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Short communication

Differential expression of microRNAs in mud crab *Scylla paramamosain* in response to white spot syndrome virus (WSSV) infection

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ABSTRACT

Till date numerous microRNAs (miRNAs) have been discovered from various organisms, including fish, shellfish and crustaceans. The miRNAs are known to regulate immune functions in crustaceans, but little is known about the role of miRNAs against viral infection in crab. We performed small RNA sequencing to characterize the differentially expressed miRNAs in WSSV infected *Scylla paramamosain*, in comparison to that in uninfected crab, at 2 h and 12 h post infection. In total, 24 host miRNAs were up-regulated and 25 host miRNAs were down-regulated in response to WSSV infection at 2 h post infection. And 27 host miRNAs were up-regulated and 30 host miRNAs were down-regulated in response to WSSV infection at 12 h post infection. Further, the gene ontology analysis revealed that many signaling pathways were mediated by these miRNAs. The integral component of membrane is the most important biological process and endocytosis pathway is the most important pathway, which indicates that endocytosis is very important for WSSV infection. This study is one important attempt at characterizing crab miRNAs that response to WSSV infection, and will help unravel the miRNA pathways involved in antiviral immunity of crab.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level and play an important role in development, homeostasis, and immunological defense function [1–4]. Mature miRNAs, which are between 18 and 25 bp in length, are transcribed as primary-miRNA molecules which contain a characteristic stem loop structure [1]. In animals, miRNAs regulate gene expression through imperfect sequence-specific binding to the 3′-untranslated regions (3′UTR) of target mRNAs and usually causing translational repression [5]. Till date numerous miRNAs have been discovered in variety of organisms including crustaceans. The crustaceans such as shrimps are cultured and are economically valuable; hence they need to be protected from infection caused by pathogens such as bacteria and viruses. WSSV is devastating to the shrimp industry and has dramatically reduced shrimp production worldwide, and cumulative mortality can reach 100% within 3–10 days [6,7]. White spot syndrome virus (WSSV), a rod-shaped virus belonging to the genus *Whispovirus* of the Nimaviridae family, has a wide host range including many species of shrimp, crab and crayfish [8,9]. All major cultivated species of penaeid shrimp can be naturally infected by WSSV [10–12], and both natural and experimental infections of mud crab has also been reported [13,14]. Therefore, efforts are targeted towards understanding the role of crustacean miRNAs which regulate target genes during host pathogen interactions. The differential expressed miRNAs were identified from shrimps during WSSV infection and most target genes of the differentially expressed miRNAs were related to immune responses [15–17]. Some reports about crayfish miRNAs highlight the function of miRNAs in the regulation of the immune response against WSSV infection in crustaceans [18–20].

Hence, the objective of this study was to identify and characterize the differentially expressed miRNAs in mud crab *S. paramamosain* in response to WSSV infection. Our study extends the knowledge of crustacean miRNA regulation, providing clues for further research on crab immunity against WSSV infection.

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Table 1
Expression patterns of up-regulated or down-regulated ($P < 0.05$) miRNAs at 2 or 12 h post infection (hpi) in *S. paramamosain*. The expression levels were compared with that of control, then get the fold change. The expression level of control was designated as 1.

miRNA name	miRNA sequence	Fold change	
		2 hpi	12 hpi
up-regulated at 2hpi and 12hpi			
tcf-miR-125	TCCCTGAGACCCTAACTTGTGA	17.46	15.63
dpu-miR-133	TTGGTCCCCTTCAACCAGCTGT	9.22	4.95
PC-5p-58435_24	TGAAAAGGTTTATTGTGC	2.96	7.71
pte-miR-10-3p_L-1_1ss23CT	AAATTCGGTTCTAGAGAGGTTT	8.07	4.30
PC-3p-250870_3	ACCGCGGGGATTGTCGAGCC	8.46	1.93
pte-miR-10-5p	TACCCTGTAGATCCGAATTTGT	2.66	2.24
tur-miR-92-3p_1ss10CT	TATTGCACTTGTCCCGGCCTGT	2.67	2.46
PC-5p-229341_3	AAAACTGAAAGACTGTATTGTGAGA	4.03	1.80
PC-3p-177634_5	GTCTTGTTCAGGACTTTGGGC	2.21	5.18
PC-3p-209112_4	CATGCCCTCACAGTCAACCCA	1.92	2.20
isc-miR-315_R-1	TTTTGATTGTTGCTCAGAAGG	1.90	2.15
isc-miR-2001_R-3_1ss13CT	TTGTGACCGTTATAATGGGC	1.67	5.44
down-regulated at 2 h and 12 h			
isc-miR-263a_R+2	AATGGCACTGGAAGAATTCACGG	0.56	0.30
aga-miR-275_R-1_1ss13AT	TCAGGTACCTGATGTAGCGCGC	0.51	0.45
PC-3p-110352_9	AGCTTGGACCCGTCAGCCTTGAG	0.29	0.26
PC-3p-120597_8	TTTAGAGGAAGGATCATTA	0.04	0.34
PC-3p-141294_6	GGAGAAACAGACTTGAGAAC	0.16	0.28
PC-3p-83134_14	TGGGACTGGCATTTCAGTGGGC	0.58	0.22
PC-3p-73396_17	AAGCCAAACGGGCGGGGA	0.05	0.19
PC-3p-274902_3	GGAGATTGTCAGCAGATCAG	0.15	0.26
PC-3p-196059_4	TGAAGATCTATGATTGAATGAT	0.04	0.35
PC-3p-266464_3	TCGTTGCCAAGTGTCTCCTGTA	0.03	0.20
PC-3p-1078032_1	ACAGGAGAAACGCTCTTGAGAACCC	0.25	0.04
PC-3p-377516_2	GTGGCGGTGGCGGTGGTGGTGGCGG	0.37	0.51
PC-3p-379063_2	TGTTTCAGGCAGTGTTGCCAAC	0.27	0.04
PC-5p-200548_4	ACCCTCACCCACCAGCCCCGCC	0.03	0.19
PC-5p-226596_3	AGACTGATTTTTGGCCCAGCAC	0.39	0.17
PC-5p-166223_5	CTTGATTCCCGAGCCTCTG	0.32	0.03
down-regulated at 2 h and up-regulated at 12 h			
PC-5p-126751_8	TAGGTTTTTGATGATGACATTG	0.42	1.92
mja-mir-6491-p3_1ss12CT	CCGGGGAAGAGTTTTCTT	0.47	2.05
bmo-miR-2779_L-2_1ss9CT	ATCCGGTTCGAAGGACCA	0.44	2.77
pte-miR-2c-3p_R-2_1ss12TG	TATCACAGCCAGCTTGATGA	0.36	2.96
dvi-mir-9719-p3_1ss7AT	TTCTAATATATATATATATAT	0.78	4.40
PC-3p-256038_3	AGTCATCATCAAAAACCTA	0.04	4.24
PC-5p-195205_4	ATTGAAATTAGGGGAGAA	0.03	4.50
PC-3p-326870_2	TTTGAGAATCTGGCAGTGGAG	0.05	3.80
PC-5p-165941_5	CTGTTCCTCCAAAATTGG	0.04	3.40
ame-mir-3765-p5_1ss9AG	CAACAGAAGATGAAGAAA	0.03	3.09
PC-5p-198553_4	AAAGCACTGATGGCGCCCTT	0.06	2.80
bmo-mir-2837-p5_1ss16TA	TATTATTATTATTATATT	0.04	2.06
up-regulated at 2 h and down-regulated at 12 h			
PC-3p-239689_3	CACCAAAGAGAGACTTCAG	1.72	0.34
dqu-miR-12-5p_R-2_1ss9GA	AGAGTATTACATCAGGTACTG	2.28	0.84
PC-3p-81282_15	ACGGACATTCTGGACAAG	2.08	0.31
PC-5p-76016_16	TTTGGACACGGGAACGATTTGG	2.19	0.15
PC-5p-270384_3	AAGAGAGAACATAAAAGC	2.90	0.03
pte-miR-125b-5p_R+1_1ss14AT	TCCCTGAGACCCTTACTTGTGA	2.74	0.63
pte-miR-2f-3p_R-1_1ss21CA	TATCACAGCCACATTTGATGAG	2.01	0.35

The expression levels were compared with that of control. The control's was designated as 1.

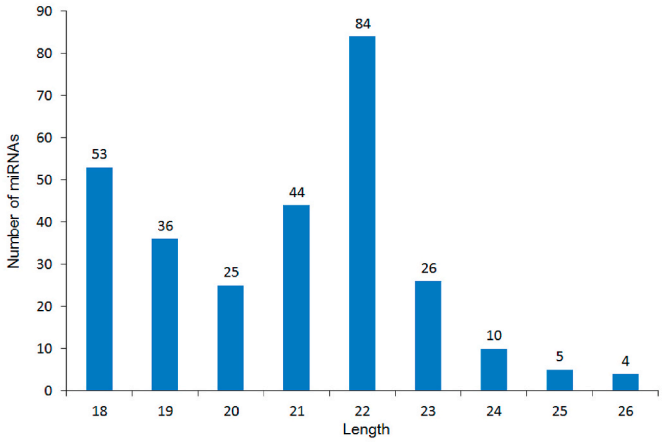

Fig. 1. Size distribution of *S. paramamosain* small RNAs found by sequencing. The symbols represented the time post-infection in hours.

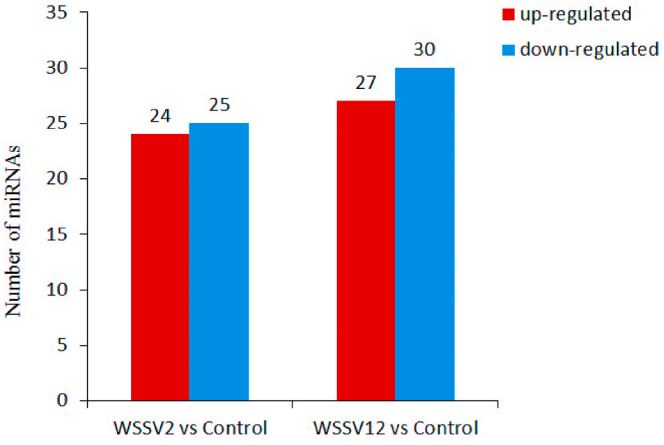

Fig. 2. The numbers represented the miRNAs up-regulated or down-regulated (more than 2 fold) compared with the control in the WSSV-infected crabs at different time post-infection (2 and 12 h).

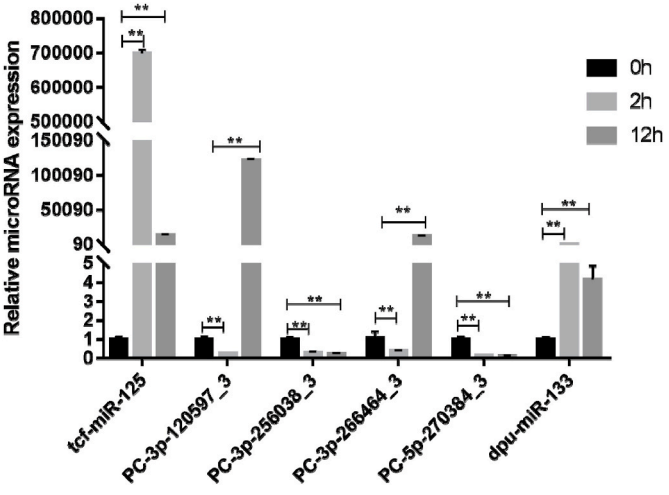

Fig. 3. The qRT-PCR results of six differentially expressed miRNAs in the immune response to WSSV in *S. paramamosain*. The miRNA expression was normalized to U6 expression level, miRNA expression of 0 h was normalized as one.

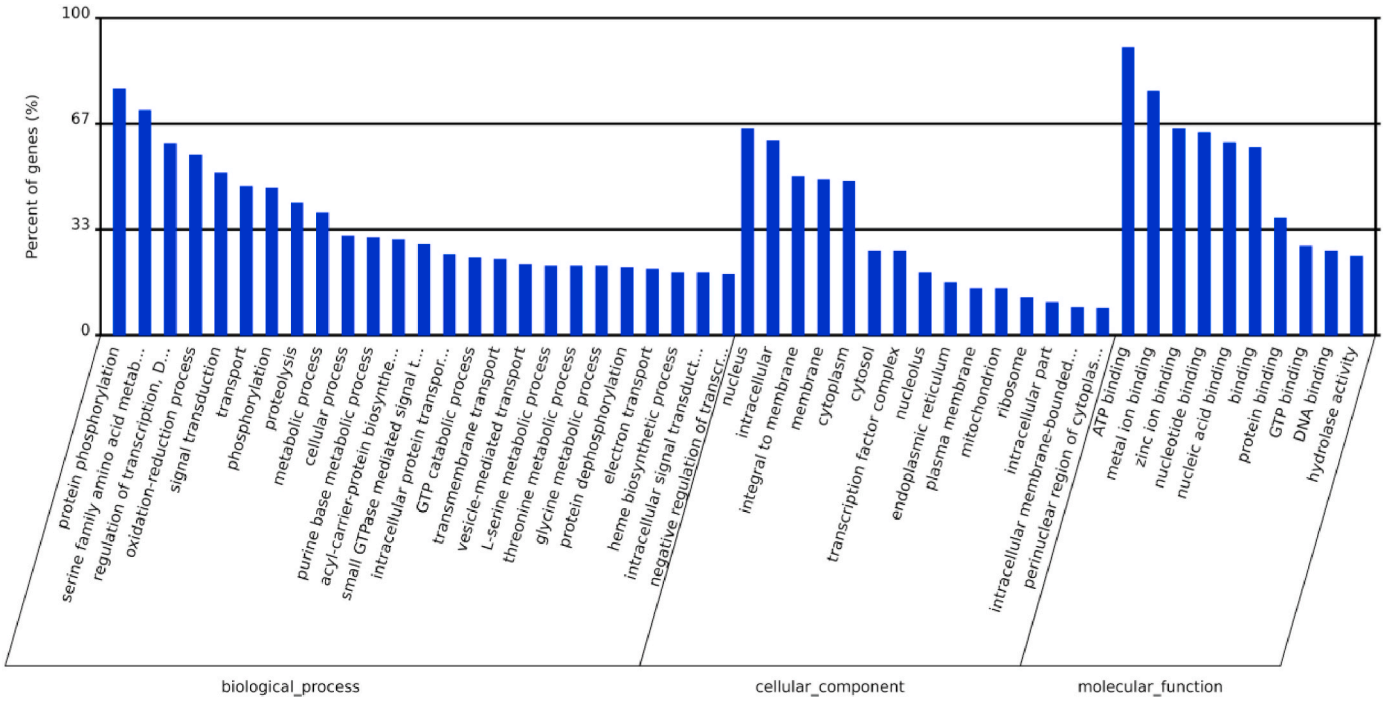

Fig. 4. Gene ontology classification of target genes of *S. paramamosain* miRNAs. By alignment to GO terms, the target genes were mainly divided into three categories with 50 functional groups: biological process (25 functional groups), cellular component (15 functional groups), and molecular function (10 functional groups). The left y-axis indicates the percentage of a specific category of genes existed in the main category.

2. Materials and methods

2.1. Crabs and tissue preparation

The normal adult *S. paramamosain* (approximately 100 g) were obtained from a aquatic product market of Hangzhou. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). The hemocytes were collected from health or challenged crabs. The samples were used immediately for RNA extraction, aiming to prevent total RNA degradation. WSSV (GenBank accession no. AF 332093.3) was purified and used in challenge experiments, as described previously [21]. The crabs were injected with 1×10^6 WSSV/crab. And the crabs were injected with hyperhaline PBS buffer were used as the control.

2.2. Sequencing of small RNAs

Total RNAs were isolated from the hemocytes of the WSSV-free and WSSV -infected crabs at different time points (2 h and 12 h) post infection by using a mirVana miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's instructions. The quantity and purity of total RNAs were monitored using a NanoDrop ND-1000 spectrophotometer (Nano Drop, Wilmington, USA) at a 260/280 ratio>2.0. The integrity of total RNAs was analyzed using an Agilent 2100 Bioanalyzer system and an RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with an RNA integrity number > 8.0. The extracted RNA samples were immediately stored at −80 °C. Subsequently 200 μg of total RNAs was separated onto a denaturing 15% polyacrylamide gel. The small RNAs (16–30 nt) were excised and dephosphorylated by alkaline phosphatase. After recovery by ethanol precipitation, the purified small RNAs were ligated sequentially to RNA adapters (5′- ACAGGUUCAGAGUUCUACAGUCCGACGAUC-3′ and 5′-UCGUAUGCCGUCUUCUGCUUG-3′). Reverse transcription and polymerase chain reaction (PCR) amplification were preformed after ligation. The resulting products were sequenced on the Genome Analyzer GA-II (Illumina, San Diego, USA) in accordance with the manufacturer's instructions.

2.3. Small RNA sequence analysis

Illumina's Genome Analyzer Pipeline software and the ACGT V3.1 program developed by LC Sciences (Houston, USA) were used for small RNA sequence analysis, as described in an earlier report [22]. The following sequences were removed: (1) sequences of the vector and adaptor, (2) low-quality sequences, (3) low-copy sequences (counts<3), (4) sequences containing more than 80% A, C, G, or T, (5) sequences containing only A and C or only G and T, (6) sequences shorter than 16 nt and longer than 26 nt, (7) sequences containing 10 repeats of any dimer, 6 repeats of any trimer, or 5 repeats of any tetramer, (8) sequences matching mRNAs, rRNA, tRNA, snRNA, snoRNA. After these sequences were removed, all the remaining high-quality sequences were used for miRNA identification. To identify conserved miRNAs that were homologous with those of other species, all highquality sequences were mapped to known mature and precursor arthropod miRNAs in miRBase 22 with an Evalue similarity cutoff of 1e-10. To characterize novel miRNA candidates in crab, the remaining high-quality sequences with no homologs in miRBase 22 were analyzed by a BLASTN search against the crab EST database in the National Center for Biotechnology Information. To reveal the differentially expressed miRNAs, the hybridization signals with the microRNA microarray were analyzed using the statistical calculation of the relevant P-values. The fold-change and P -value were calculated and used as the threshold to determine significant differences between the differentially expressed miRNAs. Statistical significance was analyzed by multiple *t*-test method. The significantly differential miRNAs ($P < 0.05$) were identified the differentially expressed miRNAs.

2.4. Quantitative real-time PCR of miRNA

Total RNA was extracted from the hemocytes of the WSSV-free and WSSV -infected crabs at 2 and 12 h post infection and quantified using a spectrophotometer (NanoDrop, Wilmington, USA). The qPCR of miRNAs was based on stem-loop real-time PCR technology and was performed as described before [23]. Less than 200 μg total RNA was used for cDNA

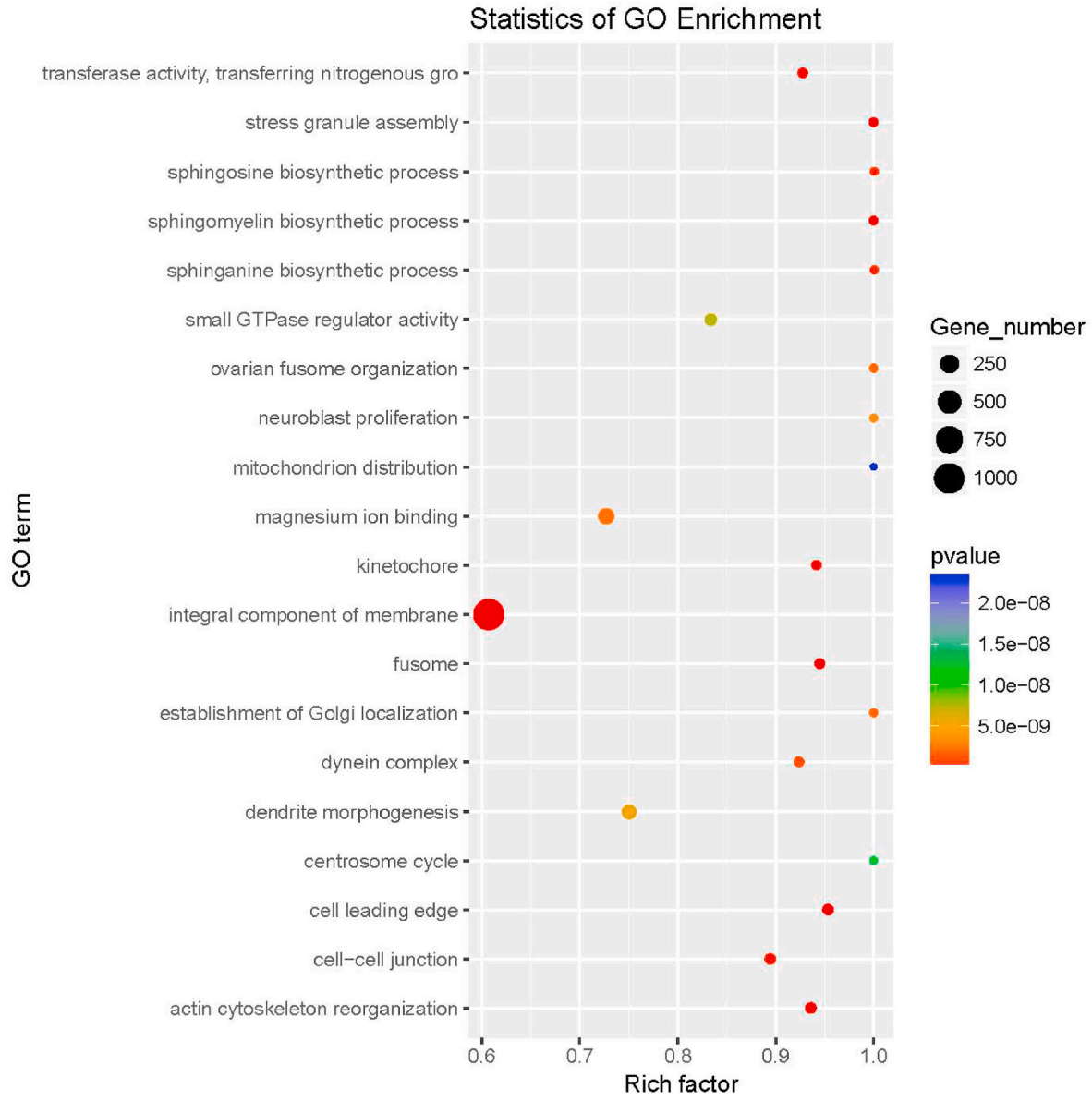

Fig. 5. GO enrichment scatter plot analysis of target genes of differentially expressed miRNAs output for GO biological process (GOBP) terms.

synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan), and the cDNA was kept at −20 °C. The SYBR Green RT-qPCR assay was carried out in Bio-Rad Two Color Real-Time PCR Detection System and the data was calculated according to the comparative CT method by office EXCEL, with the amplification of GAPDH as internal control. Designing and synthesizing of the qRT-PCR primers were entrusted to Generay Shanghai Company, based on the open read frame. The primer sequences for SYBR Green RT-PCR were shown in Table 1.

2.5. Gene ontology (GO) analysis

The coding sequences of the crab ESTs were extracted and used as queries to search the protein sequences collected by the GO database with the blast E-value <1e-5 (http://www.geneontology.org). The best hit GO IDs were assigned to the crab EST sequences. The *P*-values were corrected by false discovery rate (FDR).

3. Results

3.1. Sequence analysis of miRNAs

Based on the small RNA sequencing, the small RNA sequences of crab infected with WSSV were analyzed. The uninfected crabs were considered as controls for the analysis. The small RNA sequencing generated a total of 10–16 million raw reads. Above 80% raw reads were present at least twice and their lengths were ranged from 18 to 26 nucleotides. After removal of mRNA, rRNA, tRNA, snRNA and snoRNA, the high throughput sequencing generated a total of 2 million sequences. The data analysis showed a low proportion of long RNAs (over 2% by count), indicating that the sequencing samples were not contaminated by degraded RNA and were of high integrity. Size distribution of small RNAs of the indicated length (%) showed that 29.27% of miRNAs were of length 22 bp (Fig. 1).

3.2. Host miRNAs involved in WSSV infection

The expression profiles of miRNAs in WSSV-infected crab at various

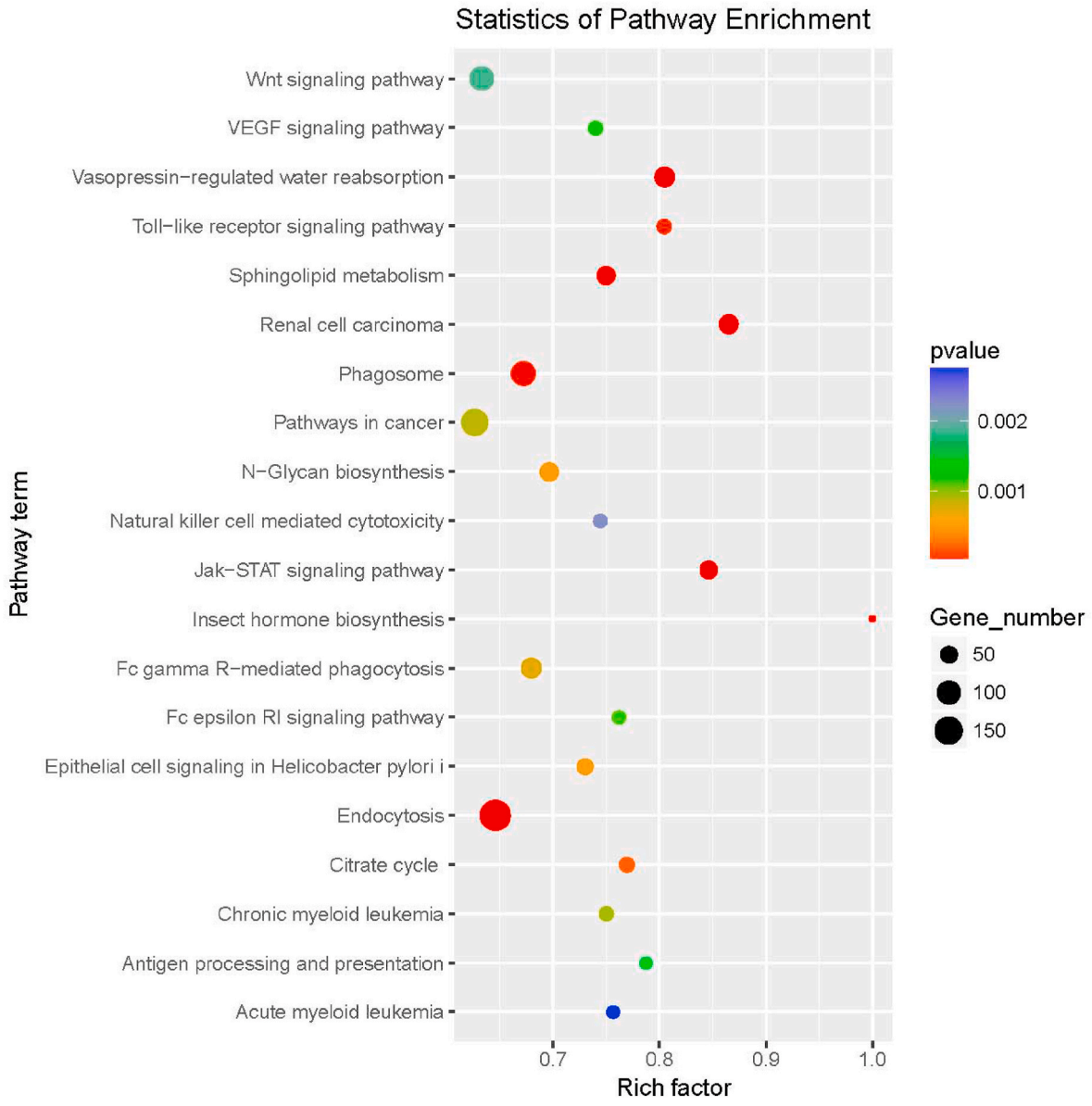

Fig. 6. KEGG enrichment scatter plot analysis for pathway enrichment of target genes of differentially expressed miRNAs.

times post infection were compared with that of control, and the miRNAs involved in the infection were identified and characterized. A *P*-value < 0.05 indicated that differences expression profiles in the miRNA counts were statistically significant. The results showed that the expression patterns of many miRNAs did not significantly change in response to WSSV infection. However, 49 and 57 host miRNAs were differentially expressed in response to WSSV infection at 2 and 12 h post infection (Fig. 2). Among these, some miRNAs were up-regulated at 2 h and 12 h post infection, like tcf-miR-125 and dpu-miR-133 (Table 1). Some miRNAs were down-regulated at 2 h and 12 h post infection, like PC-3p-120597_8 and PC-3p-266464_3 (Table 1). Some miRNAs were down-regulated at 2 h post infection and were up-regulated at 12 h post infection, like PC-3p-256038_3 (Table 1). Some miRNAs were up-regulated at 2 h post infection and were down-regulated at 12 h post infection, like PC-5p-270384_3 (Table 1). To confirm the involvement of these miRNAs in WSSV infection, six differentially expressed miRNAs were selected for stem-loop RT-qPCR. The tcf-miR-125 and dpu-miR-133 showed similar expression patterns (significantly up-regulated) to the sequencing results (Fig. 3). However, PC-3p-120597_8 and PC-3p-266464_3 did not show down-regulation at 12 h post infection, they showed up-regulated at 12 h post infection. PC-3p-256038_3 and PC-5p-270384_3 did not show up-regulation at 2 h or 12 h post infection but only showed down-regulation at 2 h and 12 h post infection. The target genes of these miRNA were shown in Table S1.

3.3. Gene ontology analysis and KEGG pathway analysis

Gene ontology classification of target genes of miRNAs showed that protein phosphorylation is the most represented biological process, nucleus is significant in cellular component, and ATP binding is over represented in molecular function (Fig. 4). To explore the potential pathways regulated by these miRNAs, we performed Gene Ontology (GO) analysis by using "GoStat" analysis tool (http://gostat.wehi.edu.au/). We took the total list of predicted and validated targets of the differentially expressed miRNAs and determined if any GO categories were over-represented within our list. The integral component of membrane is the most important biological process in the immune response to WSSV by GO enrichment scatter plot analysis (Fig. 5). In addition, KEGG enrichment scatter plot analysis showed that endocytosis is the most important pathway in the immune response to WSSV in

S. paramamosain. And the following pathways are pathways in cancer, phagosome and Wnt signaling pathway (Fig. 6).

4. Discussion

In the present investigation, we infected mud crab *S. paramamosain* with WSSV and compared the expression profiles of miRNAs in WSSV-free and WSSV infected crabs, and characterized the differentially expressed miRNAs. Our study provides the large-scale characterization of crab miRNAs in response to WSSV infection. After WSSV infection, some miRNAs were up-regulated at 2 h and 12 h post infection, like tcf-miR-125 and dpu-miR-133. In mammals, a lot of studies confirmed that miR-125 showed its important role in development [24], hematopoiesis [25], cancer and immunity [26], and directly participate in the immune response to both bacterial [27] and viral pathogens [28,29]. In invertebrate, limited researches also showed that miR-125 regulate development and innate immunity in *Drosophila* [30–33], and it was related to the skin ulceration syndrome of sea cucumber [34]. MicroRNA-133 can control cardiac hypertrophy in mouse and human models [35], regulates skeletal muscle [36] and cardiomyocyte proliferation [37], and it is related to cancer [38,39], muscle diseases [39] and cardiac diseases [40]. Other significantly differentially expressed microRNAs should also play important roles in immune regulation like miR-125 and miR-133, and they all deserves for further study.

The gene ontology classification of target genes is closed to the transcriptomic analysis of crab hemocytes in response to WSSV infection [41]. In this study, endocytosis and phagosome were also found to be the important pathways induced by miRNAs in the immune response to WSSV. These pathways including Wnt signaling pathway were all the components of phagocytosis. And endocytosis and phagosome had been found to be very important for viral infection [42–44]. WSSV-responsive miRNAs have been shown that endocytosis and phagocytosis are very important for antiviral immune responses in crustaceans [23,45,46]. Interesting, the Wnt signaling pathway also were found to be an important pathway in the immune response to *V. parahemolyticus* [47]. In our previous study, the Wnt signaling pathway is involved in the regulation of phagocytosis of virus in *Drosophila* S2 cells [48]. So we can deduce a conclusion that miRNAs would regulate the phagocytosis process in WSSV infection. The integral component of membrane is very important for WSSV entry and replication, so it became the most important biological process.

Our study shows that most of the miRNAs targeted the genes involved in immune response to WSSV infection. Further studies should investigate the molecular events in virus-host interactions mediated by miRNAs, which will help to control the WSSV infection.

Declaration of competing interest

There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the gene policies on sharing data and materials.

CRediT authorship contribution statement

Yongyong Lai: Investigation, Data curation, Writing - original draft, Validation, Software. **Qingri Jin:** Investigation, Data curation, Validation, Software. **Fei Zhu:** Conceptualization, Methodology, Investigation, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.06.055.

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