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The effect of a LysR-type transcriptional regulator gene of *Pseudomonas plecoglossicida* on the immune responses of *Epinephelus coioides*

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ABSTRACT

As an important pathogen in aquaculture, *Pseudomonas plecoglossicida* has caused heavy losses. It was determined with RNA-seq that the expression of a LysR-type transcriptional regulator gene (*L321_20267*) of *P. plecoglossicida* at 18 °C was significantly higher than that at 28 °C, which was verified by quantitative real-time PCR (qRT-PCR). RNAi significantly reduced the content of *L321*_20267 mRNA in *P. plecoglossicida*, with a maximal decrease of 90.63%. Compared with the wild-type strain, infection with the *L321_20267*-RNAi strain resulted in a 50% reduction in mortality and an onset time delay of *Epinephelus coioides*, as well as alleviation of the symptoms in *E. coioides* spleens. Compared with the wild-type strain of *P. plecoglossicida*, the *L321_20267*-RNAi strain resulted in a significant change in the spleen transcriptome of infected *E. coioides*. The results of GO and KEGG analysis showed that genes of serine hydrolase activity, the antigen processing and presentation pathway, the B cell receptor signalling pathway and the chemokine signalling pathway were most affected by the *L321_20267* gene of *P. plecoglossicida*. Meanwhile, the immune genes were related to different numbers of miRNAs and lncRNAs, and some miRNAs were related to more than one gene. The results indicated that 1. *L321_20267* is a virulence gene of *P. plecoglossicida*; 2. the upregulation of the immune pathways facilitated *E. coioides* to remove the *L321_20267*-RNAi strain compared with the wild-type strain of *P. plecoglossicida*; and 3. the immune genes were regulated by miRNA and lncRNA in a complex manner.

1. Introduction

Pseudomonas plecoglossicida, a gram-negative rod-shaped motile bacterium, was first isolated and identified from infected cultured ayu (*Plecoglossus altivelis*) suffering from bacterial haemorrhagic ascites [1]. In recent years, *P. plecoglossicida* has been related to the fulminant disease of specific marine fish, such as large yellow croaker (*Pseudosciaena crocea*) [2,3] and rainbow trout (*Oncorhynchus mykiss*) [4]. The fulminant disease caused by *P. plecoglossicida* is temperature-dependent and mainly recorded during a seawater temperature range from 15 to 20 °C. With the aim of exploring pathogenicity, the transcriptomes of *P. plecoglossicida* incubated at 12, 18 and 28 °C were sequenced [5], and the results have been deposited in the NCBI Sequence Read Archive (Accession Number SRP107111). The results showed that the expression of the *L321_20267* gene of *P. plecoglossicida* was significantly higher at 18 °C than at 28 °C.

L321_20267 encodes an unknown protein that belongs to a well-characterized group of the LysR-type transcriptional regulator (LTTR) family [6]. The conservation of this family within the genomes of extremely diverse bacteria means that LTTRs have evolved a regulatory role over genes with similarly diverse functions, which are related to quorum sensing [7], virulence [8], biocontrol activity [9], biofilm information [10] and cell division [11]. In the past 60 years, many genes, such as *scmR* [8] and *gvmR* [12], encoding LTTRs have been found to play virulence-related roles in archaea organisms. However, to our present knowledge, the effect of *L321_20267* on the pathogenicity of bacteria is still unknown.

Due to the tremendous effect of *P. plecoglossicida* on cultured fish and the underlying important role of *L321_20267* in the virulence of *P. plecoglossicida, L321_20267* was silenced by RNAi. Furthermore, the pathogenicity of the control was compared to that of *L321_20267*-RNAi *P. plecoglossicida* in *Epinephelus coioides*. The infected spleens of *E. coioides* by wild type strain and *L321_20267*-RNAi *P. plecoglossicida* were submitted to RNA-seq. The aim of this paper is to disclose the

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immune response of *E. coioides* to the *L321_20267* gene of *P. plecoglossicida*.

2. Material and methods

2.1. Bacterial strains and culture conditions

The pathogenic *P. plecoglossicida* strain (NZBD9) was isolated from the spleen of *P. crocea* suffering from white-spot disease [2]. The *P. plecoglossicida* strain was cultured in LB (Luria Bertani) medium at 18 °C or 28 °C with shaking at 220 rpm. *Escherichia coli* DH5α was acquired from TransGen Biotech from Beijing in China and was cultured in LB medium (37 °C, 220 rpm).

2.2. Construction of P. plecoglossicida RNAi strain

The construction of the RNAi strain was conducted based on methods described by Choi and Schweizer [13] and Darsigny et al. [14]. An Invitrogen Block-iT RNAi Designer (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=7085871032206845) was used to predict shRNA sequences that would lead to silencing of target genes. Five short hairpin RNA (shRNA) sequences targeting 465–556 bp, 466–557 bp, 880–931 bp, 882–933 bp and 889–940 bp downstream from the translation start site of the *L321_20267* gene were designed and synthetized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China) (Supplementary Table 1). pCM130/tac vectors were linearized by the restriction enzymes *Nsi*I and *Bsr*GI (New England Biolabs), and then the oligonucleotides were annealed and ligated to the linearized pCM130/tac vectors with T4 DNA ligase (New England Biolabs) according to the user manual. The recombinant pCM130/tac vectors were transformed into *E. coli* DH5a cells by heat shock and then extracted and transferred into *P. plecoglossicida* by electroporation [15]. Finally, qRT-PCR was used to detect the expression levels of the *L321_20267* gene of each shRNA strain to select the shRNA strains with optimal silencing efficiency for downstream experiments.

2.3. Artificial infection and sampling

Fish experiments were conducted under the recommendations in the 'Guide for the Care and Use of Laboratory Animals' established by the National Institutes of Health. The fish project was approved by the Animal Ethics Committee of Jimei University (Acceptance No. JMULAC201159). Healthy weight-matched *E. coioides* were acquired from Zhangzhou city of Fujian Province in China and were accommodated under specified pathogen-free laboratory conditions at 18 ± 1 °C for one week prior to processing. For survival tests, each *E. coioides* was intrapleurally injected with 10^3 colony forming units per gram (cfu/g) of *P. plecoglossicida* (wild-type strain or the RNAi strains) while *E. coioides* injected with PBS were used as the negative control. The mortality of infected fish was recorded daily. The dead *E. coioides* were removed in a timely manner. For tissue RNA-seq, the spleens of six *E. coioides* infected with the wild-type strain or RNAi strain of *P. plecoglossicida* were sampled at 24 h post infection (hpi), of which two spleens were mixed and used as one sample.

2.4. RNA isolation and reverse transcription

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the user manual. After digesting the mixed genomic DNA in total RNA with Turbo DNA-free DNase (Ambion, Austin, TX, USA), the RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the rRNA in total RNA was removed by the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA) following the user manuals. The quality of total RNA was verified by agarose gel electrophoresis. cDNA was synthesized using the TaKaRa PrimeScriptTM RT-PCR Kit (TaKaRa Bio Group, JAPAN) protocol in a system. The synthesized cDNA was stored at −20 °C and used as a new sample template for qRT-PCR.

2.5. qRT-PCR

qRT-PCR was conducted using a QuantStudio 6 Flex (Life Technologies). All primer sequences are provided in Supplementary Table 2. The expression of bacterial genes was normalized with *gyrB*. The expression of mRNA and lncRNA in *E. coioides* was normalized with *β-actin* [16], and miRNA was normalized with *5SrRNA* [17]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative level of gene expression.

2.6. Transcriptomic analysis

2.6.1. Library preparation and Illumina sequencing

The protocol supplied with the TruSeqTM RNA sample preparation Kit (Illumina, San Diego, CA, USA) was used for preparation of the RNA-seq library. After the rRNA-depleted RNA sample was fragmented in fragmentation buffer, a SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) was used to facilitate cDNA synthesis. After end repair, phosphorylation and poly (A) addition, Phusion DNA polymerase (NEB) was used to amplify the cDNA library. A TruSeqTM Small RNA Sample Prep Kit (Illumina) was used to build the small RNA-seq library. Library quality was validated by an Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing was performed at Majorbio Biotech Co., Ltd. (Shanghai, China) on the Illumina HiSeq4000 sequencing platform.

2.6.2. Processing and mapping of reads

Sickle (https://github.com/najoshi/sickle) and SeqPrep (https://github.com/jstjohn/SeqPrep) with default settings were used to trim and perform quality control of the raw Illumina reads. Clean data were mapped to the genome reads of *P. plecoglossicida* strain NZBD9 (NCBI RefSeq Accession Number: SRP062985) for RNA-seq using Bowtie2 [18]. Mapped reads were considered as reads of *P. plecoglossicida*, and the remaining reads were used for de novo assembly to acquire the *E. coioides* unigenes.

2.6.3. De novo assembly, detection of lncRNAs and annotation of mRNA in the host

All non-mapped clean reads to the *P. plecoglossicida* genome from the infected spleens were treated as a pool of reads. This pool of reads was assembled de novo into unigenes by Trinity [19]. All unigenes were previously aligned to the bacterial NCBI non-redundant (NR) protein database to remove as much prokaryotic contamination as possible. The detection of lncRNAs was carried out as described previously [20,21]. In addition, the clean unigenes were mapped to several databases, including the SWISS-PROT, STRING, NCBI NR protein and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases. Identification of the proteins sharing the most sequence similarity with the identified unigenes was conducted by BLASTx. Blast2GO software (http://www.blast2go.com/b2ghome) was used for annotation of Gene Ontology (GO) [22], while KEGG was used for KEGG pathway analysis (http://www.genome.jp/kegg/) [23].

2.6.4. Identification of miRNAs

The raw reads were analysed with quality control to obtain clean reads. In addition, the clean reads were BLAST searched against the Rfam database (http://Rfam.sanger.ac.uk/) to obtain annotation of the miscellaneous RNAs. rRNA, tRNA, scRNA, snoRNA, snRNA and other non-coding RNAs were filtered, and the remaining sRNAs were mapped to the zebrafish miRNA data of miRBase version 21.0 to identify the known and novel miRNAs.

2.6.5. Analysis of differential gene expression

Analysis of the expression of RNA-seq data from *E. coioides* was carried out on the basis of the reference transcriptome annotation described above (annotation of mRNA and lncRNA in *E. coioides*) and annotations from NCBI (NZ_ASJX00000000.1). After acquiring uniquely mapped read counts, the package edgeR (version 3.10.2) [24] was used to test for differentially expressed genes (DEGs) with the criteria of $|\log2^{\text{fold change}}| \geq 1$ and a false discovery rate (FDR) < 0.05.

2.6.6. Statistical analyses

All of the data were expressed as the means ± standard deviation (SD) from at least three independent experiments. Data analysis was carried out using SPSS 17.0 software (Chicago, IL, USA), and one-way analysis of variance with Dunnett's test was used. *P* values < 0.05 were considered statistically significant.

2.7. Data access

The RNA sequencing reads data were deposited in the GenBank SRA database under the accession numbers SRP152612 and SRP115064.

3. Results

3.1. Construction of the L321_20267-RNAi strain

The RNA-seq results of the relative expression level of the *L321_20267* gene in *P. plecoglossicida* cultured at 18 °C and 28 °C were highly consistent with the results of qRT-PCR (Fig. 1A). The results showed that the expression level of the *L321_20267* gene in *P. plecoglossicida* cultured at 18 °C was several times higher than that at 28 °C (Fig. 1A). All five shRNAs resulted in a reduction in the content of *L321*_20267 mRNA in *P. plecoglossicida* with different efficiencies (Fig. 1B). The reduction in the expression level of the *L321_20267* gene by *L321_20267*-shRNA889, *L321_20267*-shRNA882, *L321_20267*-shRNA880, *L321_20267*-shRNA466 and *L321_20267*-shRNA465 was 30%, 90.63%, 65%, 50% and 82%, respectively (Fig. 1B). *P. plecoglossicida,* including the *L321*_20267-shRNA-882 strain (named the *L321_20267*-RNAi strain), exhibited the best efficiency for silencing the *L321_20267* gene and was selected for further analysis.

3.1.1. The effect of the L321_20267 gene on the pathogenicity of P. plecoglossicida

Since *P. plecoglossicida* exhibited high pathogenicity against *E. coioides*, the injection of the wild-type strain of *P. plecoglossicida* resulted in the rapid death of *E. coioides*. The first mortality of *E. coioides* injected with the wild-type strain was observed 2 days post-injection (dpi), while the majority of the deaths were observed 4 dpi, and all *E. coioides* died within 6 dpi (Fig. 2A). Compared with the injection with the wild-type strain, the injection with the *L321_20267*-RNAi strain resulted in an onset time delay of 2 days and only 50% mortality of *E. coioides. E. coioides* injected with PBS were regarded as a negative control, and no deaths were observed. At 2 dpi, the spleens of *E. coioides* injected with the wild-type strain presented the typical symptoms of natural disease, with a large number of white nodules (Fig. 2B). However, no visible white nodules were observed in the spleens of *E. coioides* injected with the *L321_20267*-RNAi strain or with PBS (Fig. 2B).

3.2. Tissue RNA-Seq of the spleens of infected E. coioides

3.2.1. Differentially expressed genes (DEGs)

In the tissue RNA-Seq program, the transcriptomes of the spleens of infected *E. coioides* were catalogued. The base distribution is balanced, and N% is within the reasonable range. The reproducibility of the three biological replicates is satisfactory. The Pearson correlation coefficients between infected *E. coioides* for the three biological replicates are plotted in Fig. 3A. The difference between infected *E. coioides* is significant, and all samples are closely correlated with their respective replicates from the same biological sample group. The gene expression profile was calculated by edgeR, and DEGs subjected to the criteria of FDR < 0.05 and $|\log_2 FC| > 1$ were regarded as statistically significant. Of 61309 profiled mRNAs of the *L321_20267*-RNAi strain-infected spleens, 21873 were significantly altered in abundance in contrast with the control. In 21873 DEGs, 1548 were upregulated in response to infection with the *L321_20267*-RNAi strain, and 20323 were downregulated (Fig. 3B). Based on the criteria above, the top 50 upregulated DEGs were selected and are presented in Fig. 3C. Of the top 50 upregulated DEGs, 14 failed to match GO terms, and 36 successfully matched to 9 GO terms, which were binding, cellular process, response to stimulus, serine hydrolase activity, single-organism process, protein metabolic process, peptidase activity, and extracellular region and cell. The relationships between genes and GO terms are presented in Fig. 3D. Among the 9 GO terms, the serine hydrolase activity is the most detailed.

In terms of the KEGG database, all of the top 50 upregulated DEGs were enriched in 18 KEGG pathways. Compared with the control, all 18 KEGG pathways were upregulated in the spleens infected with the *L321_20267*-RNAi strain of *P. plecoglossicida* (Fig. 4A). Among the 18 KEGG pathways, 3 were directly related to the immune system, including antigen processing and presentation, the B cell receptor signalling pathway, and the chemokine signalling pathway. In the antigen processing and presentation pathway (Fig. 4B), genes encoding MHCII, MHCI, HSP70, HSP90, CTSB6 and TAP were highly upregulated. In the B cell receptor signalling pathway (Fig. 4C), genes encoding Igα, Igβ, BTK, CCL4 and CXCR4 were highly upregulated. In the chemokine signalling pathway (Fig. 4D), genes encoding CCL4, CCL19, IL8, IL10 CXCR4, CCR5, CCR9 and BLR1 were highly upregulated.

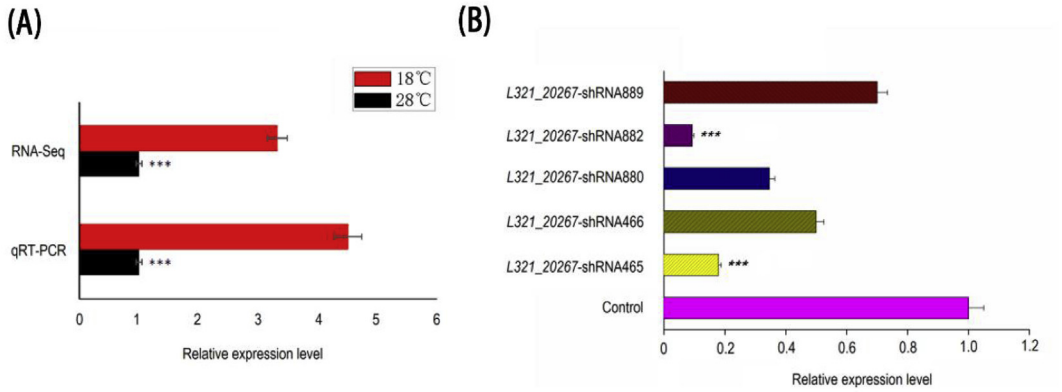

Fig. 1. Construction of the *L321_20267*-RNAi strain. (A): Relative expression of the gene *L321_20267* at 18 °C and 28 °C. (B): Silencing efficiency of 5 shRNAs targeting *L321_20267*. Data are shown as the means ± SD from three independent biological replicates. ***$P < 0.001$.

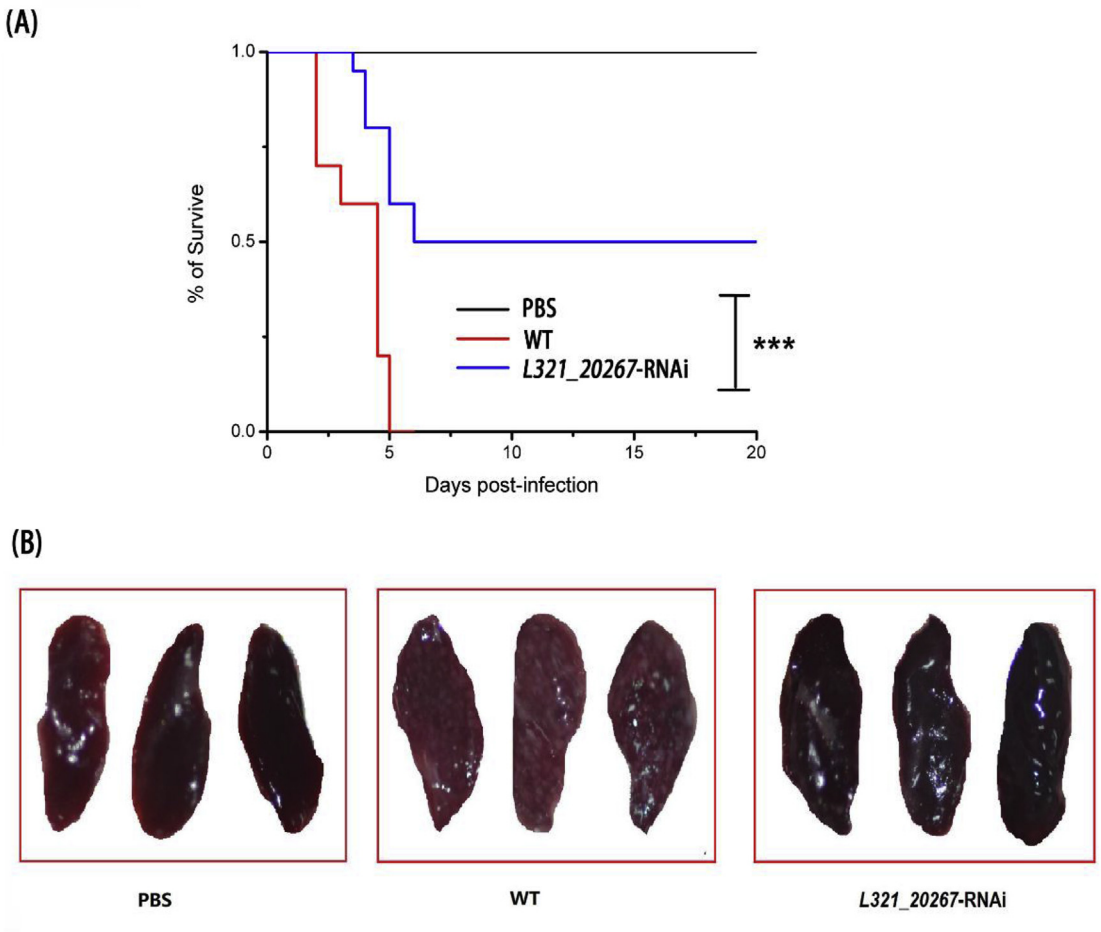

Fig. 2. The virulence of the wild-type strain and the *L321_20267*-RNAi strain of *P. plecoglossicida* to *E. coioides*. (A): Survival rate of *E. coioides* injected with *P. plecoglossicida* (WT strain, *L321_20267*-RNAi strain) or PBS. Survival of *E. coioides* was monitored for 20 days. (B): Symptoms of spleens in *E. coioides* injected with *P. plecoglossicida* (WT strain, *L321_20267*-RNAi strain) or PBS. Data are shown as the means ± SD from three independent biological replicates. ***$P < 0.001$.

3.2.2. Differentially expressed non-coding RNAs

Long chain non-coding RNAs (lncRNAs) were authenticated by Coding-Non-Coding Index (CNCI) with the criteria of CNCI < 0 and Coding Potential Calculator (CPC) with the criteria of CPC score < -1, and the collection was used for further research. Of the 29495 profiled non-coding RNAs of the *L321_20267*-RNAi strain-infected spleens, 9581 were significantly altered in abundance compared with the control. In 9581 differentially expressed non-coding RNAs, 981 were upregulated in response to infection with the *L321_20267*-RNAi strain, and 8600 were downregulated.

miRNAs were authenticated by the Rfam database. Of 605 profiled miRNAs of the *L321_20267*-RNAi strain-infected spleen, 236 were significantly altered in abundance in contrast with the control. In 236 differentially expressed miRNAs, 145 were up-regulated in response to infection with the *L321_20267*-RNAi strain, of which 56 were known miRNAs and the other 89 were novel miRNAs. Meanwhile, 91 were downregulated, of which 10 were known miRNAs and the other 81 were novel miRNAs.

Some transcripts were selected from the transcriptome and verified by qRT-PCR, including mRNA, lncRNA and miRNA.

3.2.3. Network of miRNAs-mRNAs-lncRNAs

Fig. 5 shows the interaction of 11 DEGs with lncRNAs and miRNAs. Most DEGs were directly related to different numbers of non-coding RNAs. *gzma* was targeted by 21 lncRNAs and 15 miRNAs. *slc25a14_30* was targeted by 3 lncRNAs and 12 miRNAs. *ccl19* was targeted by 11 miRNAs. *dhrs4* was targeted by 10 mRNAs and 2 miRNAs. *cxcr4* was targeted by 6 miRNAs. *cela3* was targeted by 6 miRNAs. *e3.1.27.1* was targeted by 1 lncRNA and 3 miRNAs. *cd79b* was targeted by 9 lncRNAs. *apoh, cpa1* and *far* were targeted by 1 lncRNA.

The relationships among DEGs were produced with different numbers of non-coding RNAs, in addition to *dhrs4, cd79B, apoh, cpa1* and *far*. *gzma* and *slc25a14_30* shared 5 miRNAs. *gzma* and *cxcr4* shared 2 miRNAs. *gzma* shared 1 miRNA with *cela3* and *ccl19*. *ccl19* and *e3.1.27.1* shared 3 miRNAs. *cxcr4* and *slc25a14_30* shared 4 miRNAs. Compared with the control, the expression of these non-coding RNAs was different in the spleen of *E. coioides* infected with the *L321_20267*-RNAi strain of *P. plecoglossicida.* Although 11 DEGs were upregulated during the comparison, 40 of 90 non-coding RNAs were upregulated.

4. Discussion

Pathogenic invasion causes disease, and pathogenic colonization is controlled by bacterial virulence, which is regulated by many genes [25]. Currently, a great number of genes have been certified to be involved in the virulent regulation of marine pathogenic bacteria by gene-silencing methods, for example, *exsE* of *Vibrio alginolyticus* [26]; *flrA, flrB and flrC* of *V. alginolyticus* [15]; *vtrA* and *vtrB* of *V. parahaemolyticus* [27]; and *sigX* and *L321_RS19110* of *P. plecoglossicida* [20,21]. However, to our knowledge, research on the effect of *L321_20267* on virulence

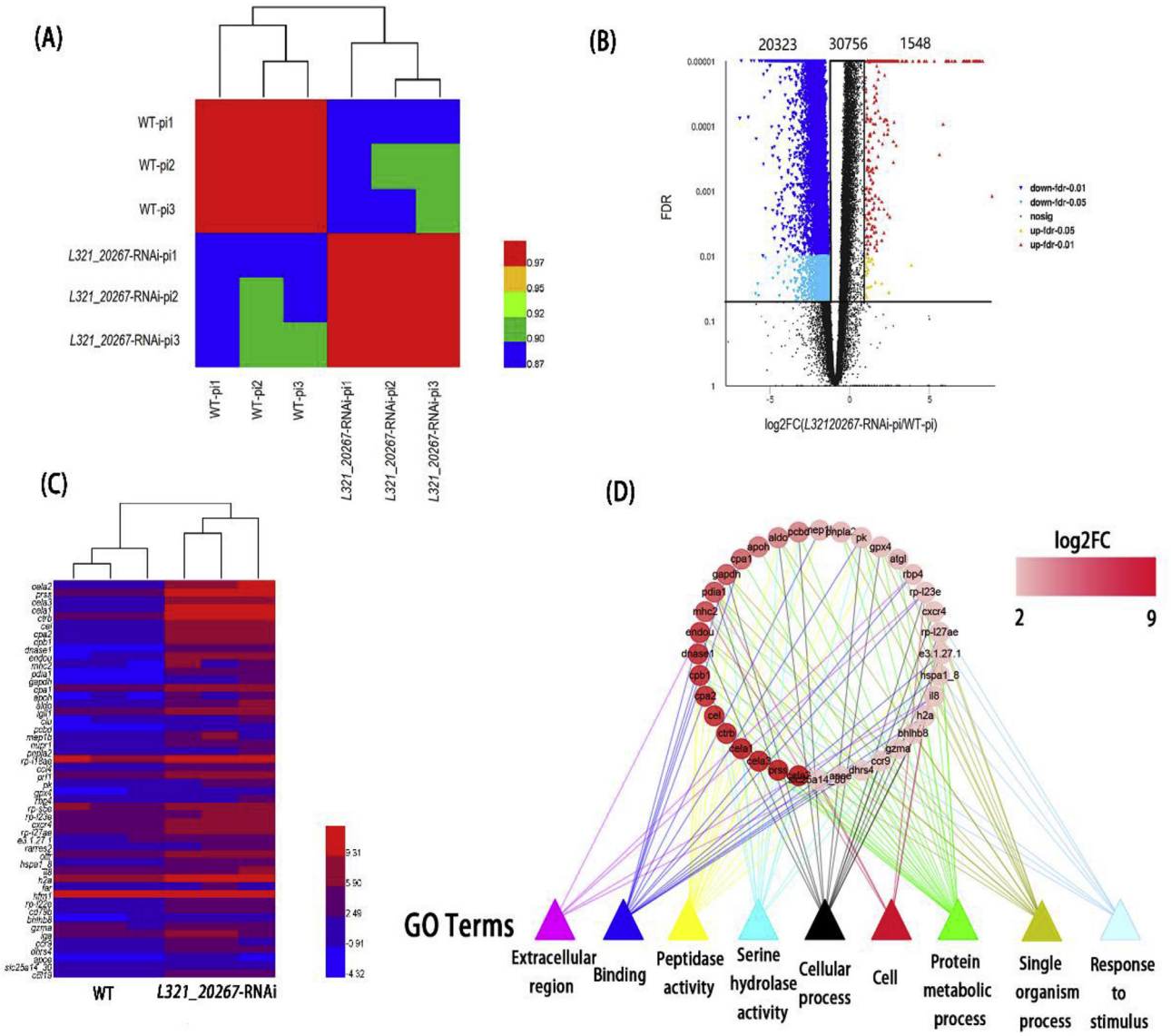

Fig. 3. Differentially expressed genes of infected *E. coioides* and GO analysis. (A): Correlation coefficient analysis of infected *E. coioides*. (B): Volcano plot obtained from *DESeq2* analysis of infected *E. coioides*. (C): Heat map of the 50 most significant upregulated genes. Values of the bars represent log2FC, adjusted FDR < 0.05; |log2FC| ≥ 1; 3 biological replicates. Colour-coding is based on log2FPKM (per kilobase of exon model per million mapped reads). Blue and red represent decreased and increased expression, respectively. (D): The relationship between DEGs and GO terms. The circle node represents the gene, and the triangle represents the respective GO terms. The redder the colour of the circle, the more upregulated the gene. The edges represent relationships of genes that are included in GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

has not been reported.

Infection with *P. plecoglossicida* was temperature-dependent, and host clinical signs were developed at 15–20 °C [5]. The expression level of *L321_20267* of *P. plecoglossicida* cultured at 18 °C was significantly higher than that at 28 °C, which indicated that *L321_20267* may be involved in the virulence of *P. plecoglossicida*. In recent years, the usage of RNA interference (RNAi) has emerged as an effective tool for the study of gene function [28]. In our present study, *L321_20267* was silenced by five different shRNAs, and the pathogen including the pCM130/tac-*L321_*20267-shRNA-882 strain (named the *L321_20267*-RNAi strain) significantly reduced the content of *L321_*20267 mRNA in *P. plecoglossicida* with a maximal decrease of 90.63%.

Compared with the infection with the wild-type strain, the infection with the *L321_20267*-RNAi strain resulted in an onset time delay of 2 days and showed only 50% mortality of *E. coioides*. The results indicated that RNAi of *L321_20267* evidently attenuates the virulence of *P. plecoglossicida*. Furthermore, no visible nodules were observed in the spleens of *E. coioides* infected with the *L321_20267*-RNAi strain or with PBS at 2 dpi. Since the spleen is the primary targeted site of *P. plecoglossicida* [1] and there are obvious differences in the symptoms of the infected spleens, the spleen is considered to be the optimal organ for further studying the immune response of *E. coioides* to *P. plecoglossicida* infection.

Recent studies have shown that pathogens can lead to great changes at the host transcriptional level during infection [29]. Moreover, a single virulence-related gene can also lead to pervasive changes at the host transcriptional level [20,21,30]. In our current work, in contrast to the wild-type strain, the *L321_20267*-RNAi strain of *P. plecoglossicida* led to a significant change in the transcriptional level of infected *E. coioides*. The results revealed the important effect of *L321_20267* in *P. plecoglossicida* on host transcripts during infection. As a result of GO and KEGG analysis, the serine hydrolase activity GO term and the antigen processing and presentation pathway, the B cell receptor signalling pathway, and the chemokine signalling pathway were significantly

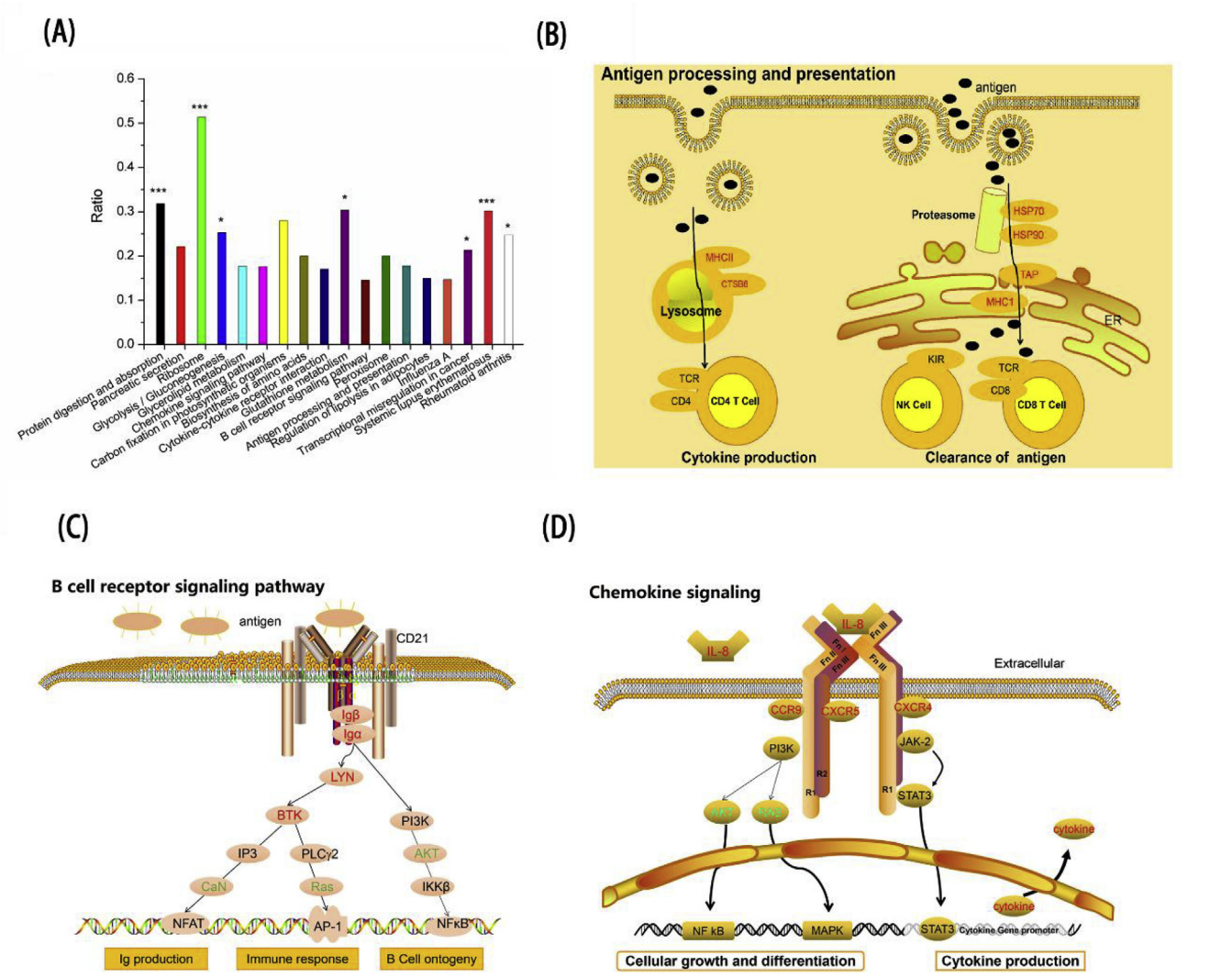

Fig. 4. The enriched KEGG pathway. (A): 18 enriched KEGG pathways, including the 50 most significantly upregulated genes. Ratio: targeted gene number/background gene number. The significance of enrichment: $*P < 0.05$, $***P < 0.001$ (B): Simplified overview of the *L321_20267*-RNAi strain interacting with the host antigen processing and presentation pathway (C): Simplified overview of the *L321_20267*-RNAi strain interacting with the host B cell receptor signalling pathway (D): Simplified overview of the *L321_20267*-RNAi strain interacting with the host chemokine signalling pathway. The genes in red letters in (B), (C), (D) are upregulated, the black represent no change and the green are downregulated in spleens infected by the *L321_20267*-RNAi strain compared with the counterpart infected by wild-type strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

affected by *L321_20267* in *P. plecoglossicida* during infection. Serine hydrolases have been mainly targeted for the development of drugs against microbial infections, such as *influenza virus* infection [31]. Neutrophil serine protease could regulate inflammatory and immune responses against the pathogen at inflammatory sites [32]. *Staphylococcus aureus* secretes a unique class of neutrophil serine protease inhibitors to resist its host immune response [33]. Antigen processing begins with the degradation of proteins into small peptides, which are presented by major histocompatibility complexes (MHCs), and recognition of MHC–peptide complexes by the immune system determines successful pathogen elimination [34]. Upon pathogen infection, effector CD8 T cells have to recognize antigenic peptides loaded in MHC-I to kill the infected cells [35]. It has been reported that constitutive expression of MHCII is restricted to professional antigen-presenting cells, which are responsible for the initiation of adaptive immune responses by priming naive T cells in secondary lymphoid organs [36]. The perfect assembly of a receptor of B cells was a prerequisite for entry into the simple compartment. B cells respond to antigens through the aggregation of the antigen receptor and of coreceptors through which immune-associated molecular or helper T cells are activated against the antigens [37]. Mice deficient in B cells are highly susceptible to *Citrobacter rodentium*-induced sepsis [38]. Chemokines are a kind of secreted molecule that is signal mediated by G protein-coupled receptors to promote cell proliferation and survival and to attract immune cells to inflammation sites [39]. Neutrophils, primary cells of the innate immune response, were found to be recruited by chemokines and their receptors to sterile wounds in *zebrafish larvae* [40]. Both T cells and natural killer cells, which require CXCR4 to be generated, were reported to have important roles in the innate immune response and adaptive immune response by producing cytokines [41,42]. In addition, the number of natural killer cells was severely reduced in the bone marrow and spleen of CXCR4 conditionally deficient mice compared with control mice [43]. Furthermore, B cells are capable of producing considerable numbers of cytokines and chemokines [44], which can play important regulatory roles in inflammation during infection. As previous studies on these pathways were conducted in response to bacterial infection, the present study provides more insight into the response of these pathways to a single gene during infection. The upregulation of these pathways in spleens infected with the *L321_20267*-RNAi strain promoted *E. coioides* to remove the *L321_20267*-RNAi strain

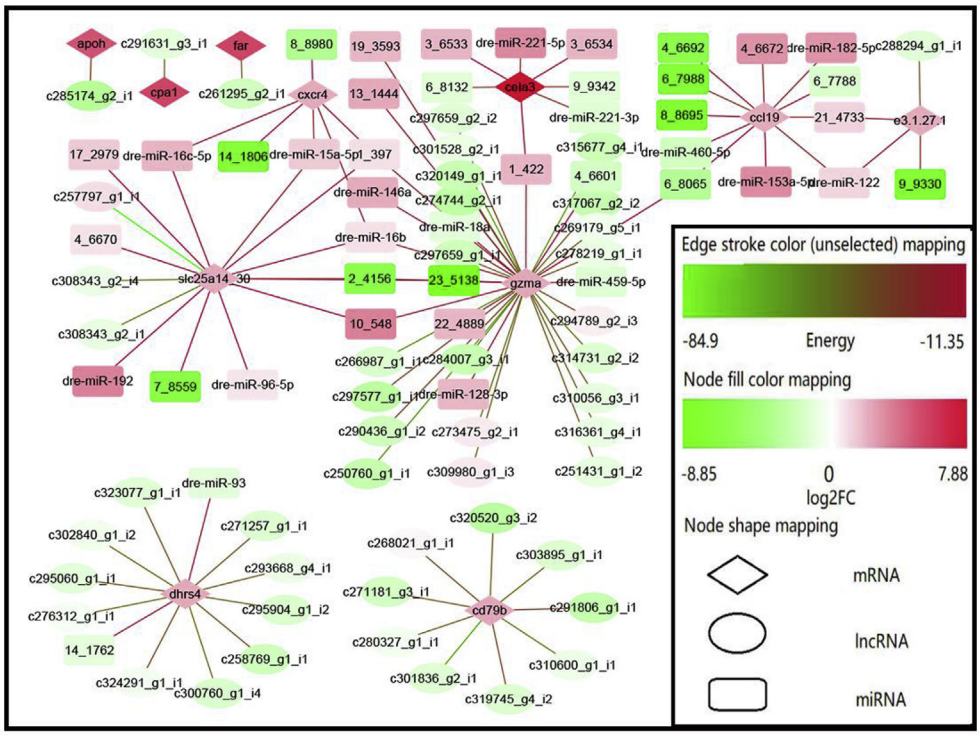

Fig. 5. The network between mRNAs and non-coding RNAs. Diamond nodes represent mRNA, elliptical nodes represent lncRNA, and rounded rectangular nodes represent miRNA. Red-coloured nodes represent upregulation, and green-colour nodes represent downregulation. The deeper the colour, the greater the change. The edge colour ranging from green to red represents the interaction energy from low to high. The smaller the energy, the closer the bond. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared with the wild-type strain of *P. plecoglossicida*. These results were in good agreement with the fact that the *L321_20267*-RNAi strain was less virulent in *E. coioides*.

The regulatory mechanism between mRNA and noncoding RNA is complex [45]. miRNAs are involved in almost all developmental and pathological processes in animals, targeting an enormous number of mRNAs [46]. miRNAs could be key regulatory mechanisms in immunity [47]. It is inferenced that lncRNAs have a competing interaction with miRNAs when targeting mRNAs [48]. In our present study, the significantly upregulated immune genes were related to different numbers of miRNAs and lncRNAs, and some miRNAs were related to more than one gene. The results indicated that immune genes were regulated by both miRNAs and lncRNAs in a complex manner.

5. Conclusion

L321_20267 is a virulent gene in *P. plecoglossicida* that plays a critical role in the pathogenicity of *P. plecoglossicida* against *E. coioides*. Compared with the wild-type strain infection, the infection of *E. coioides* with the *L321_20267*-RNAi strain resulted in the upregulation of serine hydrolase activity, the antigen processing and presentation pathway, the B cell receptor signalling pathway and the chemokine signalling pathway in the spleen. The immune genes were regulated by both lncRNAs and miRNAs in a sophisticated manner.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.051.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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