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miRNAs induced by white spot syndrome virus involve in immunity pathways in shrimp *Litopenaeus vannamei*

Wenlin Wu[a], Congjie Dai[a], Xunwei Duan[a], Cuifang Wang[a], Xiaosi Lin[a], Jiaying Ke[a], Yixuan Wang[a], Xiaobo Zhang[a,b,*], Haipeng Liu[c,d,**]

[a] Fujian Province Key Laboratory for the Development of Bioactive Material from Marine Algae, Quanzhou Normal University, Quanzhou, 362000, China
[b] School of Life Science, Zhejiang University, Hangzhou, 310004, China
[c] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, 361102, Fujian, China
[d] Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources (Xiamen University), State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen, 361102, Fujian, China

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ABSTRACT

White shrimp *Litopenaeus vannamei* are widely cultured in the world and white spot syndrome virus (WSSV) led to huge economic losses in the shrimp industry every year. In the present study, miRNAs involved in the response of shrimp *L. vannamei* to WSSV infection were obtained through the Illumina HiSeq 2500 high-throughput next-generation sequencing technique. A total number of 7 known miRNAs and 54 putative novel miRNAs were obtained. Among them, 14 DEMs were identified in the shrimp infected with WSSV. The putative target genes of these DEMs were related to host immune response or signaling pathways, indicating the importance of miRNAs in shrimp against WSSV infection. The results will provide information for further research on shrimp response to virus infection and contribute to the development of new strategies for effective protection against WSSV infections.

1. Introduction

White shrimp *Litopenaeus vannamei* (*L. vannamei*) are widely cultured marine food shrimp species in the world. In recent years, accompanying the fast development of white shrimp aquaculture, various diseases, especially viral diseases have posed severe threat to shrimp aquaculture. Among viral diseases, white spot syndrome virus (WSSV) is one of the most important viral pathogens which can cause > 90% mortality in cultured shrimp and led to huge economic losses in the shrimp industry every year [1]. Preventing and controlling the spread of WSSV has become a priority to the shrimp industry. Although great advances have been made in both pathogen study and host immune responses against the virus, there is no method or drug to restrict or treat the occurrence and spread of the virus in the field. WSSV is still a great threat to the shrimp culture. Understanding the mechanisms of the host-virus interaction might help to find new strategies and methods for WSSV control [2].

MicroRNAs (miRNAs) are a class of endogenous noncoding small RNA molecules, 19 to 25 nucleotides, typically 22 nucleotides in length. They are involved in post-transcriptional regulation of gene expression by binding primarily to the 3′UTR of target mRNAs to repress translation and/or accelerate the decay [3]. Generally, a single miRNA regulates multiple target genes, and a single gene might be regulated by multiple mature miRNAs. The discovery of miRNAs and the establishment of their role in molecular pathways has brought a huge advance in molecular biology [4]. Numerous studies have found roles for miRNAs in regulation of gene expression for important biological processes including cellular proliferation and differentiation, tissue development, and the innate and adaptive immune responses [5].

miRNAs from shrimp Marsupenaeus japonicus (*M. japonicus*) were first reported in 2011 [6]. Till present, many studies have demonstrated viral infections can alter the cellular miRNA expression profile. Huang reported shrimp *M. japonicus* miRNAs responded to WSSV infection in 2012 and revealed that immune signaling pathways were mediated by miRNAs [7]. Among these shrimp miRNAs, miR-7 could target the 3′-untranslated region (3′UTR) of the WSSV gene wsv477 and inhibited WSSV replication [8], miR-1 regulated phagocytosis [9], and miR-100 was involved in the antiviral immunity by regulating apoptosis in *M.*

* Corresponding author. Fujian Province Key Laboratory for the Development of Bioactive Material from Marine Algae, Quanzhou Normal University, Quanzhou, 362000, China.
** Corresponding author. State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, 361102, Fujian, China.
E-mail addresses: zxb0812@zju.edu.cn (X. Zhang), haipengliu@xmu.edu.cn (H. Liu).

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japonicus [10]. As the research continued, more shrimp miRNAs have been identified and their functions were studied [11–16].

In the present study, to identify miRNAs involved in the response of shrimp *L. vannamei* to WSSV infection, sequences and expression profiles of miRNAs in the gills of normal and WSSV-infected *L. vannamei* were obtained through the Illumina HiSeq 2500 high-throughput next-generation sequencing technique. Differential expressed miRNAs (DEMs) from the two libraries were identified, and their potential target genes were predicted. DEMs were further verified by the real-time RT-PCR technique. The results will extend the knowledge of miRNA regulation, provide information for further research on shrimp response virus infection and contribute to the development of new strategies for effective protection against WSSV infections.

2. Materials and methods

2.1. Shrimp culture and WSSV challenge

Shrimp *L.vannamei* (approximately 10–15 g body weight) were bought from a commercial aquaculture market in Quanzhou, Fujian Province, China. They were initially cultured in 80 L aquariums filled with air-pumped circulating seawater tanks at 26–28 °C for 7 days. They were fed with commercial diet before and during experiments. After 7 days, they were randomly divided into two groups, with each group including 30 shrimp. In the WSSV infection experiment, 3.2×10^7copies of WSSV per shrimp were intramuscularly injected into shrimp by using a syringe with a 29-gauge needle. This group was named as WSSV-infected group. 48 hs after WSSV infection, gills were collected from ten WSSV-infected shrimp and from ten control group. Control shrimp received 1x PBS injection.

2.2. Total RNA isolation

For each group, three shrimps were randomly chosen and their gills were used for total RNA isolation. Total RNA was extracted with TRIzol reagent (Invitrogen, USA) by following the manufacture's instruction and treated with DNase I (Takara, Japan) according to the manufacturer's manual to remove any genomic DNA. The concentration of total RNAs was estimated by spectrophotometry at OD_{260} (Eppendorf, Germany) and their integrity were examined by electrophoresis on a 2% agarose gel. The extracted RNAs were stored at −80 °C for later use.

2.3. Illumina sequencing

According to the conventional approach, an amount of 1.5 μg total small RNA per sample was used as input materials for the small RNA library preparation. The cDNA library was prepared according to the method and process of the Small RNA Sample Preparation Kit in accordance with the kit instructions (Illumina, RS-200-0048). Briefly, total RNA were excised and purified for 18-30 nt fragments using polyacrylamide gel electrophoresis. Next, a pair of adaptors was ligated to the 5′and 3′ends of the small RNA using T4 RNA ligase. The adapted small RNAs were then converted to cDNAs and amplified by PCR. The PCR products were purified through 4% agarose gels and were prepared for Illumina sequencing. The qualified libraries were sequenced by an Illumina HiSeq 2500 platform to generate 50-bp single-end reads [17]. To confirm the results, sequencing work was repeated three times.

2.4. Basic data analysis and miRNAs prediction

After high throughput small RNA sequencing was complete, a filtering step was carried out to remove the low quality reads, including 1) containing poly-N tails or 5′ adapter contaminants, 2) without 3′adapters or insert tags, 3) lengths shorter than 18 nucleotides and longer than 35 nucleotides, and 4) containing poly-A, T, G, or C, or low quality reads from the raw data. Then, clean small RNAs were annotated by alignment against EST of the *L. vannamei* and *Cherax. quadricarinatus* sequences and *Drosophila melanogaster* genome (NCBI Assembly GCA_000001215.4) in GenBank by Bowtie (v1.1.2) to calculate the reads in different regions of the genome distribution, allowing at most one nucleotide mismatch [18]. The clean reads were compared to the Rfam database (ftp://selab.janelia.org/pub/Rfam) to match the known small RNAs of tRNAs, rRNAs, snoRNAs, snRNAs and other non-coding RNAs [19]. Reads matching with those known small RNAs were excluded. The novel miRNA candidates were predicted using miRdeep2 software [20] with *L. vannamei* transcriptome deep sequencing data [21] based on hairpin-like secondary structure pattern.

2.5. Analysis of differentially expressed miRNAs

For differentially expressed miRNAs (DEMs) analyzes, reads per million reads (RPM, miRNA counts/total counts of each sample × 1 million) were used as the value of normalized miRNA expression levels. The microRNAs with the RPM value less than 100 were excluded for comparison due to low expression levels. Then, the fold-change (Fold change = $\log_2$(infection/control) and P-value were calculated from the normalized expression. miRNAs with $P < 0.05$ and $\log_2 > 1$ are identified as significantly DEMs. To evaluate the accuracy of expression profiling for the miRNAs, the obtained results were validated by quantitative RT-PCR (qRT-PCR) analysis. Total RNA was extracted from the gills of 3 shrimps in each group. The RNAs were subjected to reverse transcription by Transcript Green miRNA Two-Step qRT-PCR SuperMix (Transgen Biotech, Beijing, China) following the manufacture's instruction. The sequences of real-time PCR primers of selected miRNAs and U6 were showed in Table 1. The qRT-PCR of miRNAs were carried out in an Applied Biosystems Stepone-plus (Applied Biosystems, Life Technologies, USA). The data were calculated according to the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicates.

2.6. Target gene prediction of DEMs

The target genes of DEMs were predicted with miRanda [22] and RNAhybrid [23]. miRanda was used to match the entire miRNA sequences, and the parameters were set as free energy < −20 kcal/mol and score > 50. The final results predicted by the two algorithms were combined and the overlaps were calculated. Enrichment analysis of these predicted target genes were analyzed by Gene Ontology (GO) (http://www.geneontology.org/) and KEGG pathway (http://www.genome.jp/kegg/).

Table 1
Details of the primer sequence used for qPCR.

Primers	Sequences(5′-3′)
Novel-miR-1-F	TTGTGATTTCTGCCCCGT
Novel-miR-2-F	TCTCCTTAGAGTCGGGTT
Novel-miR-3-F	GATGGGGTCTGTGGCGAG
Novel-miR-4-F	TGTGTGTGTAGTTATATG
Novel-miR-5-F	AATTGGGTCTCGTGACGCCA
Novel-miR-6-F	TATGGATGTGTATATGTATATA
Novel-miR-7-F	GAAAGCTGTGCTCTTCTACGT
Novel-miR-8-F	GAGCCAAGTAGAATTGCTGGA
Novel-miR-9-F	GCGGACCATGTTATCAATTCA
Novel-miR-10-F	CAGCAGCAGCAGCAGAATC
Novel-miR-11-F	ATTGAATTTTGTCTTTTTCTTTTC
mja-miR-6489-5p-F	GGCACCGGACTGGCGCCCTT
mja-miR-6489-3p-F	CGACGGAAAGGTGTCCAAGC
mja-miR-6491-F	GCGGTAGCCCGGGGAAGA
U6-F	CGCAAGGATGACACGCAAATT
Reverse Primer	Transgen universal primer

3. Results

3.1. Small RNA annotation and length distribution analysis

Through Illumina HiSeq 2500 high-throughput small RNA sequencing, 21,877,990 and 19,460,693 raw reads were respectively obtained from two small RNA libraries representing the control group (CG) and the WSSV-challenged (WG) group. After removal of low quality reads, adapter sequences, poly A sequences, and sequences smaller than 18 nt, a total of 18,734,130 and 16,485,015 clean reads were respectively obtained for the CG library and the WG library, respectively. The length distribution of clean reads was determined. The results showed that small RNAs of 22 nt in length were the most common, followed by those of 21 nt. All the clean reads were annotated using Silva, GtRNAdb, Rfam, and Repbase and our *L. vannamei* transcriptome databases, and classified into six categories including, rRNA, tRNA, snRNA, snoRNA, miRNA and those without any annotation.

3.2. Identification of known and putative novel miRNAs

After getting rid of other classes of small RNAs (rRNA, snRNA, snoRNA, tRNA, and etc.), a total number of 7 known miRNAs and 54 putative novel miRNAs were identified with miRDeep2 software from the deep sequencing data (Table 2). The length of miRNAs of distributed from 18–25 nt. The peak size of miRNAs was 22 nt, followed by 23 nt and 24 nt (Fig. 1).

3.3. Analysis of DEMs

To identify miRNAs involved in WSSV challenge, the expression profiles of the known and novel miRNAs were examined. During the analysis, $|\log2(FC)| \geq 1$, and $FDR \leq 0.01$ indicated that differences expression profiles in the miRNA counts were statistically significant. According to the expression quantity, the most significantly expressed miRNAs were shown in Table 3. There are a total of 14 miRNAs which were significantly differentally expressed post WSSV challenge with 6 downregulated and 8 upregualted respectively.

To validate the credibility of HiSeq sequencing and bioinformatics analysis results, the expression level of the DEMs were detected by real-time RT-PCR using U6 as the internal reference. The results showed that there was a consistency between the deep sequencing and real-time RT-PCR assay (Fig. 2).

3.4. Target gene prediction and functional analysis of DEMs

Identification of the target mRNA of each miRNA could provide clues for the role of miRNA in shrimp response to WSSV infection. The putative target genes of DEMs were predicted using miRanda and RNAhybrid. The miRNA target genes were first predicted by miRanda and further tested by RNAhybrid software. A total of 52890 target genes were predicted for the DEMs by the two prediction programs. The target genes were further processed for sequence annotation by using NR、Swiss-Prot、GO、COG、KEGG、KOG and Pfam databases. The annotation results are summarized in Table 4. The gene ontology analysis based on the biological processes showed the predicted target genes could be clustered into different GO terms including biological progress, cellular component, and molecular function. These three main GO categories were further classified into 43 subcategories (Fig. 3). In the meantime, COG annotation which is important for functional annotation and evolutionary studies was conducted to predict and categorize possible roles of target genes. The target genes were functionally categorized into 24 different COG categories (Fig. 4). The most enriched COG category is “general function prediction only”, followed by “translation, ribosomal structure and biogenesis”, and “posttranslational modification, protein turnover, chaperones”.

KEGG is a pathway-based categorization of orthologous genes which provides useful information for gene function prediction [24]. To obtain more valuable information for functional prediction, all target genes were mapped to KEGG database. The annotated target genes were implicated in 50 different signaling pathways and classified into 5 different KEGG pathways, including Metabolism, Cellular Processes, Genetic Information Processing, Environmental Information Processing, and Organismal Systems (Fig. 5). Some innate immunity pathways, such as endocytosis, phagosome, natural killer cell mediated cytotoxicity, apoptosis, Toll like and NOD like receptor signaling pathway, JAK-STAT signaling pathway, and mTOR signaling pathway are also

Table 2
Identification miRNAs and their sequences.

miRNA name	Length(nt)	Sequence(5′-3′)
mja-miR-6489-5p	20	GGCACCGGACUGGCGCCCUU
mja-miR-6489-3p	23	CGACGGAAAGGUGUCCAAGCUGG
mja-miR-6491	18	GCGGUAGCCCGGGGAAGA
mja-miR-6492	19	GUUGACCGAAGCGGAGGAG
mja-miR-6493-5p	22	ACGUCCGGCAGGUUUUACCCCU
mja-miR-6493-3p	24	AGGGGGAAACCGCGCUGAGCGUUA
mja-miR-6494	20	AGGGCGCGGUGGUGAGCGUA
Novel-miR-1	18	UUGUGAUUUCUGCCCCGU
Novel-miR-2	18	UCUCCUUAGAGUCGGGUU
Novel-miR-3	19	GAUGGGUCUGUGGCGAGGA
Novel-miR-4	18	UGUGUGUGUAUGUAUAUG
Novel-miR-5	24	AAUUGGGUCUCGUGACGCCGACUU
Novel-miR-6	22	UAUGGAUGUGUAUAUGUAUAUA
Novel-miR-7	20	GAAAGCUGCUCUUCUACGUC
Novel-miR-8	25	GAGCCAAGUAGAAUUGCUGGAUGUG
Novel-miR-9	24	GCGGACCAUGUUAUCAAUUCAAGA
Novel-miR-10	19	CAGCAGCAGCAGCACAAUC
Novel-miR-11	24	AUUGAAUUUUGUCUUUUUCUUUUC
Novel-miR-12	21	UGACUAGACUCUUACUCAUCU
Novel-miR-13	25	AGGGGCUUAUCUCGAUGAAGGGACU
Novel-miR-14	23	AGACCCGAGGUCGUCGAGAACCC
Novel-miR-15	22	UGGGUCUUACUAGAAACAGUGG
Novel-miR-16	23	AAUGGAAGGCCAAUGACAAAGAC
Novel-miR-17	22	GUACCGAAGCUCUGGAUGUCAU
Novel-miR-18	24	UGACUGUUAUUGUGUGCUUGUGCC
Novel-miR-19	18	AAGGUGUGGAAUGCAGCA
Novel-miR-20	23	UGGCGAGGAGAGGAACGUCGUGU
Novel-miR-21	19	GUGGUUCCCUUCUCUGGUA
Novel-miR-22	22	UCAUUGUGUAUUCUGUAGACUG
Novel-miR-23	24	AGAGCCUGACUGAUGGGGACCUCC
Novel-miR-24	22	UGAAGGAGUAGCUUUGUGAGUC
Novel-miR-25	23	UUUCUGGCAAAGACUCUUGUACU
Novel-miR-26	22	CAAUGCCCUUGGAAAUCCCAAA
Novel-miR-27	24	GAGAAGAUUUCUGAGUGGAAGAUA
Novel-miR-28	21	UGUGUGAGUGUGAGUGUGAGU
Novel-miR-29	21	UGGAUUCAGAGUCGCCUGUUU
Novel-miR-30	23	GCUCUACCCGGGGCUGCUGUACC
Novel-miR-31	20	UUGGUGAUAUUCGUUUCUUG
Novel-miR-32	24	GAUGCUCUGGAAAGUACAGAAACA
Novel-miR-33	23	UCCUGCCUGCCUUGGAUACUGCA
Novel-miR-34	20	AUACAAACAUGACUUCUAUC
Novel-miR-35	25	UGACUUGUGUGUUGCACUCUGGGGA
Novel-miR-36	22	UCUCUCCUGUAGGUCCUCUACC
Novel-miR-37	21	UCCCUGAGACCCUUUCUUGUG
Novel-miR-38	22	CUCUGAAAGCUGUGCUCUUCCU
Novel-miR-39	19	GACGUGACGAUCGUGGACG
Novel-miR-40	23	GCAUUGAUGAUUUUCAUGGACUG
Novel-miR-41	20	UGGAUAGUAGUUCUCGUGGC
Novel-miR-42	23	CUUCUGUAGCUGUUUGAUACUGC
Novel-miR-43	23	CACUUGACGCAAGAAAUCUCUUC
Novel-miR-44	24	UCUGUCACGACUUAUAUUUUGUAC
Novel-miR-45	21	UCACAACCUCCUUGAGUGAGU
Novel-miR-46	22	UAGGAACUUCAUACCGUGCUCU
Novel-miR-47	23	GUGGCUUUUGAAGAUCUCUGAUU
Novel-miR-48	22	CAUCUGAGAGGGAUAAGACCGA
Novel-miR-49	20	UGGCUGACAACCAUAGAGCU
Novel-miR-50	22	AUUUCUCACUGCCUUGGUUUCC
Novel-miR-51	22	AUUUCGAUUGAAUCUUCAUAAC
Novel-miR-52	22	CCUGCCACCGCCUCAUGGCCUU
Novel-miR-53	22	AAGCAGAAGACCGAGGACCGGA
Novel-miR-54	22	UUGUCUCGGCUAAAUGAUGCUC

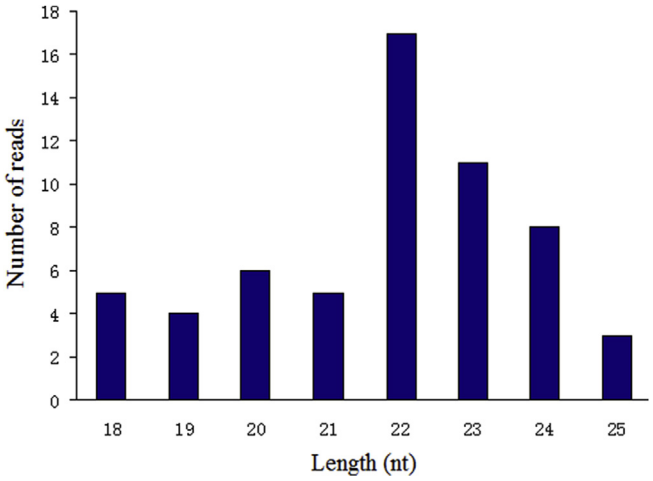

Fig. 1. The length distribution of miRNAs. The miRNAs length of *L. vannamei* distributed from 18–25 nt. The peak size of miRNAs was 22 nt, followed by 23 nt and 24 nt.

Table 3
Differentially expressed miRNAs.

miRNA name	Fold change (log2FC)
Novel-miR-1	−26.21
Novel-miR-2	−1.92
Novel-miR-3	−2.48
Novel-miR-4	+24.23
Novel-miR-5	+3.74
Novel-miR-6	+3.34
Novel-miR-7	+1.97
Novel-miR-8	+24.65
Novel-miR-9	+1.95
Novel-miR-10	+24.65
Novel-miR-11	+3.01
mja-miR-6489-5p	−1.28
mja-miR-6489-3p	−1.51
mja-miR-6491	−1.11

Table 4
Summary of the annotations of all target genes.

Database	Annotated Number	percentage (%)
Annotated in Nr database	23985	89.96
Annotated in Swiss-Prot database	15474	58.04
Annotated in GO database	12032	45.13
Annotated in COG database	11182	41.94
Annotated in KOG database	19338	72.52
Annotated in KEGG database	14579	54.68
Annotated in Pfam database	21282	78.82
All Annotated unigenes	26663	100

involved in KEGG pathways.

4. Discussion

miRNAs play essential regulatory roles in living organisms by targeting mRNA for cleavage or translational repression. More and more research results show that miRNAs play important roles in several biological processes such as development, differentiation, proliferation, apoptosis, and immunity. In recent years, numerous miRNAs have been identified to be involved in regulating genes of host and pathogenic viruses [7,8,25,26]. They play a dual role in host-virus interaction, some of these miRNAs inhibit viral replication directly or alter cellular processes to limit the virus [8,13], some miRNAs were benefit for virus infection by targeting host immunity pathway [27].

In the present study, to identify the miRNAs involved in *L. vannamei* response to WSSV infection, the miRNA expression profiles was analyzed through the Illumina Hiseq 2500 high-throughput next-generation sequencing technique. The results showed that a total number of 7 known miRNAs and 54 putative novel miRNAs were identified with a total of 14 miRNAs were significantly differentially expressed post WSSV challenge. These DEMs were further confirmed by the real-time RT-PCR technique. The results of real-time RT-PCR was consistent with the deep sequencing, indicating that the next-generation sequencing technique is efficient for miRNA expression profile analysis in shrimp *L. vannamei*.

The expression profile of host miRNAs were reported to alter in the case of viral infection [13,28,29]. In the present study, we found 14 miRNAs displayed significant expression changes upon *in vivo* during WSSV challenges, suggesting they are involved and may play important

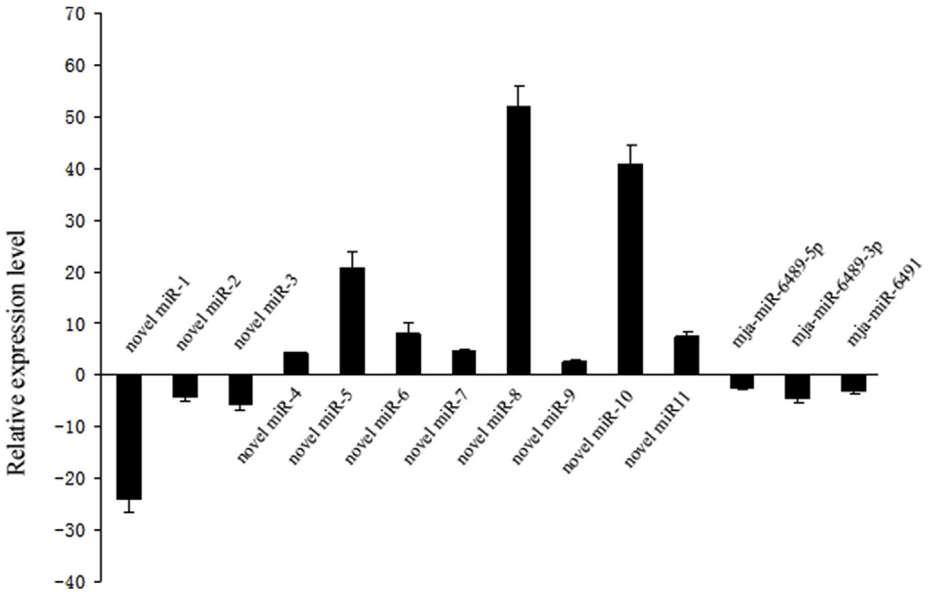

Fig. 2. The DEMs were confirmed by real-time PCR.

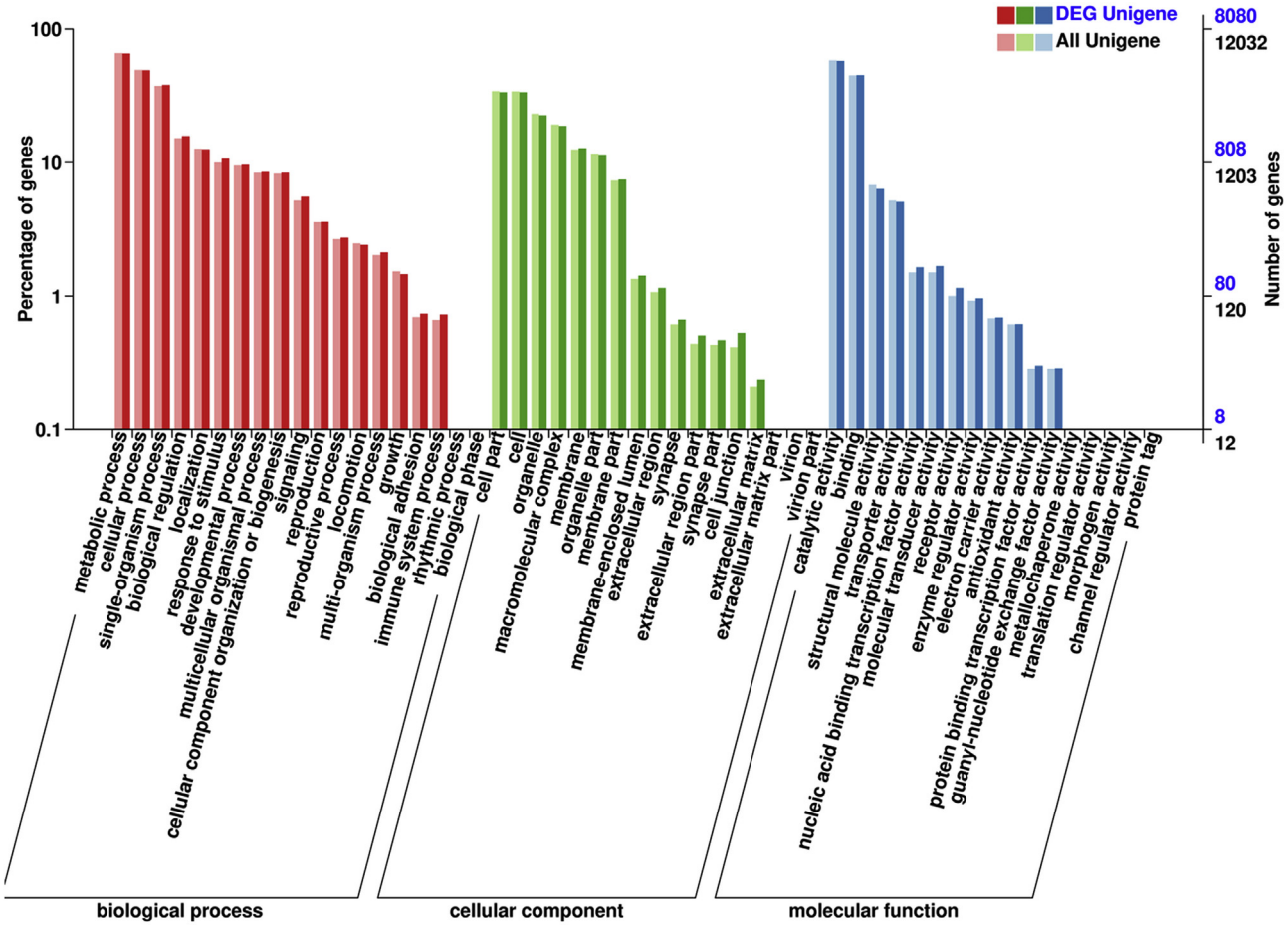

Fig. 3. Enriched GO term analysis of target genes. The top three significantly ($P < 0.05$) enriched GO terms of the predicted target genes of DEMs were metabolic process, catalytic activity, and cellular process.

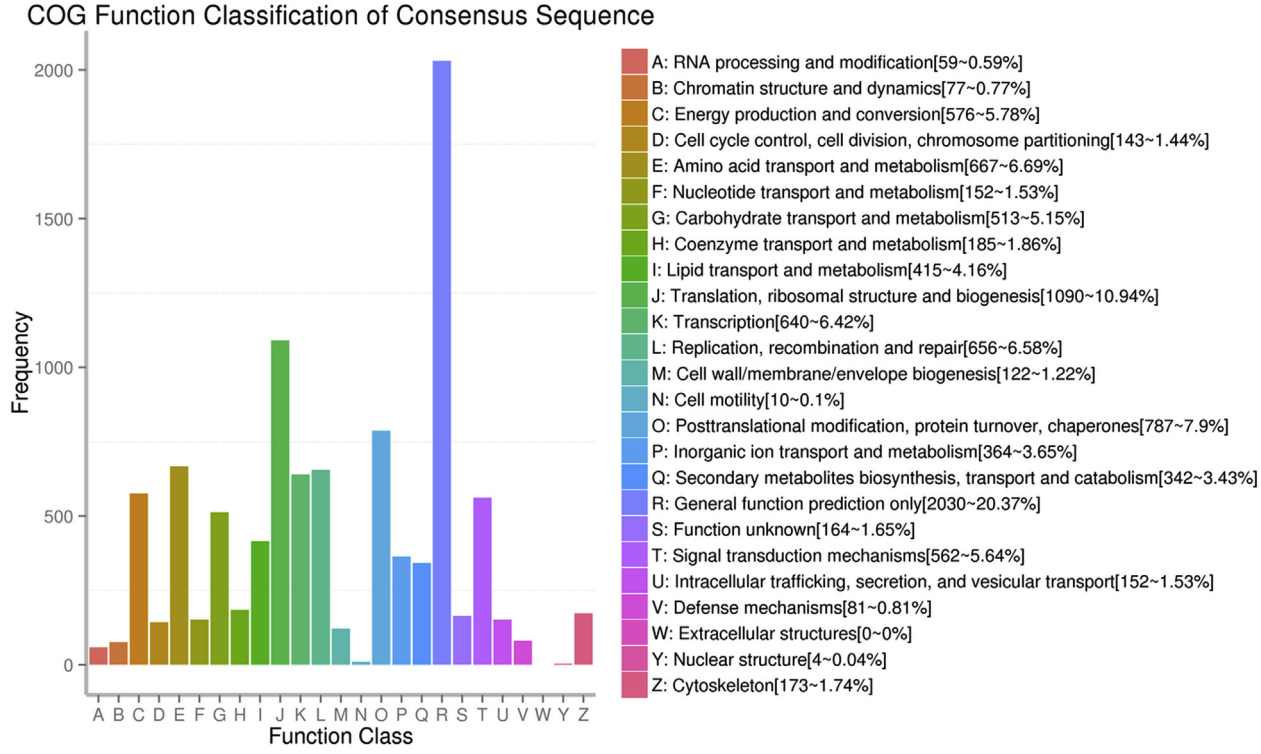

Fig. 4. COG function classification of different expression miRNAs.

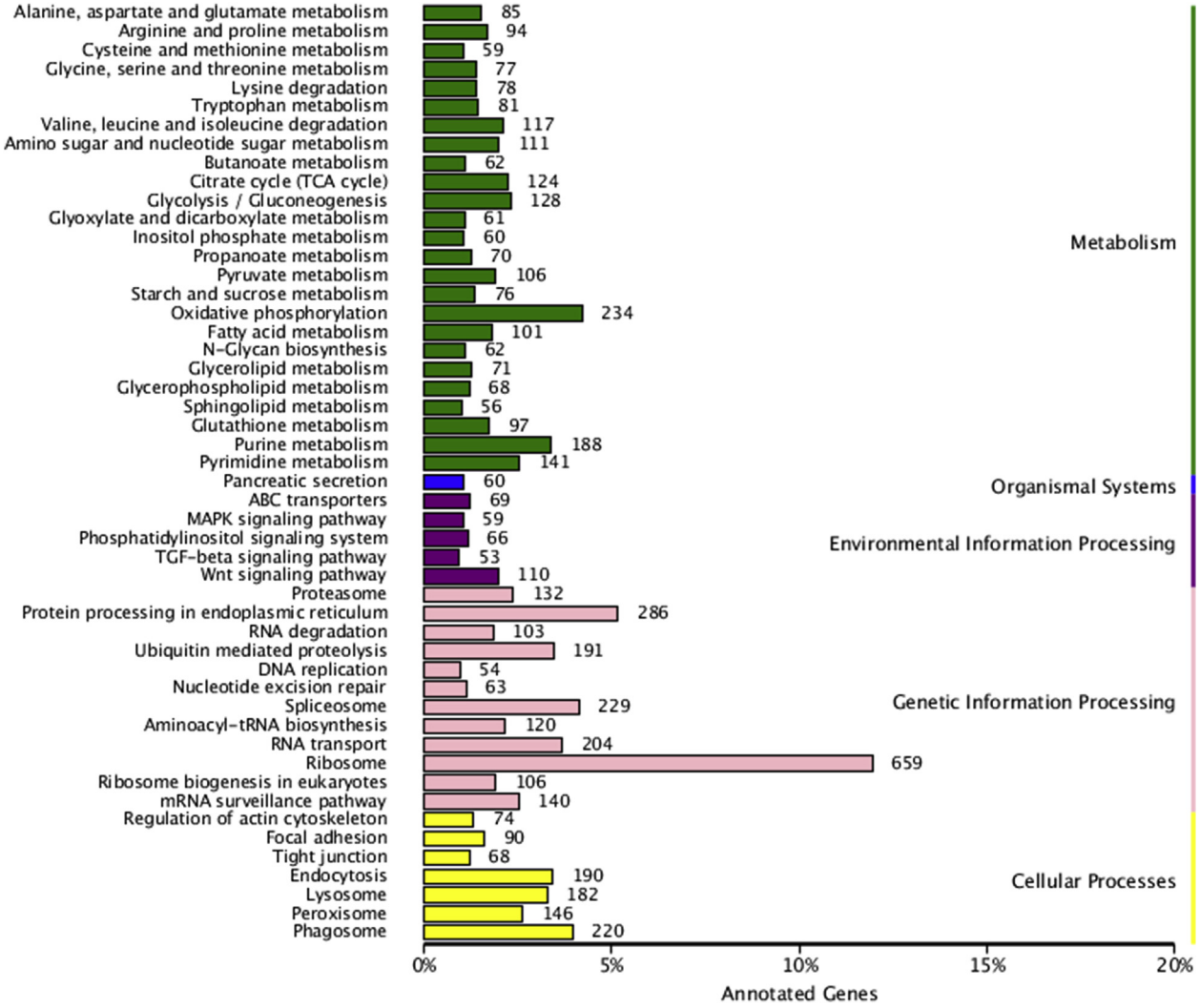

Fig. 5. Enriched KEGG pathway analysis of target genes. The top ten significantly ($P < 0.05$) enriched KEGG pathways of the predicted target genes of DEMs were ribosome (659), protein process in endoplasmic reticulum (286), oxidative phosphorylation (234), spliceosom (229), phagosome (220), RNA transport (204), ubiquitin mediated proteolysis (191), endocytosis (190), purine metabolism (188), and lysosome (182).

roles in the host-virus interactions. Because miRNAs regulate gene expression by binding to their target genes, identification of miRNA targets is necessary to uncover the miRNA's function in a certain biological condition. In our study, the most popular programs, miRanda and RNAhybrid were carried out to predict putative target genes of each different expression miRNA. Subsequently, functional annotation of these predicted targets revealed 26663 genes were annoyed by using NR、Swiss-Prot、GO、COG、KEGG、KOG and Pfam databases. The results of KEGG pathway analysis showed that target genes were enriched in a broad range biological processes, such as phagosome, endocytosis, natural killer cell mediated cytotoxicity, peroxisome, JAK-STAT pathway, autophagy, and mTOR pathway, indicating that these differential expression miRNAs were involved in immune response.

In this study, phagosome and endocytosis pathway was putative target genes of different expression miRNA. Phagocytosis or receptor-mediated endocytosis is an process that phagocytic cells engulf pathogens or antigens into phagosomes. Phagosomes then fuse with lysosomes to form phagolysosomes, where the pathogens are degraded due to respiratory or oxidative burst, low pH, action of lysosomal acid hydrolases and secretion of microbicidal substances, such as lipase, peroxidase, serine protease and hydrogen peroxide (H_2O_2) to decompose the invasive pathogens. Thus, phagocytosis is an important process of the innate immune response [30,31]. Increasing evidence indicates that phagocytosis has an important role in the crustacean immune response to pathogen infection. In shrimp, several molecules implicated in the regulation of phagocytosis have been identified to have essential functions in the host antiviral responses [32–34]. The present study showed that different expression miRNA induced by WSSV infection may target to genes of phagosome and endocytosis, indicating that miRNA profiles plays an important role in antiviral immunity of shrimp.

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, discovered in many multicellular organisms, such as mammals, fishes, insects, crustaceans, and nematodes, is firstly identified as cytokine and growth factor signaling pathway in mammal [35]. As a crucial part of innate immune system, the JAK/STAT pathway plays key role in various immune responses and inflammation in organisms. The JAK/STAT pathway contributes to antibacterial and antiviral immunity in mosquito [36,37]. In *Drosophila*, this signaling pathway plays an important role in antivirus response when challenged with *Drosophila* C virus [38]. Cytokine activation through the JAK–-STAT pathway of numerous genes has been suggested in countering viral infections in Drosophila [38,39]. A similar JAK–STAT signaling pathway was also characterized in shrimp antiviral immunity. After WSSV infection, an increased level of STAT was detected in shrimp [38–42], suggesting that JAK–STAT pathway could play an important role in defense against WSSV. Some studies indicated that WSSV

successfully exploit the shrimp JAK–STAT pathway to enhance the expression of *ie1*, and promote viral replication [40,41]. Our study reveals that miRNAs induced by WSSV infection may participate in shrimp antiviral immunity through JAK–STAT signaling pathway.

Pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs). PAMPs include lipopolysaccharides (LPS), peptidoglycan (PGN), flagellin, and microbial nucleic acids. PRRs include the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), and the retinoid acid-inducible gene-1 (RIG-1)-like receptors (RLRs). Recognition of these PAMPs by PRRs results in an inflammatory, antimicrobial response. Studies indicated that shrimp Tolls function, as pattern recognition receptors, are similar to TLRs in mammals and are able to sense microbial derived products, such as LPS and PGN. Three types of shrimp Tolls and the Spätzle/Tolls/MyD88/Pelle/TRAF6/Dorsal signaling pathway in shrimp have been reported in the literature. The activation of Toll pathway transports the signals to the NF-κB transcription factor Dorsal, and then activates the transcription of collective sets of antimicrobial peptides. This signaling pathway have been found to be involved in shrimp anti-WSSV response [43–48]. NLR signaling activates three targets after PAMP recognition, including NF-κB, MAPKs, and caspase-1. NLR signaling is very similar to that of the TLRs, with shared down-stream targets. For example, TLRs recruit adaptor proteins such as MyD88 and TRIF, which activate the MAPK and NF-κB signaling pathways. NLRs have been discovered throughout the plant and animal kingdoms. NLR played an important role in the innate immune system of the sea urchin [49,50]. In the present study, different expression miRNAs target genes involve in the signaling pathway of TLR and NLR, indicating that miRNAs regulate shrimp immune response by PRRs.

The PI3K/AKT/mTOR pathway is one of the major signaling pathways in regulating various cellular functions and biologic processes, including protein synthesis, cell cycle progression, cell survival, apoptosis, angiogenesis. Apoptosis and autophagy can be regulated by mTOR. The role of mTOR can be either pro- or anti-apoptotic. Activation of S6K, a downstream target of mTOR, inhibits the activity of pro-apoptotic factor BAD and thus reduces apoptosis [51]. Conversely, mTOR can phosphorylate p53 which enhances the transcription of Bax. The increase of this pro-apoptotic factor stimulates apoptosis [52]. Apoptosis is considered as an innate cellular response to limit viral replication and eliminate viral-infected cells [53,54]. Increasing evidence has shown that apoptosis occurs after WSSV infections in shrimp. Several caspases have been cloned and functionally characterized from multiple different shrimps. Silencing of caspase resulted in increased copy numbers of WSSV, suggesting effector caspases have protective roles in the host defense against WSSV infection [55,56]. Some shrimp apoptosis-related factors such as Dap-1, PTEN, p53, fortilin, Wnt5b, Harbl1, and HHAP were found to regulate shrimp apoptosis during WSSV infection [57–63]. In addition to proteins, shrimp also encoded miRNAs directly or indirectly regulate antiviral apoptosis. Shrimp miR-100 was up-regulated after WSSV infection and the up-regulated expressed miR-100 could inhibit apoptosis [64]. Shrimp miR-1000 targeted p53, and silencing of miR-1000 resulted in significant increases of apoptotic activity and virus infection [65]. Shrimp miR-12 could silenced the expression of BI-1 to trigger the antiviral apoptosis [66]. To counteract host apoptosis, WSSV could have taken some strategies to counter antiviral apoptosis. Firstly, WSSV encodes viral proteins with antiapoptotic activities, including WSSV449 [67,68], WSSV222 [69], VP38 [70], WSSV134 and WSSV322 [71] to suppress shrimp apoptosis-related factors. Secondly, Recent study showed that WSSV could generate their own miRNAs to control shrimp apoptosis. For example, the WSSV-miR-N24 could target shrimp caspase 8 gene, thus repressed the apoptosis of shrimp hemocytes and promoted viral replication [72]. Ren et al. found that regulation of Dorsal expression by WSSV viral miRNA, WSSV-miR-N13 and WSSV-miR-N23, suppressed the Spz-Toll-Dorsal-ALF signaling pathway in shrimp, leading to virus infection [73]. Since apoptosis is one of important antiviral immune responses in shrimp, disturbance of host apoptosis will facilitate survival and multiplication of WSSV. Our study confirmed again that miRNAs participate in shrimp antiviral apoptosis.

PI3K/AKT/mTOR pathway was found to be involve in Warburg effect during WSSV infection. The Warburg effect is a metabolic shift that facilitates the production of more energy and building blocks to meet the enormous biosynthetic requirements of cancerous and virus-infected cells. Chen et al. first found that at the viral replication stage, WSSV induces metabolic changes resembling the Warburg effect in shrimp hemocytes [74]. Su et al. further demonstrated that the PI3K-Akt-mTOR pathway was importance in triggering this WSSV-induced Warburg effect. Chemical inhibition of Akt, mTORC1 and mTORC2 suppressed the WSSV-induced Warburg effect and reduced both WSSV gene expression and viral genome replication. When the Warburg effect was suppressed by the mTOR inhibitor Torin 1, even the subsequent up-regulation of the TCA cycle was insufficient to satisfy the virus's requirements for energy and macromolecular precursors [75]. Chen et al. indicated that the PI3K-Akt-mTOR-regulated Warburg effect was acted to neutralize ROS production and reduce the host's oxidative stress defenses during WSSV infection, thereby restoring the cell to a state of redox balance and allowing the virus to successfully replicate [76]. The PI3K-Akt-mTOR pathway was also found to induce lipid biosynthesis to support WSSV morphogenesis [77].

PI3K/AKT/mTOR pathway also regulates autophagy. The activation of mTOR phosphorylates ULK1 and inhibits the initiation of autophagy [78]. Autophagy primarily functions as a protective mechanism during nutrient deprivation by degrading carbohydrates, lipids and proteins to maintain energy homeostasis. Recently, autophagy has been reported to play a crucial function in innate and adaptive immunity against invading microorganisms, and impairment of autophagy will enhance susceptibility to infection [79]. Autophagy can trigger the degradation of viruses and viral factories and promote the survival of virus-infected cells through activating innate immunity and adaptive im munity [80]. However, In the process of coevolution with their hosts, many viruses have developed a variety of strategies to destroy or manipulate the autophagy pathway. Some viruses can exploit autophagy to promote their replication. Studies reported that the host autophagy machinery is necessary for the replication of dengue, influenza A, and hepatitis C viruses [81]. In shrimp, miR-71 and miR-13b, were up-regulated in response to viral infection and autophagy inhibition by targeting their target genes [82]. In our data, miRNAs target genes in mTOR pathway and autophagy, indicating that miRNAs induced by WSSV could affect shrimp autophagy.

In conclusion, a total number of 7 known miRNAs and 54 putative novel miRNAs were obtained in the present study. Among them, 14 DEMs were identified in the shrimp infected with WSSV. The putative target genes of these DEMs were related to host immune response or signaling pathways, indicating the importance of miRNAs in shrimp against WSSV infection. These results may provide a guiding theoretical foundation for future studies about shrimp antiviral innate immunity.

Declarations of interest

None.

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