Fish and Shellfish Immunology 93 (2019) 344–353

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Mechanistic insight into the roles of *Pseudomonas plecoglossicida clpV* gene in host-pathogen interactions with *Larimichthys crocea* by dual RNA-seq

Yi Tang[a], Yujia Sun[a], Lingmin Zhao[a], Xiaojin Xu[a], Lixing Huang[a], Yingxue Qin[a], Yongquan Su[b], Ganfeng Yi[c], Qingpi Yan[a,*]

[a] Fisheries College, Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Jimei University, Xiamen, Fujian, 361021, China
[b] State Key Laboratory of Large Yellow Croaker Breeding, Ningde Fufa Aquatic Products Co., Ltd., Ningde, Fujian, 352000, China
[c] Fujian Dabeinong Aquaculture Science & Technology Co. Ltd., Zhangzhou, Fujian, 363502, China

ARTICLE INFO

Keywords:
Host-pathogen interaction
Larimichthys crocea
Pseudomonas plecoglossicida
clpV
Dual RNA-seq

ABSTRACT

Large yellow croaker (*Larimichthys crocea*) is an economical important farmed fish in China. "Visceral White Spot Disease" caused by *Pseudomonas plecoglossicida* is a disease with a high mortality rate in cage-cultured *L. crocea* in recent years and resulted in heavy economy lossess. The dual RNA-seq results of previous study showed that the expression of *clpV* gene in *P. plecoglossicida* was significantly up-regulated during infection. RNAi significantly reduced the expression of *clpV* in *P. plecoglossicida* with maximum silencing efficiency of 96.1%. Compared with the wild type strain, infection of *clpV*-RNAi strain resulted in a delayed onset time and a 25% reduction in mortality of *L. crocea*, as well as lessening the symptoms of the spleen. The results of dual RNA-seq of *L. crocea* infected by *clpV*-RNAi strain of *P. plecoglossicida* changed considerably, compared with the counterpart infected with the wild strain. The KEGG enrichment analysis showed that Cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, C-type lectin receptor signaling pathway and MAPK signaling pathway of *L. crocea* were most affected by the silence of *clpV* in *P. plecoglossicida*. RNAi of *clpV* resulted in the downregulation of genes in flagella assembly pathway and a weaker immune response of host.

1. Introduction

Pseudomonas plecoglossicida is known as a pathogen which can cause epidemic of several maricultured fish and result in heavy economy losses [1]. The epidemic of large yellow croaker (*Larimichthys crocea*) associated with *P. plecoglossicida* is named "Visceral White Spot Disease" [2], which is the most harmful disease of cultured large yellow croaker. In order to reveal the mechanism underlying the pathogenic, the spleen of *Epinephelus coioides* infected by *P. plecoglossicida* were subjected to dual RNA-seq, and the data have been deposited at the GenBank SRA database under accession number SRP115064. The comparative transcriptome analysis results showed that *clpV* gene was significant upregulated during the infection process, and was speculated to play important roles in the host-pathogen interactions.

Gene *clpV* encodes the ClpV1 family type VI secretion system (T6SS) T6SS ATPase, a factor that affects the T6SS [3,4]. In bacteria, T6SS can export macromolecules to the environment and delivers toxic effector molecules to adjacent eukaryotic cells and/or prokaryotic cells by forming needle-like structures [5,6]. T6SS plays an important role in the virulence development of various pathogens [7–10], including *Escherichia coli* [11], *Acinetobacter baumannii* [12], *Edwardsiella piscicida* [13], *Pseudomonas aeruginosa* [14,15]. The product encoded by the clpv gene is critical for the functioning of the T6SS system, ATPase ClpV disassembles the contracted sheath, which resets the systems for re-assembly of an extended sheath that is ready to eject toxin again [16]. In addition, ClpV contributes to in vitro macrophage cytotoxicity while inducing several eukaryotic proteins associated with apoptosis [17], and was recognized as a virulence gene of *P. plecoglossicida* [18].

The process of infection is a battle between the pathogen and the host, in which both side must do their best to win [19]. Both the pathogen and the host must mobilize all available resources to win this life-and-death struggle, and all changes in pathogen and host will be reflected in their respective transcriptome profiles [20]. Dual RNA-seq offers a possibility to monitor host-pathogen RNA expression profiles simultaneously [21–25].

In consideration of tenormous harmfulness of *P. plecoglossicida* to maricultured fishes and the potential important role of *clpV* in the virulence of *P. plecoglossicida*, the aim of this study is to explored the function of *clpV* in the host-pathogen interaction. In the present study, the *clpV* of *P. plecoglossicida* was knocked down by RNAi, the spleens of

* Corresponding author.
E-mail address: yanqp@jmu.edu.cn (Q. Yan).

https://doi.org/10.1016/j.fsi.2019.07.066
Received 28 April 2019; Received in revised form 16 July 2019; Accepted 24 July 2019
Available online 25 July 2019

L. crocea infected by wild-type strain or *clpV*-RNAi strain were subjected to dual RNA-seq and data of pathogen and host transcriptome were simultaneously analyzed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The pathogenic *P. plecoglossicida* strain (NZBD9) was isolated from the spleen of a large yellow croaker suffered from "Visceral White Spot Disease" [1]. All strains of *P. plecoglossicida* were routinely grown in LB (Luria Bertani) medium at 18 or 28 °C with shaking at 220 rpm. *Escherichia coli* DH5α was obtained from TransGen Biotech (Beijing, China) and grown in LB medium (37 °C, 220 rpm).

2.2. Construction of P. plecoglossicida RNAi strain

RNAi strain was constructed according to methods described by Choi and Schweizer [26]and Darsigny et al. [27]. Five short hairpin RNA sequences targeting the *clpV* gene were designed at Website (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=708587103220684543) and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China) (Table S1 in Supplementary Material). After linearizing pCM130/tac vectors with the restriction enzymes NsiI and BsrGI (New England Biolabs, USA), the oligonucleotides were annealed and ligated to the linearized pCM130/tac vectors using T4 DNA ligase (New England Biolabs) following the manufacturer's recommendations. The recombinant pCM130/tac vectors were transformed into the competent *E. coli* DH5a cells by heat shock and then were extracted and electroporated into *P. plecoglossicida* as described by Luo et al. [18]. Finally, the expression level of *clpV* of each RNAi strain was detected by quantitative real time polymerase chain reaction (qRT-PCR).

2.3. Animal infection and sampling

All animal experiments were implemented stringently under the recommendations in the "Guide for the Care and Use of Laboratory Animals" set by the National Institutes of Health. The animal protocols were approved by the Animal Ethics Committee of Jimei University (Acceptance NO JMULAC201159).

Size-matched healthy *L. crocea* were acclimatized at 18 °C for 1 week under specific pathogen-free laboratory conditions. Each fish was intrapleurally injected by wild-type strain or RNAi strain of *P. plecoglossicida* with a dose of 10^4 cfu/g. Fish that were intrapleurally injected with PBS were used as negative control. Survival of fish was monitored for 10 d. The water temperature was maintained at 18 ± 1 °C during the whole infection experiment.

For tissue dual RNA-seq, the spleens of six fish infected with injected by wild-type strain or RNAi strain of *P. plecoglossicida* were sampled at 48 h post infection (hpi). Two spleens were mixed into one sample. Spleens, livers, kidneys and bloods from 3 fish were sampled at 12, 24, 48, 72 and 96 hpi for qRT-PCR.

2.4. DNA isolation

DNA purification from spleens, livers and kidneys was accomplished with an EasyPure Marine Animal Genomic DNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's instructions. DNA was isolated from blood samples using an EasyPure Blood Genomic DNA Kit (TransGen Biotech).

2.5. RNA extraction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA samples were further purified using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA), followed by elimination of genomic DNA contamination with the Turbo DNA-free DNase (Ambion, Austin, TX, USA). The RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.6. Dual RNA-seq and transcriptome data analysis

Sequencing libraries were prepared using the TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Two Ribo-Zero rRNA Removal Kit (Epicentre) were used to remove both pathogen and host rRNAs. An Agilent 2100 Bioanalyzer (Agilent Technologies) was used to validate the library quality. The RNA-seq libraries were sequenced using the Illumina HiSeq4000 sequencing platforms at Majorbio Biotech Co., Ltd. (Shanghai, China). The trimming and quality control of raw Illumina reads were performed using Sickle (https://github.com/najoshi/sickle) and SeqPrep (https://github.com/jstjohn/SeqPrep) with the default settings. The RNA sequencing reads data were deposited at the GenBank SRA database under the accession numbers PRJNA528855 and SRP176599.

To identify the most representative biological processes, molecular annotation of differentially expressed transcripts of *L. crocea* and *P. plecoglossicida* was performed. Therefore, the Gene Ontology (GO) annotation was conducted through the BLAST2GO software (http://www.blast2go.com/b2ghome) and the enrichment analysis was performed using as reference the genomes of *L. crocea* and *P. plecoglossicida* [28]. In addition, for the *L. crocea* transcriptome data, the resulting GO enrichment analysis and visualization was performed using the clusterProfiler R package [29]. As for *P. plecoglossicida*, use Goatools (https://github.com/tanghaibao/GOatools) [30]. Finally, KEGG was used for metabolic pathway analysis [31]. Furthermore, 10 genes from bacteria and 10 genes from fish were randomly selected to verify the sequencing results by qRT-PCR (Fig. S1).

2.7. qRT-PCR

qRT-PCR was carried out using a QuantStudio 6 Flex real-time PCR system (Life Technologies, USA). All primer sequences are provided in Table S2 in Supplementary Material. The expression of the pathogen and host gene was normalized using 16s rDNA and β-actin respectively [32]. The $2-\Delta\Delta Ct$ method was used to calculate the relative level of gene expression. Three independent technical replicates were carried out for two independent preparations of RNA.

2.8. Statistical analyses

All data are presented as the means ± standard deviation (SD) from three sets of independent experiments at least. Data analysis was performed by the SPSS 17.0 software (Chicago, IL, USA), while one-way analysis of variance using Dunnett's test. *P*-values < 0.05 were considered statistically significant.

3. Result

3.1. Construction of the clpV-RNAi strain

The effects of the five shRNAs on *clpV* gene are shown in Fig. 1A. All of the five shRNAs extremely significantly reduced the mRNA level of *clpV* gene, with different efficiency (Fig. 1A). The strain containing pCM130/tac-*clpV*-shRNA- 1004 (named the *clpV*-RNAi strain) exhibited the best efficiency (silence efficiency: 96.1%) of gene silencing, and was chosen for further studies. Despite *clpV* gene has been silenced, *clpV*-RNAi strain growth rate is almost the same as wild type strain of *P. plecoglossicida* (Fig. 1B).

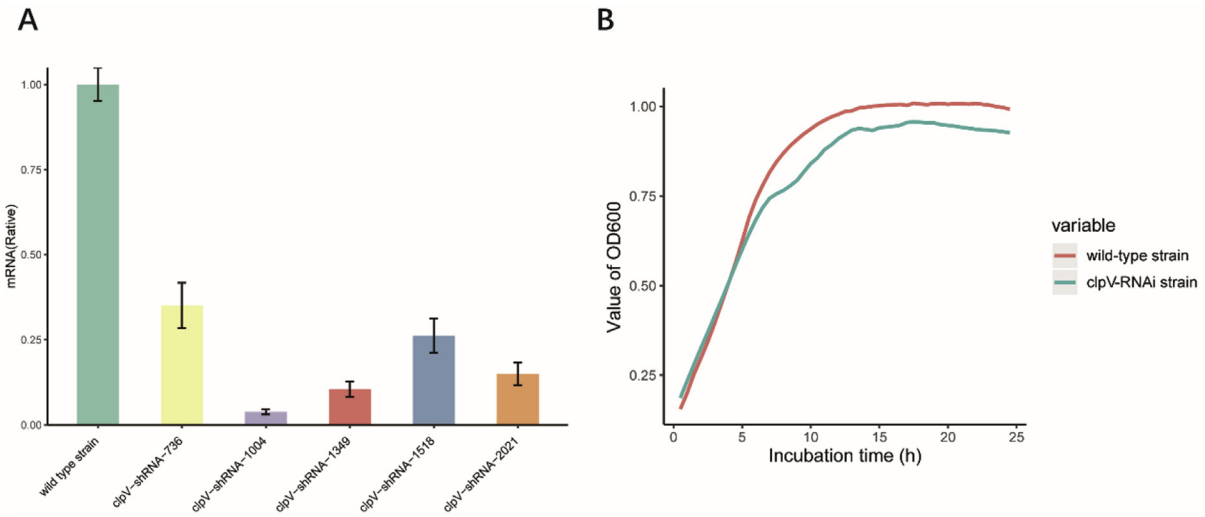

Fig. 1. Construction and growth curve of *clpV*-RNAi strain of *P. plecoglossicida*. (A) The *clpV* mRNA levels of 5 mutant strains and wild type strain. (B) Growth curve of wild-type strain and *clpV*-RNAi strain.

3.2. The effect of clpV gene on the virulence of P. plecoglossicida

Compared with the counterparts injected with wild-type strain of *P. plecoglossicida*, *L. crocea* injected with *clpV*-RNAi strain exhibited 2 days delay in the time of death and a significantly decrease in mortality (Fig. 2A). After infection by wild type strain of *P. plecoglossicida*, all *L. crocea* died in 5 days, while the counterpart which was infected by *clpV*-RNAi strain exhibited a survival rate of 25% until 10 dpi. At 72 hpi, the spleens of the *L. crocea* injected with wild type strain *P. plecoglossicida* exhibited typical symptoms (the surface of the spleen is covered with numerous white spots), however, there were only a few inconspicuous white spots or almost no white spots on the surface of spleens of the *L.*

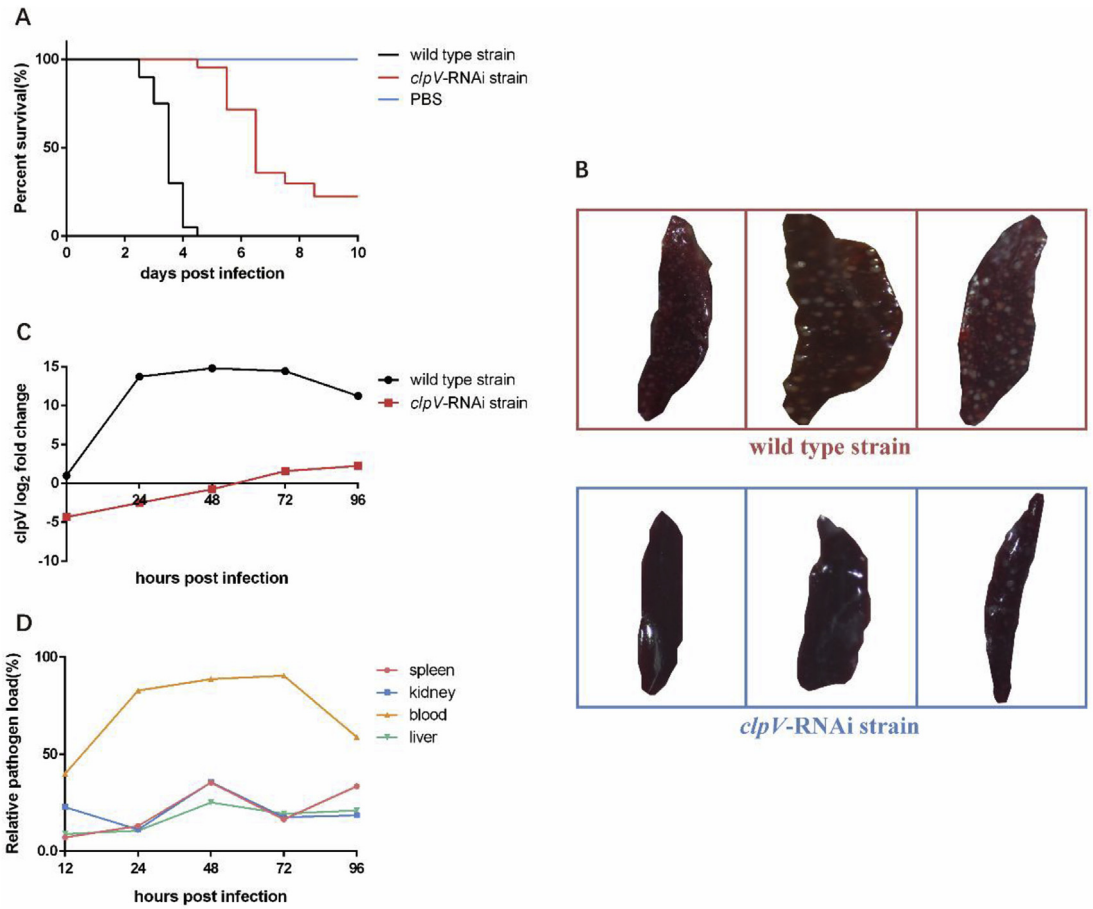

Fig. 2. The virulence of wild type strain and *clpV*-RNAi strain of *P. plecoglossicida* to *L. crocea*. (A) Survival rate of *L. crocea* post infection.(B)Symptoms of spleens of *L. crocea* infected by different strains of *P. plecoglossicida*. (C) mRNA level of *clpV* gene of *P. plecoglossicida* in the spleen of *L. crocea* during infection process. (D) The relative load of *clpV*-RNAi strain *P. plecoglossicida* in different tissues of *L. crocea* compared to wild-type strain.

crocea infected with *clpV*-RNAi strain (Fig. 2B).

Fig. 2C exhibits the mRNA level of *clpV* gene in *clpV*-RNAi strain and wild type strain of *P. plecoglossicida* in vitro and spleens at different times post-injection under 18 °C. The mRNA level of *clpV* gene of both strains were always tens of times higher in the spleens than those in vitro. The mRNA level of *clpV* gene of wild type strain were always tens of times higher than those of RNAi strain.

The relative pathogen load in different tissues of the *L. crocea* infected with *clpV*-RNAi strain compared with wild type strain are showed in Fig. 2D. The pathogen load of *clpV*-RNAi strain in each tissue at each time was lower than that of wild type strain. Among them, the ratio in blood is closest to 100%, and the ratio in other organs is less than 50% (Fig. 2D).

3.3. clpV effects on host gene expression in spleen

The spleens of *L. crocea* which infected with *clpV*-RNAi strain or wild type strain were subjected to dual RNA-seq. When comparing the transcriptome data of host infected by different strains of pathogen, there were statistically significant differences in the expression of 1138 mRNAs (FDR < 0.05 & $|\log_2 FC| \geq 1$), out of which 482 genes were upregulated in case of infected by *clpV*-RNAi strain (Fig. 3A). According to logFC, the top 50 up-regulated DEGs (exclude unnamed genes) were picked out and shown in Fig. 3B.

3.3.1. Differentially expressed genes

Functional annotation of significantly differentially expressed genes in *L. crocea* spleen was performed to define the transcriptome profile more precisely. Gene ontology (GO) and KEGG pathway enrichment analyses were conducted for differentially expressed genes (DEGs). Gene ontology (GO) analysis of the differentially expressed genes (Fig. 4A and B) recapitulated many of the findings from the enrichment analysis of dual RNA-seq data. Fig. 4A shows the top five significant GO terms with the most relevant genes and they all belong to molecular function. The more genes were associated with the node, the more important of potential roles of the network. GO classification (Fig. 4B) indicated that immune/defense response-related genes were enriched, specifically under GO terms “defense response”, “inflammatory response”, and “immune response”. The genes that enriched in the defense response and inflammatory response terms are all down-regulated. Subsequently, we found that the top 4 kegg pathway involved in immune response (Fig. 4C). Including Cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, C-type lectin receptor signaling pathway and MAPK signaling pathway. These pathways play

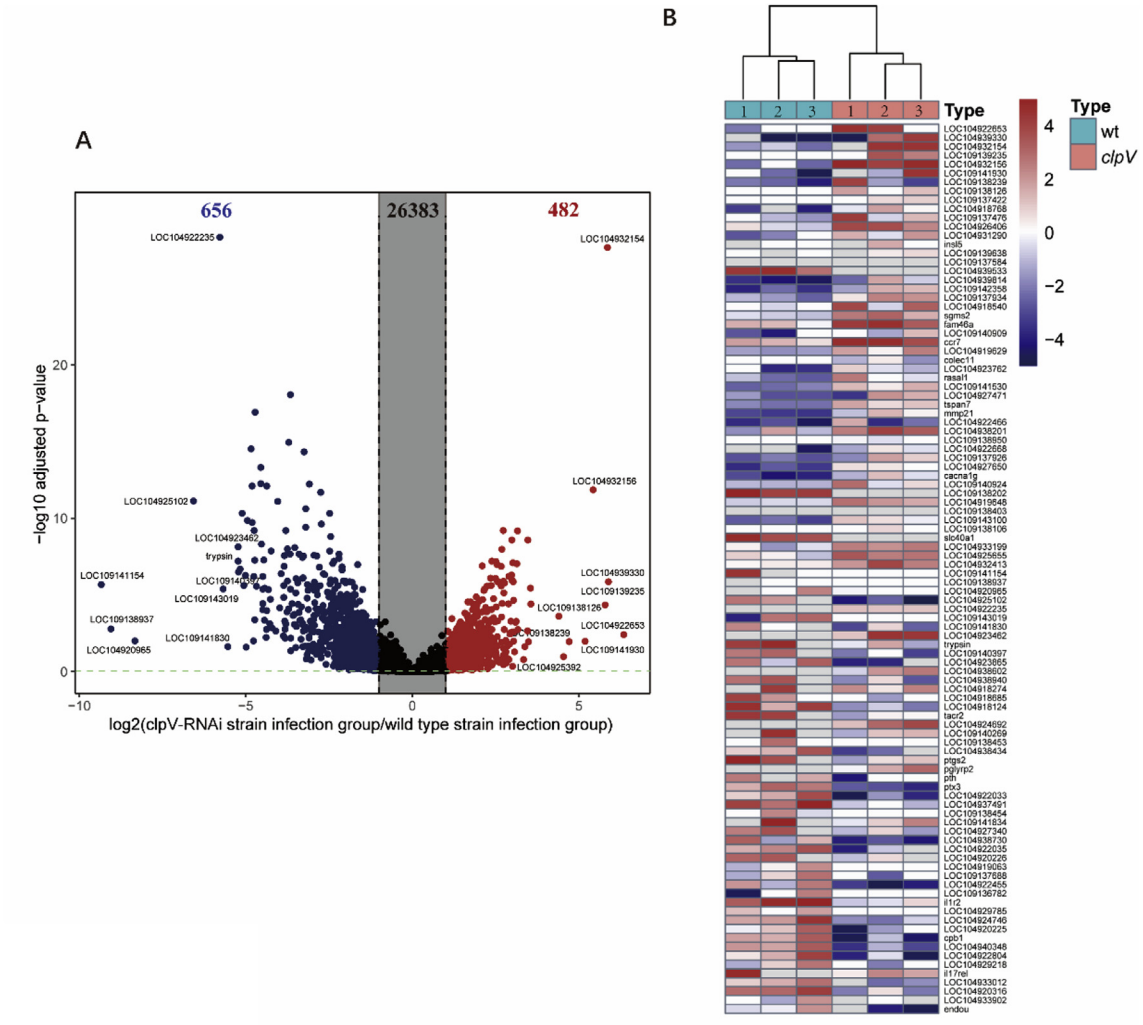

Fig. 3. The comparative analysis of host's transcriptome response to different strains of *P. plecoglossicida*.(A)Volcano plot obtained from edgeR analysis of *L. crocea*'s spleen RNA pools (*clpV*-RNAi strain infection group/wild type strain infection group). The up-regulated genes are shown as red dots, the down-regulated genes are shown as blue dots and the normal genes are shown as black dots.(B) Heat map of the top 50 enriched and depleted host transcripts. (FDR < 0.05,$|\log_2 FC| \geq 1$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

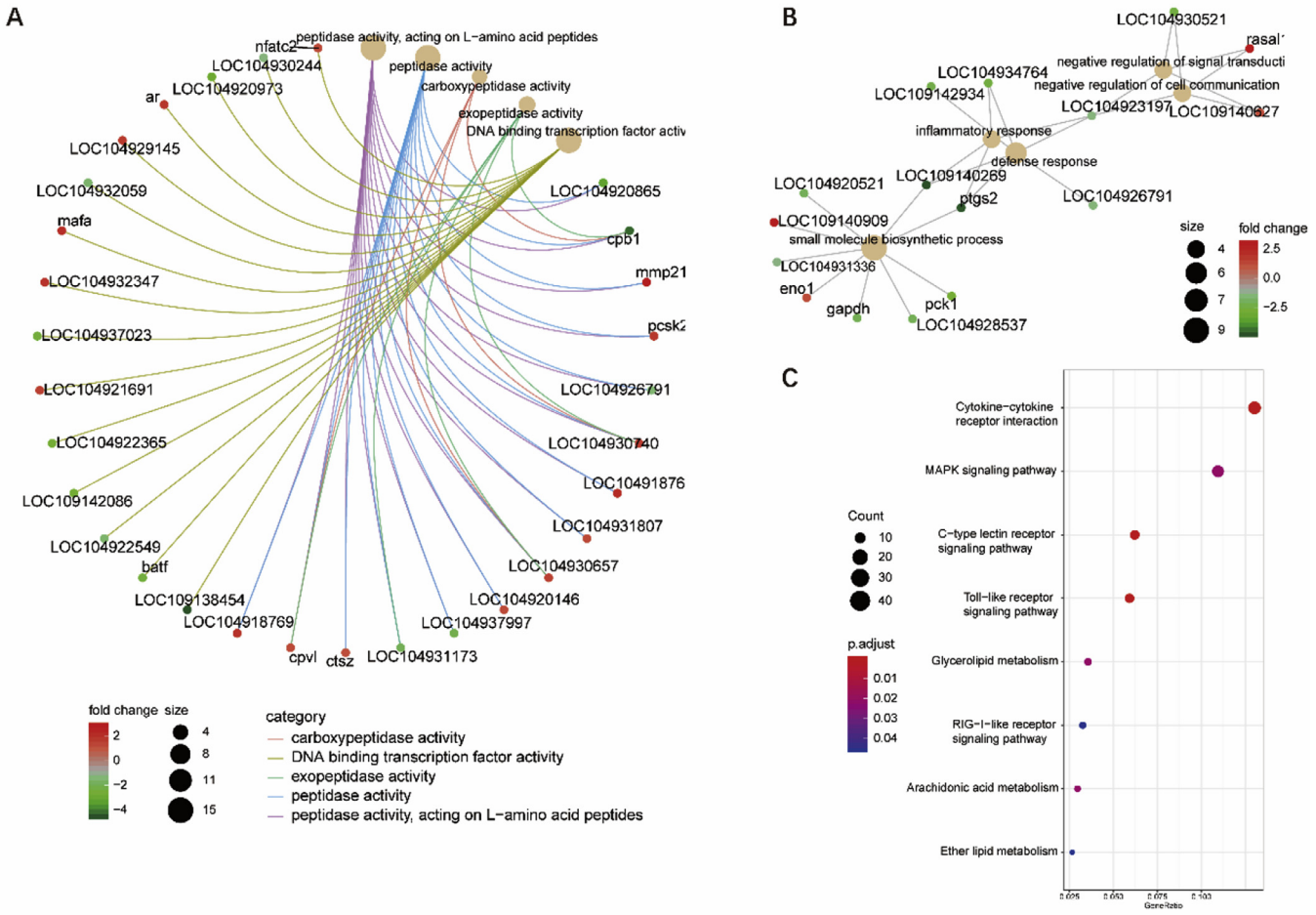

Fig. 4. Differentially expressed genes (DEGs) analysis of *L. crocea* infected by different strains of *P. plecoglossicida* (*clpV*-RNAi strain infection group/wild type strain infection group).(A)GO enrichment analysis for DEGs. (B) Biological Process. The plot depicts the connections for GO terms and their genes. Each yellow circle represents gene pathways and circle's size indicates gene count. (C) KEGG pathway enrichment analysis of the DEGs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

an important role in the host's defense against foreign enemies and regulating immune responses. Similarly, KEGG enrichment analysis also indicated that infection with different strains affects the host's immune respons.

The Cytokine-cytokine receptor interaction pathway is involved in adaptive inflammatory host defenses [33], and the gene expression heat map enriched in this pathway is shown in Fig. 5A. The C-type lectin receptor signaling pathway plays an important role in inducing the production of inflammatory cytokines and chemokines [34]. Most of the genes enriched by C-type lectin receptor signaling pathway are products of expression regulation, and Fig. 5A shows the expression of genes which are enriched in this pathway. Interestingly, most of genes are shown in blue which means down-regulated.

The enrichment of the Toll-like receptor signaling pathway is shown in Fig. 5B and MAPK signaling pathway is shown in Fig. 5C. Toll-like receptor signaling pathway is central to the innate immune system and the mediator proteins are related mainly to the induction of proinflammatory cytokines. In Toll-like receptor signaling pathway, genes, TIR domain-containing adaptor protein (TIRAP), toll-like receptor 7 (TLR7),mitogen-activated protein kinase kinase 3 (MKK3), respectively, were up-regulated. However, the inflammatory cytokines genes that are significantly enriched in this pathway are all down-regulated, and proto-oncogene protein c-fos (AP-1) and NF-kappa-B inhibitor alpha (IκBα) genes are also down-regulated. Currently, based on sequence homology and functional differences, 4 MAPK signaling pathway distinct cascades have been identified: ERK1/2, JNK/MAPK, p38-MAPK and ERK5. The data obtained indicate that DEG were seldom enriched in the ERK5 pathways, and just a few DEG were enriched in p38-MAPK and JNK/MAPK pathway. In contrast, many DEG were enriched in ERK1/2 pathway (Fig. 5C).

3.4. Analysis of pathogen transcriptome in infected spleen of L. crocea

The pathogen gene expression profile was calculated by edgeR, and the changes in the expression level met FDR < 0.05 & $|\log_2 FC| \geq 1$ were considered statistically significant different. 4988mRNAs were identified from profiled transcripts of the spleen infected by the *clpV*-RNAi strain. Compared with the spleen infected by the wild-type strain, 82 mRNAs from the spleen infected by the *clpV*-RNAi strain exhibited significant difference in abundance with 78 mRNAs down-regulated and 4 mRNAs up-regulated (Fig. 6A). The expression of all DEGs is shown in Fig. 6B

The DEGs of pathogen transcriptome were subjected to GO enrichment analysis, the top three predominant terms for 'biological process' were cellular process, cellular component organization or biogenesis, cellular component biogenesis, and ribonucleoprotein complex biogenesis; for 'cellular component' they were cytoplasmic part, cell part, and intracellular part; and for 'molecular function' they were RNA binding, structural molecule activity, and structural constituent of ribosome (Fig. 6A).

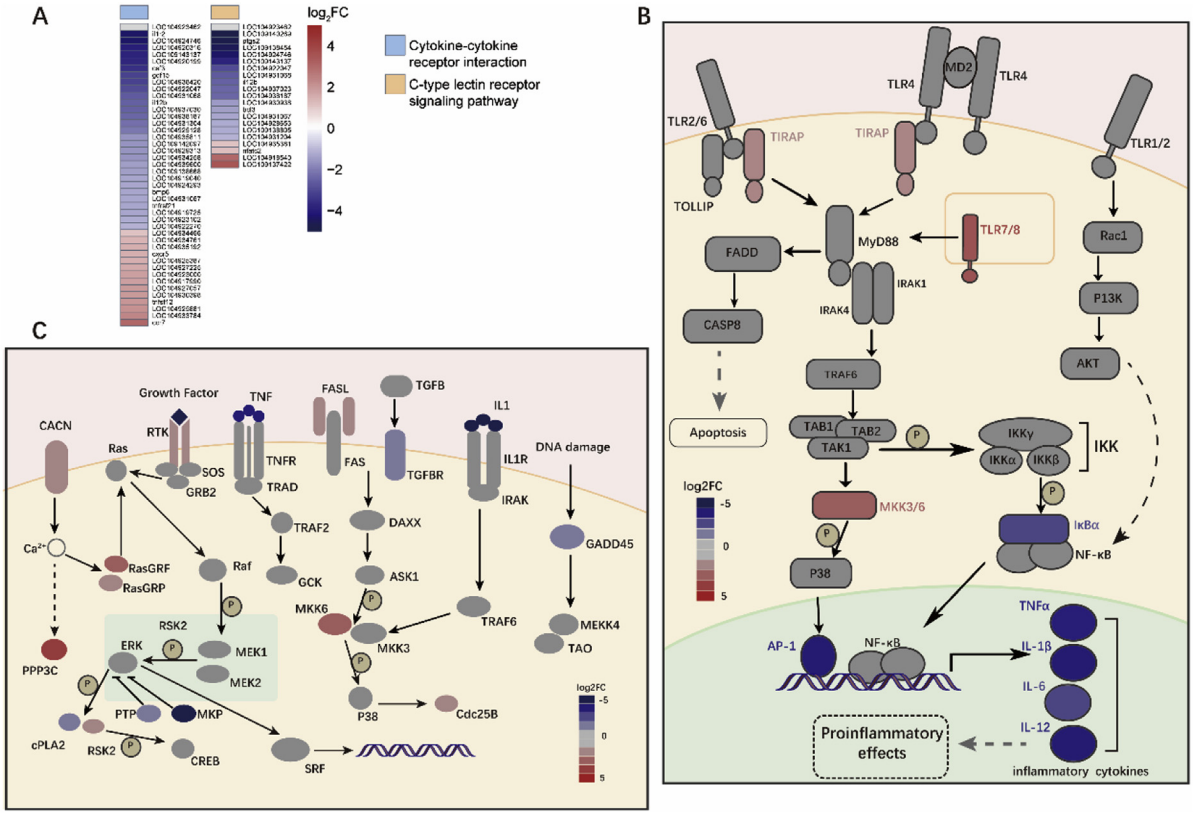

Fig. 5. Visualization of gene expression changes by pathview. Colors represent the scaled relative abundances of log2-transformed RPKM ratio (*clpV*-RNAi/WT) values. Up-regulated genes appear in red, and down-regulated genes appear in blue. (A) Differentially Expressed Genes of Cytokine-cytokine receptor interaction pathway and C-type lectin receptor signaling pathway.(B) Schematic overview of Toll-like receptor signaling pathway.(C) MAPK signaling pathway overview. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The KEGG enrichment analysis is shown in Fig. 7B. The most significant pathway for enrichment is Flaagellar assembly. Fig. 7C shows the genes involved in the construction and regulation of the flagellum, with blue lettering indicating those genes were downregulated in the data of comparative dual RNA-seq, including *L321_RS13260*, *L321_RS13325*, *L321_RS13370*, *L321_RS13425*, *L321_RS13340*, *flgD*, *L321_RS13405*, *L321_RS13380*, *flgE*, *L321_RS13385*, *L321_RS13270*, *L321_RS13355* and *L321_RS13360*.

4. Discussion

Toxicity and invasiveness are critical to the pathogenicity of pathogens and have been reported to be controlled by different genes. So far, many genes have been shown to have a strong relationship with the virulence regulation of aquatic pathogens by knockout or knockdown techniques, such as *flgE* to *Aeromonas hydrophila* [35]; *hcp1* and *vgrG1* to *Aeromonas hydrophila* [36]; *secA* and *cheB* to *Vibrio alginolyticus* [37,38]; and *ssaV* to *Edwardsiella piscicida* [39]. As for *P. plecoglossicida* which is the pathogen of large yellow croaker, some studies have mentioned that its virulence will be regulated by some genes [40,41]. However, there is no reports on the effect of *clpV* gene to host host immune response.

RNAi is a highly efficient and specific tool for studying gene function [42]. In this study, five shRNAs were designed for five different targets of the *clpV* gene. All 5 shRNA significantly reduced the expression of *clpV* gene, and the shRNA with target started at 1004 had a silencing efficiency of 96.1%. Moreover, *clpV* gene in *clpV*-RNAi strain of *P. plecoglossicida* was persistently silented during the infection process. These results indicated the stable silencing technique in this study was reliable and laid the foundation for subsequent research.

Spleen is the main target organ of *P. plecoglossicida* infection and presents typical symptoms. Therefore, the spleen was used as the main research object for studying bacterial infection of *L. crocea*. Compared with the wild strain, the survival rate of *L. crocea* infected by *clpV*-RNAi strain was significantly increased, and the onset time was also delayed. Moreover, the symptoms of the spleen also changed. The white spots on the spleen of *L. crocea* infected with the silent strain were less than those of the wild-type group, some were not even observed, and the swelling of the spleen was less likely to occur. At the same time, the pathogen load of *P. plecoglossicida* in large yellow croaker was also significantly reduced. These results indicated that *clpV* gene was a key virulence gene for *P. plecoglossicida* to infect *L. crocea*. It was reported that inactivation of *clpV* reduces the ability of pathogenic bacteria such as *Salmonella typhimurium* or *Yersinia pseudotuberculosis* to invade epithelial cells [43].

RNAi of *clpV* gene significantly affects the transcript data of pathogens during infection. GO enrichment analysis showed that pathogen's DEGs were mainly involvedin cell part, ribosomes, and energy metabolism. The *clpV* gene in enteroaggregative *Escherichia coli* encodes an ATPase for the Type VI secretion system (T6SS) which is an important machine for transporting toxins into host cells [3]. The pathogenesis of *P. aeruginosa* is related to T6SS,which consists of about 15 conserved components, including the AAA + ATPase, *ClpV* [44]. The Flaagellar assembly pathway is most significant in KEGG enrichment analysis. All of the modules enriched in this pathway are down-regulated, thereby inferring that the *clpV* gene is involved in flagella assembly and affecting the virulence of the strain. The *clpV* deletion mutant strain of *Citrobacter freundii* has less motility, cell adhesion and cytotoxicity than wild strains [45].

It is well known that infection with pathogens can cause dramatic changes in the host transcriptome [46–49]. Recently, it has been found that changes of single virulence gene can also cause significant changes in the host transcriptome [19,20]. In this research, *clpV*-RNAi strain

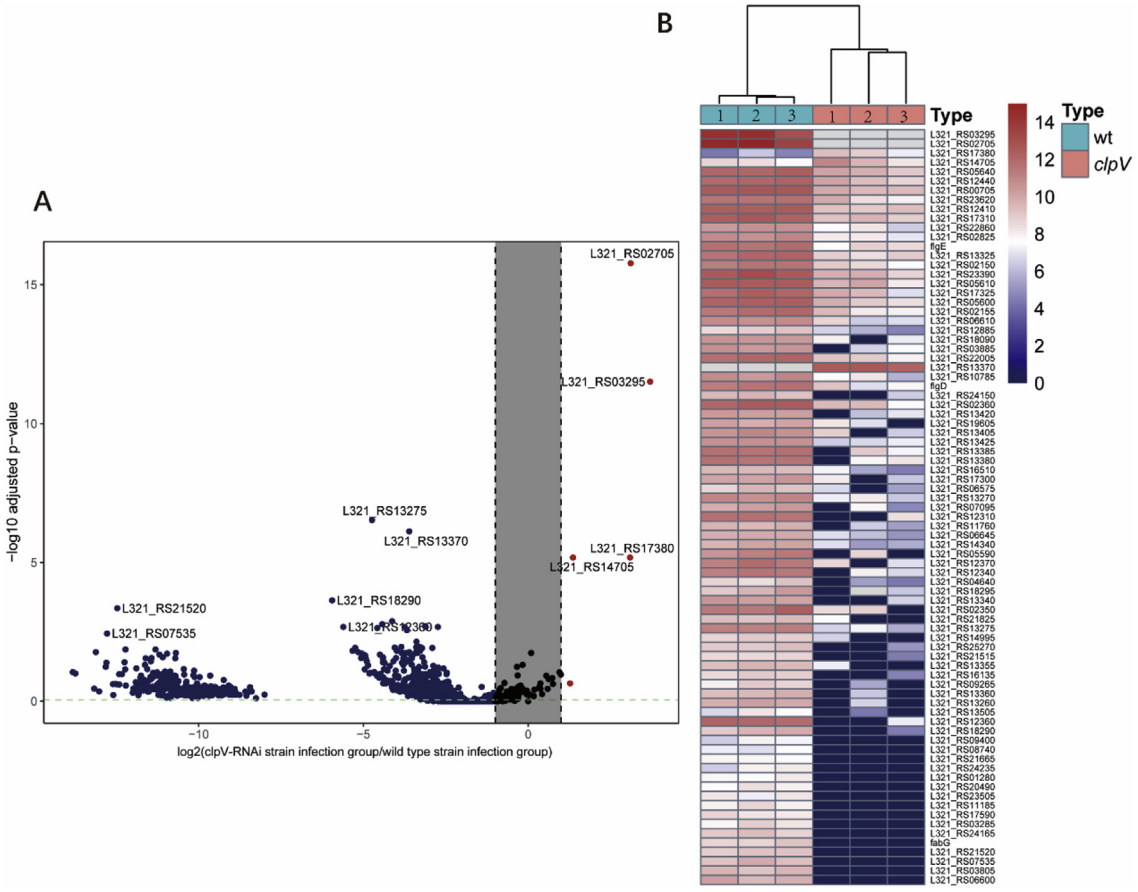

Fig. 6. Comparative analysis of *P. plecoglossicida* transcriptome between *clpV*-RNAi strain and wild type strain. (A)Volcano plot obtained from comparative Dual RNA-seq of *P. plecoglossicida*. The up-regulated genes are shown as red dots, the down-regulated genes are shown as blue dots and the normal genes are shown as black dots.(B) Heat map of all DEGs (adjusted FDR < 0.05; $|\log_2 FC| \geq 1$; 3 biological replicates) between wild-type strain and *clpV*-RNAi strain infections. Values represent $\log_2$ folds. Colors based on log-transformed transcripts FPKM mean values. The color gradient represents highly upregulated (dark red) to highly down-regulated (dark blue) genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

infected group showed significant changes compared to the wild-type infected group of large yellow croaker transcriptome. The results of GO enrichment analysis show that the most important GO terms in network relationships are GO:0070011 (peptidase activity, acting on L-amino acid peptides), GO:0008233 (peptidase activity), GO:0004180 (carboxypeptidase activity), GO:0008238 (exopeptidase activity) and GO:0003700 (DNA binding transcription factor activity). Most of them are related to peptidase activity, and many peptidases are currently considered to be associated with immune responses [51–53]. It has been reported that carboxypeptidase has immune-related functions in various aquatic organisms, such as *Oplegnathus fasciatus* [54]. In addition, carboxypeptidase plays an immune role in the complement defense mechanism of red lip mulle [55]. All genes in defense response (GO: 0006952) and inflammatory response (GO: 0006954) were down-regulated, indicating that the immune response of the *L. crocea* to *clpV*-RNAi strain infected group was relatively weak. KEGG enrichment analysis exhibited similar results of GO enrichment analysis. The top four pathways of KEGG enrichment are related to the immune response, they are Cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, C-type lectin receptor signaling pathway and MAPK signaling pathway. Notably, some classical proinflammatory genes, e.g. C-type lectin receptor signaling pathway (IL-12, IL-1β, Cox-2, TNF, IL-6), Toll-like receptor signaling pathway (TNFα, IL-1β, IL-6, IL-12, MIG), are all down-regulated. Cytokines are involved in inflammatory host defense and key modulators of inflammation. It participates in acute and chronic inflammation through a complex network of interactions [56–58]. Toll-like receptors (TLR) play a central role in the host's ability to recognize pathogens and produce immune responses, it uses proinflammatory cytokines to regulate immune responses [59–61]. C-type lectin receptors (CLRs) induced intracellular signal cascades are indispensable for the initiation and regulation of antifungal immunity [62–64]. Upon pathogen binding, CLRs trigger distinct signalling pathways that can help to activate the innate immune system, whereas some CLRs affect signalling by Toll-like receptors [65]. Many cytokine showed down-regulation in the MAPK signaling pathway. MAPK signaling pathway is crucially involved in inflammation and the regulation of cytokine production and is associated with multiple immune pathways [66]. Changes in the expression levels of these pathways in the spleen infected by the *clpV*-RNAi strain indicate that immune response of the large yellow croaker is weaker to *clpV*-RNAi strain than the wild type strain of *P. plecoglossicida*. This corollary based on bioinformatics data was consistent with the results of pathogen load. RNAi of *clpV* gene resulted in less virulence of *P. plecoglossicida* and lower pathogen load in host tissues, and lower pathogen load resulted in a weaker immune response of host.

In conclusion, *clpV* was a pathogenic gene of *P. plecoglossicida* and contributed to the pathogenicity of *P. plecoglossicida* to *L. crocea*. RNAi of *clpV* resulted in the downregulation of genes in flagella assembly pathway and lower pathogen load in host tissues as well as a weaker immune response of host.

Conflicts of interest

The authors declare that the research was conducted in the absence

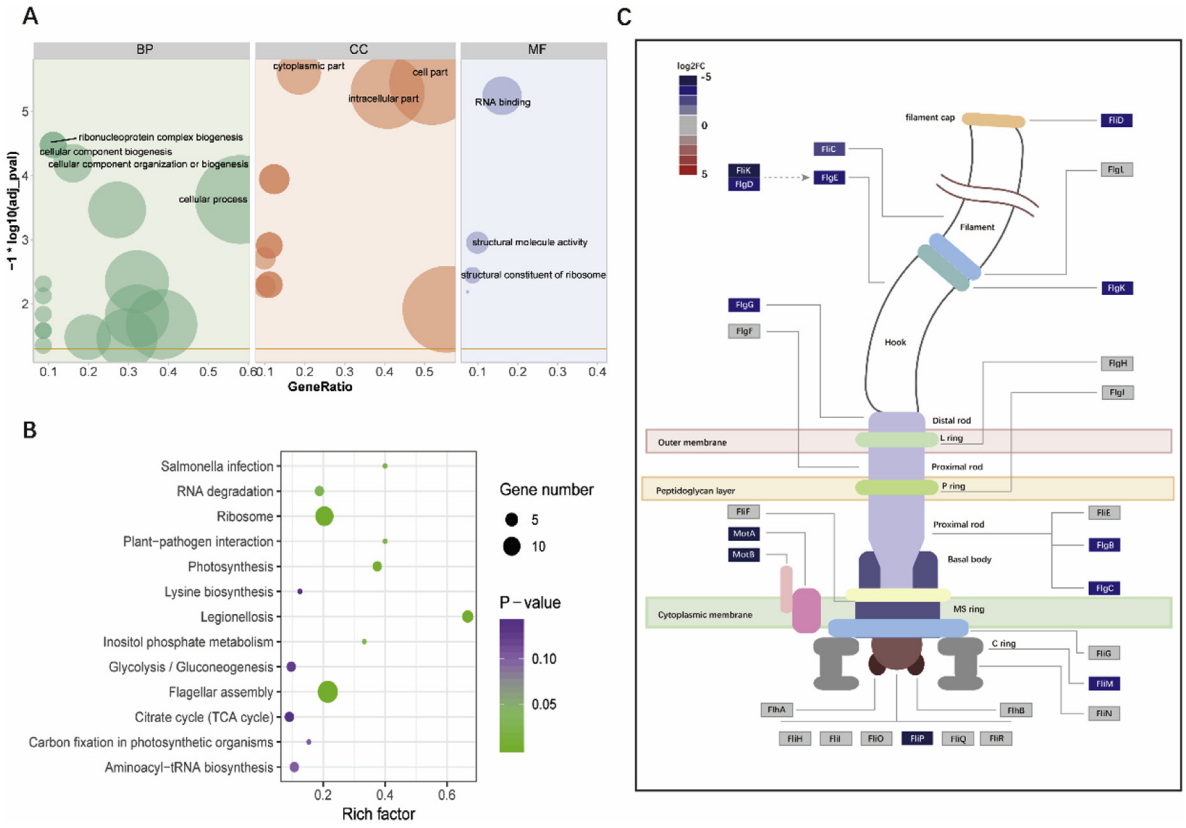

Fig. 7. DEGs enrichment analysis of pathogen transcriptome data.(A) Bubble graph of top enrichment GO terms. The size of the circles represents the number of differentially expressed genes belonging to this GO term.(B) KEGG pathway enrichment analysis of the DEGs. Rich factor refers to the ratio of the number of DEGs enriched in a KEGG pathway to the number of all the annotated genes enriched in the KEGG pathway. (C) Schematic overview of the Flagellar assembly with comparative Dual RNA-seq data *P. plecoglossicida* infected spleen; wild type strain and *clpV*-RNAi strain). The color shows the expression level of the gene, red is up-regulated, blue is down-regulated, and the darker the color, the more multiples of up-and-down.

of any commercial or financial relationships that could be construed as a potential conflict of interest.

Statement of modifications

After careful examination, we found a few mistakes in the original paper. Some supplemental materials in original submission were used only for the review of manuscript and not for publication. These supplemental materials are no longer included in the revised submission.

List of changes:

Line 108. "10^3 cfu/g" was changed to "10^4 cfu/g".
Line 138. "Sequencing statistics for each RNA-seq data are presented in Supplementary Table S2." was deleted.
Line 154. "(Fig. S1)" was added.
Line 158. "S3" was changed to "S2".
Line 218. "The results showed that the base distribution was balance, and N% was within the reasonable range (Fig. S1 in Supplementary Material). The reproducibility of the three biological duplicates was satisfactory (Fig. S2 in Supplementary Material)." was deleted.
Line 241. "(B) Biological Process." was added.
Line 243. "(B)" was changed to "(C) ". The "(C)" at the end of the sentence was deleted.
Line 280. "In Supplementary Fig. 3, the picture of this two kegg pathways are presented." was deleted
Line 303. "The reproducibility of the three biological duplicates is satisfactory (Fig. S2 in Supplementary Material)." was deleted.
Line 447. Most of supplemental materials were deleted. Only two supplemental tables are retained. "Supplementary Table 3" renamed as "Supplementary Table 2". "Supplementary Fig. 4" renamed as "Supplementary Fig. 1". The number of the corresponding supplementary material file has also changed.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under contract No. 31672694.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.066.

References

[1] L. Huang, Y. Zuo, Q. Jiang, et al., A metabolomic investigation into the temperature-dependent virulence of *Pseudomonas plecoglossicida* from large yellow croaker (*Pseudosciaena crocea*), J. Fish Dis. 42 (3) (2019) 431–446, https://doi.org/10.1111/jfd.12957.
[2] J. Zhang, Y. Wang, H. Guo, et al., Identification and characterization of a phospholipase A1 activity type three secreted protein, PP_ExoU from *Pseudomonas plecoglossicida* NB2011, the causative agent of visceral granulomas disease in large yellow croaker (*Larimichthys crocea*), J. Fish Dis. 40 (6) (2017) 831–840, https://doi.org/10.1111/jfd.12565.
[3] B. Douzi, Y.R. Brunet, S. Spinelli, et al., Structure and specificity of the Type VI secretion system ClpV-TssC interaction in enteroaggregative *Escherichia coli*, Sci. Rep. 6 (2016) 34405, https://doi.org/10.1038/srep34405.
[4] A. Filloux, The type VI secretion system: a tubular story, EMBO J. 28 (4) (2009) 309–310, https://doi.org/10.1038/emboj.2008.301.
[5] M. Basler, J.J. Mekalanos, Type 6 secretion dynamics within and between bacterial cells, Science 337 (6096) (2012) 815, https://doi.org/10.1126/science.1222901.
[6] N.M. Bleumink-Pluym, L.B. van Alphen, L.I. Bouwman, et al., Identification of a functional type VI secretion system in Campylobacter jejuni conferring capsule

polysaccharide sensitive cytotoxicity, PLoS Pathog. 9 (5) (2013) e1003393, , https://doi.org/10.1371/journal.ppat.1003393.
[7] F.R. Cianfanelli, L. Monlezun, S.J. Coulthurst, Aim, load, fire: the type VI secretion system, a bacterial nanoweapon, Trends Microbiol. 24 (1) (2016) 51–62, https://doi.org/10.1016/j.tim.2015.10.005.
[8] A.B. Russell, S.B. Peterson, J.D. Mougous, Type VI secretion system effectors: poisons with a purpose, Nat. Rev. Microbiol. 12 (2) (2014) 137–148, https://doi.org/10.1038/nrmicro3185.
[9] M. Basler, M. Pilhofer, G.P. Henderson, et al., Type VI secretion requires a dynamic contractile phage tail-like structure, Nature 483 (7388) (2012) 182–186, https://doi.org/10.1038/nature10846.
[10] E. Cascales, The type VI secretion toolkit, EMBO Rep. 9 (8) (2008) 735–741, https://doi.org/10.1038/embor.2008.131.
[11] C.G. Aas, F. Drablos, K. Haugum, et al., Comparative transcriptome profiling reveals a potential role of type VI secretion system and fimbriae in virulence of non-O157 shiga toxin-producing *Escherichia coli*, Front. Microbiol. 9 (2018) 1416, https://doi.org/10.3389/fmicb.2018.01416.
[12] G.D. Repizo, S. Gagne, M.L. Foucault-Grunenwald, et al., Differential role of the T6SS in acinetobacter baumannii virulence, PLoS One 10 (9) (2015) e0138265, , https://doi.org/10.1371/journal.pone.0138265.
[13] K. Yin, Y. Guan, R. Ma, et al., Critical role for a promoter discriminator in RpoS control of virulence in Edwardsiella piscicida, PLoS Pathog. 14 (8) (2018) e1007272, , https://doi.org/10.1371/journal.ppat.1007272.
[14] L. Chen, Y. Zou, P. She, et al., Composition, function, and regulation of T6SS in *Pseudomonas aeruginosa*, Microbiol. Res. 172 (2015) 19–25, https://doi.org/10.1016/j.micres.2015.01.004.
[15] J. Corbitt, J.S. Yeo, C.I. Davis, et al., T6SS dynamics reveals a novel secretion mechanism in Pseudomonas aeruginosa, J. Bacteriol. (2018), https://doi.org/10.1128/JB.00744-17.
[16] A. Forster, S. Planamente, E. Manoli, et al., Coevolution of the ATPase ClpV, the sheath proteins TssB and TssC, and the accessory protein TagJ/HsiE1 distinguishes type VI secretion classes, J. Biol. Chem. 289 (47) (2014) 33032–33043, https://doi.org/10.1074/jbc.M114.600510.
[17] L.S. Weyrich, O.Y. Rolin, S.J. Muse, et al., A Type VI secretion system encoding locus is required for Bordetella bronchiseptica immunomodulation and persistence *in vivo*, PLoS One 7 (10) (2012) e45892, , https://doi.org/10.1371/journal.pone.0045892.
[18] G. Luo, X. Xu, L. Zhao, et al., clpV is a key virulence gene during in vivo *Pseudomonas plecoglossicida* infection, J. Fish Dis. (2019), https://doi.org/10.1111/jfd.13001.
[19] Y. Sun, G. Luo, L. Zhao, et al., Integration of RNAi and RNA-seq reveals the immune responses of *Epinephelus coioides* to *sigX* gene of *Pseudomonas plecoglossicida*, Front. Immunol. 9 (2018) 1624, https://doi.org/10.3389/fimmu.2018.01624.
[20] B. Zhang, G. Luo, L. Zhao, et al., Integration of RNAi and RNA-seq uncovers the immune responses of *Epinephelus coioides* to *L321_RS19110* gene of *Pseudomonas plecoglossicida*, Fish Shellfish Immunol. 81 (2018) 121–129, https://doi.org/10.1016/j.fsi.2018.06.051.
[21] R. Aprianto, J. Slager, S. Holsappel, et al., Time-resolved dual RNA-seq reveals extensive rewiring of lung epithelial and pneumococcal transcriptomes during early infection, Genome Biol. 17 (1) (2016) 198, https://doi.org/10.1186/s13059-016-1054-5.
[22] A.J. Westermann, K.U. Forstner, F. Amman, et al., Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions, Nature 529 (7587) (2016) 496–501, https://doi.org/10.1038/nature16547.
[23] A.M. Nuss, M. Beckstette, M. Pimenova, et al., Tissue dual RNA-seq allows fast discovery of infection-specific functions and riboregulators shaping host-pathogen transcriptomes, Proc. Natl. Acad. Sci. U. S. A. 114 (5) (2017) E791–E800, https://doi.org/10.1073/pnas.1613405114.
[24] Y. Sun, Z. Zhuang, X. Wang, et al., Dual RNA-seq reveals the effect of the *flgM* gene of *Pseudomonas plecoglossicida* on the immune response of *Epinephelus coioides*, Fish Shellfish Immunol. 87 (2019) 515–523, https://doi.org/10.1016/j.fsi.2019.01.041.
[25] B. Zhang, Z. Zhuang, X. Wang, et al., Dual RNA-Seq reveals the role of a transcriptional regulator gene in pathogen-host interactions between *Pseudomonas plecoglossicida* and *Epinephelus coioides*, Fish Shellfish Immunol. 87 (2019) 778–787, https://doi.org/10.1016/j.fsi.2019.02.025.
[26] K.H. Choi, H.P. Schweizer, mini-Tn7 insertion in bacteria with single attTn7 sites: example Pseudomonas aeruginosa, Nat. Protoc. 1 (1) (2006) 153–161, https://doi.org/10.1038/nprot.2006.24.
[27] M. Darsigny, J.P. Babeu, E.G. Seidman, et al., Hepatocyte nuclear factor-4alpha promotes gut neoplasia in mice and protects against the production of reactive oxygen species, Cancer Res. 70 (22) (2010) 9423–9433, https://doi.org/10.1158/0008-5472.CAN-10-1697.
[28] A. Conesa, S. Gotz, J.M. Garcia-Gomez, et al., Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research, Bioinformatics 21 (18) (2005) 3674–3676, https://doi.org/10.1093/bioinformatics/bti610.
[29] G. Yu, L.G. Wang, Y. Han, et al., clusterProfiler: an R package for comparing biological themes among gene clusters, OMICS 16 (5) (2012) 284–287, https://doi.org/10.1089/omi.2011.0118.
[30] D.V. Klopfenstein, L. Zhang, B.S. Pedersen, et al., GOATOOLS: a Python library for Gene Ontology analyses, Sci. Rep. 8 (1) (2018) 10872, https://doi.org/10.1038/s41598-018-28948-z.
[31] M. Kanehisa, S. Goto, KEGG: kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (1) (2000) 27–30.
[32] W. Chen, L. Yi, S. Feng, et al., Characterization of microRNAs in orange-spotted grouper (Epinephelus coioides) fin cells upon red-spotted grouper nervous necrosis virus infection, Fish Shellfish Immunol. 63 (2017) 228–236, https://doi.org/10.1016/j.fsi.2017.02.031.
[33] M.D. Turner, B. Nedjai, T. Hurst, et al., Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease, Biochim. Biophys. Acta 1843 (11) (2014) 2563–2582, https://doi.org/10.1016/j.bbamcr.2014.05.014.
[34] M. Gaestel, A. Kotlyarov, M. Kracht, Targeting innate immunity protein kinase signalling in inflammation, Nat. Rev. Drug Discov. 8 (6) (2009) 480–499, https://doi.org/10.1038/nrd2829.
[35] Y. Qin, G. Lin, W. Chen, et al., Flagellar motility is necessary for *Aeromonas hydrophila* adhesion, Microb. Pathog. 98 (2016) 160–166, https://doi.org/10.1016/j.micpath.2016.07.006.
[36] H.C. Tekedar, H. Abdelhamed, S. Kumru, et al., Comparative genomics of *Aeromonas hydrophila* secretion systems and mutational analysis of hcp1 and vgrG1 genes from T6SS, Front. Microbiol. 9 (2018) 3216, https://doi.org/10.3389/fmicb.2018.03216.
[37] L. Guo, L. Huang, Y. Su, et al., *secA, secD, secF, yajC*, and *yidC* contribute to the adhesion regulation of *Vibrio alginolyticus*, Microbiologyopen 7 (2) (2018) e00551, , https://doi.org/10.1002/mbo3.551.
[38] L. Huang, L. Wang, X. Lin, et al., *mcp, aer, cheB*, and *cheV* contribute to the regulation of *Vibrio alginolyticus* (ND-01) adhesion under gradients of environmental factors, Microbiologyopen 6 (6) (2017), https://doi.org/10.1002/mbo3.517.
[39] A. Edrees, H. Abdelhamed, S.W. Nho, et al., Construction and evaluation of type III secretion system mutants of the catfish pathogen *Edwardsiella piscicida*, J. Fish Dis. 41 (5) (2018) 805–816, https://doi.org/10.1111/jfd.12784.
[40] R. Tang, G. Luo, L. Zhao, et al., The effect of a LysR-type transcriptional regulator gene of *Pseudomonas plecoglossicida* on the immune responses of *Epinephelus coioides*, Fish Shellfish Immunol. 89 (2019) 420–427, https://doi.org/10.1016/j.fsi.2019.03.051.
[41] L. Huang, Y. Zhang, R. He, et al., Phenotypic characterization, virulence, and immunogenicity of *Pseudomonas plecoglossicida rpoE* knock-down strain, Fish Shellfish Immunol. 87 (2019) 772–777, https://doi.org/10.1016/j.fsi.2019.02.028.
[42] G.J. Hannon, RNA interference, Nature 418 (6894) (2002) 244–251, https://doi.org/10.1038/418244a.
[43] C. Schlieker, H. Zentgraf, P. Dersch, et al., ClpV, a unique Hsp100/Clp member of pathogenic proteobacteria, Biol. Chem. 386 (11) (2005) 1115–1127, https://doi.org/10.1515/BC.2005.128.
[44] A. Hachani, N.S. Lossi, A. Hamilton, et al., Type VI secretion system in Pseudomonas aeruginosa: secretion and multimerization of VgrG proteins, J. Biol. Chem. 286 (14) (2011) 12317–12327, https://doi.org/10.1074/jbc.M110.193045.
[45] L. Liu, S. Hao, R. Lan, et al., The type VI secretion system modulates flagellar gene expression and secretion in Citrobacter freundii and contributes to adhesion and cytotoxicity to host cells, Infect. Immun. 83 (7) (2015) 2596–2604, https://doi.org/10.1128/IAI.03071-14.
[46] M.D. Baldissera, C.F. Souza, N.B. Bottari, et al., Purinergic signalling displays an anti-inflammatory profile in the spleen of fish experimentally infected with Aeromonas caviae: modulation of the immune response, J. Fish Dis. 41 (4) (2018) 683–687, https://doi.org/10.1111/jfd.12773.
[47] Q. Zhang, C. Ji, J. Ren, et al., Differential transcriptome analysis of zebrafish (*Danio rerio*) larvae challenged by *Vibrio parahaemolyticus*, J. Fish Dis. 41 (7) (2018) 1049–1062, https://doi.org/10.1111/jfd.12796.
[48] N. Chen, J. Jiang, X. Gao, et al., Histopathological analysis and the immune related gene expression profiles of Mandarin fish (*Siniperca chuatsi*) infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 83 (2018) 410–415, https://doi.org/10.1016/j.fsi.2018.09.023.
[49] X. Gao, D. Pi, N. Chen, et al., Survival, virulent characteristics, and transcriptomic analyses of the pathogenic *Vibrio anguillarum* under starvation stress, Front Cell Infect Microbiol 8 (2018) 389, https://doi.org/10.3389/fcimb.2018.00389.
[51] Q. Fu, N. Yang, C. Gao, et al., Characterization, expression signatures and microbial binding analysis of cathepsin A in turbot, Scophthalmus maximus L.(SmCTSA), Fish Shellfish Immunol. 81 (2018) 21–28, https://doi.org/10.1016/j.fsi.2018.07.002.
[52] S. Urban, K. Textoris-Taube, B. Reimann, et al., The efficiency of human cytomegalovirus pp65(495-503) CD8+ T cell epitope generation is determined by the balanced activities of cytosolic and endoplasmic reticulum-resident peptidases, J. Immunol. 189 (2) (2012) 529–538, https://doi.org/10.4049/jimmunol.1101886.
[53] Y. Waumans, L. Baerts, K. Kehoe, et al., The dipeptidyl peptidase family, prolyl oligopeptidase, and prolyl carboxypeptidase in the immune system and inflammatory disease, including atherosclerosis, Front. Immunol. 6 (2015) 387, https://doi.org/10.3389/fimmu.2015.00387.
[54] G.I. Godahewa, W.D. Wickramaarachchi, I. Whang, et al., Two carboxypeptidase counterparts from rock bream (*Oplegnathus fasciatus*): molecular characterization, genomic arrangement and immune responses upon pathogenic stresses, Vet. Immunol. Immunopathol. 162 (3–4) (2014) 180–191, https://doi.org/10.1016/j.vetimm.2014.10.005.
[55] N.C.N. Perera, G.I. Godahewa, S. Jung, et al., Identification and characterization of a carboxypeptidase N1 from red lip mullet (*Liza haematocheila*); revealing its immune relevance, Fish Shellfish Immunol. 84 (2019) 223–232, https://doi.org/10.1016/j.fsi.2018.10.010.
[56] W.P. Arend, G. Palmer, C. Gabay, IL-1, IL-18, and IL-33 families of cytokines, Immunol. Rev. 223 (2008) 20–38, https://doi.org/10.1111/j.1600-065X.2008.00624.x.
[57] D. Boraschi, D. Lucchesi, S. Hainzl, et al., IL-37: a new anti-inflammatory cytokine of the IL-1 family, Eur. Cytokine Netw. 22 (3) (2011) 127–147, https://doi.org/10.1684/ecn.2011.0288.
[58] M. Gouwy, S. Struyf, P. Proost, et al., Synergy in cytokine and chemokine networks amplifies the inflammatory response, Cytokine Growth Factor Rev. 16 (6) (2005) 561–580, https://doi.org/10.1016/j.cytogfr.2005.03.005.
[59] I. Balan, M.C. Beattie, T.K. O'Buckley, et al., Endogenous neurosteroid

(3alpha,5alpha)3-hydroxypregnan-20-one inhibits toll-like-4 receptor activation and pro-inflammatory signaling in macrophages and brain, Sci. Rep. 9 (1) (2019) 1220, https://doi.org/10.1038/s41598-018-37409-6.
[60] T. Kawai, S. Akira, Toll-like receptors and their crosstalk with other innate receptors in infection and immunity, Immunity 34 (5) (2011) 637–650, https://doi.org/10.1016/j.immuni.2011.05.006.
[61] S. Uematsu, S. Akira, Toll-like receptors and innate immunity, J. Mol. Med. (Berl.) 84 (9) (2006) 712–725, https://doi.org/10.1007/s00109-006-0084-y.
[62] J. Tang, G. Lin, W.Y. Langdon, et al., Regulation of C-type lectin receptor-mediated antifungal immunity, Front. Immunol. 9 (2018) 123, https://doi.org/10.3389/fimmu.2018.00123.
[63] X.W. Zhang, X. Man, X. Huang, et al., Identification of a C-type lectin possessing both antibacterial and antiviral activities from red swamp crayfish, Fish Shellfish Immunol. 77 (2018) 22–30, https://doi.org/10.1016/j.fsi.2018.03.015.
[64] D. Xue, W. Guang-Hua, S. Yan-Li, et al., Black rockfish C-type lectin, SsCTL4: a pattern recognition receptor that promotes bactericidal activity and virus escape from host immune defense, Fish Shellfish Immunol. 79 (2018) 340–350, https://doi.org/10.1016/j.fsi.2018.05.033.
[65] S. Vautier, D.M. MacCallum, G.D. Brown, C-type lectin receptors and cytokines in fungal immunity, Cytokine 58 (1) (2012) 89–99, https://doi.org/10.1016/j.cyto.2011.08.031.
[66] K. Newton, V.M. Dixit, Signaling in innate immunity and inflammation, Cold Spring Harb Perspect Biol 4 (3) (2012), https://doi.org/10.1101/cshperspect.a006049.