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De novo transcriptome sequencing in *Frankliniella occidentalis* to identify genes involved in plant virus transmission and insecticide resistance

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ABSTRACT

The western flower thrips (WFT), *Frankliniella occidentalis*, a world-wide invasive insect, causes agricultural damage by directly feeding and by indirectly vectoring Tospoviruses, such as *Tomato spotted wilt virus* (TSWV). We characterized the transcriptome of WFT and analyzed global gene expression of WFT response to TSWV infection using Illumina sequencing platform. We compiled 59,932 unigenes, and identified 36,339 unigenes by similarity analysis against public databases, most of which were annotated using gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Within these annotated transcripts, we collected 278 sequences related to insecticide resistance. GO and KEGG analysis of different expression genes between TSWV-infected and non-infected WFT population revealed that TSWV can regulate cellular process and immune response, which might lead to low virus titers in thrips cells and no detrimental effects on *F. occidentalis*. This data-set not only enriches genomic resource for WFT, but also benefits research into its molecular genetics and functional genomics.

1. Introduction

The western flower thrips, *Frankliniella occidentalis* (Pergande), is an invasive species and the most economically important pest within the insect order Thysanoptera, which includes more than 5500 described thrips species [1]. *F. occidentalis* is haplodiploid, unfertilized eggs develop into haploid males, and fertilized eggs turn into diploid females. Adults develop from eggs, after passing through two instar larvae, pre-pupae and pupae in approximately two weeks [2]. Its short life cycle, high fecundity, thigmotatic behavior, and emergence of resistant strains have contributed to the success of *F. occidentalis* as an invasive species worldwide [1]. *F. occidentalis* was first reported in a greenhouse in Beijing, China in 2003 [3]. Since then, it has rapidly spread to several other provinces in China [4]. *F. occidentalis* causes agricultural damage not only by directly feeding on crops/plants, but also by indirectly vectoring plant viruses, Tospoviruses [1], the only plant-infecting genus in the virus family *Bunyaviridae* [5].

Tospoviruses are segmented, single-stranded and ambisense RNA viruses. Tomato spotted wilt virus (TSWV), a typical species of Tospoviruses, is most effectively transmitted by *F. occidentalis*, resulting in major economic losses annually worldwide [6]. TSWV segmented genome is composed of three single-stranded RNAs, S (2.9 kb, ambisense), M (4.8 kb, ambisense), and L (8.9 kb, negative). The L genome section encodes the RNA-dependent RNA polymerase (RdRp), which is associated with virus replication [7]. The M genome section encodes a nonstructural protein (NSm) that is involved in cell-to-cell movement in host plant, and encodes a precursor of two glycoproteins (GPs) [8]. The S genome section encodes a nonstructural protein (NSs) that is known to be a suppressor of silencing, and encodes the nucleocapsid (N) protein [9].

The interaction between *F. occidentalis* and TSWV is very complicated. TSWV is transmitted by *F. occidentalis* in persistent propagative fashion. It is known to move through the whole thrips body and replicate in different organs of the insect vector, from midgut epithelia and muscle cells surrounding the alimentary canal into putative ligaments joining the midgut to the salivary gland, and finally into the salivary glands. Subsequently, viruses are secreted from the salivary gland and infect the plant host during thrips feeding by non-destructive, brief probes [10]. TSWV is most efficiently acquired by young first instar larvae, and transmitted by late second instar larvae and adults of *F. occidentalis* [6]. However, TSWV is not transmitted transovarially [11], each new generation of thrips must acquire the virus as larvae. Although a relatively low virus titer in *F. occidentalis* body, with the exception of the salivary glands, and no cytopathological changes are found in the insect body after TSWV acquisition [11], TSWV can alter the feeding behavior of infected male thrips, and consequently, improve its transmission efficiency among host plants [12]. On the other hand, *F. occidentalis* achieved an accelerated performance on TSWV-infected plants compared with non-infected plants [13], resulting in larger number of vectors. Obviously, there has been a mutualistic interaction established between *F. occidentalis* and TSWV [6]. However, the molecular mechanisms underlying the mutualistic interactions between TSWV and *F. occidentalis* have been poorly

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understood and the global transcriptional response of *F. occidentalis* to TSWV infection remains unknown.

At present, there are 13,839 expressed sequence tags (EST) generated from first instar larvae by Rotenberg and Whitfield [14], 904 nucleotide sequences and 532 proteins for *F. occidentalis* deposited in GenBank by 2012, which were sequenced by conventional methods for gene cloning and sequencing. Recently, next-generation high-throughput sequencing techniques, such as Illumina sequencing and 454 Pyrosequencing, have been employed to investigate the genetic background of insects, such as *Plutella xylostella* [15], *Nilaparvata lugens* [16] and *Cimex lectularius* [17]. Nevertheless, the available genetic data of *F. occidentalis* (only 15,275 sequences stored in NCBI) are insufficient for studying the molecular mechanism of interactions between TSWV and *F. occidentalis*, and the molecular mechanism of insecticide resistance.

The plant virus TSWV activates the immune system of *F. occidentalis* in the larval stage [18], and a few proteins that responded to TSWV in first instar larvae of *F. occidentalis* are detected [19]. A few studies have attempted to elucidate the mechanism of insecticide resistance in *F. occidentalis* by cloning and characterizing the genes that encode for insecticide effect target [20] and key detoxification enzyme [21]. However, this slow research process has limited progress toward our fuller understanding of the genetic background of *F. occidentalis*, the molecular mechanism of TSWV interacting with *F. occidentalis*, and the molecular mechanism of insecticide resistance of *F. occidentalis*.

To obtain detailed and general data, we employed the Illumina sequencing platform to obtain the transcriptome of *F. occidentalis* throughout its life cycle, identified a total of 59,932 unigenes from *F. occidentalis*, and comparative analysis of the expression profiles of TSWV-infected and non-infected *F. occidentalis*, indicated that some disease infection, substance transport, insect immune systems and salivary secretion pathways were activated. Our objectives were to enrich the gene resource of *F. occidentalis* with these data, and to aid in the discovery of genes related to the molecular mechanism of interactions between TSWV and *F. occidentalis* and insecticide resistance of *F. occidentalis*, which could help to establish new strategies for controlling *F. occidentalis* and its vectoring viruses. In addition, by successfully sequencing this species' transcriptome, we believe that it can be a potential representative and ideal model for studying virus transmission, insecticide resistance, and annotation of genomes of other thrips species.

2. Results and discussions

2.1. Illumina paired-end sequencing and de novo assembly

We pooled equally RNA from each of the eight samples (TSWV-infected and TSWV non-infected first instar, second instar, pre-pupae and pupae, and adult of *F. occidentalis*) for transcriptome analysis by using the Illumina paired-end sequencing technology to obtain deep coverage of the transcriptome. Our sequencing approach produced independent reads from either end of a DNA fragment. A total of 51,391,358 clean reads with the length of 90–100 bp were generated after trimming analysis by in-house perl scripts. The clean reads was *de novo* assembled by SOAP de-novo program. A total of 192,285 contigs were assembled with an average length of 227 bp and an N50 of 358 bp. The length of contigs ranged from 60 to 7404 bp. The length of 109,314 (56.85%) contigs was more than 100 bp (Table 1). Then, using pair-end reads following joining and gap-filling steps, the contigs were further assembled into 80,235 scaffolds with an average length of 456 bp and an N50 of 767 bp (Table 1). 60,272 (75.12%) scaffolds with length were ranged from 100 to 500 bp. To further shorten the remaining gaps, we accumulated the paired-end reads with one end mapped on the unique contig and the other end located in the gap region and performed local assembly with the unmapped end to fill in the small gaps within the scaffolds. Such scaffolds comprising least number of Ns and not being stretched on either end were confirmed as unigenes. During this approach, more than 90% were filled in, and only 0.043 Mb gaps (0.12% of total unigene sequences) were not closed yet (Fig. S1). Finally the de novo assembly 59,932 unigenes were obtained with an average length of 565 bp and an N50 of 851 bp, which had a total length of 33.86 Mb (Table 1). The length of assembled unigenes ranged from 150 to 9860 bp. The length of 40,025 unigenes (66.78%) varied from 150 to 500 bp, the length of 11,565 unigenes (19.30%) ranged from 501 to 1000 bp, and the length of 8342 unigenes (13.92%) was more than 1000 bp (Table 1). These unigenes were prepared as transcriptome database for finding virus transmission and insecticide resistance related genes or pathways.

Table 1
Length of distribution of assembled contigs, scaffolds and unigenes.

Nucleotide length (bp)	Contigs	Scaffold	Unigene
75–100	82,970	0	0
101–200	52,398	32,179	12,225
201–300	20,761	14,493	14,354
301–400	11,037	8482	8387
401–500	6471	5123	5111
501–600	4452	3716	3687
601–700	3231	2713	2705
701–800	2194	2111	2097
801–900	1777	1706	1701
901–1000	1345	1351	1334
1001–1200	1878	2090	2103
1201–1400	1192	1489	1460
1401–1600	837	1082	1074
1601–1800	563	804	813
1801–2000	363	592	587
2001–2500	450	970	969
2501–3000	192	543	544
>3000	174	791	781
Total	192,285	80,235	59,932
Minimum length (bp)	60	100	150
Maximum length (bp)	7404	9860	9860
N50(bp)	358	767	851
Average length (bp)	227	456	565
Total nucleotide length (bp)	43,648,695	36,587,160	33,861,580

2.2. Functional annotation

For validation and annotation of assembled unigenes, sequence similarity search was conducted on sequence-based and domain-based alignments. Sequence-based alignments were carried out in non-redundant protein database (nr) at NCBI, Swiss-Prot/Uniprot, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using BLASTx algorithm with an E value threshold of 10^{-5}. Domain based alignments were performed against the InterPro, Pfam and COG data bases. Altogether, a total of 24,670 unigenes were annotated by sequence-based alignments (Fig. 1A), and an additional 11,669 unigenes were annotated by domain-based alignments (Figs. 1B, C). Overall, 36,339 unique sequence-based or domain-based annotations using the six selected public databases were assigned to *F. ocidentalis* unigenes (60.6% of the assembled unigenes). Among them, 6310 unigenes had hits in all six public databases with relatively defined functional annotations (Table 2). Consequently, the results in this study indicated that the Illumina paired-end sequencing project generated a considerable part of *F. ocidentalis* genes. Of all the 36,339 unigenes, 25 were uniquely mapped with more than 6000 reads, which represented the most abundant transcripts in the *F. occidentalis* cDNA library (Table S1).

We compared the similarity of *F. occidentalis* genes with those of the other arthropods whose complete or draft genomes are available, and the top 20 species were shown in Fig. S2. A. The largest number of database matched was from *Tribolium castaneum* (Coleoptera) (17.28%), followed by *Harpegnathos saltator* (Hymenoptera) (9.02%),

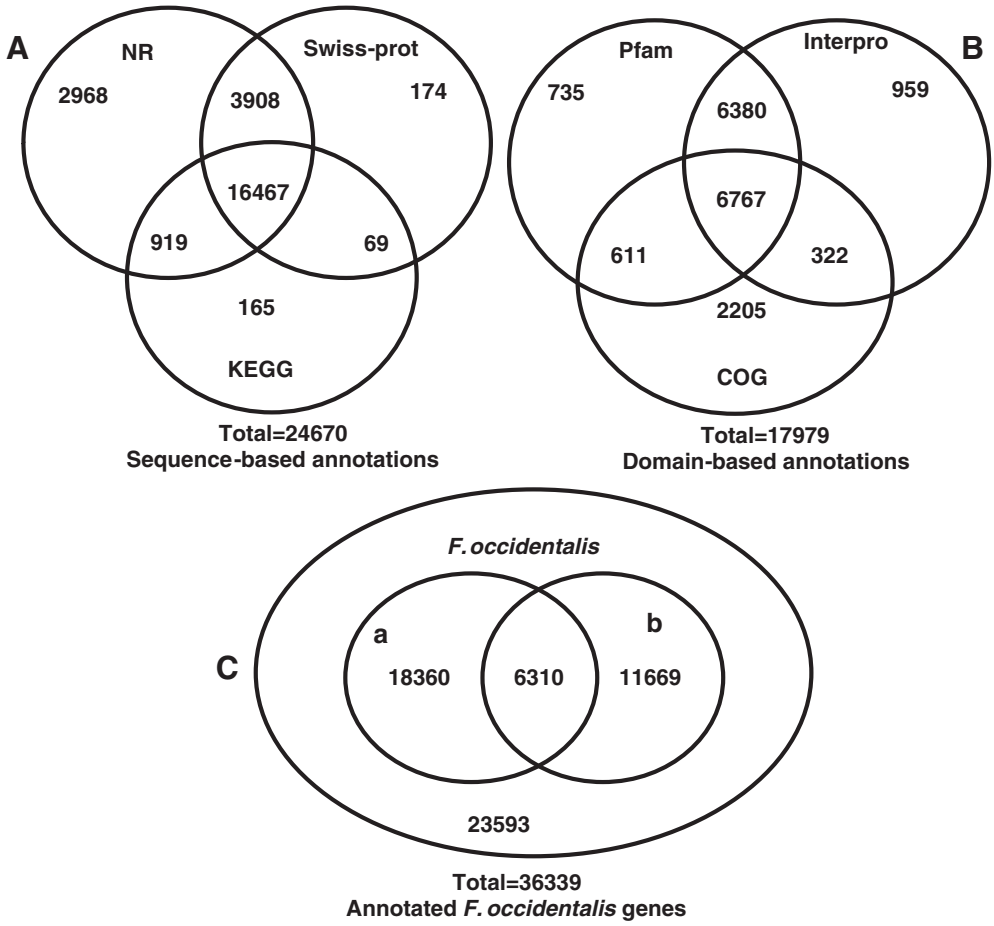

Fig. 1. Distribution of similarity search results. (A) The sum of unique best BLASTX hits in the nr, Swiss-Prot and KEGG databases, respectively. The cutoff e-value $\leq 10^{-5}$. The overlap regions among the three circles contain the number of unigenes shared in three databases. (B) The integration of unique similarity search results against the InterPro, Pfam and COGs databases, respectively. The cutoff e-value $\leq 10^{-5}$. The overlap regions among the three circles contain the number of unigenes shared in three databases. (C) Number of all annotated *Frankliniella occidentalis* unigenes in both unique sequence-based annotations and unique domain-based annotations. The circle "a" and "b" represent the two subsets of *F. occidentalis* unigenes with sequence-based annotations (24,670 counts in Fig. 1A) and domain-based annotations (17,979 counts in Fig. 1B), respectively.

Acromyrmex echinatior (Hymenoptera) (7.97%), *Camponotus floridanus* (Hymenoptera) (7.86%), *Pediculus humanus* (Anoplura) (5.16%), and *Solenopsis invicta* (Hymenoptera) (4.48%) with all other species less than 5%. In addition, we identified the similarity of *F. occidentalis* sequences in the current study to the 15,275 *F. occidentalis* sequences available in NCBI database, by using a Blastx analysis of our *F. occidentalis* sequences against the NCBI database. There were 9157 distinct sequences with the highest homology to genes from *F. occidentalis* in previous study [14] (Fig. S2. B), supporting the robustness and validity of our RNA-Seq based approach. Our sequencing in this study makes more detailed and general genetic data available that will facilitate large-scale discovery and utilization of genetic resources for *F. occidentalis*.

2.3. Gene ontology and pathway analysis

We utilized gene ontology assignment programs for functional categorization of the annotated unigenes. In total, 7432 unigenes were assigned to three gene ontology classes: biological process, cellular component and molecular function, with 41,960 functional terms (Fig. 2), because multiple terms were assigned to the same transcript. Metabolic process, binding and cell or cell part were the vast majority

Table 2
Summary of annotations of the *F. occidentalis* unigenes.

	Sequences (n)	Annotations (n)	Functional classification
All assembled unigenes	59,932	–	–
Unique gene annotations against NR	24,262	24,262	18,808 protein accessions
Gene annotations against Swiss-Prot	20,618	20,618	12,496 protein accessions
Gene annotations against KEGG	17,620	17,620	220 pathways
Gene annotations against InterPro	14,428	14,428	4337 domains/families
Gene annotations against Pfam	14,493	14,493	3676 domains/families
Gene annotations against COG	9905	22,965	25 categories
GO annotations for NR protein hits	7432	41,960	3 main categories 77 sub-categories
GO annotations for InterPro protein hits	12,047	29,053	3 main categories 46 sub-categories
All annotated unigenes	36,339	–	–
Unigenes matching all six databases	6310	–	–

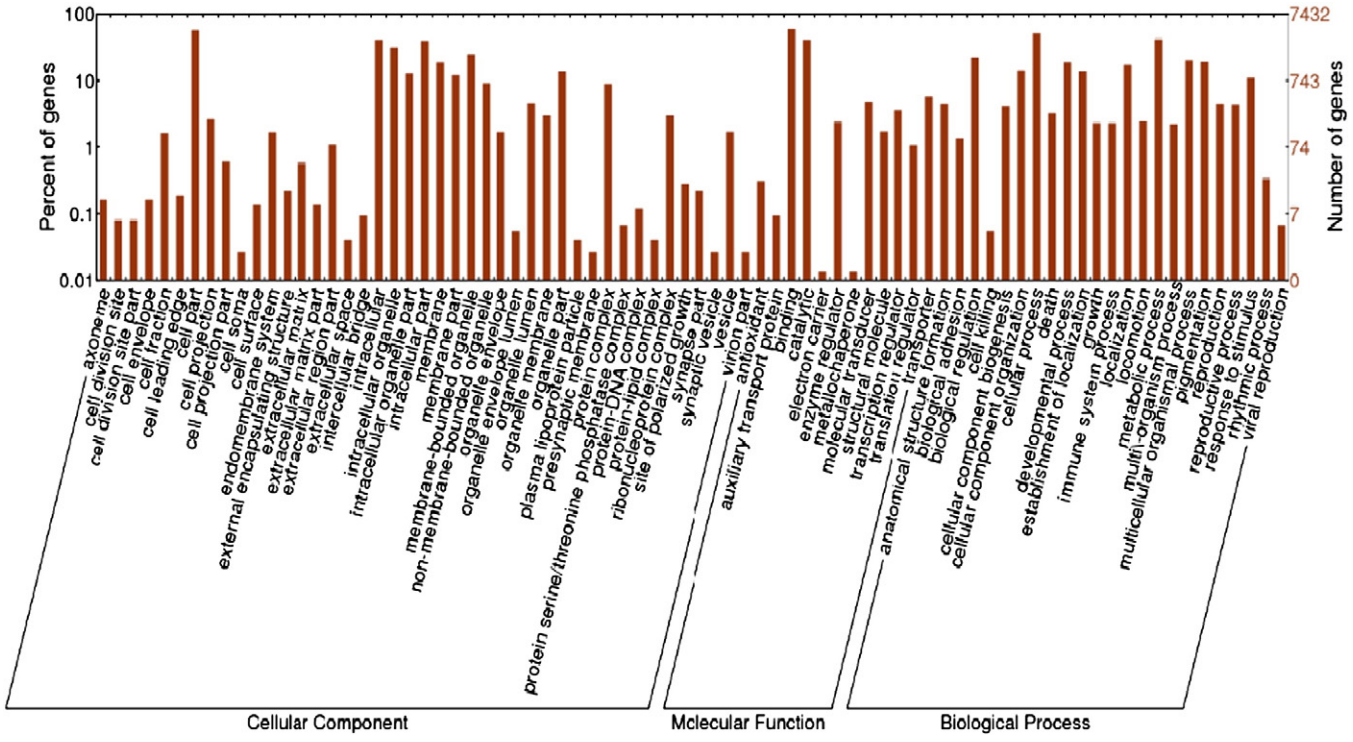

Fig. 2. Gene ontology classification of assembled unigenes. The results are summarized in three main categories: biological process, cellular component and molecular function. In total, 7432 unigenes with BLAST matches to known proteins were assigned to gene ontology.

categories from each GO domain, which suggested that the life cycle of *F. occidentalis* is prominently governed by gene related metabolism, molecular interaction and cellular structure, perhaps indicating a shift in cellular metabolic state and process to support virus replication and diffusion in thrips. In contrast, among the categories with the fewest members were cell killing and viral reproduction of the biological process ontology, virion or virion part of the cellular component ontology, and auxiliary transport protein activity, metallochaperone activity of the molecular function ontology. In addition, 171 unigenes were assigned to the immune system process, which imply that the vector, *F. occidentalis*, provides immune defense against TSWV.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database records networks (pathways) of molecular interactions in these cells, as well as their variants specific to particular organisms. Pathway-based analysis helps to further understand the biological functions of genes. To identify the biological pathways in *F. occidentalis* organisms, we mapped the annotated sequences to the KEGG database. Out of the 59,932 unigenes, 17,620 could be matched in the database and assigned to 220 KEGG pathways (Table 2 and Fig. 1A). Among them, the metabolic pathway was the largest group, followed by amebiasis, spliceosome, and vibrio cholerae infection pathway. Given that amebiasis and vibrio cholerae infection pathways are two sub-pathways involved in the human infectious diseases (http://www.genome.jp/kegg-bin/get_htext?query=05146&htext=br08901.keg), we speculated that TSWV infection of *F. occidentalis* was partially similar to human infectious disease. The EST database of TSWV non-infected first instar *F. occidentalis* reported in previous study showed that 92 sequences are involved in the endocytic pathway, 74 sequences in innate immunity, 40 sequences in virus infection and 16 sequences possibly functioned in RNAi [14]. In the current study, 524 unigenes can be mapped into endocytic pathway, 336 unigenes are involved in tissue secretion, e.g., salivary secretion (Fig. 3), an important process for transmission of virus from vector to host [22,23]. Moreover, we identified plentiful gene families that encode key enzymes in immune systems and RNAi (Fig. 3). Therefore, this broad coverage by these genes provides more abundant information for examining the mechanism of TSWV entry, infection, replication, diffusion in thrips vector and vector immune response to TSWV infection in *F. occidentalis*.

2.4. Identification of transcripts of TSWV

To investigate the transcript of TSWV in *F. occidentalis*, each TSWV ORF sequence was searched against our EST library. The abundance of TSWV transcripts is shown in Fig. S3. RdRP was the most abundant transcript (11 unigenes in total) that is experimentally demonstrated as a replication, transcription, 'cap-snatching', and genomic strand selection protein, it is believed to function cooperatively with host-encoded factors [24]. The dominant presence of the RdRP transcript suggested that replication and assembly of TSWV are active in the insect after TSWV invades *F. occidentalis*. Therefore, that would be interesting to further identify the host factors that cooperate with RdRP for

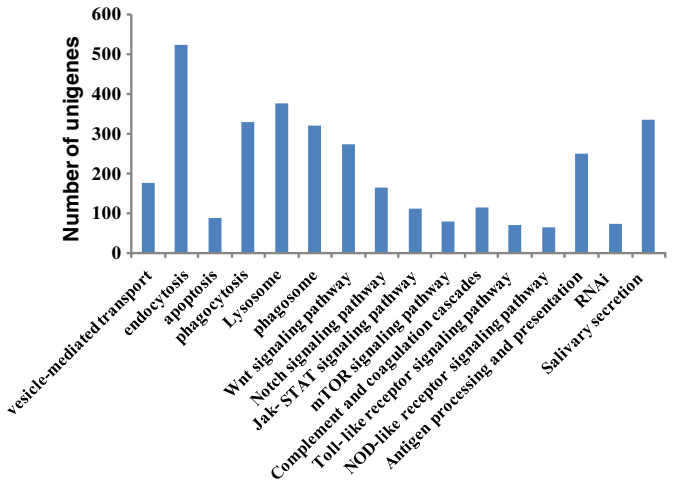

Fig. 3. Unigenes from *Frankliniella occidentalis* related to virus infection and response to viral infection.

TSWV propagation in the vector. The other transcripts, including NSs and N, were both present in two transcripts in *F. occidentalis*, suggesting that N and NSs play important roles in the TSWV infection cycle. The N protein, as part of the RNP, serves as structural protein and may also have some regulatory role in modulating the transcription, in replication and virus particle assembly. The nonstructural protein, NSs has been shown to be a suppressor of RNA silencing [9], The presence of the NSs transcript suggests that TSWV might suppress the immune response of the host insect as well as that of the host plant. Therefore, it would be interesting to identify the host cellular target(s) suppressed by TSWV NSs in future studies. In addition, we failed to identify transcripts of the movement protein NSm, which agrees with the existing hypothesis that the movement protein usually functions in planta during virus spread to neighboring cells through the plasmodesmata [25], and thus its expression might be shut down or remain at a very low level within the insect. Glycoprotein, which plays a critical role in determining the ability of virus transmission by thrips [26], was absent in our library. The reason may be that the virus titer was too low to be detected in the gene trascription in the vector's body.

2.5. Detection of sequences related to the insecticide targets and metabolism resistance

F. occidentalis is highly resistant to insecticides [27], its mechanisms of insecticide resistance are associated with decreased penetration [28], metabolic resistance [29] and target resistance [30]. So, we analyzed the sequences related to insecticide targets and metabolism, and compared our data with sequences from NCBI nucleotides and EST database. As shown in Table 3, we identified a number of sequences which are homologous to enzymes related to conventional detoxification enzymes, such as carboxylesterase, glutathione S-transferase, cytochrome P450; and putative insecticide targets, such as sodium channel, acetylcholinesterase, GABA receptor and nicotinic acetylcholine receptor. Except for cytochrome P450 and sodium channel which have been documented [20,22], all putative insecticide resistant genes were identified for the first time in *F. occidentalis*. In total, we obtained 21 nicotinic acetylcholine receptor related sequences. After removing redundant sequences, we identified 11 different nicotinic acetylcholine receptor subunits (Table S2). Because an insect genome usually comprises 10 to 11 nicotinic acetylcholine receptor subunits [31], our database potentially provides a complete collection of such genes in *F. occidentalis*, which will provide primary molecular information for research on *F. occidentalis* resistance to spinosad, the most effective and environmentally-friendly insecticide against *F. occidentalis*, so far [30]. The main action targets of spinosad are nicotinic acetylcholine receptors [32] and its resistant mechanism in thrips is target resistance [30].

2.6. Analysis of gene expression differences between TSWV-infected and non-infected F. occidentalis population

To specifically identify genes related to TSWV infection in *F. occidentalis*, we performed genome-wide expression profiling comparisons to examine gene activity changes between TSWV-infected and non-infected *F. occidentalis*. Because single-end (SE) RNA-Seq on the Illumina platform rapidly and economically delivers massive high-quality data, and is widely used for gene expression analysis [33], we pooled equally RNA from each of four samples (TSWV-infected first instar, second instar, pre-pupae and pupae, and adults of *F. occidentalis*), and equally RNA from each of four samples (TSWV-non-infected first instar, second instar, pre-pupae and pupae, and adults of *F. occidentalis*), respectively, to establish SE RNA-Seq libraries for comparison of gene expression profile analysis. A total of 14.73 and 13.35 million clean reads (50-bp each) were generated from TSWV-infected and non-infected groups respectively. (Table S3). The expressions of two libraries were quantified by read mapping analysis using 59,932 unigenes from the transcriptome as reference database. 7,611,410 (51.65%) and 6,356,049 (47.61%) of all clean reads were mapped to the entire reference database. 57,208 (95.45%) and 56,465 (94.22%) read-mapped genes were matched in the transcriptome reference database.

Comparison of different expressed genes in TSWV-infected and non-infected *F. occidentalis* populations showed that 1454 genes were significantly (FDR$<$0.001, |log2 ratio|$\geq$1) different between the two groups. Among them, 661 genes were upregulated and 793 genes were downregulated in the TSWV-infected *F. occidentalis* compared with non-infected *F. occidentalis* (Fig. 4A; Table S4). Correlation analysis of the two libraries reflected the extent to which the *F. occidentalis* is affected by the TSWV; the Pearson correlation coefficient of the two libraries was low (0.454), suggesting a substantial effect of the virus on the gene expression profile of *F. occidentalis* (Fig. 4B). To validate the RNA-Seq data, we compared the gene expression profiles of the TSWV-infected and non-infected thrips using qPCR. Among 36 randomly selected different expression genes, the majority exhibited a consistent expression pattern between RNA-Seq and qPCR (Fig. 4C and Table S5), indicating that RNA-seq data are reliable.

2.7. Functional annotation of differentially expressed genes

KEGG and GO analysis showed that transcripts up-regulated in TSWV-infected *F. occidentalis* (FDR$<$0.001, log2 ratio$\geq$1) were significantly ($p<0.01$) enriched in several functional categories: environmental information processing pathways, cellular processes, infection diseases, and developmental process. In contrast, transcripts down-regulated in TSWV-infected *F. occidentalis* (FDR$<$0.001, log2 ratio≤-1) were significantly ($p<0.01$) enriched in the functional categories of genetic information processing, organismal systems, ribosome

Table 3
Genes related to the insecticide targets and metabolism.

Gene name	Sequences had a hit with nr database (n)	Known sequences from NCBI nucleotide database (n)	Known sequences from NCBI EST database (n)
Carboxylesterase	15	0	
Catalase	3	0	0
Cytochrome P450	133	34	0
Glutathione S-transferase	19	0	0
NADH dehydrogenase	32	0	0
Trypsin	28	0	0
Superoxide dismutase	5	0	0
Acetylcholinesterase	4	0	0
GABA receptor	11	0	0
Nicotinic acetylcholine receptor	21	0	0
Sodium channel	7	9	0

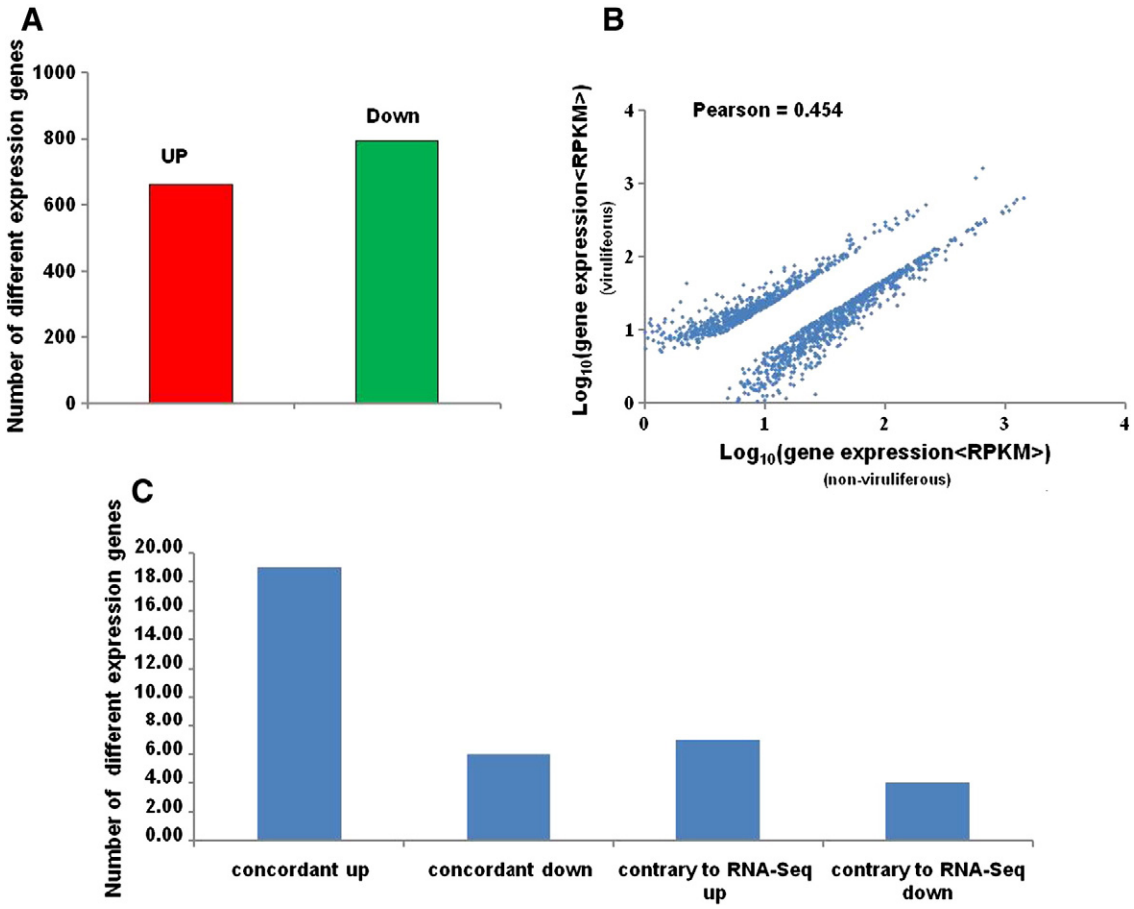

Fig. 4. Analysis of differentially expressed genes between TSWV-infected and non-infected *F. occidentalis* population. (A) Summary of the numbers of differentially expressed genes in the TSWV infected thrips. "FDR < 0.001 and the absolute value of $\log_2$ratio ≥ 1" were used as the threshold to judge the significance of gene expression difference. (B) Correlation analysis of two libraries. The Pearson correlation coefficient for two libraries is shown in the upper left corner of the plot. (C) Comparison of RNA-Seq data and qPCR results. "Concordant up" means that genes in the TSWV infected thrips were up-regulated for both RNA-Seq and qPCR analyses. "Concordant down" means that genes in the TSWV infected thrips were down-regulated for both RNA-Seq and qPCR analyses. "Contrary to RNA-Seq up" means that genes in the TSWV infected thrips were up-regulated for RNA-Seq, but were down-regulated for qPCR analyses. "Contrary to RNA-Seq down" means that genes in the TSWV infected thrips were down-regulated for RNA-Seq, but were up-regulated for qPCR analyses.

and other intracellular structure (Fig. 5). The most enriched pathway of the TSWV infected population up-regulated transcripts was the vibrio cholerae infection and amebiasis pathway involving human disease infection (Fig. 5 and Table S6). We found high expression of the mucin, phospholipase C, tight junction protein ZO-1, collagen, laminin and vinculin in TSWV-infected *F. occidentalis* (Table S6). The prevalence of mucin in TSWV-infected *F. occidentalis* indicated that mucin is likely to play important roles in TSWV infection, replication, and diffusion in bodies of thrips vectors and in TSWV transmission from thrips to plant. This is consistent with previous studies which indicated that rotavirus infection induced the expression of host mucin genes involved in defense against rotavirus infection [34]. With the increase of mucin, greater number of virus particles were perhaps trapped in the mucus of the midgut or other tissues and organelles, resulting in low virus titers into thrips cells. and subsequently, no detrimental effects on the life cycle or cytopathological changes in the *F. occidentalis* after infection by TSWV [11].

The pathway enrichment analysis showed that 5 differentially expressed genes enriched in the proteasome pathway were down-regulated (Table S6). Interestingly, 5 genes were down-regulated and one gene was up-regulated in the ubiquitin-mediated proteolysis pathway (Table S6). Proteasomes can degrade protein substrates marked for degradation by the attachment of ubiquitin moiety, forming the ubiquitin–proteasome pathway [35]. The ubiquitin–proteasome pathway is involved in the regulation of the cell cycle, metabolic adaptation, and immune response through degrading short-lived proteins and regulatory proteins that control these cellular functions [36]. All of these pathways were regulated in TSWV-infected thrips (Table S6), which provide good condition for virus replication in vector body.

For the primary metabolism, 37 genes were up-regulated and 96 genes were down-regulated in the TSWV-infected thrips (Table S6). Most genes involved in ribosome, spliceosome, amino acid metabolism, and carbohydrate metabolism were down-regulated in TSWV-infected thrips, indicating that the synthesis of protein, and amino acid and carbohydrate metabolism were inhibited by TSWV infection in thrips. However, most genes involved in lipid metabolism were up-regulated, which indicated that lipid metabolism was active in the TSWV-infected thrips. A similar effect has been reported for protein production in insects infected by the virus [37,38]. Thus primary metabolism of thrips can be disturbed by TSWV, which might be due to the invasion of the insect tissues by the virus. This was consistent with the results of TYLCCNV invading whitefly, *Bemisia tabaci* [39].

Among the differentially expressed genes between TSWV-infected and non-infected thrips, many related to cellular process and immune responses are up-regulated in TSWV-infected thrips, such as endocytosis, lysosome, phagosome, Toll pathway, the JAK/STAT pathway, notch pathway and RNA interference pathway (Table S6). We found six sequences in *F. occidentalis*, with putative homology to proteins enriched in endocytosis, four of which were up-regulated and two were down-regulated (Table S6). Based on this, we speculated that the endocytic pathway plays a crucial role in intracellular or intercellular transport of TSWV particles in the vector. The reason is that the endocytic

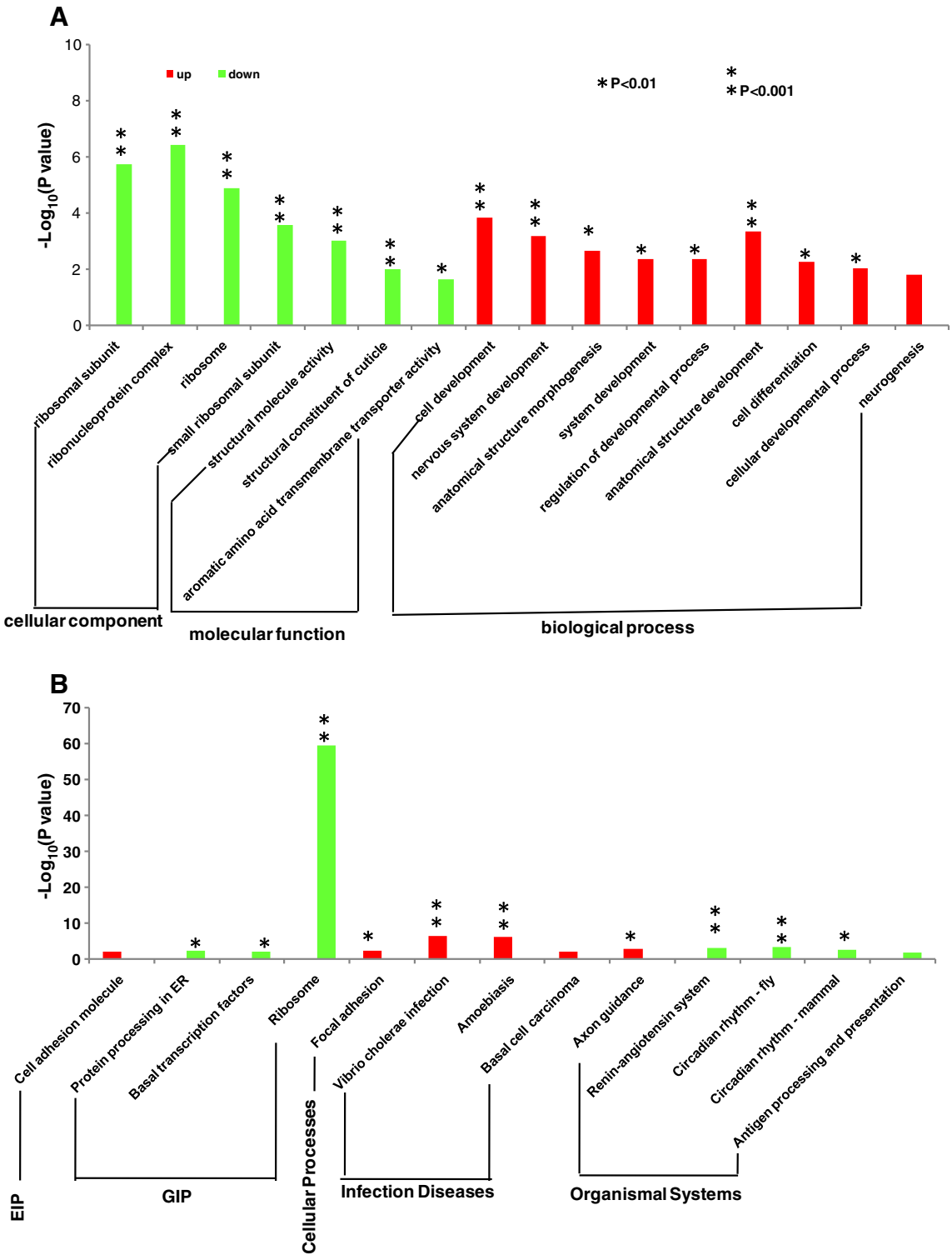

Fig. 5. GO and KEGG enrichment differentially expressed genes between TSWV infected and non-infected *F. occidentalis* population. (A) GO enrichment, (B) KEGG enrichment. The Y axis is $-\log_{10}$ transformation of the p value calculated in enrichment test. EIP Environmental Information Processing; GIP Genetic Information Processing.

pathway, a vesicle-mediated mechanism of host cell entry and intracellular transport, is utilized by many members of the family *Bunyaviridae* that infect animals [40–42]. Lysosome and phagosome, in collaboration with autophagy, have been shown to play a direct antiviral role against viruses [42–45]. For the TSWV-infected thrips, most genes involved in lysosome and phagosome function were significantly up-regulated (Table S6), suggesting that these pathways were activated in the TSWV-infected thrips. Apart from the up-regulation of most genes involved in endocytosis and autophagy, genes involved in humoral response, such as complement and coagulation were also up-regulated (Table S6). This suggests that TSWV can trigger both the humoral and cellular immune responses of thrips, which may result in the degradation of virions and low virus titers in the body of the vector.

Genes involved in immune systems and signaling transduction: Toll pathway, RNA interference pathway, JAK/STAT pathway, and notch pathway were up-regulated (Table S6), suggesting that the

TSWV might activate the immune system and signaling transduction in the vector's body. The Toll pathway plays a critical role in inhibition of virus replication in insects [46,47]. In the EST libraries of *F. occidentalis* in this study, homologs of Toll pathway gene transcripts, including tuberin and Toll-7-like protein were significantly up-regulated in TSWV-infected *F. occidentalis* (Table S6), indicating that TSWV may trigger this pathway, which confirmed the results of a previous study [18]. However, in *B. tabaci*, the Toll pathway can be suppressed by TYLCCNV infection [39]. Previous research has indicated that activation of the notch pathway is involved with establishment of virus persistence in vector cells [48] and host cells [49]. Here we found that four genes involved in the notch pathway were up-regulated (Table S6), which may be related to the degradation of virions in the insect vector. Moreover, gene expression profile between TSWV-infected and non-infected in this study, was partially different as reported by Medeiros et al. [18] and Badillo-Vargas et al. [19]. In these studies they used different virus isolates and thrips populations than the ones in this study. Furthermore, they conducted a macroarray analysis of subtractive cDNA libraries of TSWV-infected second instar [18] and proteomic (using two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometry) different analysis between TSWV-infected and non-infected first instar [19]. In addition, there were many transcripts related to salivary secretion, such as salivary gland secretion 1 and salivary gland secretion 3, Na-K-Cl cotransporter (NKCC1) and calcium activated potassium channel (MaxiK) [50] that were significantly up-regulated (Table S6). Based on the foregoing, we were putative that the thrips vectoring virus would increase salivary secretion and benefit to virus transmission from vector to host plant, because salivary gland secretion is necessary for virus transmission by vectors [22,23].

3. Conclusions

We employed the Illumina sequencing platform to collect ESTs from TSWV-infected and non-infected *F. occidentalis*, obtained the transcriptome of *F. occidentalis* at an unprecedented depth (4.36 gigabase pairs) and produced 59,932 assembled unigenes with 36,339 unigenes obtaining annotation. These findings provide a substantial contribution to existing sequence resources for thrips and are likely to accelerate insecticide resistance mechanism research in *F. occidentalis*. Comparative genomic analysis advanced a repertoire of candidate genes that might be involved in the *F. occidentalis*–TSWV interaction. For example, some disease infection genes, such as amebiasis, vibrio cholerae infection and a set of putative innate immunity regulatory genes, such as RNA interference, JAK/STAT, Toll pathway and notch pathway, might play crucial roles in viral infection and resistance to viral infection. The findings will allow us to improve molecular strategies, such as RNAi-based pest and virus disease control, for incorporating into integrated WFT and TSWV management program.

4. Materials and methods

4.1. Virus isolates, insect populations and plant

The TSWV-YN isolate was original collected in Yuxi county, Yunnan province, South-western PR China, and maintained by thrips-mediated passages on potted *Datura stramonium* plants grown under greenhouse conditions. Only plants with obvious symptoms were selected for experiments approximately 2 to 3 weeks post inoculation. A *F. occidentalis* colony was collected from Beijing, PR China, in 2003, and reared with fresh green bean pods *Phaseolus vulgaris* in a climate-controlled chamber (27 ± 1 °C, 16 L: 8 days).

4.2. Sample preparation and RNA isolation

To prepare TSWV-infected and non-infected *F. occidentalis* samples, adult thrips reared on green bean pods (about 3 days after eclosion) were transferred to TSWV-infected and non-infected *D. stramonium* plants, which were kept in separate growth chambers for 24 h (at 27 ± 1 °C, 16 L: 8 days), for thrips oviposition. Then, TSWV-infected and non-infected *D. stramonium* plants were placed in climate-chambers separately, after removing the adult thrips. After about 3 days, thrips eggs in the TSWV-infected or non-infected *D. stramonium* plants hatched. The newly hatched first instars were maintained on TSWV-infected or non-infected *D. stramonium* plants for 48 h. The thrips larvae were then transferred to fresh bean pods of *P. vulgaris* without TSWV, for rearing to adulthood. The acquisition efficiency (number of thrips infected with TSWV) of our laboratory colony was in the range of 30–60%, therefore thrips cohort fed with TSWV-infected and non-infected *D. stramonium* plants represented both TSWV-infected and non-infected *F. occidentalis*. Approximately 1000 TSWV-infected and TSWV non-infected first instar larvae (L1), second instar larvae (L2), pre-pupae and pupae (P), and adults (A) of *F. occidentalis* (except for eggs, which are usually deposited on the surface of plant leaf, is inconvenient to collect),were collected respectively. Therefore, there were eight *F. occidentalis* samples. Two biological replicates for all of the *F. occidentalis* samples were conducted and processed independently. One replicate was used in the gene expression profile analysis and Illumina sequencing, and the other was used for the quantitative PCR (qPCR) analysis. Total RNA was isolated individually from each sample using SV total RNA isolation system (Promega) according to the manufacturer's protocol. RNA integrity was confirmed using the 2100 Bioanalyzer (Agilent Technologies) with a minimum RNA integrated number value of 8.

4.3. RNA-seq library preparation and sequencing

Three pooled samples of total RNA were prepared prior to mRNA isolation. The first sample was pooled by 1.25 μg of RNA from each of the eight samples (TSWV-infected and TSWV non-infected first instar, second instar, pre-pupae and pupae, and adult), to establish paired-end RNA-seq library for transcriptome analysis. The second sample was pooled by 1.25 μg of RNA from each of the four samples (TSWV-infected first instar, second instar, pre-pupae and pupae, and adult), and the third sample was pooled by 1.25 μg RNA from each of the four samples (TSWV-non-infected first instar, second instar, pre-pupae and pupae, and adult), to establish single-end (SE) RNA-seq libraries for comparison of gene expression profile analysis between TSWV-infected and non-infected thrips population. Enrichment of mRNA, fragment interruption, addition of adapters, size selection and PCR amplification and RNA-Seq were performed by staff at Beijing Genome Institute (BGI) (Shenzhen, China). Poly (A) mRNA was isolated using oligo dT beads, and then broken into short fragments. The one paired-end (PE) RNA-seq library and two single-end (SE) libraries were prepared following Illumina's protocols and sequenced on the Illumina HiSeq™ 2000 platform.

4.4. Analysis of Illumina sequencing results

Raw reads produced from sequencing machines contain dirty reads which contain adapters, unknown or low quality bases. These data will negatively affect any bioinformatic analysis. Therefore, dirty raw reads (i.e., reads with adaptors, reads with unknown nucleotides larger than 5%, low quality reads) were discarded. *De novo* assembly of the short reads (from paired-end RNA-seq library) into transcriptome was carried out with short reads assembling program–SOAP de novo at the parameters of "-K 29 -M 2 -L 50" by BGI. The meaning and selection principles of the parameters were available on Internet (http://soap.genomics.org.cn/soapdenovo.html). The SOAP *de novo* firstly combined

reads with certain length of overlap to form longer fragments without N, which were called contigs. Then the reads were mapped back to contigs with paired-end reads, to detect contigs from the same transcript as well as the distances between these contigs. Next, SOAP *de novo* connected the contigs using N to represent unknown sequences between each two contigs, and scaffolds were made. Paired-end reads were used again for gap filling of scaffolds to get sequences with least Ns and could not be extended on either end. Such scaffolds were defined as unigenes. Unigene annotation was performed with a sequence based BLAST search against nr, Swiss-Prot/Uniprot and KEGG database, and a domain based BLAST search against COG, InterPro and Pfam database, with an e-value cut-off of $1.0E^{-5}$. Gene ontology terms were assigned by Blast2GO through a search of the database. The data sets are available at NCBI Short Read Archive (SRA) with the accession number: SRA052229.

4.5. Expression profiling

4.5.1. Gene expression value measurement

Gene expression profiling was measured by mapping reads (generated by single-end libraries RNA-Seq) to the reference transcriptome (generated by pair-end libraries RNA-Seq) using SOAP at the parameters of "-m 0 -x 1000 -s 28 -l 32 -v 2 -r 2" by BGI. The meaning and selection principles of the parameters are available on the Internet (http://soap.genomics.org.cn/soapaligner.html), Mismatches of no more than 2 bases were allowed in the alignment. Prior to mapping reads to the reference database, we filtered all sequences to remove adaptor sequence, reads in which unknown bases are more than 10%, low quality reads (the percentage of the low quality bases of quality value ≤ 5 is more than 50% in a read). Then, the RPKM value for each transcript was measured in reads per kilo base of transcript sequence per million mapped reads. To exclude the bias caused by different RNA output between samples, we adopted a TMM (trimmed mean of M values) method to calculate a normalization factor introduced by Robinson, using calcNorm Factors function in edge R package.

4.5.2. Evaluation of different expressed genes in TSWV infected and non-infected thrips populations

A statistical analysis of the frequency of each read in the different single-end libraries was performed to compare gene-expression in different treatments (TSWV-infected and non-infected populations). FDR (False Discovery Rate) was used to determine the threshold of P value in multiple test and analysis. We used FDR$<1.0E^{-3}$ as the threshold and $|\log2 \text{ ratio (TSWV-infected/non-infected)}| \geq 1$ to judge the significance of gene expression difference. For enrichment analysis, we mapped all differentially expressed genes to terms in GO and KEGG database, and looked for significantly ($p \leq 0.01$) enriched GO and KEGG terms compared with the genome background (the whole *F. occidentalis* transcriptome in this study).

4.6. qPCR analysis

To confirm the results of the differentially expressed genes analyses by RNA-Seq, the expression levels of 36 randomly selected genes were quantified and compared between TSWV-infected and non-infected population using qPCR. The cDNA was synthesized using the SYBR PrimeScript reverse transcription-PCR (RT-PCR) kit II (Shine Gene). The qPCRs were carried out on the FTC2000 (Canada) fast real-time PCR system with SYBR green detection. Each gene was analyzed in triplicate, after which the average threshold cycle (CT) was calculated. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of 18S was as an endogenous control.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2013.02.005.

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