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Dual RNA-seq uncovers the immune response of *Larimichthys crocea* to the *secY* gene of *Pseudomonas plecoglossicida* from the perspective of host-pathogen interactions

Luying Wang[a], Yunjia Sun[a], Lingmin Zhao[a], Xiaojin Xu[a], Lixing Huang[a], Yingxue Qin[a], Yongquan Su[b], Jiaonan Zhang[c], Qingpi Yan[a,b,*]

[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, Fujian, 352000, China*
[c] *Key Laboratory of Special Aquatic Feed for Fujian, Fujian Tianma Technology Company Limited, Fuzhou, Fujian, 350308, China*

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ABSTRACT

Pseudomonas plecoglossicida is a Gram-negative aerobic bacterium that causes high mortality and serious economic losses in some commercial marine fish. Expression of *secY* was found to be significantly upregulated at 18 °C compared to 28 °C by RNA-seq and qRT-PCR. All five tested recombinant vectors (pCM130/tac + shRNA) significantly reduced *secY* mRNA levels in *P. plecoglossicida*. The recombinant vector encoding shRNA-1165 exhibited the best gene-silencing efficiency, 82.4% and was used to create an RNAi strain for further studies. Compared with the wildtype strain, infections of *Larimichthys crocea* with the RNAi strain resulted in a 2-day delay in onset time and a 35% reduction in mortality, as well as the alleviation of spleen symptoms. The spleens of *L. crocea* infected by the wild type or RNAi strain of *P. plecoglossicida* were subjected to dual RNA-seq at 2 dpi. Compared with the wildtype strain, infection of *P. plecoglossicida* with the RNAi strain resulted in significant changes in the transcriptomes of both host and pathogen. KEGG analysis showed that the complement and coagulation cascade and the Toll-like receptor signalling pathway were the most enriched host pathways. In the pathogen, genes of the “Sec secretion system” were significantly downregulated. This downregulation of “Sec secretion system” genes hindered the secretion of bacterial proteins and reduced the virulence of *P. plecoglossicida*. Thus, it was easier for *L. crocea* to clear the RNAi strain of *P. plecoglossicida*, and the immune response was similarly reduced. The results indicated that *secY* was a virulence gene of *P. plecoglossicida* and played roles in the host-pathogen interactions of *L. crocea* and *P. plecoglossicida*.

1. Introduction

Pseudomonas plecoglossicida, a Gram-negative, aerobic and rod-shaped bacterium, poses a serious threat to cultured ayu (*Plecoglossus altivelis*) in Japan, causing an emerging bacterial disease with bacterial haemorrhagic ascites [1,2]. Recently, *P. plecoglossicida* has been associated with fulminating infectious diseases of several marine teleosts, such as *Epinephelus coioides* and *Larimichthys crocea* [3–5]. The pathogenicity of *P. plecoglossicida* has attracted attention, and several virulence genes have been identified [6–8]. To further reveal the molecular mechanism of *P. plecoglossicida* pathogenesis, the transcriptional profile of *P. plecoglossicida* during infection was determined, and the data were deposited with a registration number of SRP115064 in the NCBI SRA database. The data showed that the *secY* gene of *P. plecoglossicida* was expressed at a significantly high level throughout the infection. Therefore, it is assumed that *secY* play roles in the pathogenicity of *P. plecoglossicida*, which highlights the importance of studying the host immune response to the *secY* gene of *P. plecoglossicida.*

The infection process is a competition between pathogens and hosts [5,9]. To win this competition, both pathogen and host must go all out, and all changes will be reflected in their respective transcriptional profiles [10]. Therefore, it is important to simultaneously monitor the RNA expression profiles of the two interacting species to study host immune responses and pathogen virulence mechanisms in context. Dual RNA-seq is a new approach to monitor both host and pathogen RNA expression profiles simultaneously [11]. Initially, dual RNA-seq was

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[*] Corresponding author. Fisheries College, Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Jimei University, Xiamen, Fujian, 361021, China.

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

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successfully applied to pathogen-host cell infection models, and it showed unique advantages over traditional RNA-seq by successfully revealing a large number of previously unknown interaction characteristics and identifying several key genes of interaction [12,13]. Later, dual RNA-seq was successfully applied to studies of in vivo host-pathogen interactions, and it again revealed a large number of previously unknown interaction characteristics [11,14]. Recently, dual RNA-seq has been increasingly used to study the functions of single virulence genes in host-pathogen interactions, which provides a powerful tool for the intensive study of the function of a specific virulence gene [15–18]. At present, low pathogen load in infected tissues is the main factor limiting the wide application of dual RNA-seq. With the progress in sequencing technology, dual RNA-seq will be more frequently used in the study of host-pathogen interactions.

The *secY* gene encodes the protein SecY, it has been proposed that SecY contains 10 transmembrane segments, with five regions exposed to the periplasm and six cytoplasmic regions, including the amino- and carboxyterminal regions [19]. SecY has been reported to be a transmembrane protein and the most important component of the Sec protein secretion system [20,21]. The SecY protein can act post-translationally, enhancing the translocation of completed pro-OmpA polypeptide chains across the plasma membrane, thereby promoting bacterial protein export [22]. Up to now, research on *secY* function has been very limited, and there is no report on host immune responses to the *secY* gene of pathogens.

In light of the great threat of *P. plecoglossicida* to aquaculture [3–5] and the potential important role of *secY* in the virulence of *P. plecoglossicida,* the aim of this study was to evaluate the function of *secY* gene by exploring the immune responses of *L. crocea* to the *secY* gene of *P. plecoglossicida* from the perspective of host-pathogen interactions. The *secY* gene of *P. plecoglossicida* was silenced stably by RNAi, the virulence of the wildtype strain and a *secY*-RNAi strain of *P. plecoglossicida* were compared, the spleens of *L. crocea* infected by a wildtype strain or by mutant *P. plecoglossicida* were subjected to dual RNA-seq, and the data were compared and comprehensively analysed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The experimental strain was wildtype *P. plecoglossicida* (NZBD9), which was isolated from diseased *L. crocea* and was confirmed to be pathogenic by artificial infection [4]. *P. plecoglossicida* was cultured in Luria-Bertani broth or LB agar medium at a temperature of 18 °C–28 °C at 220 rpm. *Escherichia coli* DH5α was obtained from TransGen Biotech (Beijing, China) and was cultured in LB medium (37 °C, 220 rpm).

2.2. Construction of P. plecoglossicida RNAi strains

RNAi strains were constructed according to the experimental methods used by Choi, Schweizer and Darsigny et al. [23,24]. The full-length sequence of the *secY* gene was entered into the online software (http://rnaidesigner.thermofisher.com/rnaiexpress/setOption.do?designOption=shrna&pid=7085871032206845), five pairs of specific oligonucleotide sequences for shRNA formation were designed (Supplementary Table 1, and the sequences were sent to Shanghai Jierui Bioengineering Co., Ltd. for synthesis. The oligonucleotide pairs were annealed and ligated to vector pCM130/tac, which had been linearized with the restriction enzymes NsiI (R3127S) and BsrGI (R3575S) in Performance Buffer according to the TIME-SAVER$^{\mathrm{TM}}$ Protocol (New England Biolabs), using T4 DNA ligase (New England Biolabs). *E. coli* competent cells were prepared by the classical $CaCl_2$ method of Mandel and Higa [25]. Each recombinant pCM130/tac vector was transformed into competent *E. coli* DH5α cells by heat shock, and then the recombinant plasmid was electrotransformed into *P. plecoglossicida* competent cells [26]. Finally, the presence of *secY* in five constructed *secY*-RNAi *P. plecoglossicida* strains was verified by qRT-PCR [27].

2.3. Artificial infection and sampling

All laboratory animals were operated on according to the guidelines in the “Guidelines for the Care and Use of Laboratory Animals” developed by the National Institutes of Health. The animal experiments were approved by Jimei University Animal Ethics Committee (Acceptance NO JMULAC201159).

Healthy *L. crocea* of equivalent weight and size were obtained from Ningde (Fujian, China) and were kept for one week under pathogen-free laboratory conditions at a water temperature of 18 °C. To determine the survival curve, wildtype strain or RNAi strain *P. plecoglossicida* were injected into the base of the pectoral fin of *L. crocea*, with 10^4 cfu injected per gram of fish body weight. *L. crocea* in the control group were injected with PBS buffer. The water temperature during infection was 18 ± 1 °C. Then, three groups of fish were fed in the water, the symptoms were observed daily, and the survival was recorded. For tissue RNA-Seq, spleens infected with wildtype strain and RNAi strain *L. crocea* were randomly sampled at 48 h. A total of 6 spleens were harvested, and two spleens were combined per sample. For tissue distribution assays, spleen, liver, kidney, and blood from three *L. crocea* were randomly sampled at 24, 48, 72, and 96 h after injection.

2.4. DNA isolation

DNA was purified from blood samples using EasyPure Blood Genomic DNA Kit (TransGen Biotech, Beijing, China). DNA purification from spleen, liver and kidney was performed according to the instructions of the EasyPure Marine Animal (TransGen Biotech).

2.5. RNA isolation and reverse transcription

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and mixed genomic DNA in the total RNA was digested with Turbo DNA-free DNase (Ambion, Austin, TX, USA). RNA quality was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA), while the rRNA in total RNA was removed using the Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. The quality of total RNA was checked by agarose gel electrophoresis. The cDNA was synthesized by TaKaRa PrimeScript™ RT-PCR Kit (TaKaRa Bio Group, Japan) according to the included protocol. The synthesized cDNA was used as a new sample template for qRT-PCR and then stored at −20 °C until use.

2.6. Quantitative real-time PCR

qRT-PCR was performed using QuantStudio 6 Flex (Life Technologies). All primer sequences were designed using Primer Premier 5.0 and are listed in Supplemental Table 2. Reaction mixtures of 10 μL per tube (UltraFlux® i PCR 8-strip Low Profile 0.1 mL with individually attached flat cap) comprised 5 μL of qMix (Life Technologies), 0.25 μL of forward primer, 0.25 μL of reverse primer, 0.5 μL of diluted template DNA and 4 μL of nuclease-free water. The abundance of *P. plecoglossicida* in each sample was estimated using the copy number of the *gyrB* gene. Gene expression of *P. plecoglossicida* and of *L. crocea* were normalized using the 16S RNA gene and the *β-actin* gene, respectively. Three replicates were performed for each treatment, and the relative levels of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method [28]. High-throughput sequencing results were verified by qRT-PCR (Supplementary Fig. 1).

2.7. Transcriptomic analysis

2.7.1. Library preparation and sequencing

The RNA-seq libraries were prepared using protocols supplied with the TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA, USA). RNA quality was determined by a 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA samples ($OD260/280 = 1.8–2.2$, $OD260/230 \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$, $> 10\,\mu g$) were used to construct sequencing libraries. In brief, the rRNA-depleted RNA samples were fragmented in fragmentation buffer, and cDNA synthesis was conducted using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). After end repair, phosphorylation and poly (A) addition, the cDNA libraries were amplified using Phusion DNA polymerase (NEB). Sequencing was performed on the Illumina HiSeq4000 sequencing platform at Majorbio Biotech Co., Ltd. (Shanghai, China).

2.7.2. Sequence data processing and mapping of reads

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. For RNA-seq, clean data were mapped to the genome of *P. plecoglossicida* strain NB2011 (NCBI RefSeq accession number: NZ_ASJX00000000.1) using Bowtie2 [29]. Clean data were mapped to the genome of *L. crocea* (NCBI RefSeq accession number: GCF_000972845.2) using Hisat2 [30].

2.7.3. Differentially expressed genes and enrichment analysis

The software used for differential expression was edgeR, which performs differential expression calculations based on gene readcount data that is based on a negative binomial distribution model [31,32]. The screening criteria for significant differentially expressed genes were as follows: $FDR < 0.05$ and $|\log_2 FC| >= 1$. The differentially expressed genes were then subjected to enrichment analysis by hypergeometric distribution testing using Goatools software (https://github.com/tanghaibao/goatools) and KOBAS [33,34] (http://kobas.cbi.pku.edu.cn/home.do).

2.7.4. Statistical analyses

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

2.8. Data access

The RNA sequence data were deposited at the GenBank SRA database under the accession numbers SRP190337 and SRP176599.

3. Results

3.1. Construction of RNAi strains

Expression levels of *secY* were significantly downregulated in the five RNAi strains, at 24.1%, 27.3%, 41.9%, 17.6%, and 26.6% of the wildtype levels. Among them, *secY*-RNAi-1165 had the lowest expression level of *secY* and the best silencing effect; therefore *secY*-RNAi-1165 (hereafter referred to as *secY*-RNAi) was selected for subsequent experiments (Fig. 1a). Although the *secY* gene was silenced, the growth curve was not significantly different from that of the wild strain (Fig. 1b).

3.2. Effect of the *secY* gene on the survival of *L. crocea*

Compared with infection with the wildtype strain, the death time of *L. crocea* injected with the *secY*-RNAi strain was delayed by 2 d, and the mortality was reduced by 35% (Fig. 2a). *L. crocea* had a large number of white nodules on the surface of the spleen 60 h after injection with the wildtype strain, while the spleen symptoms of *L. crocea* injected with the *secY*-RNAi strain were significantly lighter, with only a small number of unremarkable white spots (Fig. 2b). The difference in abundance between wildtype strain and silencing strain in *L. crocea* tissues varied by organ and by time. Although both strains increased in *L. crocea* tissue with infection time, at most points after injection, the abundance of the *secY*-RNAi strain in spleen, liver, kidney and blood was significantly reduced compared to the wildtype strain. At 72 h, the abundance of the *secY*-RNAi strain in the spleen was close to that of the wildtype strain (Fig. 2c). In both strains, *secY* gene expression was higher in vivo than in vitro. The expression level of the *secY* gene in the wildtype strain-infected spleen peaked at 48 hpi and then gradually decreased. During infections with the *secY*-RNAi strain, expression gradually increased until 96 hpi (Fig. 2d).

3.3. Analysis of transcriptome data of spleen of *L. crocea* after infection

3.3.1. Quality of transcriptional data

High quality sequencing results were the basis for the transcriptome data analysis. The A/T/G/C base content was evenly distributed, and N % is within a reasonable range. Q20 was the main criterion for assessing the quality of reads, with $Q20 > 97\%$ for the sequencing data of each sample. The base error rate of the sequencing data was $< 0.1\%$. Among three replicate samples, $r > 0.9$ (Fig. 3a).

3.3.2. Differential expression gene analysis

In total, 27,520 mRNAs were obtained from the transcriptome of the *L. crocea* spleen infected with the *secY*-RNAi strain. The software used for differential expression analysis was edgeR, and the screening criteria for significant differentially expressed genes were $FDR < 0.05$ and $|\log_2 FC| >= 1$. Compared with the spleen infected with the wildtype strain, the spleen infected with the *secY*-RNAi strain had 1189 differently expressed mRNAs, of which 432 mRNAs were upregulated and 757 mRNAs were downregulated (Fig. 3b). The heat map shows genes whose expression levels vary significantly by more than two folds for each sample. Among the set of differentially expressed genes, the top 50 upregulated and downregulated genes were selected according to the $\log_2 FC$ (Fig. 3c). The gene with the greatest fold change among the upregulated genes was *LOC104939330* ($\log_2 FC = 7.09$); among the downregulated genes, *LOC*104922235 had the greatest change ($\log_2 FC = -6.9$).

Following the GO annotation conventions, DEGs fall into three categories: biological processes, cellular components, and molecular functions. A total of 340 GO terms were enriched, of which 87 were significantly enriched. Statistical analysis of the top 10 enriched terms and of the DEGs of the three types of GO shown in Fig. 4 shows that the GO terms peptidase activity, acting on L-amino acid peptides; extracellular region; and immune response occurred significantly after *P. plecoglossicida* infection. Most of the differentially expressed genes annotated with these GO terms were significantly downregulated.

According to the KEGG database, a total of 280 KEGG pathways were enriched, of which 34 were significantly enriched. Most of the DEGs represented 16 immune-related KEGG pathways. All of these 16 pathways were altered in the spleen infected with the *secY* RNAi strain compared to the spleen infected with the wild type strain (Fig. 5a). According to the P-value of Fisher's exact test, enrichment of ko04620, which represents the Toll-like receptor signalling pathway, was significant. In addition, ko04610, the Complement and coagulation cascades pathway, had the greatest number of differentially expressed genes and had a higher ratio than the other pathways.

According to the calculated Complement and coagulation cascades heat map (Fig. 5b), there were 18 differentially expressed genes in the spleen that were enriched in the this pathway. Among them, 15 genes were significantly down-regulated in the RNAi strain-infected

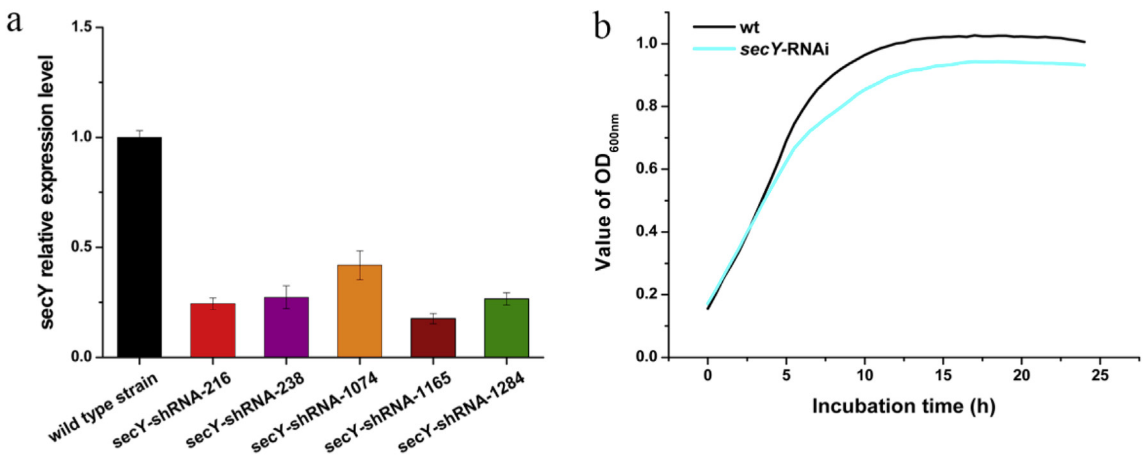

Fig. 1. Construction of RNAi strains of *P. plecoglossicida*. (a): The *secY* expression level of 5 *secY*-RNAi silencing strains. (b): Growth curves of *P. plecoglossicida* strains.

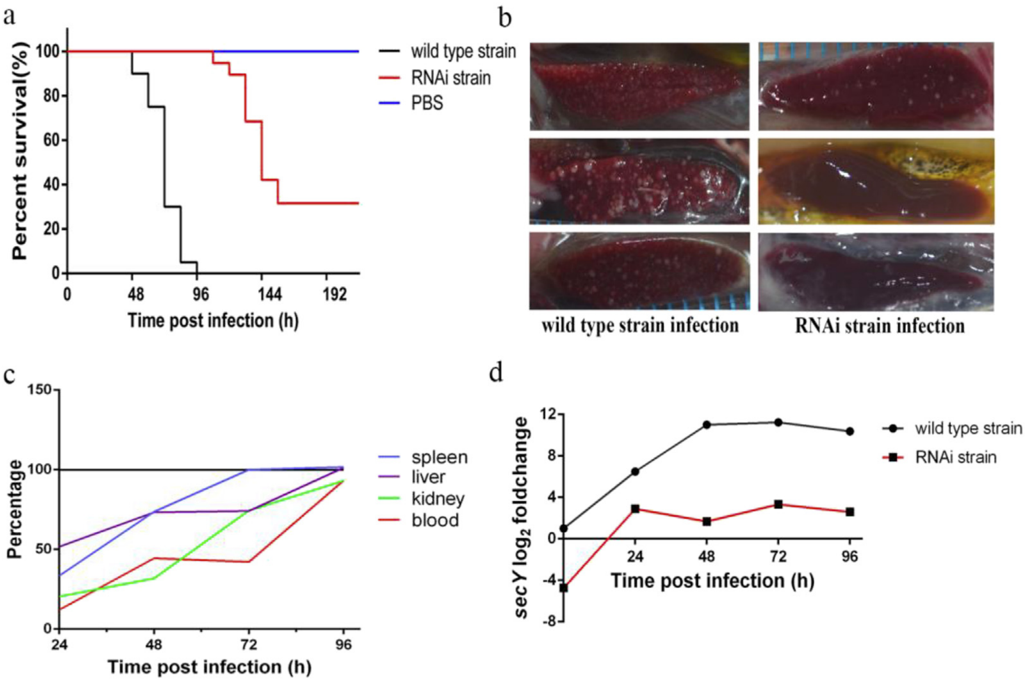

Fig. 2. The virulence of *P. plecoglossicida* to *L. crocea.* (a): Survival rate of *L. crocea* infected by *P. plecoglossicida*. (b): Symptoms of spleen after infection of *L. crocea* by *P. plecoglossicida* (c): Spatial and temporal relative distribution of the *secY*-RNAi strain compared to the wildtype strain. (d) Expression levels of the *secY* gene in the *secY*-RNAi strain and the wildtype strain at 18 °C in vitro and in spleen at different time points after injection.

compared to wildtype strain-infected spleens (*c8b, LOC104937094, LOC104936275, LOC104938602, LOC104928194, LOC104936421, plat, LOC104926132, c6, LOC104927237, LOC104919991, LOC104922750, LOC104930424, LOC104936957, LOC104926791*), with only 3 up-regulated (*LOC104928322, LOC104938336, LOC104938335*). According to the Toll-like receptor signalling pathway heat map, spleen infected with silencing strains showed significant changes in the TLR signalling pathway, with only MKK3/6 and MKK1/2 upregulated while TLR5 and inflammatory cytokines IκBα, AP -1, TNFα, IL-1β, IL-6, IL-12, IL-8, and MIP-1β were downregulated (Fig. 6).

3.4. The host-responsive mRNA repertoire of P. plecoglossicidais

3.4.1. Quality of transcriptional data

The A/T/G/C base content was evenly distributed, and N% is within a reasonable range. Q20 was the main criterion for assessing the quality of reads, with Q20 > 97% for each sample sequencing data, the base error rate of the sequencing data was < 0.1%. Reproducibility of three biological replicates was satisfactory.

3.4.2. Differential gene expression analysis

The software used for differential expression analysis was edgeR, and the screening criteria for significant differences in gene expression were as follows: FDR < 0.05 and $|\log_2 FC| > = 1$. In total, 4988 mRNAs were obtained from the spleen transcriptome of *L. crocea* infected with *secY*-RNAi strain. Compared with the spleen infected with the wildtype strain, the spleen infected with the *secY*-RNAi strain had 115 mRNAs with expression differences, among which 7 were upregulated and 108 were down regulated (Fig. 7a). The heat map shows genes whose expression levels vary significantly by more than two-fold for each sample. According to the $\log_2$FC, all differential genes that were up-regulated and down-regulated were selected. The gene with the greatest fold change among the up-regulated genes is *L321_RS17380* ($\log_2$FC = 4.48); among the downregulated genes, *L321_RS*18300 had the greatest change ($\log_2$FC = −15.38) (Fig. 7b). The protein export transport channel pathway map of the “Sec secretory system” of *P. plecoglossicida* in the host spleen is known (Fig. 7c), and the expression of *secY* ($\log_2$FC = −2.62) and of *secG* ($\log_2$FC = −3.44) are significantly down-regulated compared to the wildtype strain.

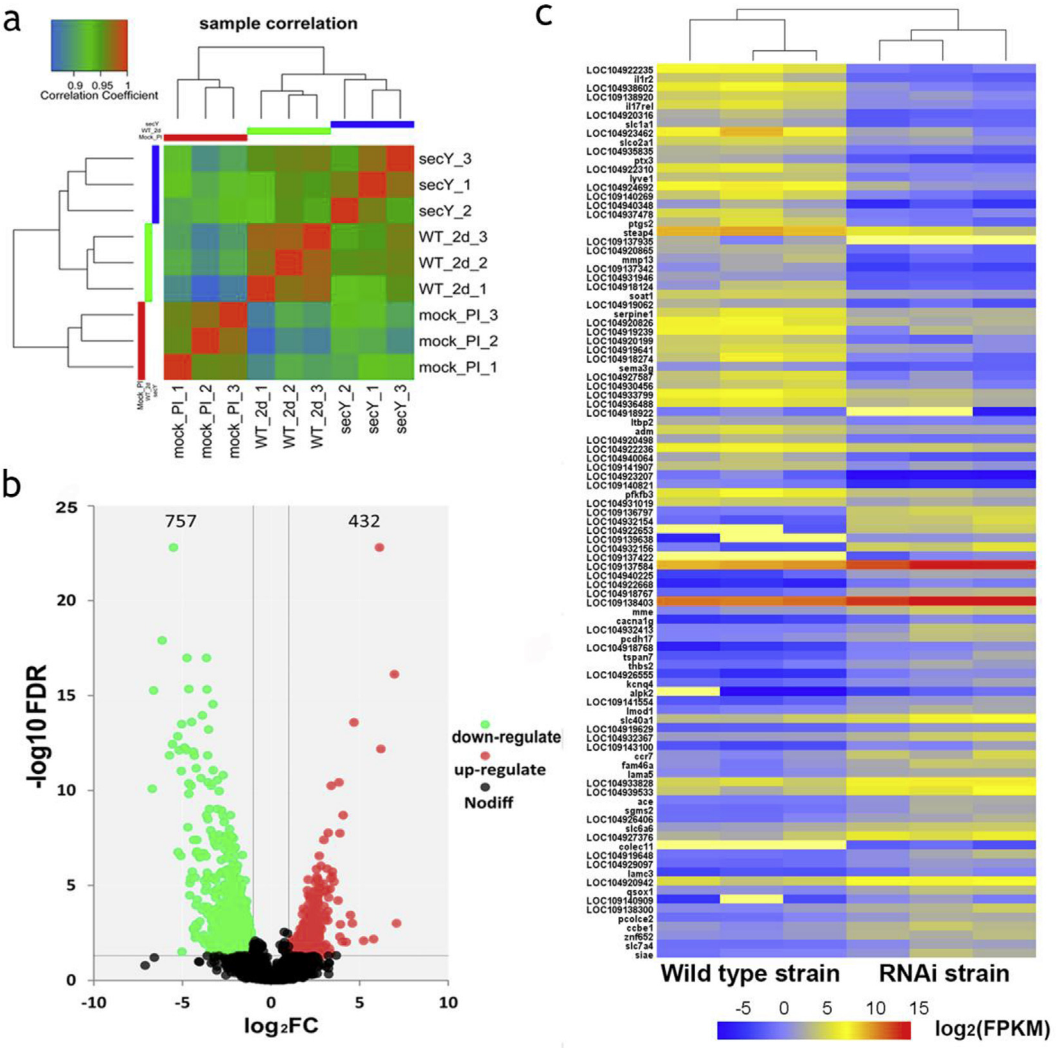

Fig. 3. Comparative transcriptional analysis of *L. crocea* spleens. (a): Reproducibility of transcriptional data (The Pearson correlation coefficient (r) > 0.9). (b): Volcano plot obtained from edgeR analysis of infected *L. crocea* RNA pools. (c): Heat map of the top 50 upregulated and downregulated genes from host transcripts (FDR < 0.05, $|\log_2 FC| \geq 1$).

4. Discussion

RNA interference can be used to experimentally manipulate gene expression and determine gene function on a genome-wide scale [35,36]. The application of RNA interference technology to different aquatic pathogens helps to explore their pathogenic mechanisms [37,38]. In this study, RNA interference technology targeting five different sites was used to stably silence *secY*. The results showed that the five shRNA silencing strains significantly reduced the content of *secY* mRNA. The best silencing strain was *secY*-shRNA-1165, with a silencing efficiency of 82.4%; however, the growth rate of the *secY*-RNAi strain compared to the wildtype strain was only slightly decreased, and this suggests that the effect of *secY* on the pathogenicity of *P. plecoglossicida* is not simply due to a defect in growth. This strain was selected for subsequent research.

Previous studies have shown that many genes are involved in the virulence regulation of aquatic pathogenic bacteria. For example, *oppABCDF* contributes to the pathogenesis mechanisms of *Vibrio alginolyticus*, including adhesion, biofilm production and haemolytic activity [39]. In this study, we constructed a stable *secY*-silencing strain and artificially infected *L. crocea*. The results showed that, compared with *L. crocea* infected with the wildtype strain, the time of death was delayed, the mortality rate was significantly reduced, and the symptoms of visceral white spot disease were significantly improved; there were only a few, slightly white nodules appearing in the spleen and a density that was significantly reduced. At the same time, the distribution of the *secY* RNAi strain in the spleen, liver, kidney and blood of the host was significantly lower than that of the wildtype strain. In addition, the expression level of *secY* was very high during the process of wildtype-strain infection. These results suggest that *secY* may be involved in the regulation of virulence and infection of *P. plecoglossicida*.

Invasion by the pathogen will cause a host immune response, which will affect the host's transcriptome, and the pathogen will also change its own transcriptome [11,40]. Transcriptome analysis is an important method for understanding the mechanisms of pathogens [41,42]. In this study, compared with wildtype levels of *secY*, silencing of *P. plecoglossicida secY* resulted in significant changes in the transcriptomes of infected *L. crocea*. There were 1189 significant differences in the expression of host genes in the spleen; 432 were upregulated and 757 were downregulated in the *secY*-RNAi strain infections compared to the wild type infections. At the same time, the transcriptional profile of *P. plecoglossicida* in the spleen was significantly changed. Compared with wildtype strains in the spleen, the expression of 115 genes by the *secY*-RNAi strain were significantly different; 7 were upregulated and 108 were downregulated. The results showed that the *secY* gene of *P.*

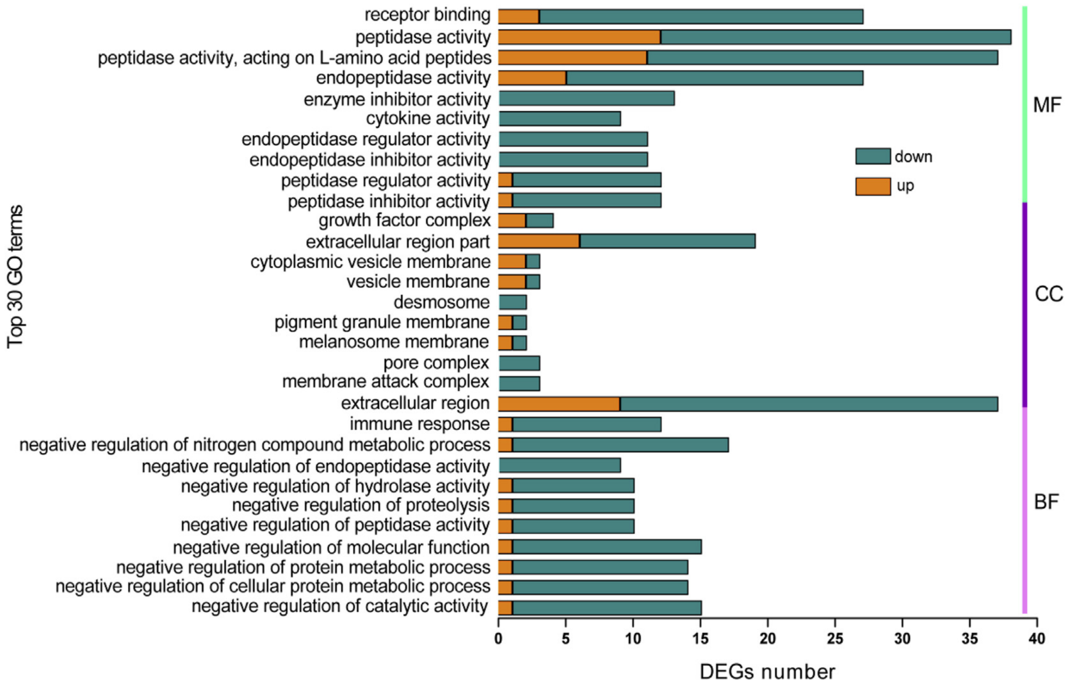

Fig. 4. Gene ontology (GO) enrichment for differentially expressed genes (DEGs).Blue indicates a GO term for a differentially expressed gene that was down-regulated, and orange indicates a GO term for a differentially expressed gene that was upregulated. The horizontal axis represents the number of differentially expressed genes, and the vertical axis represents the term name.

plecoglossicida plays an important role in infection of *L. crocea*.

Enrichment analysis of GO functions of differentially expressed genes can identify the enrichment of different gene functions and clarify the differences between samples at the level of gene function [43,44]. The results of the GO enrichment analysis of the host are shown in Fig. 5. Compared to spleen injected with the wildtype strain, most of the GO-enriched differentially expressed host genes were significantly downregulated in spleen injected with the RNAi strain. KEGG analysis of infected spleens showed significant enrichment of 16 immune-related pathways. Prominent among them are the Toll-like receptor signalling pathway (ko04620) and the Complement and coagulation cascades pathway (ko04610). Toll-like receptors (TLRs) are important

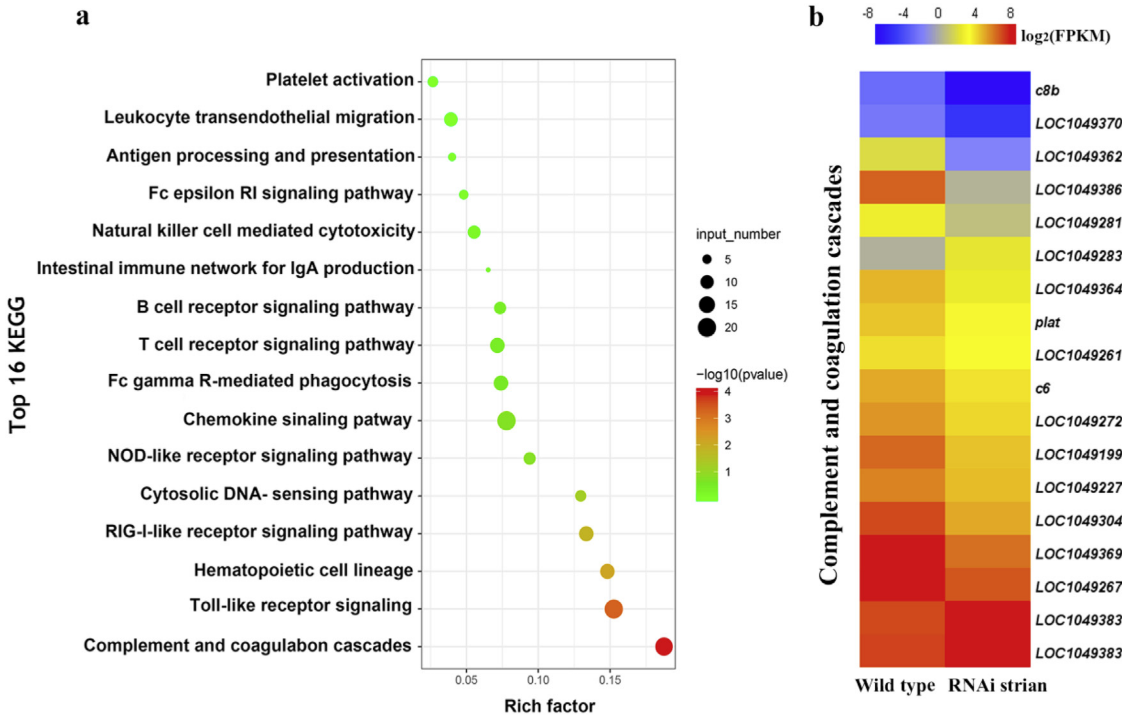

Fig. 5. KEGG pathway analysis of host spleen.
(a): Bubble graph of the top 16 enriched immune-related KEGG pathways; the x-axis represents the enrichment factor, and the y-axis indicates the corresponding KEGG pathway. (b): Heat map of enriched genes in Complement and coagulation cascades.

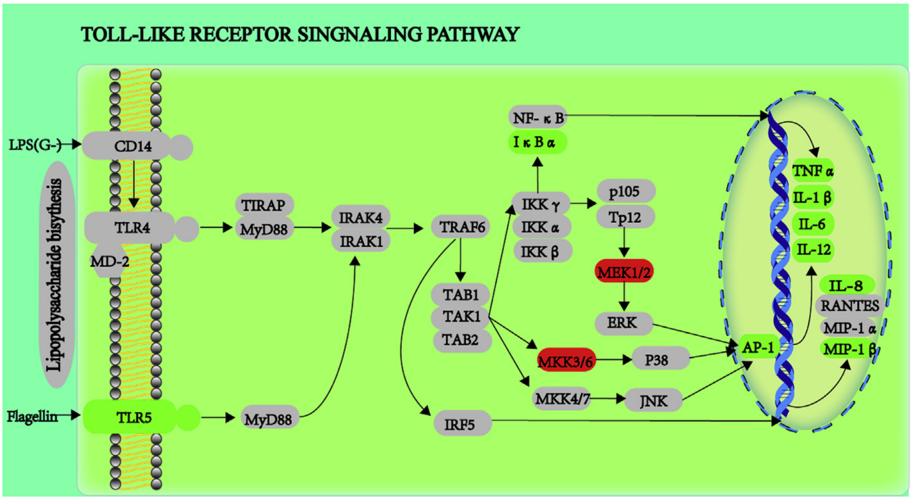

Fig. 6. TLR signalling pathway of *L. crocea*.Compared to wild type strains, the up-regulated genes and down-regulated genes of the RNAi strains are indicated by red and green rectangular boxes, respectively; grey rectangle indicates no significant change.

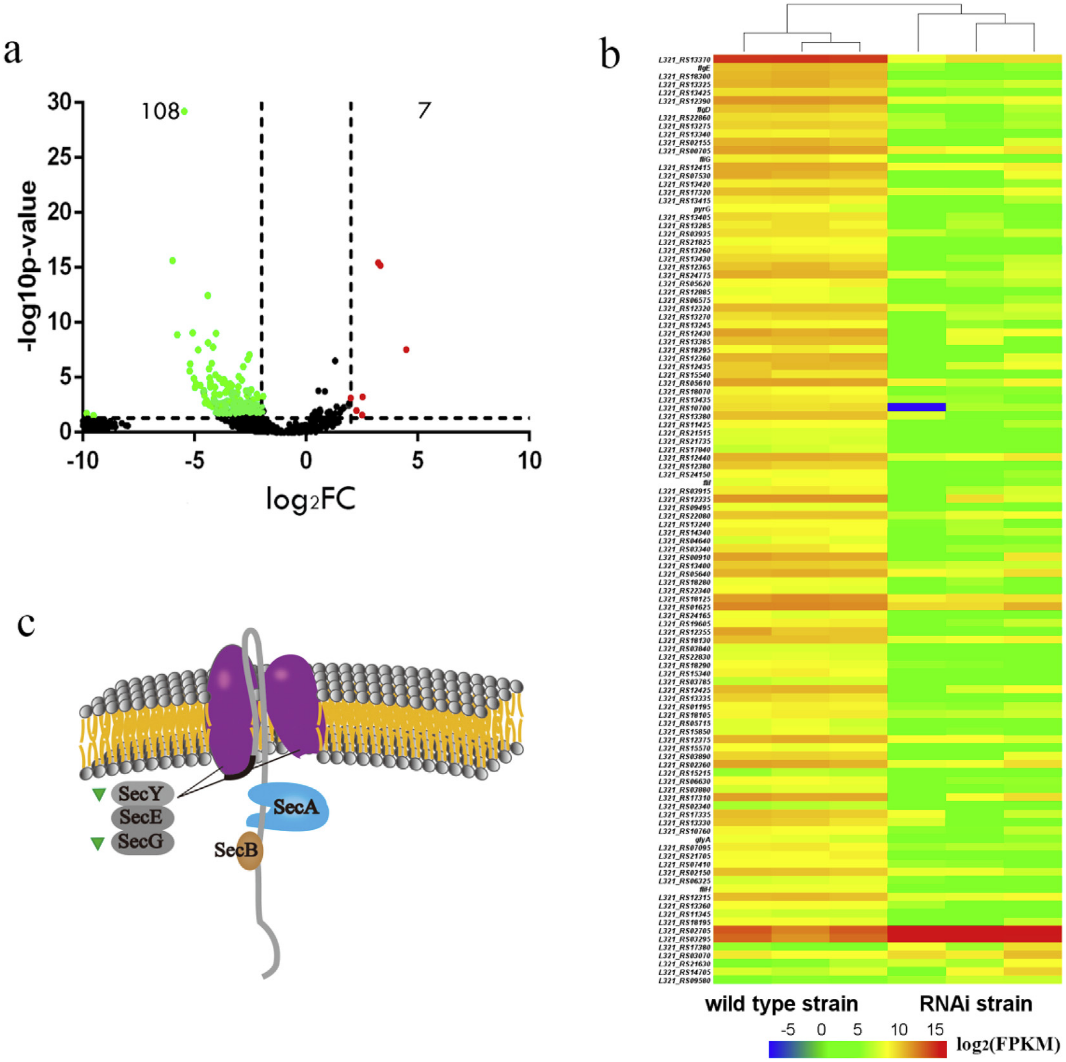

Fig. 7. DEGs enrichment analysis of pathogen transcriptome data. (a): Volcano plot obtained from edgeR analysis of infected *L. crocea* RNA pools. (b): Heat map of differentially expressed genes based on transcripts of bacterial strain in the host spleen (FDR < 0.05, $|\log_2 FC| \geq 1$), (c): Protein export transport channel pathway map of the "Sec secretory system" of *P. plecoglossicida* in the host spleen; *secY*, *secE* and *secG* form the SecYEG complex. The red triangle on the left side of the gene indicates that the gene was up-regulated, and the green triangle indicates that the gene was down-regulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and trigger signalling pathways, which activate immune cells to respond to pathogen infections [45,46]. Compared with *L. crocea* infected with the wildtype strain, in RNAi strain infections, the expression of inflammatory cytokines was significantly downregulated in the MyD88-dependent branch of the TLR signalling pathway, including TNFα, IL-1β, IL-6, IL-12, IL-8, MIP -1β. Among them, TNFα, IL-1β, IL-6 and IL-12 are indirectly linked to pro-inflammatory effects, and IL-8 and MIP-1β are indirectly linked to chemotactic effects. The complement system, a highly regulated network that helps antibodies clear pathogens, interacts with the coagulation system and with “first line defences” during microbial invasion [47–49]. Most of the differentially expressed genes in the Complement and coagulation cascades pathway were significantly downregulated in spleens infected with RNAi strains compared to spleens infected with wildtype strains. In addition, compared with the wildtype strain in the host spleen, the expression of *secE* ($\log_2 FC = -3.44$) was significantly downregulated in addition to *secY* ($\log_2 FC = -2.62$) in the RNAi strain. This may be because the SecYEG complex is the transport channel of “Sec secretion system” [50]. As the largest protein in the complex, SecY constitutes the core of the translocation machinery and plays a key role in the process of substrate output [51,52]. The above results may be due to the fact that the immune response of *L. crocea* infected with the RNAi strain is stimulated less intensely because the virulence of the silenced strain is reduced.

In conclusion, *secY* is a virulence gene of *P. plecoglossicida* that contributes greatly to the virulence of *P. plecoglossicida* during the infection of *L. crocea*. Silencing of the *secY* gene affects the “Sec secretion system” of the pathogen, thereby reducing the virulence of the pathogen during the infection of the *L. crocea*. Infection of *L. crocea* with the *secY*-RNAi strain resulted in a downregulation of genes in the host's Toll-like receptor signalling pathway and Complement and coagulation cascades pathway compared to infection with the wild-type strain.

Conflicts of interest

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

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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.2019.08.040.

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