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The shrimp hormone receptor acts as an anti-apoptosis and anti-inflammatory factor in innate immunity

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ABSTRACT

Previously, we found that the expression of several genes, including *HR*, varied in *Drosophila melanogaster* after white spot syndrome virus (WSSV) infection. In this present study, we further investigated the role of *HR* in Kuruma shrimp, *Marsupenaeus japonicus* and determined its anti-apoptosis and anti-inflammation role in the innate immune system. We successfully identified a partial sequence (866 bp in length) of the *M. japonicus* hormone receptor ligand binding domain (*mjHR_LBD/mjHR*). The 5′ end of *mjHR* was successfully obtained; the open reading frame (ORF) ran from 33 to 701 bp, and encoded a protein containing 222 amino acids. *mjHR* belonged to the ligand binding domain of hormone receptors, was most likely part of a nuclear hormone receptor, and shared a close evolutionary relationship with other arthropods, such as insects. *mjHR* was expressed predominantly in immunity tissues such as gills, hemolymph and the hepatopancreas. WSSV infection could cause the down-regulation of *mjHR*, while infection with *Vibrio alginolyticus* could cause significant up-regulation of *mjHR*. The expression of *mjHR* was knocked down by dsRNA expressed by an engineered LITMUS 38i-HR plasmid. Virus and bacteria challenge experiment showed that the mortality of WSSV-infected shrimps was elevated in the absence of HR while the mortality of shrimps infected with *V. alginolyticus* was slightly reduced. Phenoloxidase (PO) activity, phagocytosis and apoptosis were promoted, while superoxide dismutase (SOD) activity was impaired, indicating that *mjHR* functions in an anti-apoptosis and anti-inflammation manner to prevent shrimp death caused by an over-load of immunity responses. Differences between mjHR expression and mortality change after WSSV or *V. alginolyticus* infection indicated that there was a different strategy for viruses or bacteria when confronted with the innate immune system.

1. Introduction

The nuclear hormone receptor (NHR) is ligand-regulated sequence-specific transcription factor that may activate or repress gene expression, contains a N-terminal DNA-binding domain and a C-terminal ligand binding domain (LBD). The DNA-binding domain of NHR is relatively more conserved than the LBD [1], which binds to small molecular including lipids, hormones, vitamins and amino acid metabolites. The LBD is also known to modulate the structural conformation of NHRs [2]; with certain ligands, NHRs promote the assembly of nuclear receptor coactivators and drive down-stream target gene transcription. Without certain ligands, NHRs promote the assembly of nuclear receptor corepressors and inhibit transcription [2]. A report recently reviewed the known functions of NHRs in the immunity of mammals [3]; generally, the mammal NHRs function to regulate a variety of cellular processes including the mevalonate pathway, apoptosis, T-cell response and development-induced autoimmune disease, secondary development of lymphoid organs, and the immunity response in some non-immune tissues. Another study pointed that NHRs could also regulate microRNAs [4]. In invertebrates, especially arthropods, our knowledge of NHRs is much less completed. While the basic structure of NHRs in invertebrates shows good conservation with mammalian NHRs, the function of NHRs in different insect tissues varies greatly. In *Caenorhabditis elegans*, NHR CHR3 participates in four larval molt stages as a critical regulator, which acts in a strikingly similar manner as DHR3 in the regulation of *Drosophila* metamorphosis [1]. In *C. elegans, nhr-49* is known to influence 13 genes associated with energy metabolism, and functions as a key regulator to control fat consumption and maintain a normal balance of fatty acid saturation; indeed, *C. elegans*, in which *nhr-49* was deleted, exhibited a high fat content and a short life span [5]. In *Drosophila*, the NHRs DHR3 and DHR39 were reported to be induced in the third instar larval organs, and function as early puff genes to direct the early stages of metamorphosis; the DNA-binding properties of seven other *Drosophila* NHRs were also described to participate in ecdysteroid response [6].

In our previous study, we used a DNA microarray to identify a

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potential link between the expression of HR and innate immunity in *Drosophila melanogaster* [7]. In this present study, we aimed to further our research on *Marsupeneaus japonicus HR*. We successfully cloned a partial sequence of the mjHR LBD, detected the expression of this domain in multiple tissues before and after viral/bacterial infection, and knocked down its expression using dsRNA produced by engineered LIMTUS 38i-HR bacteria. We found that mjHR functions as a critical regulator of balance in the shrimp innate immune system.

2. Methods and materials

2.1. Shrimp and pathogen preparation

Healthy juvenile shrimps weight 10–12 g were purchased from a local sea food market, and were temporarily kept in artificial sea water in laboratory condition. Healthy shrimps were kept in a 70 L PMMA tank in WSSV-free area, then based on experiment requirement, they were randomly divided into different groups and kept in 35 L tanks in experimental area. Room temperature and water temperature were set at 24 °C and 22 °C separately. Shrimps were killed by immersing in cold sea water. Shrimp hemolymph was draw from the ventral joint of carapace and abdomen with sterilized disposal syringe. Shrimp tissues include gill, muscle, heart, hepatopancreas, sex gland, digest organ and gut were taken. These samples were frozen in liquid nitrogen and stored at −80 °C, or immediately homogenized in lysis solution for total RNA extraction.

Pathogens used for this research include WSSV (white spot syndrome virus) and *Vibiro alginolyticus*. The extraction and preparation of WSSV injection solution was described in a previous research [8]. To infect shrimps, WSSV extraction was diluted with sterilized high saline PBS to a density of 1×10^5 virus copies per mL. *V. alginolyticus* was kindly provided by Jimei University, Xiamen, China. To infect shrimp, *V. alginolyticus* was recovered on a vibrio selective culture medium to guarantee its pathogenicity. Alive vibrio lawn was diffused in sterilized high saline PBS and gradually diluted to appropriately 1.0×10^5 per mL. Each shrimp individual would receive 100 μL of pathogen injection solution, separately. Control group was treated with high saline PBS only.

2.2. Rapid amplification of cDNA ends (RACE)

Shrimp hemolymph was draw and centrifuged at 800 g × 10min, 4 °C to collect hemocytes. Total RNA was isolated immediately using the mirVana miRNA™ Isolation Kit (Ambion, USA), according to the manufacturer's protocol. The RACE experiment was performed using the 5′–3′ RACE Kit (Roche) according to manufacturer's protocol. Briefly, target specific cDNA was synthesized and applied for PCR and nest-PCR. Specific Primer (SP) for 5′RACE, Gene-specific Primer (GSP) and Nest Gene-specific Primer (NGSP) for 3′RACE was designed based on the core sequence of *mjHR*. Primer sequences were listed in Table 1, primer sets were marked with arrows in Fig. 1. PCR product was analyzed by 1% agarose gel and the target fragment was cut and purified with DNA Gel Purification Kit (Genery, China). Subsequently, purified product was attached to a pMD-19 (Simple) vector (Takara, Japan) for sequencing (Biosune, China).

2.3. Nucleotide sequence and bioinformatics analyses

Similarities in the nucleotide sequences were analyzed by NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST/). Protein prediction was performed using the online ORF Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). Multiple sequence alignment was performed by DNAMAN 6.0. The phylogenetic tree based on the amino acid sequences was performed by MEGA 7. The functional domains were predicted by the SMART database (http://smart.embl-heidelberg.de/).

Table 1
Primer sequences and purpose.

Name	Nucleotide Sequence (5′ to 3′)	purpose
3′ race GSP	CGGAGAAAATGGGATGAAGACA	gene specific for 3′RACE
3′ race NGSP	ACAGAACCATTGCTCTAATATCG	nest gene specific primer for 3′RACE
5'race SP1	CAATCAAAAGCCTTGCTGTAGTC	first specific primer for 5′RACE
5'race SP2	CTCAAATTGGCAATGTGGTCTC	second specific primer for 5′RACE
5'race SP3	TGCGATATTAGAGCAATGGTTC	third specific primer 5′RACE
HR-*Eco*RI-F	CCGGAATTCGTCTACTCAACCGAGC	primer for L38i-HR construction with restriction enzyme cutting site
HR-*Bam*HI-R	CGCGGATCCCTCCAGACAGAGCGATCG	
realtime-F	TGTGAATCAGAACTGCGTGGT	primer for HR gene expression
realtime-R	CAAGCCTAACTCTGTGTCTGTCA	
GAPDH-F	GGTGCCGAGTACATCGTTGAGTC	primer for GAPDH internal control
GAPDH-R	GGCAGTTGGTAGTGCAAGAGGC	
*pro*PO-F	TTCTACCGCTGGCATAAGTTTGT	primer for proPO gene expression
*pro*PO-R	TATCTGCCTCGTCGTTCCTCAC	
myosin-F	GCCCAGGTCAAGAAGGACAAGGA	primer for myosin gene expression
myosin-R	AAGACGCTCACCAAGGGACAGGA	
hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	primer for hemocyanin gene expression
hemocyanin-R	AACGGACGGTAAGTTGATGATGT	
Rho-F	GTGATGGTGCCTGTGGTAAA	primer for Rho gene expression
Rho-R	GCCTCAATCTGTCATAGTCCTC	
QM-F	CGTCACAAGGAGCAGGTTATT	primer for QM gene expression
QM-R	GGGACCATGTTCAGGGAGA	
L-type lectin-F	ATGTTATGCCATCTGCCTCGTATTT	primer for L-type lectin gene expression
L-type lectin-R	CTTTCGCTGCTGCTCTTTCTGTT	
IMD-F	ATTCATCCGTCTACCTCCCTACA	primer for IMD gene expression
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC	
*p*53-F	TTCCTGCCTGGCTGACTCTA	primer for p53 gene expression
*p*53-R	CACCCAATCTTCCAACATCACAT	
Toll like receptor-F	CCACCTAAAGTCATCATCGCCAGTA	primer for Toll like receptor gene expression
Toll like receptor-R	TATTCATTCACCACAGCCCACAA	
TNF-α-F	ACAGACGGTCCAGAGTCCCAAAG	primer for TNF-alpha gene expression
TNF-α-R	GCGACGAAGTGAGCCACAGTAA	
STAT-F	TGGCAGGATGGATAGAAGACAAG	primer for STAT gene expression
STAT-R	TGAATAAGCTGGGATACGAGGGA	
*Rab*7-F	TCATTAGGTGTTGCATTTTATCGC	primer for Rab7 gene expression
*Rab*7-R	AGGCTTGAATTAGGAACTCGTC	
MAPK-F	CGCATCACTGTTGAGGAGG	primer for MAPK gene expression
MAPK-R	GCAGGTCATCAAGTTCCATCT	

2.4. Expression analysis by SYBR green real-time qPCR

The gene expression was analyzed by Two-step real-time quantitative PCR using SYBR Green. Total RNA of different tissues of health/infected shrimps was extracted by EASY spin tissue/cell RNA extraction kit (Aidlab, China), 200 ng total RNA was applied for cDNA synthesis by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The two-step real-time quantitative PCR was performed in Bio-Rad Two Color Real-Time PCR Detection System. PCR data was analyzed by the $2^{-\Delta\Delta Ct}$ comparative Ct method and represented as relative value, the amplification cycles of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Data difference significant was analyzed by one-way analysis of variance (ANOVA). SYBR Green primers were designed and synthesized by Generay (Shanghai, China), primer sequences of mjHR were listed in Table 1 and marked with arrows in Fig. 1. The real-time experiment was performed in

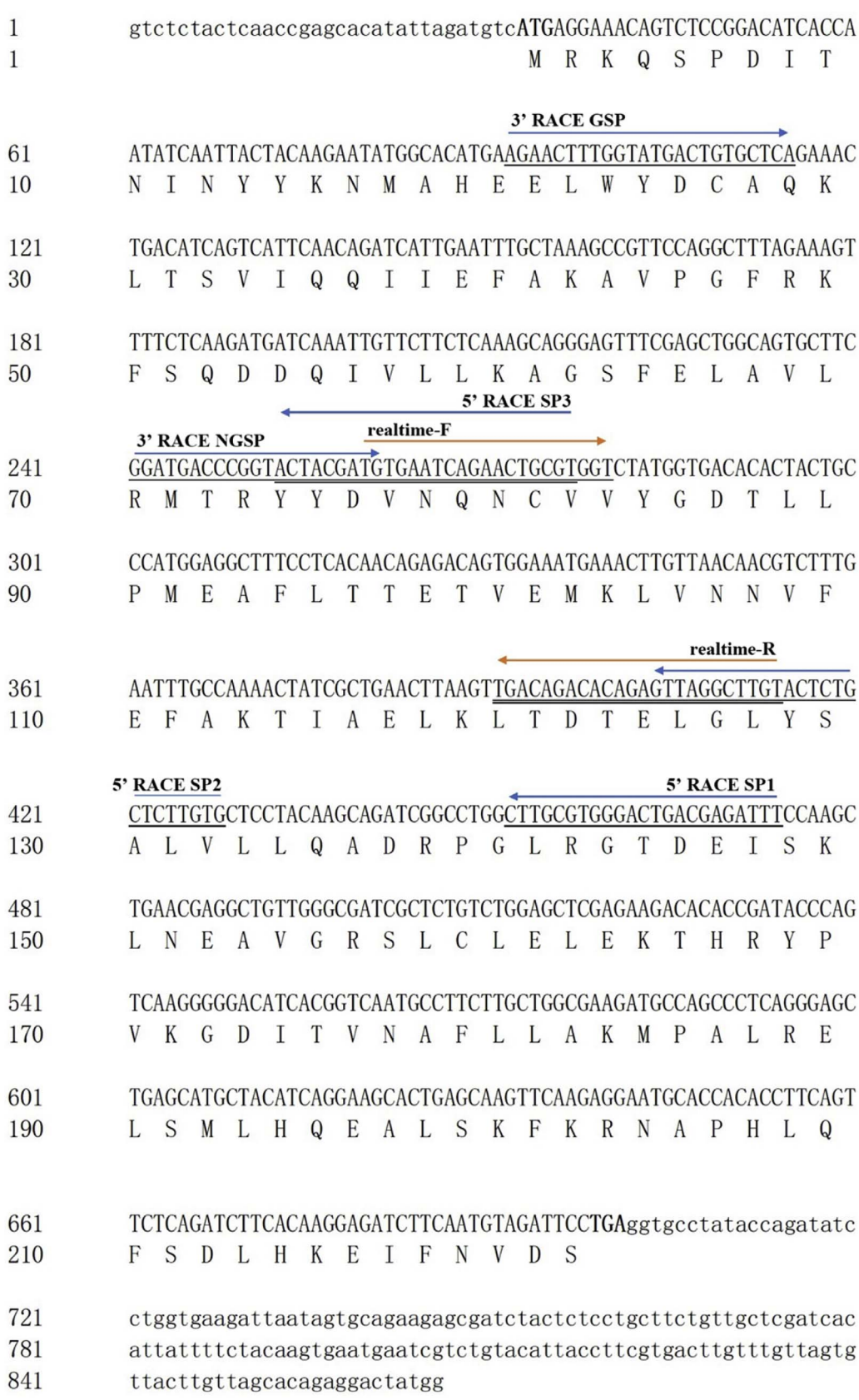

Fig. 1. Nucleotide, ORF and deduced amino acid sequences of *mjHR*. The nucleotide sequence is displayed in the 5′–3′ direction and numbered on the left. The deduced amino acid sequence is shown using a single capital letter code denoting each amino acid. The 3′-untranslated region (UTR) and 5′-UTR sequences are shown in lowercase letters. Codons are numbered at the left starting from the initiation codon (ATG) to the termination codon (TGA), which are marked use bold font. RACE primers are marked with blue arrows and real-time quantitative PCR primers are marked with orange arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

triplicate, each treatment included at least three shrimps.

2.5. dsRNA-mediated silencing of gene expression

Gene expression knock down was achieved by double strain RNA (dsRNA) mediated RNA interference. The dsRNA of mjHR was designed and constructed as reported in our previous research [9]. Briefly, a forward primer with *Eco*RI restriction site and a reverse primer with *Bam*HI restriction site was designed based on the 5′ end sequence of cloned mjHR. PCR was performed to amplify the target sequence with specific restriction sites. After digested by specific restriction enzymes, target gene was inset into LIMTUS 38i Vector (NEB, MA, USA) to construct LIMTUS 38i-HR plasmid. DNA sequencing was performed to verify its accuracy. The LITMUS 38i-HR (L38i-HR) plasmid was then inducted into a RNase free HT115 (DE3) engineering bacteria to express mjHR dsRNA *in vitro*. Meanwhile, a LIMTUS 38i-EGFP (L38i-EGFP) plasmid was also constructed as the negative control to determine the specific of L38i-HR. The engineering bacteria of L38i-HR or L38i-EGFP was cultured in LB medium containing 100 μg/mL ampicillin, IPTG was added when the OD600 reached approximately 0.6 to induce expression. After approximately 4 h of expression, total RNA was extracted by mir Vana miRNA™ Isolation Kit and annealing process (gradient temperature falling from 95 °C to 25 °C in 45 min) was performed. Finally, the annealed dsRNA was precipitated with 5 M sodium acetate and anhydrous alcohol and stored at −80 °C. To treat shrimps, precipitated dsRNA was dissolved in RNase-Free water and the concentration was determined. Each shrimp was treated with 100 μL of PBS containing 5–20 μg dsRNA immediately after dsRNA preparation to avoid degradation according to our previous research [10]. Each treatment included at least three shrimps. 24 h after treatment, hemocytes of PBS treated shrimps, L38i-HR treated shrimps, or the L38i-EGFP treated shrimps were collected separately. The mjHR expression was detected to confirm the knock down effect or L38i-HR.

2.6. Pathogen challenge and mortality count

Healthy shrimps were randomly distributed into different groups, each group contained over 20 individuals. Three control groups were treated with PBS, L38i-HR, L38i-EGFP, separately. Three virus infection groups were treated with WSSV, the mixture of WSSV and L38i-HR, the mixture of WSSV and L38i-EGFP, separately. Three bacterial infection groups were treated with *V. alginolyticus*, the mixture of *V. alginolyticus* and L38i-HR, the mixture of *V. alginolyticus* and L38i-EGFP, separately. Shrimp populations of each treatment groups were counted twice a day at 8:00 a.m. and 20:00 p.m., shrimps were fed at the same time and the dead individuals were fished out. One third of the polluted sea water was pumped out and replaced with prepared clean sea water each day. Mortality data was analyzed by one-way analysis of variance (ANOVA) by GraphPad Prism 6.

2.7. Detection of immune gene expression and immunological parameters

Shrimps were treated with L38i-HR, L38i-EGFP, or high saline PBS, separately. 24 h after treatment, shrimp hemocyte RNAs were extracted and cDNA of each sample was synthesized as described in Section 2.4. The expression of thirteen known innate immune pathways related genes, proPO (prophenoloxidase), myosin (myosin light chain), hemocyanin, Rho (Rho GTPase), QM, L-lectin (L-type lectin), IMD (immune deficiency pathway), p53, Toll like receptor, TNF-alpha (tumor necrosis factor-α), STAT, Rab7 and MAPK (mitogen-activated protein kinase) were detected to predict which pathways or immune factors might be regulated by *mjHR*.

To detect immune parameters, shrimps were randomly separated into 3 × 2 groups. 3 vertical groups represented dsRNA treatments, were treated with sterilized high saline PBS, L38i-HR, or L38i-EGFP, separately. 2 cross groups represented infection treatments, were treated with WSSV or *V. alginolyticus*, separately. DsRNA treatment and infection treatment were performed simultaneously as described in Section 2.6. At 24 h, 48 h post infection, shrimp hemolymph of each group was collected for immune parameters as described in our previous researches. Briefly, shrimp hemolymph was draw 1:1 with anticoagulant, 75 μL mixture was immobilized with 25 μL 4% paraformaldehyde for total hemocyte count (THC). 100 μL hemolymph was mixed with 900 μL 50 mM PB (phosphate buffer) for SOD extraction [11]. 500–1000 μL hemolymph was centrifuged to remove hemocytes for PO activity detection. THC was counted under a microscope using hemocytometer, each sample was counted for four times [12]. SOD was detected using optimized NBT photoreduction method [12]. PO activity was measured by the ability of hemolymph serum to promote L-DOPA oxidation [13].

2.8. Apoptosis rate counting by flow cytometry

Apoptosis rate was detected using Annexin V (Invitrogen, USA) based on a previous research [12]. Briefly, healthy or L38i-HR treated shrimp were injected with PBS, WSSV, or *V. alginolyticus* for infection, separately. 24 h post injection, shrimp hemolymph of each treatment group was draw and centrifuged at 800 g × 5 min, 4 °C to collect shrimp hemocytes. Stain the hemocytes with Annexin V-FITC and PI in high saline PBS diluted 1 x Binding solution for 15min in room temperature. Then the hemocytes of each treatment group was detected by flow cytometry, the fluorescent intensity of PI represents the late stage of apoptosis cells, died cells, or cell fragment, the fluorescent of FITC represents the late and early stage of apoptosis. Live cells showed no fluorescent positive.

2.9. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). The statistical differences were estimated by one-way analysis of variance (one-way ANOVA) followed by least-significant differences (LSD) and Duncan's multiple range test. All statistical analyses were carried out using SPSS Statistics version 19 and GraphPad Prism 6. A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$).

3. Results

3.1. Sequence, bioinformatics and phylogenetic analysis

In our previous research, we identified the gene expression profile of S2 cells treated with inactivated/non-inactivated white spot syndrome virus (WSSV), showing that 120 genes, including the hormone receptor (HR) gene, participated in the virus defense system of invertebrate immunity [7]. In the present study, we carried out further research to identify the potential function of *mjHR* in the shrimp innate immune system. Based on the core sequence of *mjHR*, rapid-amplification of cDNA ends (RACE) technology was applied to obtain the full-length sequence. RACE allowed us to determine an 866 bp sequence of *mjHR*; the ORF1 ran from 33 bp and ended at 701 bp, thus encoding a protein of 222 amino acids.

The 5′-end was successfully cloned but we failed to clone the full sequence of the 3′-end, including the poly(A) tail. Nevertheless, identification of the 5′-end nucleotide acid sequence met the requirements for our expression inhibition experiment. Fig. 1 illustrates the nucleotides, ORF and the deduced amino acid sequences of partial *mjHR* cDNA, RACE and the primers used for real-time quantitative PCR.

Next, we used SMART to predict the functional protein domains of *mjHR*; prediction results indicated that the *mjHR* protein belonged to the LBD of HRs, and started at amino acid 28 and ended at amino acid 191. This functional domain can be found in growth HRs or NHRs. The NHR consists of two parts, a DNA binding domain and an LBD [14]; the

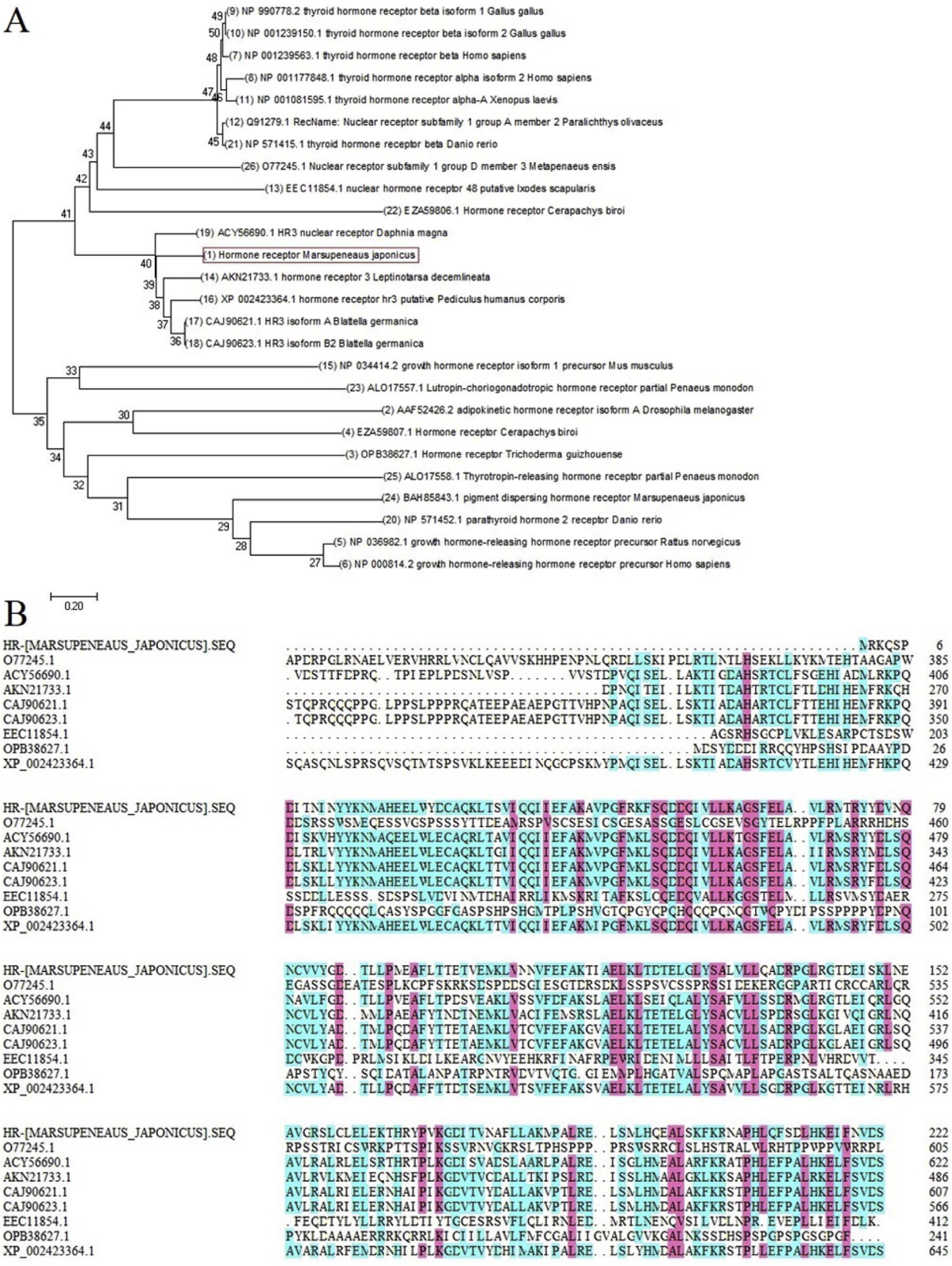

Fig. 2. A. The phylogenetic tree of HRs from different organisms based on amino acid sequence BLAST. Species names, types and GenBank accession numbers of HRs are listed on the right of the tree. The target gene is highlighted in the red frame; B. the amino acid sequence alignment of 10 arthropods; the GenBank accession numbers of the different species are listed on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth HR features one more domain, identified as a transactivation domain [15]. The LBD represents the C-terminal domain, responsible for the ligand binding step and the induction of downstream HR signaling.

A phylogenetic tree constructed using MEGA7, and using the neighbor-joining method (Fig. 2A), showed two large branches of HRs from multiple species. One branch consisted of the steroid HR-like thyroid HR and the NHR. The other branch consisted of the steroid HR

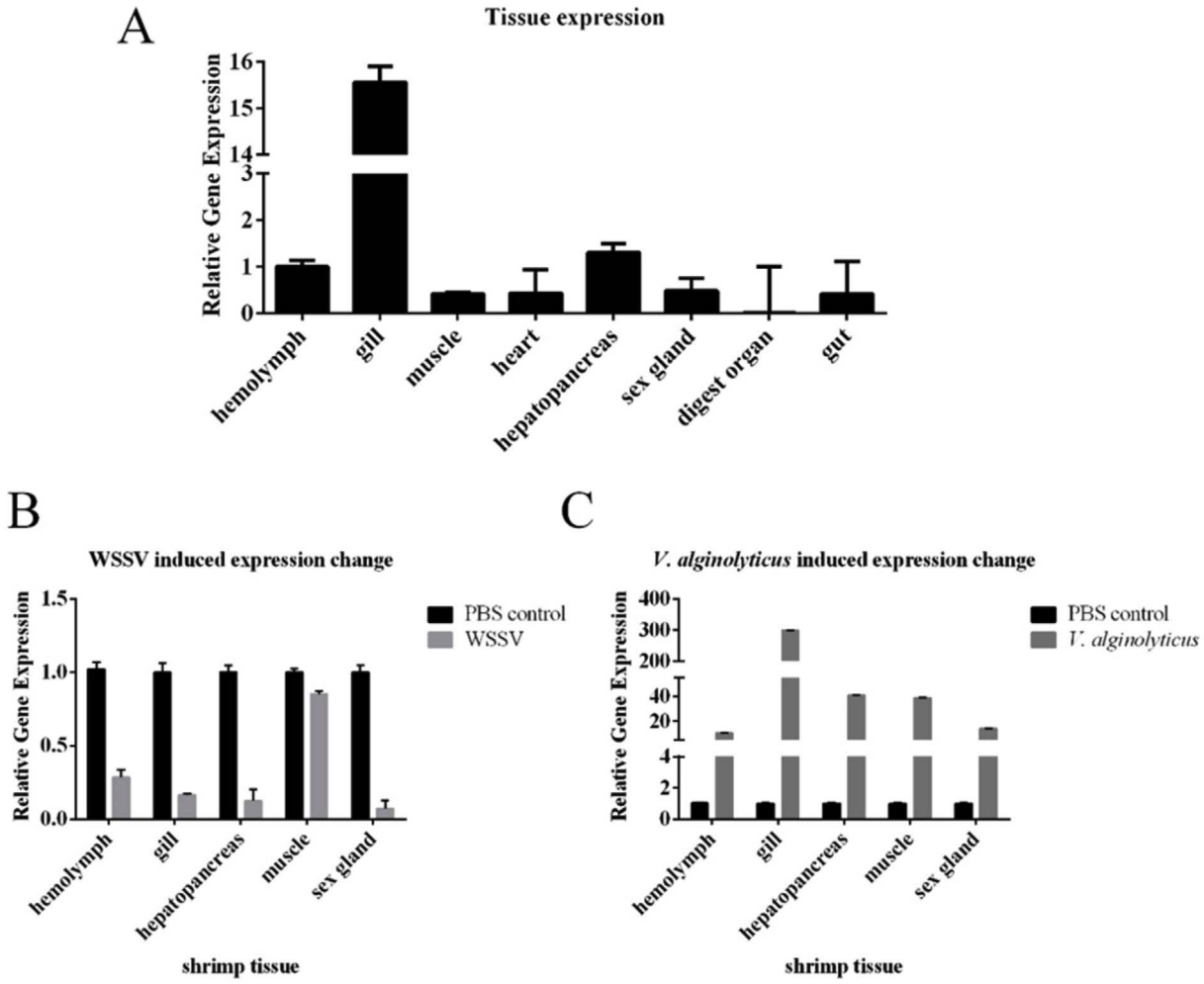

Fig. 3. Tissue expression differences and stress response. A. The expression of *mjHR* in eight tissues from healthy shrimps; B. The expression of *mjHR* in five tissues from WSSV challenged shrimps, and C. The expression in five tissues from *V. alginolyticus* challenged shrimps. Expression characterization of *mjHR* in different tissues was revealed by real-time quantitative PCR. The amount of *mjHR* mRNA was normalized to the *GAPDH* transcript level. In healthy shrimps, *mjHR* expression in hemolymph was indexed as 1. In infected shrimps, *mjHR* expression of all tissues of PBS control was indicated as 1. Data are shown as means ± standard deviation of three separate individuals for each tissue.

including the growth HR and the sexual HR. In the first branch, HRs of *Chordata* species were separated from *Arthropoda* species. Our target protein, *mjHR*, showed a closer relationship with arthropod NHRs. The thyrotropin-releasing HR of another shrimp, *Peneaus monodon*, was located on the other branch of steroid HRs, quite apart from *mjHR*. This result indicated that the *mjHR* we had identified was more like an NHR than a growth HR. Because of the lack of a DNA binding domain or transactivation domain, this assumption, however, is not valid and requires further verification.

Based on the phylogenetic tree, a protein BLAST and amino acid alignment (Fig. 2B) was performed to discover the evolutionary conservation of *mjHR*. Protein alignment showed that the similarity of 26 species on the N-J tree was 19.67%, the similarity of six thyroid HRs and 10 NHRs was 25.27%, while the similarity across 10 arthropod species was 38.83%. However, the similarity of *mjHR* with five other insect HRs showed a relatively higher similarity of 52.28%. Fig. 2 shows the amino acid alignment of *mjHR* with nine other arthropods. This result indicated that the *mjHR* LBD was not a protein with high evolutionary conservation, it shared a relatively closer relationship with insect HRs, and it was much more likely to be an NHR.

3.2. Tissue expression differences and stress response

Real-time PCR was used to detect the levels of *mjHR* expression in seven different tissues (Fig. 3A); this experiment showed that *mjHR* was expressed predominantly in the gills, hepatopancreas and hemolymph, particularly in the gills; in the muscle, heart, sex gland and gut, the expression of HR was relatively lower, and in the digestive organ we did not detect HR expression.

Next, the shrimps were challenged with WSSV or *V. alginolyticus*. Real-time qPCR results showed that after WSSV infection, *mjHR* expression in the hemolymph, gill, hepatopancreas and sex gland were significantly down-regulated; there was no change detected in the muscle. After *V. alginolyticus* infection, *mjHR* expression in the hemolymph, gill, hepatopancreas, muscle and sex gland were significantly up-regulated. Since the hemolymph and gills are the main tissues of invertebrate innate immunity, higher levels of expression in these tissues indicated involvement of the HR in shrimp immunity. *mjHR* expression was far higher in the gills than any other tissue tested. The variation in expression after the WSSV (Fig. 3B) or *V. alginolyticus* (Fig. 3C) challenge indicated that the *mjHR* may play different roles in the anti-viral or anti-bacterial process, or, that the virus or the bacteria affected HR expression in different ways. Different GHRs have been identified from different immune cells including lymphocytes, macrophages, monocytes, granulocytes and other non-lymphoid cells of the hepar, spleen, thymus and bursae of mammals, birds and fish [16]. Consequently, our present results indicating tissue expression of the NHR in shrimp and shows variation from these vertebrates.

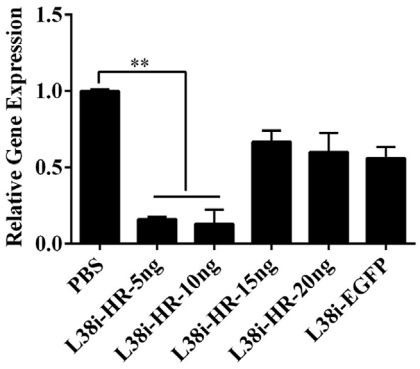

Fig. 4. *mjHR* knock down by dsRNA expressed in engineered L38i-HR bacteria and the inhibitory effect at different treatment doses.

3.3. mjHR RNAi by dsRNA

To inhibit *mjHR* expression, we first engineered a LITMUS 38i-HR vector. A 515 bp length of HR sequence was successfully inserted into the LITMUS 38i vector. The LITMUS 38i-HR (L38i-HR) vector was then transformed into RNase-free TH5α engineering bacteria to express HR dsRNA *in vitro*. L38i-EGFP vector was also constructed as a negative control to determine the specific expression profile of the bare L38i-HR vector. Expressed L38i-HR dsRNA was applied to inhibit shrimp HR. Just 24 h post-treatment, the expression of *mjHR* was detected (Fig. 4). Data showed that 5 ng and 10 ng of L38i-HR dsRNA per shrimp could effectively inhibit *HR* expression. Over 15 ng of L38i-HR dsRNA showed no knock down effect. L38i-EGFP dsRNA showed no influence upon *HR* expression. Furthermore, the expression of *mjHR* was successfully inhibited by L38i-HR dsRNA.

3.4. Mortality count and the detection of virus copies

The cumulative mortality of the control groups showed no statistical difference, indicating that HR deficiency would not affect healthy shrimp (data not shown). The cumulative mortality of shrimps infected with either WSSV or *V. alginolyticus* is shown in Fig. 5 In the WSSV-infected groups, the mortality of normal shrimps increased rapidly from 36 h to 96 h, and reached 100% at 108 h post treatment. The mortality of shrimps in which HR had been inhibited started from 12 h post treatment, and reached 100% at 84 h, 1 d earlier than normal shrimps; furthermore, the mortality count was constantly higher than that of normal shrimps for the duration of infection. We also determined the number of WSSV copies in challenged shrimps. At 12 h after WSSV treatment, the number of WSSV copies in normal and HR inhibited shrimps started to increase. From 24 to 48 h, the number of WSSV copies in HR inhibited shrimps was constantly higher than normal shrimp; of particular note was the fact that 24 h post-treatment, the number of WSSV copies (Fig. 5) of the HR inhibited shrimps was nearly 10 times higher. At 60 h, the number of WSSV copies in the two groups showed no difference, while at 72 h, the number of WSSV copies in HR inhibited shrimps was lower than that in normal shrimps. Nevertheless, the mortality of HR inhibited shrimps had not recovered by the 72 h time-point.

In the groups of shrimps infected with *V. alginolyticus*, at 12 h post treatment, the mortality of both normal shrimps and HR inhibited shrimps started to increase. The mortality of normal shrimps reached 100% at 96 h, while 12 h later, the mortality of HR inhibited shrimps reached 100%. For the duration of infection, the mortality count of HR inhibited shrimps was slightly lower, although the final mortality showed no statistical difference. L38i-EGFP groups showed no significant difference compared to the infection groups. To present the difference between the L38i-HR groups and infection groups more clearly, the data for the L38i-EGFP groups are not shown. These results showed that HR deficiency in shrimps could promote death caused by WSSV; the number of viral copies was higher, indicating that the shrimp HR played a positive role in the anti-viral process. HR deficiency could also have a negative effect upon the anti-bacterial process although the influence was not as strong as for the anti-viral process.

3.5. Effect of mjHR on the expression of immune genes and immunological parameters

To determine the potential influence of *mjHR* on the innate immune system, real-time quantitative PCR was conducted to detect the expression of thirteen genes involved in the immune pathways of normal shrimps or HR inhibited shrimps. When the HR was inhibited, the relative expression of *myosin, hemocyanin,* QM protein, IMD (immune deficiency) pathway gene, TNF-α and MAPK were significantly up-regulated, while the relative expression of L-lectin was down-regulated (Fig. 6). The expression of *pro*PO, Rho, *p*53, Toll-like receptor, STAT and Rab7 showed no significant difference.

Three innate immunological parameters, total hemocyte count (THC), PO activity and SOD activity, were detected to determine the influence of *mjHR* on the basic innate immune system (Fig. 6). In the WSSV challenge groups, the THC of normal or HR inhibited shrimps both decreased after WSSV infection, but showed no significant difference between the two groups. The same result was obtained in the *V. alginolyticus* challenge groups, indicating that HR deficiency had no influence on hemocyte number. The PO activity of normal or HR inhibited shrimps were both decreased slightly at 24 h after WSSV infection; at 48 h, the PO activity of normal shrimps increased slightly, while the PO activity of HR inhibited shrimps increased significantly, indicating that the viral infection was getting worse. At 24 h after *V. alginolyticus* infection, the PO activity of normal shrimps showed no significant change; at 48 h, when bacteria replication had increased, the PO activity had also increased. However, the PO activity of HR inhibited shrimps increased significantly at 24 h. At 48 h, the PO activity of HR inhibited shrimps dropped slightly lower than that of normal shrimps. The changes in PO activity caused by the virus or vibrio were both affected by the HR; when the HR was lacking, there was more PO enzyme activity, indicating that the infection was worse. HR mainly affected the late phase of WSSV infection and the early phase of Vibrio infection.

In the WSSV challenge groups, the SOD activity of normal shrimps at 24 h had increased significantly, while the HR inhibited shrimps showed less SOD activity than normal shrimps. At 48 h, the SOD activity of normal shrimps had fallen, while the HR inhibited shrimps showed no variation compared to the 24 h time-point. In the groups that had been challenged with *V. alginolyticus*, a similar result was obtained. SOD activity represents the ability to eliminate cytotoxic reactive oxygen species (ROS) produced by the host defense system [17]; ROS could eliminate foreign particles but redundant ROS would result in oxidase stress and cause harm to the host cells [18]. In the absence of *mjHR*, the SOD activity of shrimp hemolymph showed no significant variation compared to the control group, while the SOD activity of normal shrimps either increased or decreased in order to regulate the ROS density after infection. This result indicated that HR could negatively affect the activity of SOD and therefore influence the ability of the host to contain the oxidase stress.

3.6. Effect of mjHR on hemocyte apoptosis

The mean apoptosis rate of the PBS control was 12%, compared to 11.6% in the L38i-HR control; these values were not significantly different, indicating that the knock down of HR would not affect apoptosis in healthy shrimps. In groups of shrimp infected with WSSV, the apoptosis rate of normal shrimps was approximately 65%; when *mjHR* expression was inhibited, the apoptosis rate increased to 80%, approximately 20% higher. In groups infected with *V. alginolyticus*, the

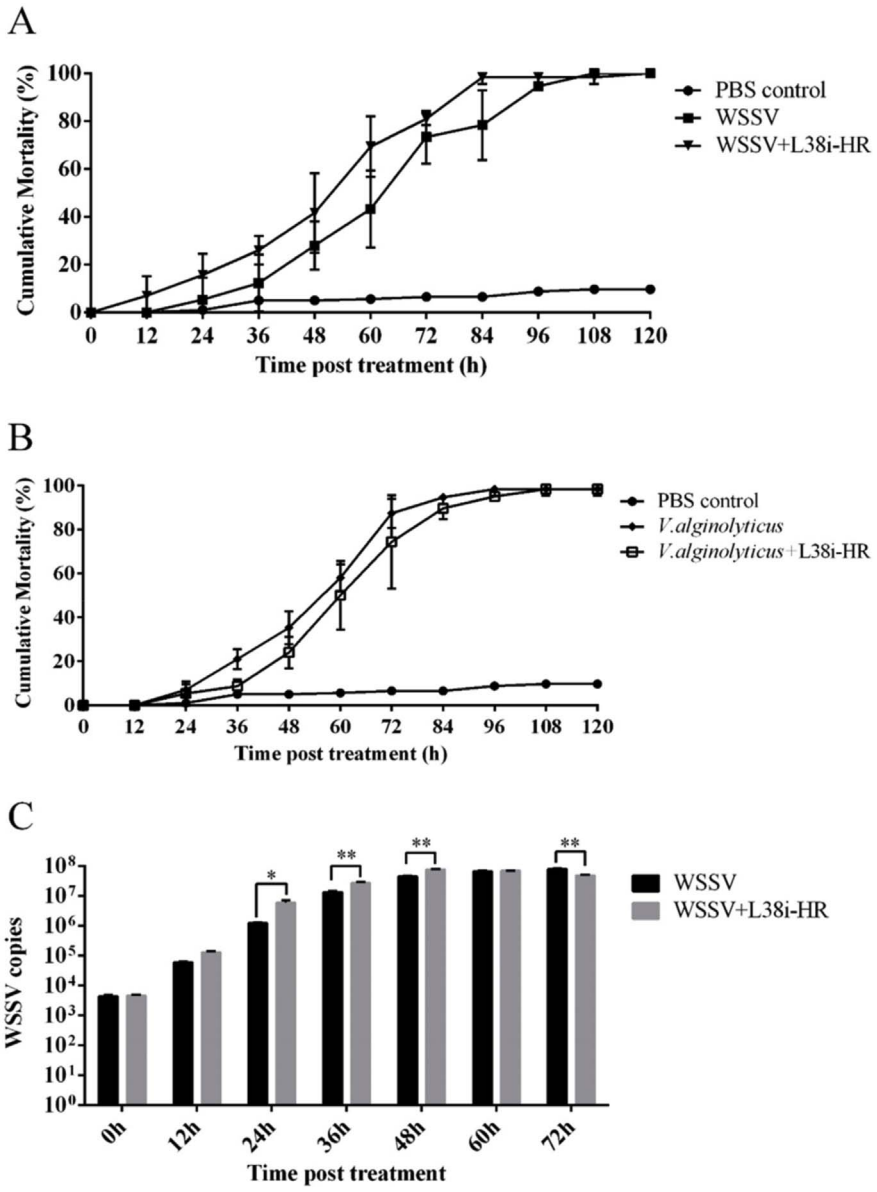

Fig. 5. The cumulative mortality and number of WSSV copies in challenged shrimps. A, the mortality of shrimps challenged with WSSV; B. the mortality of shrimps challenged with *V. alginolyticus*; C. The number of WSSV copies in shrimps challenged with WSSV. The treatment solutions are shown on the left. Each point shows the mean value of triplicate assays with the standard deviation. The number of WSSV copies represents the virus copies in 200 ng gDNA of shrimp gill tissue.

apoptosis rate of normal shrimps was approximately 58%; when *mjHR* expression was inhibited, the apoptosis rate increased to approximately 70%. L38i-EGFP treatment showed no significant effect in either group (Fig. 7).

Comparing the scatter plot of WSSV infection groups, the early stage of apoptosis decreased while the late stage of apoptosis increased, indicating that not only the number of apoptotic cells had increased but also that the apoptotic process had accelerated. Comparing the scatter plot of *V. alginolyticus* infection groups, the early stage of apoptosis and the late stage of apoptosis were both increased. This might suggest that the promotion of apoptosis by the virus and bacteria occurred in different ways (see Fig. 7).

4. Discussion

The LBD of *M. japonicus* HR (*mjHR*) contained 222 amino acids, and was predominantly expressed in immunity tissue include the gills, hemolymph and hepatopancreas, but especially in the gills. When challenged with WSSV, *mjHR* expression was down-regulated in multiple tissues. In contrast, when challenged with *V. alginolyticus*, *mjHR* expression was up-regulated in multiple tissues. An *mjHR*-specific plasmid, LITMUS 38i-HR, was constructed and successfully expressed in engineering bacteria HT-115 to synthesis dsRNA *in vitro*. With a suitable dose treatment (5–10 ng per shrimp) of L38i-HR dsRNA, we found that *mjHR* expression had been significantly knocked down.

After mjHR inhibition, multiple immune pathways and immunological parameters were analyzed. The IMD and Toll signaling pathway have both been considered as major regulators of the invertebrate innate immune response [19]. The IMD pathway is mainly responsible for responses to Gram-negative bacteria and the expression of antimicrobial peptide (AMP) which helps to eliminate bacteria. In contrast, the Toll-like receptor is mainly responsible for Gram-positive bacteria and can also induce AMPs and mediate the NK-κB pathway [18]. Other than AMPs, the IMD pathway could also promote apoptosis to a certain extent [20]. L-type lectin is one of the known pattern recognition receptors in shrimp hemolymph. The carbohydrate recognition domain of lectins could bind to specific carbohydrate sequences on different cell surfaces to enhance hemocyte phagocytosis, leading to bacterial agglutination and opsonization [18]. Virus infection would also raise the expression of several shrimp lectins. After HR expression was inhibited, L-type lectin was down-regulated, indicating that the pathogen/host recognition process might be positively influenced by

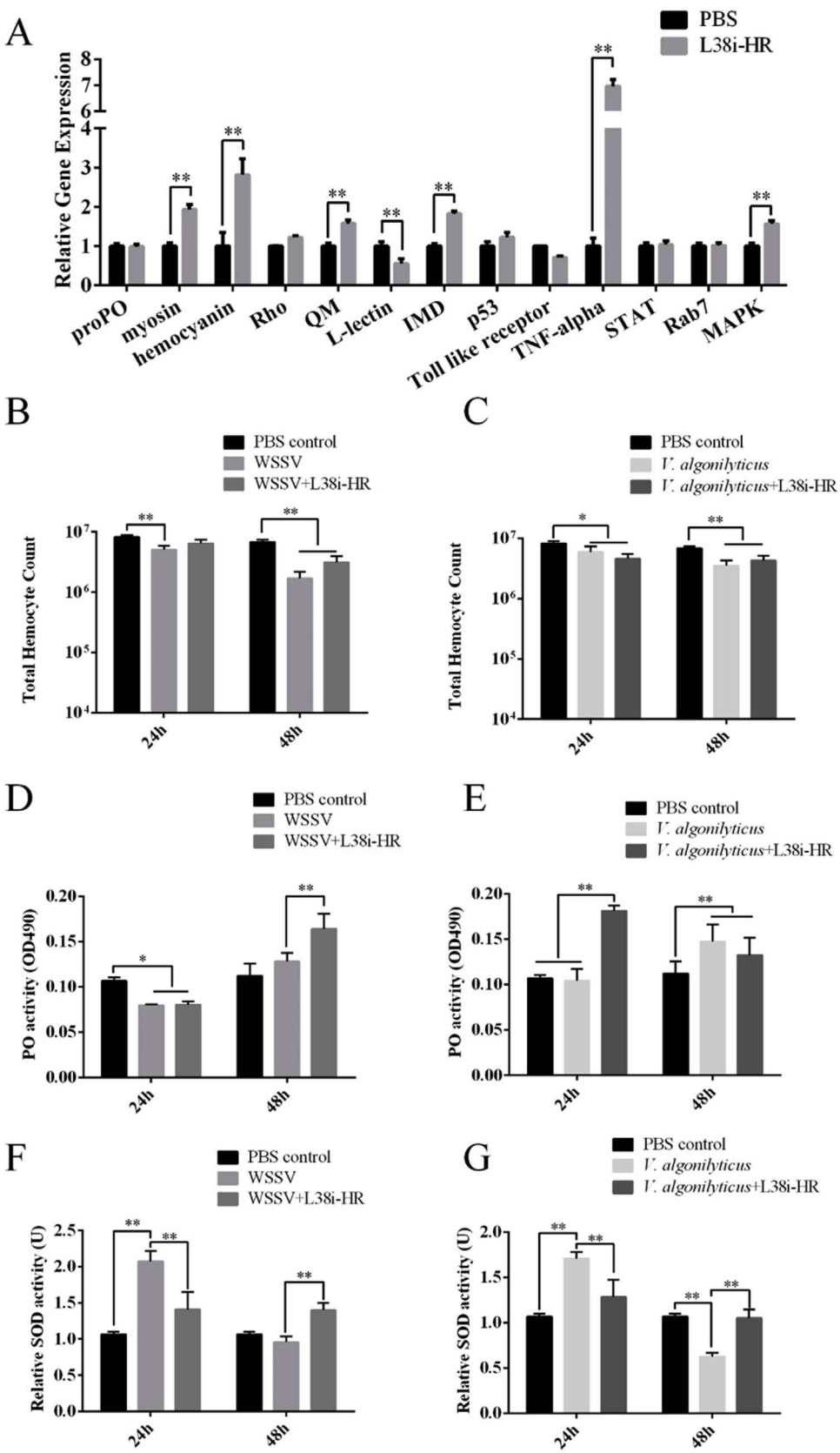

Fig. 6. Expression of genes related to the immune pathway and the analysis of immune parameters. A, Real-time RT-PCR analysis of nine immune genes (proPO, myosin, hemocyanin, Rho, QM, L-lectin, IMD, p53, Toll like receptor, TNF-α, STAT, Rab7 and MAPK) expression in the hemocytes of shrimps treated with L38i-HR; B, THC of shrimps infected with WSSV; C, THC of shrimps infected with *V. alginolyticus*; D, PO activity of shrimps infected with WSSV; E, PO activity of shrimps infected with *V. alginolyticus*; F, SOD activity of shrimps infected with WSSV; G, SOD activity of shrimps infected with *V. alginolyticus*; PO activity is shown as OD490. SOD activity of the PBS control was indexed as 1, and SOD activity units of other treatments represent a relative value.

the HR.

Hemocyanin is the carrier of oxygen [21] and provides energy throughout the shrimp body. In arthropods, hemocyanin can be activated by clotting enzyme to function as a substitute for prophenoloxidase [22], and cooperate with AMPs to participate in the innate immunity response to pathogen infection and condition-caused stress. QM has been confirmed to interact with hemocyanin and myosin gene in shrimp. The QM gene could regulate the activity of PO [23].

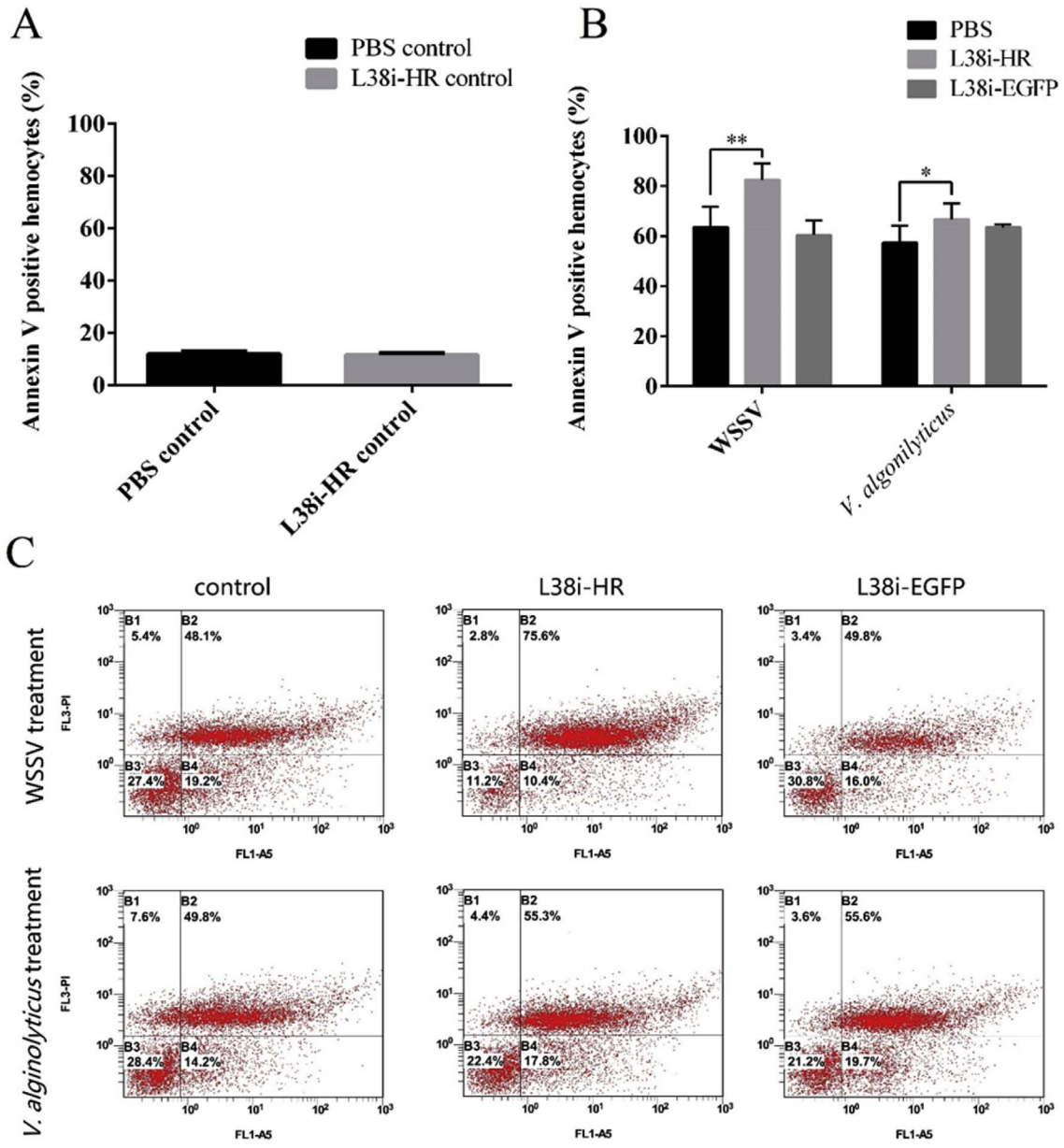

Fig. 7. Flow cytometry assay of hemocyte apoptosis. A, apoptosis rate of non-pathogenic groups; B, apoptosis rate of pathogen-infected groups. The proportion (%) of Annexin V positive hemocytes represent the apoptosis rate. In the bar chart, each column represents the mean value of triplicate experiments while the scatter plot below shows the result of one of the triplicate experiments. In the scatter plot, B1 represents cell fragments and dead cells, B2 represents late stage apoptosis hemocytes, B3 represents alive and normal hemocytes, and B4 represents early stage apoptosis hemocytes. The origin scatter plot of the controls is not shown.

RPPs recognize bacteria or virus patterns to initiate the proPO system, a non-self-recognition system in arthropods [24]. Research has shown that proPO is activated by prophenoloxidase-activating enzyme to convert to mature PO [17]. PO is the key enzyme, also the terminal enzyme, in the proPO activation system, which can oxidize phenols into quinones, and non-specifically crosslink neighboring molecules to form melanin in the process of melanization, one of the most important components of the immune defense system [18]. Our data showed that the expression of proPO showed no significant variation while the expression of QM protein, hemocyanin and myosin genes increased significantly. The PO activity of HR inhibited shrimps was significantly increased. These results suggested that *mjHR* had no impact on proPO, but could interact with QM protein, hemocyanin and myosin to regulate the production of PO enzyme.

The MAPK subfamily includes Ser/Thr protein kinases which can be triggered by the pathogen pattern recognition process and regulate downstream events to regulate the production of pro-inflammatory cytokines [25]. TNF- α is one of the pro-inflammatory cytokines that can promote inflammation and apoptotic responses; in mammals, the up-regulation of TNF- α can lead to several neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [26]. In our study, the expression of MAPK and TNF- α were significantly up-regulated, indicating that pro-inflammation and apoptosis might be negatively regulated by *mjHR*.

STAT and the JAK/STAT pathway plays an important role in the shrimp antiviral response [17,18]. Toll-like receptor is also part of an essential signaling pathway, the NK-κB pathway, which can protect the host against viruses in both insects and mammals [18,27]. These two pathways showed no significant variation after HR was inhibited, indicating that there was little impact of HR inhibition upon these two major anti-virus processes. However, our subsequent experiments relating to mortality, WSSV copies, and virus-induced apoptosis, showed

that the virus replication had increased and that virus-induced apoptosis had increased significantly. These results indicated that either the WSSV escaped the JAK/STAT pathway and/or the Toll-like receptor mediated NK-κB pathway, or that there were other pathways involved in the anti-virus process with important functions that could possibly be regulated by the HR. Considering there is so little known about the innate immunity system, we are willing to believe that there are other highly efficient pathways waiting to be identified and explored.

Generally, mjHR can down-regulate the IMD pathway to inhibit the Gram-negative bacteria induced IMD pathway to potentially inhibit the production of immune factors such as AMPs. Hemocyanin, which can cooperate with AMPs to activate innate immunity, and myosin, QM protein, were also down-regulated, leading to a restriction of PO activity in the proPO system to negatively regulate the process of inflammation following pathogen recognition. High level inflammation could cause damage of the normal hemocytes and neurological system. MAPK regulates pro-inflammation cytokines such as TNF-α which can promote inflammation and apoptosis. In our study, the expression of MAPK and TNF-α were both down-regulated by *mjHR*, indicating a negative role for *mjHR* in inflammation and apoptosis. Similarly, the JNK (a member of the MAPK subfamily) signal pathway in HeLa cells was found to be inhibited by the NHR [28]. Meanwhile, an acknowledged apoptosis indication gene, *p*53 [26], was not affected. On the other hand, L-type lectin was up-regulated by *mjHR*, indicating a positive role for *mjHR* in the pathogen recognition process. One known anti-virus pathway gene, STAT, was not affected by *mjHR*.

Nevertheless, *mjHR* had a significant impact on SOD activity in that it helped SOD to restrict the ROS produced by inflammatory and apoptotic processes to protect the host cells during the anti-pathogen progress. The double edge effect of ROS has been well acknowledged [29,30]. It appears that the HR functions much like a supervisor and watches over the immunity responses, such that immunity can be restricted in relatively safer zones. In the absence of HR, the immunity response appeared to be out of control, causing damage to both the pathogen and the host cells; the balance of immune responses determines life and death of the host. The up-regulated expression of HR after *V. alginolyticus* infection would satisfy this theory, although the down-regulation of HR after WSSV infection was much more interesting. It has been accepted that many viruses could take advantage of the host protein for its own proliferation; the same strategy has been found in WSSV. A study has shown demonstrated that WSSV could produce at least two anti-apoptosis proteins to block cell apoptosis and complete the viral replication cycle [31]; after virus replication was complete, WSSV can produce pro-apoptosis factors to promote cell death, thus releasing newly-generated virus particles. This theory has been questioned by some researchers, although there was no doubt that extensive apoptosis would impair the host cell and cause dysfunction in the infected tissue. In our analysis of apoptosis, both the virus and the bacteria induced a high apoptosis rate; when *mjHR* expression was inhibited, apoptosis in the hemocyte was induced, although acceleration of the apoptotic process was observed only in the group challenged with the virus, indicating that WSSV could intentionally promote apoptosis. This result was consistent with the expression changes of MAPK and TNF-α, the over expression of which, could cause several neuronal diseases [26]. We further found that the expression of p53 showed no variation; however, in humans, it has been shown that the HR can act as a neuroprotective compound to inhibit HIV-1 Tat-induced p53 expression and thus reduce neuronal death [32]. Three NHRs, NR4A1, NR4A2 and NR4A3 have been reported to play a role in the process of cell survival and apoptosis [33]; these can then can either activate genes responsible for cell survival or cell apoptosis, or translocate to the cytoplasm to target the mitochondria to cause cell apoptosis via Bcl-2 binding. The anti-apoptotic and pro-apoptotic function of the NHR may occur simultaneously or separately. Based on our present results, shrimp HR predominantly perform its anti-apoptotic function in shrimp immunity to prevent apoptosis-induced shrimp death.

In the infection experiments, PO activity and apoptosis increased, while SOD activity decreased; this was the case after *mjHR* inhibition with both WSSV infection and *V. alginolyticus* infection. However, the mortality count for WSSV or *V. alginolyticus* infection showed different results, as the mortality of WSSV-infected shrimp significantly increased, while the mortality of *V. alginolyticus* slightly decreased. The mechanism of infection for the virus and bacteria appeared to be different. One special feature of WSSV infection was that it could take advantage of the host protein, even the apoptosis process, to avoid host immunity and promote its own proliferation. The differential expression of *mjHR* after WSSV/*V. alginolyticus* infection also provided a clue. Based on the functional assumption of *mjHR*, we suspected that WSSV could use *mjHR* by limiting *mjHR* expression or by interacting with the LBD to disturb its basic function, thus promoting hemocyte inflammation and apoptosis to accelerate shrimp death. In mice liver cells, *Salmonella typhimurium* was reported to use the expression of liver orphan NHR NR3B3 to produce hepcidin, which circulates in the blood and binds to receptors in macrophages to increase iron loads in these cells, thus promoting the growth of some intracellular microbes and indicating that hepcidin induction by NR3B3 could act as a bacterial evasion mechanism for *S. typhimurium* [34]. Perhaps the critical function of the HR as regulators has made HRs a main target of the escape mechanism of virus and/or bacteria.

In conclusion, *mjHR* generally plays a positive role in the shrimp innate immune system. *mjHR* functions as an anti-apoptosis and anti-inflammatory factor to prevent pathogen-induced program cell death, and promotes SOD activity to eliminate ROS, thus keeping the host safe from these non-specific defense systems. Thus, during bacterial infection, the expression of HR was elevated to watch over the immune responses. However, WSSV could take advantage of this mechanism to inhibit HR function, and thus accelerate shrimp death. Our study provides new evidence of WSSV-shrimp interaction during virus infection, and has brought new insights into the shrimp innate immune system. Other than promoting an immune response, we identified that the immune response is controlled in a safe region and that the mechanisms underlying the shrimp innate immune system is worthy of further exploration.

Conflicts of 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.

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