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Galectin-9 activates host immune response and improve immunoprotection of *Onychostoma macrolepis* against *Aeromonas hydrophila* infection

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ABSTRACT

Galectin-9 (Gal-9) belongs to a family of the glycan-binding proteins (GBPs) and is known to restrict bacterial activity via interacting with pathogen associated molecular pattern (PAMPs). However, the underlying immune mechanism of endogenous Gal-9 on fish against bacterial infection is still unclear. In this study, effects of Gal-9 from *Onychostoma macrolepis* (*Om*Gal-9) on expression of immune-related genes were measured by HEK293T. The immune response of *O. macrolepis* with *Om*Gal-9 overexpression to *Aeromonas hydrophila* (*A. hydrophila*) infection (1.65×10^8 CFU/mL) was evaluated by tissue bacterial load, fish survival rate and transcriptome analysis. The results showed that *Om*Gal-9 displayed a punctate distribution in the nucleus and cytoplasm of HEK293T cells. Compared to cells transfected with the empty vector (EV group), recombinant plasmid pEGFP-Gal9 treatment (Gal9 group) significantly down-regulated the expression of immune-related genes *TNFα*, *STAT3*, *MyD88, LCK,* and *p52* of HEK293T cells stimulated with LPS at 24 h, while up-regulated *IκBα* and *caspase-1* ($P < 0.05$). The activities of catalase (CAT), superoxide dismutase (SOD), the total antioxidant capacity (T-AOC), alkaline phosphatase (AKP), acid phosphatase (ACP), and lysozyme (LZM) of *O. macrolepis* were significantly increased on 7 days in Gal9 group compared to EV group ($P < 0.05$). The bacterial load of liver, spleen, and kidney of *O. macrolepis* infected with *A. hydrophila* in Gal9 group at 24 h was significantly lower than that in EV group ($P < 0.05$), and the survival rate had increased from 15 % to 35 %. A comparative transcriptome analysis between the Gal9 and EV group identified 305 differentially expressed genes (DEGs). The analysis showed that *Om*Gal-9 might play an important regulatory role in glycolysis/gluconeogenesis, fatty acid degradation, and ascorbate and aldarate metabolism. Moreover, the immune-related DEGs were predominantly enriched in eleven pathways, with the most important three of them being linked to innate immunity: NOD-like, C-type lectin and Toll-like receptor signaling pathway. Taking together, *Om*Gal-9 can enhance the resistance of fish to bacterial diseases by improving immune system function and activating immune-related pathways.

1. Introduction

Innate immunity and adaptive immunity are two categories of immune system, which liaise to protect the host and maintain host health. As lower vertebrates, fish were more likely to rely on innate immunity to keep the balance of internal environment, remove pathogens, and trigger adaptive immune response [1]. Innate immunity of fish can response immediately to pathogen but has limited capacity to recognize antigens. Lectin are considered to be a class of glycoproteins without enzymatic activity, which plays an important role in biological defense, cells recognition, and cellular adhesion through specific recognition of binding glycoprotein and glycoconjugates [2].

Galectins are a family of animal lectins with carbohydrate-recognition domain (CRD) that can identify specific glycoconjugates to attach pathogen-associated molecular patterns (PAMPs) on the cellular surface to regulate cells differentiation and immune homeostasis [3]. The difference of sugar-binding sites determines the function and types of galectins. Based on sugar-binding structures, galectins include three groups: proto-type (galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) has two CRDs, forming non-covalent homodimers; chimera-type (galectin-3) existed pentamers with a carboxyl-terminal CRD and a N-terminal glycine, tyrosine, and proline rich domain; tandem repeat type

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Table 1
Primers used in this study.

Primers	Nucleotide Sequence (5'→3')	Comment
EGal-9-F	CATGGCTTTTTATCAGCAACAACCG	Protein expression
EGal-9-R	TTAAGCCTGCACTAAAGTCAGCT	Protein expression
TNFα-F	CACAGTGAAGTGCTGGCAAC	RT-PCR
TNFα-R	AGGAAGGCCTAAGGTCCACT	RT-PCR
STAT3-F	GGTGCCTGTGGGAAGAATCA	RT-PCR
STAT3-R	GCATCTTCTGCCTGGTCACT	RT-PCR
MyD88-F	CCACACTTGATGACCCCCTG	RT-PCR
MyD88-R	GGCGGCACCTCTTTTCGAT	RT-PCR
p38-F	TACGTGGCCACTAGGTGGTA	RT-PCR
p38-R	TCATGGCTTGGCATCCTGTT	RT-PCR
caspase-1-F	ATCCGTTCCATGGGTGAAGG	RT-PCR
caspase-1-R	CCTGTGCCCCTTTCGGAATA	RT-PCR
p52-F	AGCTGCACACAGCCGGAAA	RT-PCR
p52-R	CTGCTTAGGCTGTTCCACGA	RT-PCR
IκBα-F	GAAGTGATCCGCCAGGTGAA	RT-PCR
IκBα-R	CTCACAGGCAAGGTGTAGGG	RT-PCR
LCK-F	CCAGGGAGAGGTGGTGAAAC	RT-PCR
LCK-R	TTCTGGGGCTTCTGGGTCT	RT-PCR
OmCASP1-F	CATAATGTCCCACGGCAAAA	RT-PCR
OmCASP1-R	TCATCAGGCTCGCCATCACT	RT-PCR
OmLy-6B-F	AGTTGGTTCCGCTAAGGTG	RT-PCR
OmLy-6B-R	TCACAGCACTGGGCAGATAC	RT-PCR
OmCLM-1-F	GGGATACTGACAGGGCTTGG	RT-PCR
OmCLM-1-R	CATCTTTGGGCTGCGTTC	RT-PCR
OmLEAP-2-F	GCCTGGTGCCTTGTGTTTTT	RT-PCR
OmLEAP-2-R	TAACGGGGTCATTCGAGCTG	RT-PCR
OmJIP-1-F	ACTGAGGTCCAGACGCAATG	RT-PCR
OmJIP-1-R	CCTGTGGGCAGCATCAGTAA	RT-PCR
OmIL12-F	GGAATGGGGTGAGAACAGGAA	RT-PCR
OmIL12-R	TGATGTTGCTTCCACCAGGG	RT-PCR
OmSTAT1-F	CCCTGGAGACCCTTGGTGAT	RT-PCR
OmSTAT1-R	TCAGAGTTCAGCGCTCGTTT	RT-PCR
OmIL-21R-F	TACAGTGGAACGTGGAGTGAC	RT-PCR
OmIL-21R-R	GCAGGAATGGACATAAGCAG	RT-PCR
OmC3-F	AAATCAACGCCGAGCAGC	RT-PCR
OmC3-R	TTTTCGCCACCCACCATC	RT-PCR
OmTNFSF-5-F	TGTGTGATGTCTGTCCTGGC	RT-PCR
OmTNFSF-5-R	ACTCAGCATCAGCCGTATCG	RT-PCR
GAPDH-F	TTCTTTTGCGTCGCCAGCC	RT-PCR Control
GAPDH-R	CCGTTCTCAGCCTTGACGGT	RT-PCR Control
β-actin-F	AAGGATTCCTATGTGGGCGAC	RT-PCR Control
β-actin-R	CGTACAGGGATAGCACAGCC	RT-PCR Control
Om-GAPDH-F	TGCTATCACCGTCTTCAGCG	RT-PCR Control
Om-GAPDH-R	ACATAGGGGCATCTGCACTG	RT-PCR Control
Om-β-actin-F	TGACCCACACTGTACCCATC	RT-PCR Control
Om-β-actin-R	CGGACAATTTCACTCTCGGC	RT-PCR Control

(galectin-4, -6, -8, -9, and -12) has two CRDs connected by a functional linker peptide. Gal-9 has two distinct carbohydrate recognition domains which can form rich multivalent lattices with cell-surface glycoconjugates [4]. Thus, it is known as a versatile immunomodulator, and shows a high level of complexity in mediating immune regulation [5,6]. However, research of immune regulation in Gal-9 were main focused on mice and human. Gal-9 in mice macrophages can degrade Nod-like receptor protein (NLRP) to attenuate the NLRP3-dependent inflammation [7]. As an eosinophilic activator, Gal-9 can induce eosinophilic recruitment and superoxide production in human [8]. Human Gal-9 can also activate apoptosis of T cells through the calcium-calpain-caspase-1 pathway [9]. Thus, Gal-9 can endow immune cell with antimicrobial, anti-inflammatory, or immunoregulatory functions at the cross-road of innate and adaptive immune responses. However, regulation role of fish Gal-9 on the innate and adaptive immunity has few reports.

To date, researches of fish Gal-9 mainly focus on the gene identification and antimicrobial test. *Pelteobagrus fulvidraco*, *Paralichthys olivaceus* and *Labeo rohita* can resist pathogenic bacterial infection by increasing of Gal-9 expression, demonstrating that Gal-9 can bind to glycoconjugates of diverse bacteria surface and function as pattern recognition receptor (PRR) in the innate immune system [10–12]. Gal-9 identified from *Larimichthys crocea*, *Carassius auratus* and *Oreochromis niloticus* indicated specific binding to Gram-negative bacteria and Gram-positive bacteria in vitro [13,14]. Moreover, the ability of potential antiviral immunity of Gal-9 were revealed in *Siniperca chuatsi* [15]. Thus, Gal-9 was considered as potential bioactive immunostimulants, and then the effect of Gal-9 on immune system of fish need to be evaluated. Overexpression is an effective method to study the function of genes in fish. Niu et al. reported that Gal-8 overexpression improved the resistance of *O. niloticus* to *Streptococcus agalactiae* infection [16]. Recombinant NK-lysin overexpression improved the survival rate of *O. niloticus* after infection with *S. agalactiae* [17]. Overexpression of liver expression antimicrobial peptide 2 (LEAP-2) in *Sebastes schlegelii* can reduce the tissue bacterial load and increase the expression of inflammatory factors after *Listonella anguillarum* infection [18]. In word, overexpression can provide effective way to study functional role of Gal-9 in modulating the fish immune response to bacterial infection.

Onychostoma macrolepis belonged to the family Cyprinidae, Barbinae, *Onychostoma* inhabits the flowing freshwater in North China [19]. Previous studies have focus on the geographical distribution and genetic diversity of *O. macrolepis*. In recent year, the artificial breeding of *O. macrolepis* made the breakthrough progress, and disease resistance traits and farming breeding are urgent issues which need studying. According to our previous study, *Om*Gal-9 exhibits obvious binding activity to a variety of pathogenic bacteria in vitro [20]. *Aeromonas hydrophila* as an important pathogen can cause bacterial septicemia in fish, but its immune prevention and treatment are still encountering challenges and obstacles [21,22]. Thus, *Om*Gal-9 exhibits potential applications as a bioactive immunostimulants on inhibiting *A. hydrophila* infection. In the present study, we firstly assessed immune response of HEK293T cells transfected with *Om*Gal-9 to LPS stimulation. Next, the effect of *Om*Gal-9 overexpression on activity of non-specific immune and antioxidant enzymes of *O. macrolepis* were tested. At last, transcriptome was used to identify the key pathways and molecules involved in the immune regulation of *Om*Gal-9 after *A. hydrophila* infection, and at same time the bacterial load, and mortality of *A. hydrophila* infection fish were measured.

2. Materials and methods

2.1. Plasmid construction

The specific primers (Table 1) of *Om*Gal-9 designed by Primer Premier 6.0 were used to amplify *Om*Gal-9 ORF sequence. The pEGFP-C1 vector plasmid (TaKaRa Bio Technology) was dissected with *Eco*RI and *Xba*I enzymes, and then ligated *Om*Gal-9 ORF according to ClonExpress®II one-step cloning instructions (Vazyme, China) to construct recombinant plasmid pEGFP-Gal9. The junction product was transformed into DH5α (TIANGEN, Beijing, China) and sent to Tsingke Biotechnology Co., Ltd for sequencing. All plasmids were extracted using EndoFree Mini Plasmid Kit II (TIANGEN, Beijing, China) in compliance with manufacturer's instructions.

2.2. Subcellular localization of OmGal-9

Human embryonic kidney 293 T cells (HEK293T; China Center for Type Culture Collection, Wuhan) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) with 10 % fetal bovine serum (FBS; Biological Industries, Israel), 100 U/mL penicillin and 100 mg/mL streptomycin (Solarbio, China) under 37 °C and 95 % air humidity condition with 5 % CO_2.

To examine the subcellular distribution of *Om*Gal-9 in HEK293T, the cells were seeded into 6-well plates with 1×10^6 cells per well until the cells grew to a certain density (70 %). The pEGFP-Gal9 was transfected into HEK293T cells by QuickShuttle transfection reagent (Biodragon, China) according to the manufacturer's instructions. After 24 h post transfection, the cells were washed with PBS and later fixed with immunostaining fixative (4 % paraformaldehyde), and then stained with l μg/mL 4,6-diamidino-2-phenylindole (DAPI) for 10 min. After washing,

the cells which expressed *Om*Gal-9 were adding anti-fluorescence quenching sealing solution (BioTek, USA) and observed using the fluorescent microscope Cytation 5.

2.3. LPS stimulation

HEK293T cells transfected with the empty plasmid pEGFP-C1 and recombinant plasmid pEGFP-Gal9 were planted in 24-well plates to cultivate for 72 h, and then stimulated with LPS (100 μg/mL) and physiological saline as control, respectively. The cells were collected at 24 h and extracted RNA using the Trizol Reagent Kit (TIANGEN, Beijing, China). The cDNA was synthesized using the Fastking RT Kit with gDNase (TIANGEN, Beijing, China). Immune-related regulatory factors involved in inflammatory pathways including tumor necrosis factor α (*TNFα*), signal transducers and activators of transcription 3 (*STAT3*), lymphocyte-specific protein tyrosine kinase (*LCK*), Inhibitor of nuclear factor-κBα (*IκBα*), Myeloid differentiation factor 88 (*MyD88*), and the key regulatory genes related to cell apoptosis *caspase-1*, *p38*, *p52* were detected by RT-qPCR. Specific primers were designed using National Center of Biotechnology Information and shown in Tab. 1. The RT-qPCR reaction volume was 20 μL, including 10 μL of 2 × UltraSYBR Mixture (CWBIO, China), 2 μL of cDNA, 1 μL of each primer, and 6 μL of nuclease-free water. The program was performed as follows: 10 min at 95 °C, 34 cycles of 10 s at 95 °C, 30 s at 60 °C and 32 s at 72 °C. The *GAPDH* and *β-actin* were used as internal control for the reference genes. All reactions were performed in triplicates, and the data were analyzed using the $2^{-\Delta\Delta}$CT method.

2.4. Overexpression of OmGal-9 in O. macrolepis

The *O. macrolepis* were obtained from Tianyuan ecological farm in Zhenping town, Shaanxi Province, China. The fish were acclimatized for two weeks with constant temperature control breeding system containing 20 L tap water (22 ± 2 °C, pH 8.0 ± 0.2) equipped with internal biofilter and aeration device. According to overexpression protocol described by Niu [16], a total of 60 healthy fish (body length: 11.5 ± 0.8 cm) were randomly divided into 3 groups (control group, EV group, and Gal9 group). The EV and Gal9 groups intramuscular injected 50 μL empty vector pEGFP-C1 and recombinant plasmid pEGFP-Gal9 (200 μg/mL), respectively, and control group injected 50 μL physiological saline. Three fish were randomly selected from each group on 7 days after plasmid treatment for blood collection from caudal vein to detect serum enzymes activities. All fish procedures were conducted with the approval of the Committee of Laboratory Animal Experimentation at Northwest A&F University and were in full compliance with the guidelines of the Animal Experiment Committee.

2.5. Sample collection and detection

To examine the expression of green fluorescent protein of transfected plasmid, the spleen tissue of *O. macrolepis* were collected on 7 days in control group, EV group, and Gal9 group. Spleen samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. The frozen spleen were sectioned to detect the fluorescence signal by fluorescence microscope (ZEISS, Germany). Fresh bloods samples from the 2.4 section remained at room temperature to clot and were centrifuged at 1500 g for 15 min. The supernatant solution was transferred to new 1.5 mL tubes and kept at −80 °C for further biochemical analysis. Serum were used to detect activities of antioxidant and immunity related enzymes by the corresponding protease detection kit (Nanjing Jiancheng, China). Catalase (CAT) activity was measured by Xanthine oxidation method. Superoxide dismutase (SOD) activity was detected using the Visible light method. ABTS system was used to determinate the total antioxidant capacity (T-AOC). Alkaline phosphatase (AKP) and acid phosphatase (ACP) activities were tested with Spectrophotometric method, and detection of lysozyme (LZM) activity was used Turbidimetric method.

2.6. Infection experiment

To investigate the role of *Om*Gal-9 in immune response of fish to bacterial infection, experimental fish were injected intraperitoneally with 100 μL A. *hydrophila* (1.65×10^8 CFU/mL) suspended in PBS on the 7 days of treatment with plasmid pEGFP-Gal9 and pEGFP-C1. The liver, spleen, and kidney of three fish were collected and weighed under aseptic condition after 24 h post infection (hpi). Then, the tissues were homogenated and diluted to 10^{-8} fold in sterile PBS (pH=7.2). 100 μL solution of tissues were plated on LB solid medium to culture at 37 °C for 12 h. Colony-forming units (CFU) in all plates were calculated and multiplied by the dilution factor. The mortality was recorded from 0 to 24 h after *A. hydrophila* infection.

2.7. RNA sequencing, DEGs identification and KEGG enrichment analysis

The three liver tissue samples separately were collected from EV and Gal9 groups at 24 h after *A. hydrophila* infection for transcriptome sequencing, and samples were rapidly placed in liquid nitrogen and then stored at −80 °C. Total RNA was extracted according to manufacturer's procedure and the cleaved RNA fragments were reverse-transcribed to create the cDNA. The cDNA library was sequenced with Illumina Novaseq™ 6000 sequence platform (LC-Bio Technology CO., Ltd, Hangzhou, China). Raw reads with low quality, adapter contamination, and containing Poly-A were processed using Fastqc software to obtain clean reads. These clean reads were then aligned to the *O. macrolepis* reference genome (GenBank: GCA_012432095.1) using STAR software. Differentially expressed genes (DEGs) between different groups were obtained by DESeq2 software based on default criteria with the parameter of false discovery rate below 0.05 and absolute fold change ≥2. DEGs were subjected to enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using phyper package of R.3.6.1 software [23,24].

2.8. Verification of illumina sequencing data by RT-qPCR

To validate the results of transcriptome sequencing data, ten immune-related DEGs including caspase-1 (*CASP1*), lymphocyte antigen 6 B (*Ly-6B*), CMRF-35-like malecule 1 (*CLM-1*), liver-expressed antimicrobial peptide 2 (*LEAP-2*), C-Jun-amino-terminal kinase-interacting protein 1 (*JIP-1*), interleukin 12 (*IL-12*), signal transducer and activator of transcription 1 (*STAT1*), interleukin 21 receptor (*IL-21R*), complement component 3 (*C3*), tumor necrosis factor superfamily member 5 (*TNFRSF-5*) were selected for RT-qPCR analysis (seen methods 2.3), and the relative expression of the target genes were calculated as fold changes by normalizing to *β-actin* and *GAPDH*. The obtained results were analyzed using the $2^{-\Delta\Delta}$CT method. Specific primers were shown in Table 1.

2.9. Statistical analysis

All analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA) and SPSS 12 software (Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA, followed by post-hoc analysis with the Tukey-Kramer test. $P < 0.05$ were considered statistically significant, and $P < 0.01$, $P < 0.001$ considered highly significant.

3. Result

3.1. Effects of OmGal-9 overexpression on cellular immune factors

3.1.1. Subcellular localization of OmGal-9

The recombinant plasmids were constructed and successfully

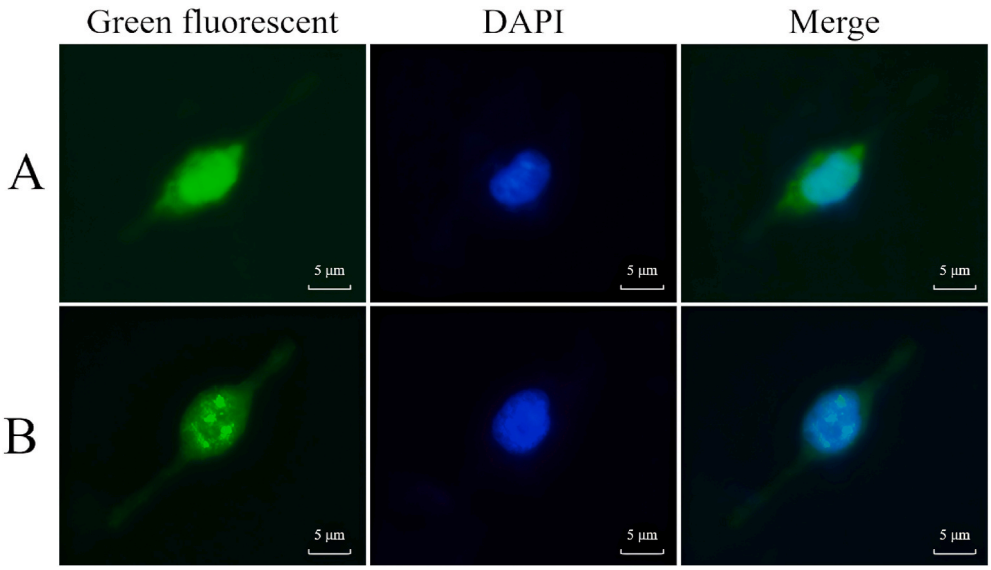

Fig. 1. Subcellular localization of *Om*Gal-9 in HEK293T cells. HEK293T cells with transfected pEGFP-C1 (lane A) and pEGFP-Gal9 (lane B). The left are pEGFP-C1 and pEGFP-Gal9 fusion protein with green fluorescence, the middle are the cell nucleus stained with DAPI, and the right are the combined images panels.

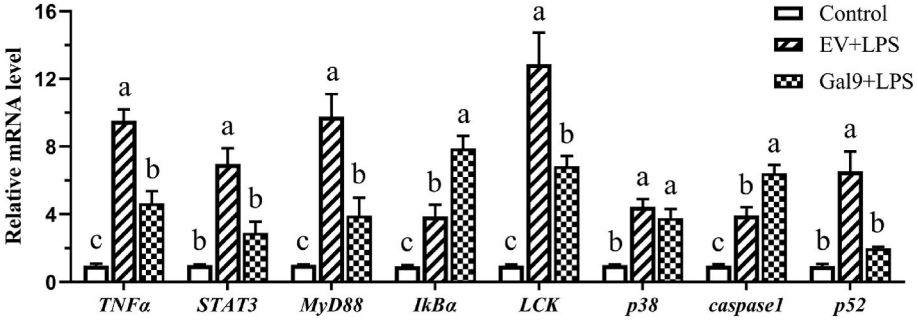

Fig. 2. Expression level of immune-related genes in HEK293T cells with *Om*Gal-9 overexpression against LPS stimulation at 24 h.

transfected into HEK293T cells. As shown in Fig. 1, green and blue fluorescence represented the location of transfected plasmid and cell nucleus, respectively. *Om*Gal-9-EGFP fusion protein was expressed in HEK293T cells, and mainly localized in the cytoplasm and nucleus with punctate distribution.

3.1.2. Transfection HEK293T cell effected expression of immune-related genes

At 24 h after LPS stimulation, expression level of immune regulatory factors *TNFα*, *STAT3*, and *LCK* in HEK293T cell transfected with *Om*Gal-9 were significantly down-regulated compared with cell transfected with empty vector, while *IκBα* was significantly up-regulated ($P < 0.05$). Transfection with *Om*Gal-9 significantly up-regulated *caspase-1* expression and down-regulated *p52* of HEK293T cell compare to empty vector transfected group ($P < 0.05$), but there was not affected with *p38* (Fig. 2).

3.2. Overexpression of OmGal-9 promotes immunity of O. macrolepis

3.2.1. Improvement of antioxidant and immune capacity

Histological examination of spleen tissue of *O. macrolepis* showed that green fluorescence was widely distributed in EV group and Gal9 group, while no fluorescence was observed in the PBS group on 7 days (Fig. 3A). The activities of the serum antioxidant enzymes CAT, SOD ($P < 0.05$), and T-AOC ($P < 0.001$) were significantly higher in *Om*Gal-9 overexpression group than that of PBS group, and the similar changes were also found in immune enzyme ACP, LZM ($P < 0.01$), and AKP ($P < 0.001$) (Fig. 3B).

3.2.2. Bacterial load and survival rate

*Om*Gal-9 overexpression significantly decreased the bacterial load of liver, spleen, and kidney in *O. macrolepis* after *A. hydrophila* infection ($P < 0.05$) (Fig. 4A). The bacterial load of liver in the control group, EV group, and Gal9 group were 1.62×10^7 CFU/g, 2.62×10^7 CFU/g and 3.15×10^6 CFU/g, respectively. In spleen, the number of bacteria in Gal9 group (1.09×10^4 CFU/g) was remarkably lower than that in the control group (1.47×10^5 CFU/g) and EV group (6.83×10^4 CFU/g). The bacteria load of kidney was 4.11×10^7 CFU/g in control group and 1.87×10^7 CFU/g in EV group, while there was only 1.52×10^6 CFU/g in Gal9 group. After *A. hydrophila* infection, the survival rates has no significant difference between control (10 %) and EV group (15 %), while Gal-9 group was significantly increased to 35 % ($P < 0.05$) (Fig. 4B).

3.3. Liver transcriptomic analysis of O. macrolepis with OmGal-9 overexpression response to A. hydrophila infection

3.3.1. DEGs identification and KEGG pathway analysis

A total of 305 DEGs were identified between Gal9 and EV groups. 134 DEGs were up-regulated and 171 DEGs were down-regulated (Fig. 5A). Heat map clustering based on the gene expression of 305 DEGs demonstrated that 3 samples from each group were clustered together (Fig. 5B).

KEGG pathway analysis showed that the DEGs involved in five main

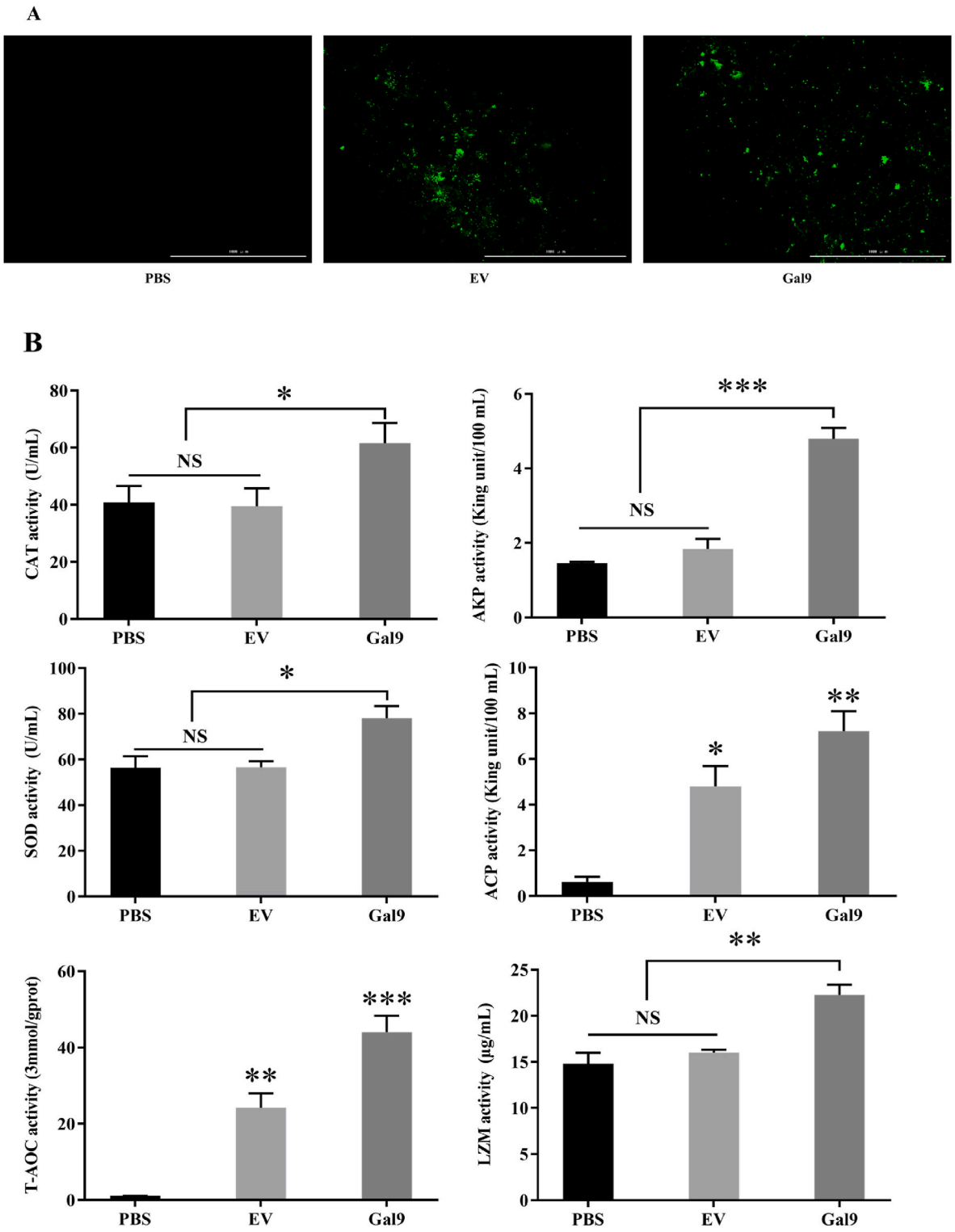

Fig. 3. Determination of green fluorescence signals in the spleen (A) and detection of serum enzymes (B) of *O. macrolepis* after *Om*Gal-9 overexpression. Fish were injected with PBS (PBS), pEGFP-C1 (EV), and pEGFP-Gal9 (Gal9), respectively. Asterisks (*) representative of significant difference (* means $P < 0.05$; ** means $P < 0.01$; *** means $P < 0.001$).

categories, including metabolism, genetic information processing, environmental information processing, cellular processes, and organismal systems (Fig. 6A). The pathways with the most DEGs were environmental information processing (37; 28.46 %), followed by metabolism (32; 24.62 %), cellular processes (32; 24.62 %), organismal systems (16; 12.31 %), drug development (7; 5.38 %), and genetic information processing (6; 3.08 %). The top 20 KEGG pathways are presented in Fig. 6B, in all 9 related to metabolism, 6 related to organismal systems, 3 to environmental information processing, and 2 to genetic information processing. Specifically, the KEGG pathways mainly enriched in glycolysis/gluconeogenesis (ko00010), fatty acid degradation (ko00071), ascorbate and aldarate metabolism (ko00053) (Fig. 6B).

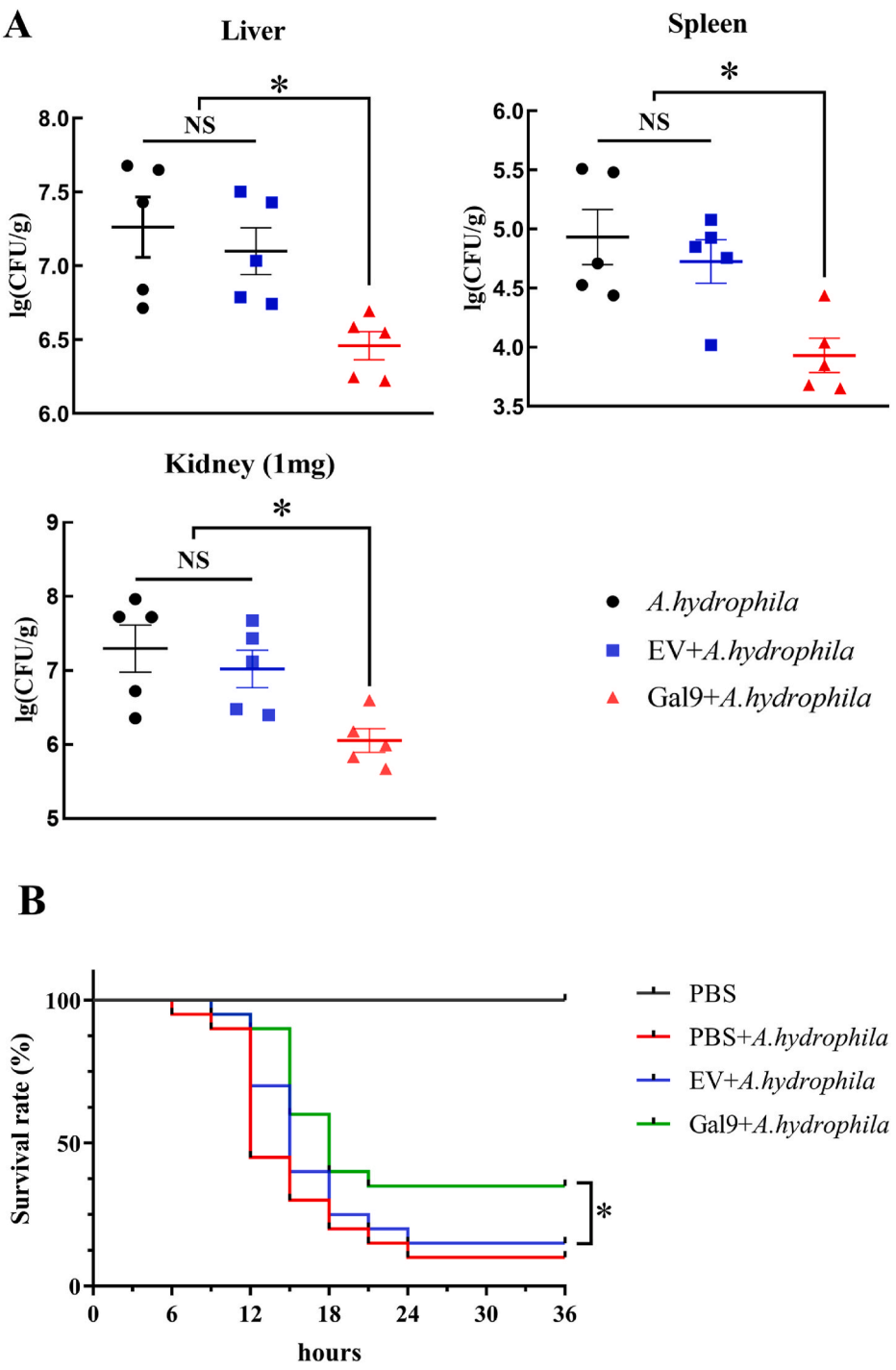

Fig. 4. Effect of *Om*Gal-9 overexpression on bacterial load (A) and survival rate (B) after *A. hydrophila* infection. Colony numbers were normalized to PBS group. Asterisks (*) representative of significant difference (* means $P < 0.05$).

3.3.2. Analysis of differentially expressed immune-related genes

Eleven immunity pathways that DEGs involved were identified (Fig. 7A). Eight DEGs were enriched in the NOD-like receptor signaling pathway, followed by the C-type lectin receptor signaling pathway (4 DEGs), Toll-like receptor signaling pathway (3 DEGs), RIG-I-like receptor signaling pathway (2 DEGs), Complement and coagulation cascades (1 DEGs), and Cytosolic DNA-sensing pathway (1 DEGs). DEGs from NOD-like receptor pathway were used to construct gene pathways network to reveal the regulatory role of *Om*Gal-9 on immunity of fish (Fig. 7B). In NOD-like receptor pathway, *NLRP3*, *NLRP12*, *p38*, *PLCβ*, and *HSP90* were significantly down-regulated, while *CASP1* and *NLRP1* were up-regulated in *O. macrolepis* with *Om*Gal-9 overexpression compare with fish without *Om*Gal-9 overexpression. Genes related to immune response and inflammatory process were screened from DEGs, among which *CLM-1*, *Ly-6B*, and *LEAP-2* were up-regulated, while *TNFRSF-5*, *IL-12*, *IL-21R*, *C3*, *STAT1*, *JIP-1* were down-regulated.

3.3.3. DEGs verification using RT-qPCR

To confirm the accuracy and reliability of DEGs, the expression profiles of the 10 immune-related DEGs were measured. The RT-qPCR results demonstrated that the expressions of all tested DEGs comprising four up-regulated (*CASP-1*, *CLM-1*, *Ly-6B*, and *LEAP-2*) and

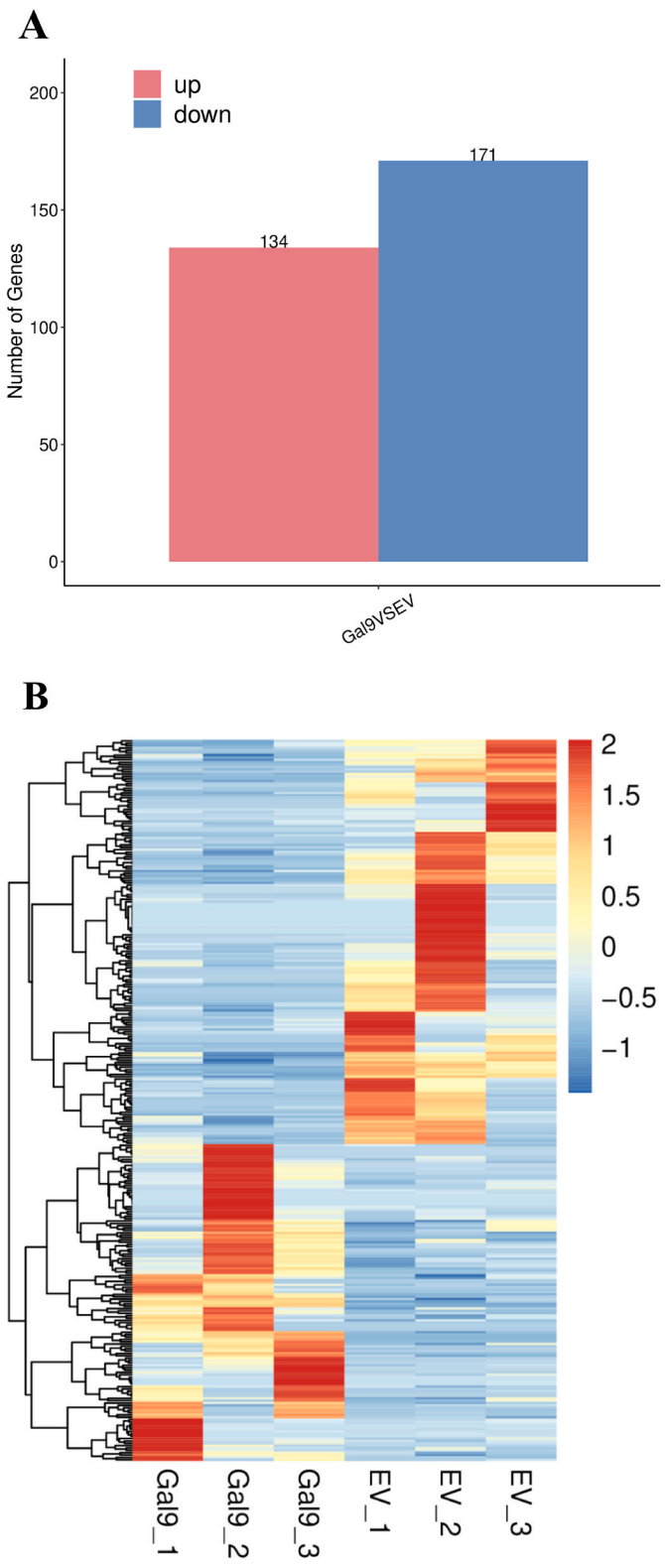

Fig. 5. Analysis of differential gene expression between EV vs Gal9 group. (A) Number of differential genes, red bar represented up-regulated genes, and blue bar represented down-regulated genes; (B) Hierarchical cluster analysis of differentially expressed genes and sample relations. The expression level of genes in different samples was represented by different colors. The redder the color, the higher the expression level, and the bluer the color, the lower the expression level in the heatmap.

six down-regulated (*TNFRSF-5*, *IL-12*, *IL-21R*, *C3*, *STAT1*, and *JIP-1*) exhibited significant differences between the EV and Gal9 groups (Fig. 8). The results showed that the expression pattern of the selected gene was consistent with that of RNA-Seq analysis, which indicated that the result was reliable.

4. Discussion

The frequent outbreaks of bacterial septicemia caused by *A. hydrophila* in recent years has led to huge economic losses to aquaculture in China [25]. In recent years, bioactive molecules were used to enhance aquatic animals' health in many ways such as antimicrobial, antioxidant, immunostimulant, and antiinflammatory properties. Selecting of bioactive molecules is a new way for inhibiting *A. hydrophila* infection. Galectins from different animals exhibited distinct ability to resist pathogenic bacteria infection. Previous study reported that Gal-9 was synthesized on free ribosomes and then partially engaged in protein-protein interactions to regulate intracellular activities in cytoplasm, and partially secreted to the cell surface where interacts with glycoproteins and glycolipids through multivalency cross-linking [26,27]. Notably, *Om*Gal-9 has a punctate distribution in the cytoplasm of HEK293T. The same results were reported in *Nibea albiflora* kidney cells that Gal-9 can form secretory granules in the cytoplasm [28]. In human macrophages, galectin is recruited in phagolysosomes to form punctate structures [29]. We speculated that this uneven distribution may be related to the aggregation and joint function of Gal-9 on some organelles in fish.

Acute inflammatory response is considered to be a crucial component of the innate immune defense mechanism. TNFα, STAT3, MyD88, and IκBα are important regulatory factors of innate immune signaling pathway. *Om*Gal-9 can regulate the expression of immune genes (*TNFα*, *STAT3*, *MyD88* and *IκBα*) and then protects the body from excessive inflammation. Down-regulation of TNFα can inhibited the NF-κB and MAPK signaling pathways to decrease expression of pro-inflammatory mediators related genes [30]. Decrease of STAT3 can inhibited the production of a variety of inflammatory cytokines [31]. When LPS stimulates, MyD88 is activated by the TLR4 and then induces the release of IL-1β, IL-6, and TNFα, ultimately inducing the production of inflammation [32]. IκBα is the up-stream inhibitory regulator of NF-κB, the up-regulation of IκBα inhibits the activity of NF-κB signaling pathway in the late stage of inflammation [33]. LCK is the first signaling molecule to activate T lymphocyte receptors in adaptive immunity, suggesting Gal-9 can inhibit LCK-mediated T cell activation [34]. Taken together, Gal-9 can rapidly recognize and bind to pathogens, minimizing organ damage caused by inflammation. *Om*Gal-9 overexpression up-regulated expression of *caspase-1*, and down-regulated *p52* against LPS simulation. Caspase-1 was reported to play an important role in mediating adaptive and innate immunity, suggesting that *Om*Gal-9 can indirectly affect the adaptive and innate immunity of body [35,36]. Previous study showed that LPS up-regulated the expression of p52 and enhanced the activation of NF-κB in human colonic epithelial cells [37]. *Om*Gal-9 had no effect on p38 expression, while transfection of Gal-7 can activate p38 MAPK to affect tumorigenesis and tumor metastasis [38], indicating that different subtypes of galectins have different regulatory mechanisms on the pathway. Collectively, Gal-9 overexpression inhibited LPS-induced inflammation and relieve excessive immune stress by mediating in innate immunity, and cell apoptosis.

In fish, non-specific immunity is required for maintaining dynamic equilibrium, preventing microbial invasion, eliminating various pathogens, and generating adaptive immune responses. Pathogenic bacteria or various stress factors cause organisms to create oxidative stress responses, which release harmful free radicals. The antioxidant system can protect the organism to avoid these destruction [39]. Based on the results, *Om*Gal-9 overexpression increased the activities of serum antioxidant enzyme to resist bacterial infection. SOD can inhibit the formation of oxygen free radicals by CAT detoxification. T-AOC reflects the ability

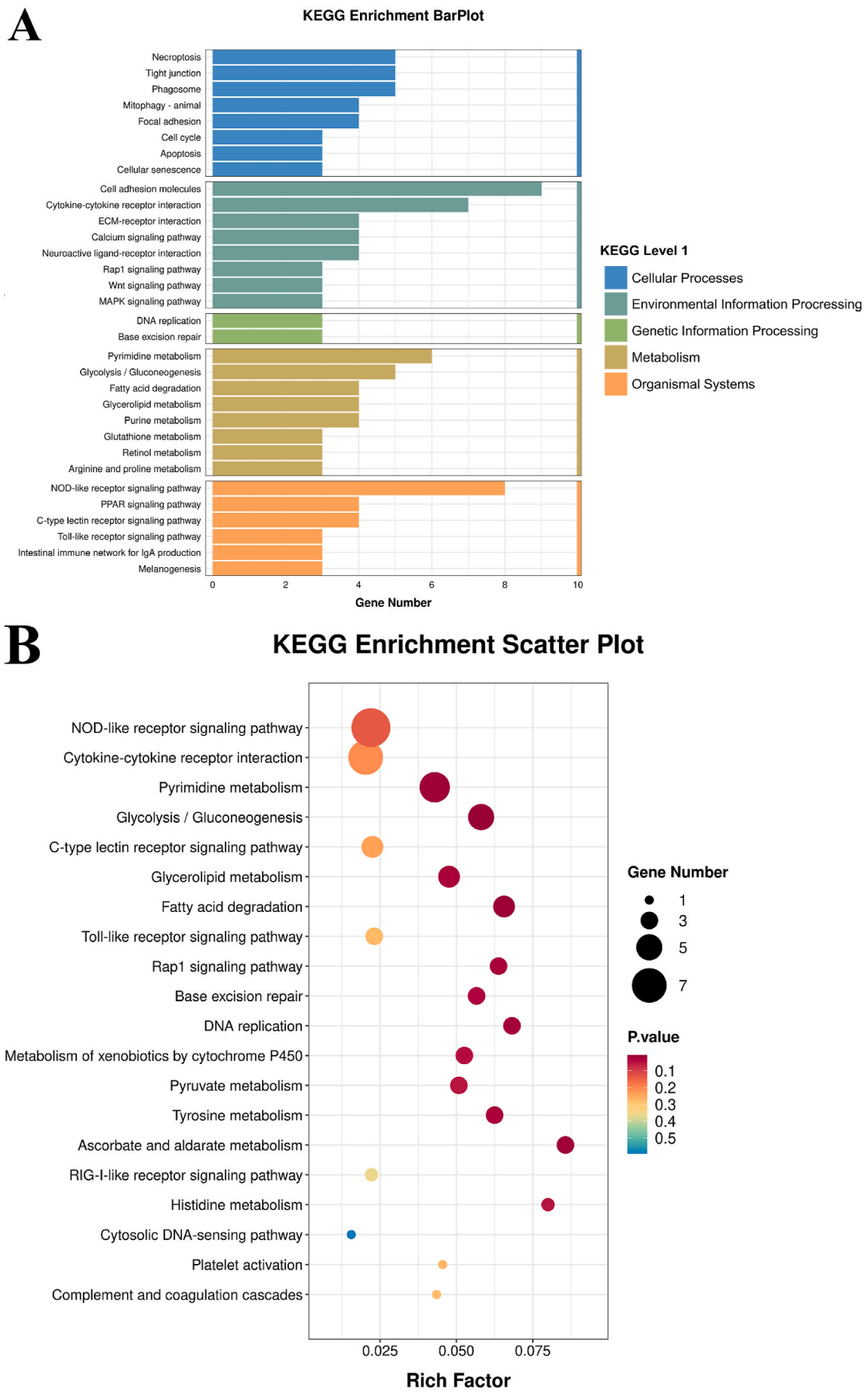

Fig. 6. Pathway enrichment of DEGs between EV and Gal9 group by KEGG analysis. (A) KEGG secondary pathway terms classification. The Y-axis indicates the pathway classification and the X-axis indicates the number of DEGs. (B) Scatterplot of the top 20 KEGG enriched pathways for differentially expressed genes. The X-axis represents the corresponding enrichment factor, and the Y-axis represents various pathways.

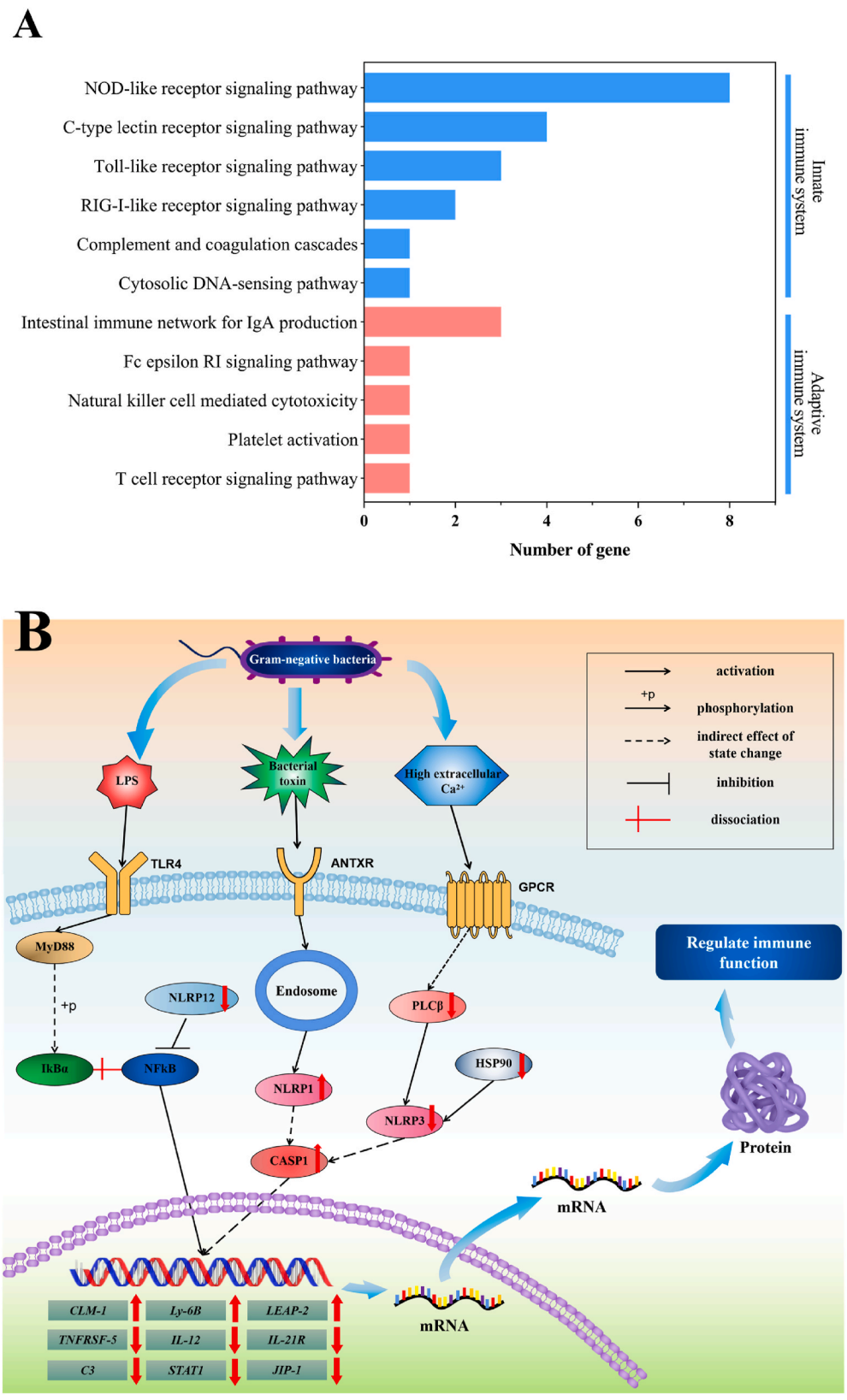

Fig. 7. Analysis of DEGs involved in immune system pathway. (A) Total of 26 DEGs were grouped into the innate immune (blue) and adaptive immune (red) according to KEGG analysis. (B) Schematic illustration of DEGs involved in the NOD-like receptor signaling pathway. Red arrows indicate up-regulated or down-regulated gene expression.

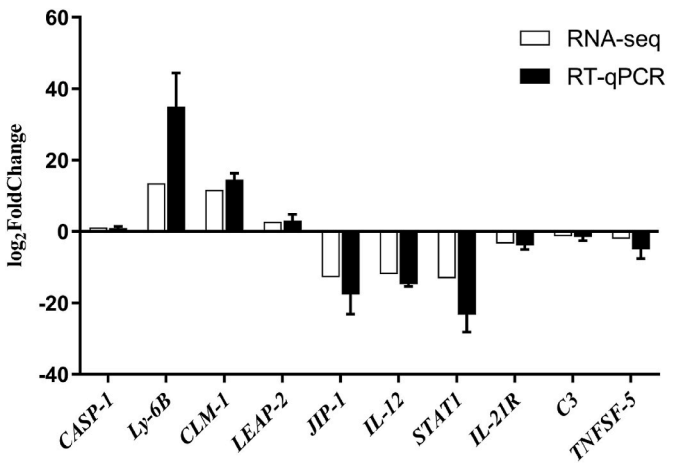

Fig. 8. Comparative verification of the expression profiles of RNA-seq to RT-qPCR.

to resist stress and the state of free radical metabolism [40]. AKP and ACP can eliminate and digest pathogens. *Om*Gal-9 overexpression enhanced production of the antimicrobial factors. LZM plays anti-inflammatory and antibacterial roles in non-specific immunity of fish mainly by PGN degradation in the bacterial cell wall [41]. Considering the decrease of bacterial load in the liver, spleen, and kidney in *O. macrolepis* with Gal-9 overexpression. We hypothesized that Gal-9 can activate the antioxidant system and inflammation factors to limit bacterial colonization, thus it might be promising for applying as a molecular adjuvant in the creation of vaccine against bacterial infection.

Gal-9 overexpression enriched the glycolysis/gluconeogenesis, fatty acid degradation, and ascorbate and aldarate metabolism. Glycolytic/gluconeogenesis pathway is a major checkpoint mediating the energy requirements of T cell division. Human Gal-9 was reported that can activate apoptosis of T cells [9]. Research found that Gal-9 promotes glucose uptake, glycolysis and glucose storage in mouse CD8 (+)T cell [42]. In fatty acid degradation, prokaryotic recombinant human Gal-9 can promote free fatty acids, glucose consumption and lactate release in AML cell lines, thereby causing changes in lipid metabolism and protecting cells from oxidative stress [43]. Ascorbate and aldarate metabolism is known to be part of a pathway related to antioxidant defense that is able to effectively scavenge superoxide and enhances the immunity [44]. Therefore, increase of ascorbate and aldarate metabolism may support that *Om*Gal-9 overexpression could enhance the antioxidant capacity. Gal-9 can promote the formation of the NLRP3/p62 complex in NOD-like receptor signaling pathway by interacting with NLRP3, thereby promoting the p62-dependent autophagy degradation of NLRP3 in cells and limiting inflammation [7]. Gal-9 could enhance leukocyte recruitment via activating platelets through interaction with C-type lectin-like receptor 2 in C-type lectin receptor signaling pathway [45]. In macrophage, Gal-9 reduced IL-6 production by inhibiting TLR4-mediated NF-κB signaling pathway [46]. Thus, Gal-9 involves in the regulation of inflammation-related signaling pathways.

Overexpression of *Om*Gal-9 can inhibit inhibiting inflammation by decreasing the expression of *TNFRSF-5*, *IL-12*, *IL-21R*, and *C3*. TNFRSF-5 can lead the production of pro-inflammatory factors and chemokines in the target tissues, and then leading to tissue destruction and inflammation [47]. Production of IL-12 can be initiated by TLR ligation alone, and augmented in the presence of secondary pro-inflammatory signaling [48]. IL-21 R can promote macrophage polarization towards M1 phenotype to play a pro-inflammatory role and enhance neutrophil inflammation by regulating the TLR/MyD88 signal pathway [49]. When foreign antigens such as bacteria, viruses, and endotoxin enter the blood, they will activate complement C3 in the blood and then induce inflammatory immunity [50]. Overexpression of *Om*Gal-9 can enhance the secretion of anti-inflammation and antimicrobial cytokines such as *CLM-1*, *Ly-6B*, and *LEAP-2*. CLM-1, acts as an anti-inflammatory molecule, regulates interactions between dendritic and T cells in a multiple sclerosis mouse model [51]. The up-regulation of Ly-6B can delineates activation of neutrophils and monocytes when the tissues are in state of infection or inflammation [52]. LEAP-2 has been found to play a role in a variety of bacterial diseases [53].

5. Conclusion

*Om*Gal-9 mainly localized in the nucleus and cytoplasm with punctate distribution. *Om*Gal-9 overexpression increased antioxidant activity of health fish, and decreased tissues bacterial load and mortality rates of infected fish. The enriched KEGG pathways mainly include glycolysis/gluconeogenesis, fatty acid degradation, ascorbate and aldarate metabolism. The immune-related DEGs affected by *Om*Gal-9 overexpression were mainly enriched in NOD-like receptor, Toll-like receptor and C-type lectin receptor signaling pathways. Collectively, Gal-9 overexpression improves invading pathogen recognition of fish and regulates immune pathway genes to inhibit inflammation response.

CRediT authorship contribution statement

Hongzhou Xu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation. Jingfei Long: Formal analysis, Methodology, Software. Xiaoyu Qi: Data curation, Software, Validation. Ping Li: Conceptualization, Funding acquisition, Resources. Chenyang Yan: Software, Validation, Visualization. Kuiquan Pan: Data curation, Software. Yuanjiang Jin: Formal analysis. Haixia Liu: Funding acquisition, Methodology, Project administration, Supervision, Visualization, Validation, Writing - review & editing. All authors read and approved the final version of the manuscript.

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Declaration of competing interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

Data availability

No data was used for the research described in the article.

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