPH Salivary protein genes

Salivary protein genes which were categorized as those involved in sugar metabolism, detoxification, suppression of plant defense responses, immunity-related responses, general digestion, and other phytophagy processes were selected in the sialotranscripome annotation of WBPH. The longest open reading frame (ORF) of each unigene was determined using the ExPASY Translate Tool (http://web.expasy.org/translate/). The sequences of all candidate salivary protein were checked on the BLASTx program at the NCBI by manually.

Verification of the salivary protein sequences by cloning and sequencing

Gene-specific primers designed by Primer 5.0 program were used to clone the ORF or partial sequences of each salivary protein gene (see Supplementary Material: S1 Table). The total RNA from the whole adult was extracted by using RNAiso plus Reagent (TaKaRa, Dalian, China) and template cDNA was synthesized using the Fast Quant RT kit (TIANGEN, Beijing, China). Each PCR reaction (25 μl volume) was carried out with 200 ng cDNA and the cycling conditions were set at 95°C for 3 min with 35 cycles of 94°C for 30 sec, 57°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were gel-purified and cloned into the pGEM®-T Easy Vector (Promega Corporation, Madison, USA), and the insert was
sequenced with standard M13 primers. Then all sequences of candidate salivary protein genes were manually checked by the BLASTx program at the NCBI.

Real-time qPCR measurement

The relative transcript abundance of salivary protein genes in different tissues (SG, head, gut, MT, and RB) and in the salivary gland of different developmental stages and sexes (2\textsuperscript{nd}, 3\textsuperscript{rd} instar, 4\textsuperscript{th}, 5\textsuperscript{th} instar, female adult, male adult) were analysed by qPCR using an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Due to the tissues (SG, gut, MT) were infinitesimal, total RNA was extracted by using the PureLink RNA Mini Kit (Life technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA of other body parts (head and RB) was extracted by using RNAiso plus Reagent (TaKaRa, Dalian, China). For each total RNA sample, 0.6 μg of RNA was reverse-transcribed to cDNAs by the Fast Quant RT Kit (TIANGEN, Beijing, China). Two references genes Ribosomal protein L9 (GeneBank Acc. KP735523) and Ribosomal protein L10 (GeneBank Acc. KP735524) were used for normalization, as they were stably expressed at different tissues and development stages [21]. The primers of target and reference genes were designed with the program primer3 web (version 4.0.0) (http://primer3.ut.ee/) and listed in Supplementary Material: S2 Table. The specificity and efficiency of each primer was validated by analysing standard curves with a five-fold cDNA dilution series. Each qPCR reaction was conducted in a 20 μl mixture containing 10 μl of Bester\textsuperscript{th} SybrGreen qPCR mastermix (DBI\textsuperscript{th} Bioscience, Germany), 0.4 μl of each primer (10 μM), 0.04 μl of 50 × ROX Reference Dye, 4 μl of sample cDNA, and 5.16 μl of sterilized H2O. The qPCR cycling parameters were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 31 sec, melt curves stages at 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Negative controls without template were included in each experiment. To check reproducibility, the qPCR reaction for each sample was performed in three technical replicates and two biological replicates. The comparative 2^{-ΔΔCT} method [22] was used to calculate the relative quantification between tissues and salivary glands in development stages and sexes of WBPH. Data analysis was performed using the SPSS Statistics 19.0 software (IBM SPSS Statistics Inc., Chicago, IL, USA). A one-way nested analysis of variance (ANOVA) and Duncan’s new multiple range test (p < 0.05) were used to calculate the relative expression of each target gene. The values were presented as the mean ± SE when applicable.

Results

Illumina HiSeq sequencing and reads assembly

The cDNA libraries of salivary gland of WBPH were sequenced with Illumina HiSeq. A total of 99,820,374 raw reads were produced from salivary gland samples. After trimming the adaptor sequences and removing low quality sequences, 97,174,264 clean reads were remained for following assembly and produced 65,595 (mean length 874 bp) transcripts, respectively. The longest transcript of each gene is a unigene, and the number of unigenes was 51,842 (mean length 746 bp) (Table 1). The transcriptome raw reads had been deposited with NCBI SRA database (accession number: SRR3211109).

Functional annotation of the WBPH salivary gland unigenes

In total, all of 51,842 unigenes matched known proteins within all databases, through using Nr, Nt, KO, SwissProt, PFAM, GO and KOG. Through annotation by BLASTx and BLASTn program with the E-value cut-off of 1.0E-5, 17,767 of the 51,842 unigenes (34.27%) had BLASTx hits in the Nr databases and 4,068 (7.84%) had BLASTn hits in the Nt databases. The species
distributions of the best BLASTx hit for each sequence are shown in Fig 1A. The highest percentage of unigene sequences were matched with *Zootermopsis nevadensis* (26.0%), followed by *Acyrthosiphon pisum* (6.5%), *Diaphorina citri* (6.0%), *Tribolium castaneum* (6.0%) and *N. lugens* (4.1%). A similar pattern of highest similarity with *T. castaneum* in the BLAST annotation was also reported for the transcriptomes of *S. furcifera*, *N. lugens* and *N. lugens* salivary glands, with a similarity of 16.17%, 18.89% and 14.34% with *T. castaneum*, respectively [2, 23, 24].

GO assignments were used to functionally classify the predicted proteins. The GO terms were functionally grouped into three primary divisions: ‘biological process,’ ‘molecular function’ and ‘cellular component.’ Among the 51,824 unigenes, 14,753 (28.45%) could be assigned to various GO terms and annotated, and they were categorised into 55 functional groups (Fig 1B). To investigate the biological pathways that were active in the salivary glands, the sequences were assigned to the reference canonical pathways in the KEGG (Fig 2). A total of 7,876 unigenes were mapped separately to a total of 261 pathways, the top 32 of which were depicted in Fig 2. Among these pathways, the largest number of sequences (2,548 unigenes) was involved in ‘metabolic pathways’, which confirmed that metabolism is important in the salivary glands. The GO annotations and KO classifications indicated that the salivary glands were active in metabolism, binding and signal transduction.

**Identification of WBPH salivary proteins**

We identified 32 putative salivary protein genes in the WBPH Illumina HiSeq sialotranscriptome data (Table 2). 24 of 32 putative salivary protein genes (except serine protease snake-4, serine protease snake-6, serine protease-11, cytochrome P450 CYPSF04, putative acetylcholinesterase 1, prostatic acid phosphatase, calcium/calmodulin-dependent protein kinase 2 isform X2 and calcium/calmodulin-dependent protein kinase type 1 isoform X3) have intact ORF with lengths ranging from 1050 bp to 2805 bp. Membrane-bound trehalase (GeneBank Acc. AFO54713.1) and NADPH-cytochrome P450 reductase (GeneBank Acc. AHO93009.1) have been identified in WBPH. All of the 32 salivary proteins were manually checked by the BLASTx program and then named according to the highest protein similarities with the high amino acid identities (48%–100%) of the best BLASTx hit at NCBI. The nucleotide sequences of the 32 salivary protein genes were confirmed by cloning and sequencing, and then deposited in the GenBank under the accession numbers KU764420 to KU764449 except membrane-bound trehalase and NADPH-cytochrome P450 reductase, which were further aligned to analysis and found to hit 100% with the deposited ones.

**Tissue-specific expression patterns**

The expression of the 32 WBPH salivary protein genes in different tissues (SG, head, gut, MT, and RB) were determined by using real-time qPCR. With the expression level in MT for

| Table 1. An overview of sequencing and assembly process. |
|-----------------------------------------------|
| Total size | 14.58 G |
| Total number of raw reads | 99,820,374 |
| Total number of clean reads | 97,174,264 |
| Total number of transcripts | 65,595 |
| Transcripts mean length | 874 bp |
| Total number of unigenes | 51,842 |
| Unigenes mean length | 746 bp |

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reference, all of the salivary protein genes were expressed in all tissues, but their expression levels had significant differences (Fig 3).

In this study, we identified six genes involved in sugar metabolism. The alpha-glucosidase family 31 and neutral alpha-glucosidase AB were expressed higher in gut than in other tissues. The transcript of lysosomal alpha-glucosidase was higher in the gut and RB than in other tissues. The expression level of soluble trehalase was the highest in MT among the different tissues, and membrane-bound trehalase had the same expression level in the different tissues except in SG which was the lowest. The transcript of UDP-N-acetylglucosamine pyrophosphorylase was higher in gut than in other tissues.

We investigated 14 salivary protein genes involved in detoxification and inhibition of plant defences. Glucose dehydrogenase [acceptor] had significantly 950.0 times higher expression levels in the head than in other tissues, while its expression levels in other tissues had no

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Fig 1. Summary for the annotation of WBPH salivary gland transcriptome. (A) Species distribution of best BLASTx hits of salivary gland transcriptome. (B) Gene Ontology (GO) classifications of WBPH salivary gland unigenes according to their involement in biological process, cellular component and molecular function.

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significant difference. The expression level of alkaline phosphatase-like isoform X1 was higher both in the gut and MT than in the head, RB and SG. Multicopper oxidase 4 expressed mainly in SG, and the expression level of multicopper oxidase 6 was about 4.0 times higher in SG and head than in MT. The transcript of catalase was the highest in the gut, while the expression levels of cytochrome P450 SF, cytochrome P450 CYPSF01, cytochrome P450 CYPSF02 and cytochrome P450 CYPSF04 was about 1.8, 16.5, 22.0 and 41.0 times higher in head than in MT. The expression levels of cytochrome P450 CYPSF03, NADPH-cytochrome P450 reductase and putative acetylcholinesterase 1 were the highest in MT among the different tissues. Carboxylesterase showed an expression level of about 43.0 times higher in SG than in MT.

In this study, four genes involved in immune related and one gene involved in general digestion were identified. Prophenoloxidase activating factor 2 had a lower expression levels in SG than in other four tissues, while serine protease snake-2 had significantly 5.0 times higher expression levels in the head than in other tissues, while its expression levels in other tissues had no significant difference. Serine protease snake-4 was expressed higher in RB compared with in other tissues. The expression levels of serine protease snake-6 and serine protease-11 were about 20.0 and 6.5 times higher in head than in MT. however angiotensin converting enzyme was expressed the lowest in the gut among the five tissues.

Seven other proteins involved in signal pathway and metabolism were investigated in this study. Uridine phosphorylase 1 isoform X2 showed an expression level of about 15.0 times higher in SG than in MT. The transcripts of putative phosphorylase b kinase regulatory subunit
### Table 2. Genes of interest identified in the *S. furcifera* sialotranscriptome.

| Gene name                                      | Accession number | Query length (bp) | ORF (aa) | Complete-ness | Blastx annotation                                 | Score | E-value | % Identify |
|------------------------------------------------|------------------|-------------------|----------|---------------|---------------------------------------------------|-------|---------|------------|
| Sugar metabolism                               |                  |                   |          |               |                                                   |       |         |            |
| Alpha-glucosidase family 31                    | KU764420         | 2630              | 677      | Complete      | gb|AIA09350.1|Alpha-glucosidase family 31, partial [Periplaneta americana] | 578   | 0       | 46%        |
| Neutral alpha-glucosidase AB                   | KU764421         | 3687              | 935      | Complete      | gb|KDR15365.1|Neutral alpha-glucosidase AB [Zootermopsis nevadensis] | 1144  | 0       | 59%        |
| Lysosomal alpha-glucosidase                    | KU764423         | 3885              | 926      | Complete      | gb|KDR14932.1|Lysosomal alpha-glucosidase [Zootermopsis nevadensis] | 953   | 0       | 51%        |
| Soluble trehalase                              | KU764425         | 2813              | 575      | Complete      | gb|AFL03409.1|soluble trehalase [Laodelphax striatella] | 1092  | 0       | 95%        |
| Membrane-bound trehalase                       | AFO54713.1       | 5052              | 613      | Complete      | gb|AFO54713.1|membrane-bound trehalase [Sogatella furcifera] | 1199  | 0       | 99%        |
| UDP-N-acetylglucosamine pyrophosphorylase      | KU764442         | 3166              | 489      | Complete      | gb|AEI88647.1|UDP-N-acetylglucosamine pyrophosphorylase [Nilaparvata lugens] | 751   | 0       | 97%        |
| Detoxification and inhibition of plant defenses|                  |                   |          |               |                                                   |       |         |            |
| Glucose dehydrogenase [acceptor]              | KU764422         | 5499              | 624      | Complete      | gb|KDR15959.1|Glucose dehydrogenase [acceptor], partial [Zootermopsis nevadensis] | 1123  | 0       | 52%        |
| Alkaline phosphatase-like isoform X1           | KU764424         | 2068              | 524      | Complete      | ref|XP_012278502.1|PREDICTED: alkaline phosphatase-like isoform X1 [Orussus abietinus] | 544   | 0       | 53%        |
| Multicopper oxidase 4                          | KU764431         | 2965              | 721      | Complete      | gb|AKN21382.1|multicopper oxidase 4 [Nilaparvata lugens] | 1174  | 0       | 79%        |
| Multicopper oxidase 6                          | KU764432         | 3214              | 630      | Complete      | gb|AKN21384.1|multicopper oxidase 6 [Nilaparvata lugens] | 1213  | 0       | 91%        |
| Catalase                                       | KU764433         | 1860              | 503      | Complete      | emb|CCO56224.1|catalase [Nilaparvata lugens] | 978   | 0       | 95%        |
| Cytochrome P450 SF                             | KU764434         | 2408              | 540      | Complete      | gb|AGN52753.1|cytochrome P450 [Laodelphax striatella] | 901   | 0       | 85%        |
| Cytochrome P450 CYP6FJ1v2                      | KU764435         | 1960              | 546      | Complete      | gb|AFU86439.1|cytochrome P450 [Laodelphax striatella] | 971   | 0       | 87%        |
| Cytochrome P450 CYP6FJ1v2                      | KU764436         | 2120              | 503      | Complete      | gb|AIW79999.3|cytochrome P450 [Laodelphax striatella] | 896   | 0       | 83%        |
| Cytochrome P450 CYP6BD10v2                     | KU764437         | 2403              | 519      | Complete      | gb|AFU86445.1|cytochrome P450 [Laodelphax striatella] | 1020  | 0       | 93%        |
| Cytochrome P450 CYP6K29v                       | KU764438         | 1229              | 307      | partial       | gb|ACX54783.1|cytochrome P450 [Laodelphax striatella] | 502   | 7e-173  | 87%        |
| NADPH-cytochrome P450 reductase                | AHM93009.1       | 3613              | 677      | Complete      | gb|AAM93009.1|NADPH-cytochrome P450 reductase [Sogatella furcifera] | 1332  | 0       | 100%       |
| Putative acetylcholinesterase 1                | KU764439         | 1585              | 483      | partial       | gb|ADR73026.1|putative acetylcholinesterase 1 [Laodelphax striatella] | 832   | 0       | 83%        |
| Angiotensin converting enzyme                  | KU764440         | 3803              | 664      | Complete      | gb|AGC79111.1|angiotensin converting enzyme [Nilaparvata lugens] | 1234  | 0       | 93%        |
| Carboxylesterase                              | KU764441         | 4793              | 570      | Complete      | gb|AFN66415.1|carboxylesterase, partial [Laodelphax striatella] | 781   | 0       | 93%        |
| Immune related                                |                  |                   |          |               |                                                   |       |         |            |
| Prophenoloxidase activating factor 2           | KU764426         | 2250              | 451      | Complete      | gb|AID60320.1|prophenoloxidase activating factor 2, partial [Nilaparvata lugens] | 560   | 0       | 86%        |

(Continued)
beta were higher in gut and RB than in other tissues. The expression levels of prostatic acid phosphatase-like isoform X1 and prostatic acid phosphatase were about 9.5 and 18.0 times higher in head than in MT, while the latter was also expressed higher in gut than in SG, RB and MT. The expression level of Rac-GTP binding protein had no difference in head, gut and MT. The expression level of calcium/calmodulin-dependent protein kinase 2 isoform X2 was the highest in gut and the lowest in MT compared with other tissues.

Development and sex-specific expression patterns

The transcript abundances of 32 salivary protein genes in SG of WBPH 2nd-3rd instar nymph, 4th-5th instar nymph, female and male adult were investigated by real-time qPCR. The significantly differentiation of development and sex-specific expressions of 32 salivary protein genes were shown in Fig 4. With the expression level in SG of 2nd-3rd instar nymph for reference, most of the salivary proteins had similar expression levels in SG of developmental stages and sexes, except multicopper oxidase 4, cytochrome P450 CYP5F01, cytochrome P450 CYP5F02,
NADPH-cytochrome P450 reductase, putative acetylcholinesterase 1, and Rac-GTP binding protein. The expression level of multicopper oxidase 4 was increased from the 2nd-3rd nymph to adult stages. The relatively transcript levels of cytochrome P450 CYPSF01 and NADPH-cytochrome P450 reductase were higher in adult than in instar. Cytochrome P450 CYPSF02
showed a lower expression level in 4th-5th instar than that in other development stages. The putative acetylcholinesterase 1 was expressed higher in the stages of the 2nd-3rd instar and female adult than those in the stages of the 4th-5th instar and male adult. Rac-GTP binding protein had a lower expression level at the stages of 2nd-3rd and 4th-5th instar than that in adult.
stage. Except putative acetylcholinesterase 1, the expression levels of other 31 enzymes had no difference between female and male adult.

**Discussion**

Salivary enzymes of Hemiptera played a key role between the insect and plant. Despite there are some studies, but more thorough system of related gene research is less. In this study, we obtained 51,407 unigenes with mean lengths of 647 bp, which are a major genomic resource for investigating the salivary glands of the WBPH. The total number of unigenes obtained was much higher than the numbers of salivary gland unigenes identified in *N. lugens* (43,312 unigenes) [2] and in *B. tabaci* (13,615 unigenes) [5], which reflects the sequencing depth and coverage. In this study, 32 salivary protein genes were studied using RT-PCR and real-time qPCR in order to comprehensive evaluation of the WBPH sialotranscripome.

**Tissue-specific expression patterns**

Alpha-glucosidase family 31, neutral alpha-glucosidase AB, and lysosomal alpha-glucosidase were the enzyme types typically involved in sugar metabolism and potentially in detoxification and plant defence suppression [13]. The three glucosidases were all found in the WBPH sialotranscriptome. The gene transcripts of alpha-glucosidase family 31 and neutral alpha-glucosidase AB were detected at the highest level in the gut followed by SG and MT. Consistent with our results, α-glucosidase was active in the midgut and salivary, but the expression levels of α-glucosidase was found significantly higher in the midgut than in salivary gland of *Brachynema germari* [25]. Lysosomal alpha-glucosidase-like had a high expression levels both in gut and RB, followed by SG and MT, but was hardly detected in the head. It was suggested that these enzymes may have a role of digestion and detoxification in gut.

Trehalases play a key role in various trehalose-associated physiological processes in insects, including fight metabolism [26], chitin synthesis during molting [27, 28], and development [29]. There were two types of trehalases (soluble trehalases and membrane-bound trehalase) in insect, but they may have different roles in the physiological process [28, 30]. In this study, we found the transcripts of soluble trehalases were the highest in MT and the lowest in the head. The expression level of membrane-bound trehalase was lower in SG than in other tissues. The transcript of soluble trehalases in different tissues of *Aphis glycines* was higher in the gut compared to in the integument, fat body and embryo [31], the expressions of soluble trehalases were similar with the findings in *Spodoptera frugiperda* and *B. mori* [28, 32].

UDP-N-acetylglucosamine pyrophosphorylase (UAP) was an enzyme of chitin biosynthetic pathway which began with trehalose [33]. UAP for the development of *L. migratoria* showed that both *LmUAP1* and *LmUAP2* were widely expressed in all the major tissues besides chitin-containing tissues [34], especially *LmUAP1* had the highest expression levels in the integument and the highest levels of *LmUAP2* was found in the fat bodies of the 5th instar. In this study, UAP was identified to have the highest transcripts in the gut of WBPH, and followed by RB, head, SG and MT. Uridine phosphorylase 1 isoform X2 showed the highest expression level in SG among the five tissues in WBPH.

Glucose dehydrogenase was also found in the saliva of *D. noxia* [35], *Myzus persicae* [36], and *A. pisum* [37], and it was also identified to have higher expression level in the head than in other tissues (thorax and abdomen) of *Apis mellifera* [38].

Alkaline phosphatase played a key role in several biological processes and responded to stress, pathogenesis, or infection [39–44]. Alkaline phosphatase-like isoform X1 was identified in the WBPH. The transcripts of the enzyme were higher in the gut and MT followed by in the head, RB and SG. The expression of alkaline phosphatase in the silkworm was confined mainly
in the gut tissue [42]. Two forms (soluble and membrane-bound) of alkaline phosphatase (sALP and mALP) was identified in N. lugens [17], however alkaline phosphatase-like isoform X1 in WBPH was difficult to judge belong to sALP or mALP, because both sALP and mALP show approximately the same degree of sequences identity (43.74%-45.97%) to the alkaline phosphatase-like isoform X1. The functions of alkaline phosphatase-like isoform X1 in WBPH will be investigated in future.

There were multiple enzymes of laccases in the multicopper oxidase (MCO) family. The largest subgroup of MCOs of laccases is present in plants, fungi, bacteria and insects [14]. In this study, qPCR revealed MCO4 was expressed primarily in SG, which is similar to that of the MCO4 in N. lugens [14]. MCO6 was expressed higher in SG, head and RB among WBPH tissues, the result was similar to that in N. lugens [14]. When N. lugens was injected of MCO6 dsRNA, it led to shedding failure of old cuticles, wing deformities and a high mortality, similar to MCO6 in T. castaneum and Anopheles gambiae, these was a strong evidence of MCO6 might play a role during molting [14, 45]. But the functions of MCO6 in WBPH need further to be clarified.

Catalase is the main enzyme that decomposes hydrogen peroxide (H₂O₂) at high concentrations. H₂O₂ is a part of the free radicals system that mediates important physiological roles including signalling and defence. In this study, we found that the transcript of catalase had the maximum expression level in the gut and MT followed by RB, SG and head. Catalase of N. lugens had the higher activity in the gut than salivary gland and whole body extracts, and the activity of catalase was higher in the salivary gland of BPH fed on a hopper-resistant rice variety than a susceptible rice variety, which revealed that the catalase had a most obvious role of direct toxicity from H₂O₂ [46].

Cytochrome P450s (CYRs) might be the major detoxification enzymes when the insect adapted to its chemical environment [47]. In this study, five sequences coding for CYRs were identified, and most of the transcripts were found to be expressed in the head of adult WBPH except CYP5F03 which were expressed highly in MT. The expression levels of 64 CYPs were identified in Dendroctonus armandi, suggesting that most of the CYPs were expressed in the larvae, pupae and adult [47]. The widely different expression patterns of CYPs in WBPH suggested that CYPs might have multiple functions in the different tissues. NADPH–cytochrome P450 reductase (CPR) (EC 1.6.2.4) is an essential enzyme which may play a specific function in P450-mediated detoxification pathways and other physiological processes in insects [48]. The transcripts of NADPH–cytochrome P450 reductase in WBPH were most abundant in MT in adult and were the lowest in SG of 2nd–3rd instar nymphs. While in N. lugens, the expression levels of NADPH-cytochrome P450 reductase (NlCPR) were the highest in the abdomen in adults and in 1st instar nymphs [49].

Acetylcholinesterase (AChE, EC 3.1.1.7) was a key enzyme that regulated the level of the neurotransmitter acetylcholine and terminated nerve impulses in the nervous system of insects [50]. The transcripts of putative acetylcholinesterase 1 in WBPH were most abundant in MT and the lowest in SG and gut compared with other tissues. AChE1 was identified to express abundantly in most tissues of Chilo suppressalis [51]. The AChE1 was expressed higher than AChE2 in different tissues of N. lugens, suggesting that it may be the main target of organophosphorus insecticides [52]. Insect angiotensin converting enzyme (ACE) is a zinc metallopeptidase and can activate a variety of small to medium size peptide hormones by cleavage of C-terminal dipeptides and dipeptideamides [16]. ACE has been characterised in many insects such as Bombyz mori, D. melanogaster, Locusta migratoria, Musca domestica, Spodoptera littoralis and N. lugens [53–58]. High expression level of ACE was found in RB and MT followed by SG, head and gut of WBPH. The transcripts of ACE were highly expressed in the hemolymph
and reproductive tissues of both sexes in the adult *M. sexta* than in the brain, gut and fat body [16]

Carboxylesterase (CarE) was a ubiquitous enzyme of many functions in animals, plants and microorganism [59, 60]. It had crucial roles in xenobiotic detoxification, pheromone degradation, neurogenesis and developmental regulations [61]. It was verified that the expression levels of the enzymes were high in larvae maxilla of *B. mori*, suggesting that they may degrade plant volatiles or other xenobiotics [61]. We detected the carboxylesterase gene had a significant high expression level in SG of WBPH. The expression pattern suggested that it may play a role in detoxifying plant allelochemicals or degrading the plant-cell in the interaction of insect and plant.

Serine proteases play very important roles in the innate immune responses of invertebrate animals. These proteases are mainly involved in the processes of melanin formation and hemolymph coagulation against the infections of foreign pathogens [15]. A family of serine proteases has been discovered in invertebrates, and shown to include embryonic development and immune responses of diverse biological processes [62]. The serine protease snake 2 and 4 were also reported in *N. lugens* which have the similar expression patterns [15]. These serine protease genes had high expression levels in head, except snake 4 which was higher in RB than in the other tissues. The transcripts of prophenoloxidase activating factor 2 had no difference among these tissues except in SG which was lower. Prophenoloxidase activating factor 2 that had catalytic function is one of clip domain family of serine proteases [63, 64], Clip-domain serine proteases (CLIPs) played an important role in mediating innate immunity, called proPO activation cascade, embryonic development and hemolymph clotting in arthropods [65]. These results suggested that the serine protease genes might have functions in the different tissues.

Four phosphatases of uridine phosphorylase 1 isoform X2, putative phosphorylase b kinase regulatory subunit beta, prostatic acid phosphatase-like isoform X1 and prostatic acid phosphatase were identified in WBPH. Phosphorylase b kinase (PhK) controls glycogen degradation via phosphorylation of glycogen phosphorylase b (GPb) [66]. The transcripts of putative phosphorylase b kinase regulatory subunit beta were higher both in gut and RB compared with three other tissues in WBPH. The transcripts of prostatic acid phosphatase-like isoform X1 and prostatic acid phosphatase were both higher in the head than in other tissues, while prostatic acid phosphatase was also expressed highly in the gut.

The Ras superfamily of GTP-binding proteins was subdivided into Ras, Rho, Ran, Arf and Rab proteins [67], and it had a variety role to regulate the cellular activities. The Rac proteins belonged to Rho family, which played a role in the processes of assembling the actin cytoskeleton and the members of phagocyte NADPH oxidase [68]. In WBPH, Rac-GTP binding protein showed higher expression level in the head, gut and MT than in SG and RB.

Calcium/calmodulin-dependent protein kinase 2 (CaMKII) is an essential signalling kinase involved in neuronal plasticity in insects [69], different calcium/calmodulin-dependent protein kinase 2 isoforms have been cloned in *D. melanogaster* [70], *Apis florae* [71], *Bombus terrestris* [72], *M. sexta* [73, 74] and *Periplaneta americana* [69]. The transcripts of calcium/calmodulin-dependent protein kinase 2 isoform X2 in WBPH were detected in all tissues, and the expression levels of which were higher in the gut, SG and head compared with others tissues. The calcium/calmodulin-dependent protein kinase type 1 isoform X3 showed similar expression pattern.

**Development and sex-specific expression patterns**

In this study, we obtained WBPH development and sex-specific expression profile data of 32 salivary protein genes of SG for 2nd-3rd instar nymphs, 4th-5th instar nymphs, female and male...
adults. 26 of 32 salivary protein genes (except MCO4, NADPH-cytochrome P450 reductase, cytochrome P450 CYPSF01, cytochrome P450 CYPSF02, putative acetylcholinesterase 1 and Rac-GTP binding protein) had similar expression levels in SG of developmental stages and sexes, suggesting that they were like to have multiple functions in SG of all developmental stages and sexes. Except cytochrome P450 CYPSF02 and putative acetylcholinesterase 1, the expression levels of other four salivary protein genes were higher in adult stages than in nymph stages, while the sexes had no difference between them, suggesting that these enzymes may play a more important role in SG of adult. The transcripts expression levels of cytochrome P450 CYPSF02 were lower in 4th-5th instar than that in other development stages. Putative acetylcholinesterase 1 was expressed higher in SG of 2nd-3rd instar and female adult than in 4th-5th instar and male adult. 31 of 32 salivary protein genes (except putative acetylcholinesterase 1) showed no difference of expression levels between the female and male adult, suggesting that these enzymes had similar expression levels in SG of sexes. The detailed functions and relationships between the salivary protein genes in SG of development stages and sexes were needed to be further clarified.

Supporting Information

S1 Table. Gene specific primers used for molecular cloning of WBPH salivary protein genes.
(XLSX)

S2 Table. Primers used in real-time qPCR for determination expression level of WBPH salivary protein genes.
(XLSX)

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Author Contributions

Conceived and designed the experiments: YDL. Performed the experiments: ZL. Analyzed the data: ZL YDL. Contributed reagents/materials/analysis tools: XKA MLH. Wrote the paper: YDL.

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