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Comprehensive transcriptomics and proteomics analysis of *Carassius auratus* gills in response to *Aeromonas hydrophila*

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ABSTRACT

As one of the mucosal barriers, fish gills represent the first line of defense against pathogen infection. However, the exact mechanism of gill mucosal immune response to bacterial infection still needs further investigation in fish. Here, to investigate pathological changes and molecular mechanisms of the mucosal immune response in the gills of crucian carp (*Carassius auratus*) challenged by *Aeromonas hydrophila*, the transcriptomics and proteomics were performed by using multi-omics analyses of RNA-seq coupled with iTRAQ techniques. The results demonstrated gill immune response were mostly related to the activation of complement and coagulation cascades, antigen processing and presentation, phagosome, NOD-like receptor (NLR) and nuclear factor κB (NFκB) signaling pathway. Selected 21 immune-related DEGs (ie., *Clam, nfyal, snrpf, acin1b, psme, sf3b5, rbm8a, rbm25, prpf18, g3bp2, snrpd3l, tecrem-2, cfl-A, C7, lysC, ddx5, hsp90, α-2M, C9, C3* and *slc4a1a*) were verified for their immune roles in the *A. hydrophila* infection *via* using qRT-PCR assay. Meanwhile, some complement (C3, C7, C9, CFD, DF and FH) and antigen presenting (HSP90, MHC II, CALR, CANX and PSME) proteins were significantly participated in the process of defense against infections in gill tissues, and protein-protein interaction (PPI) network displayed the immune signaling pathways and interactions among these DEPs. The correlation analysis indicated that the iTRAQ and qRT-PCR results was significantly correlated (Pearson's correlation coefficient = 0.70, $p < 0.01$). To our knowledge, the transcriptomics and proteomics of gills firstly identified by multi-omics analyses contribute to understanding on the molecular mechanisms of local mucosal immunity in cyprinid species.

1. Introduction

Fish mucosal immunology has been paid extensive attention because it plays a very critical role in the innate immunological system [1,2]. Among all the mucosal tissues, the gills are multifunctional tissue and represent the first line of defence and primary surface barrier against the invasion of pathogens [3]. In fish, gills continuously exposure to pathogens owing to the direct contact with microbial-rich water environment, and its molecular immune mechanism need to be further explored for the mucosal defences against the pathogenic attacks [4]. In recent decades, the gene expressions of gills upon pathogen infection were reported in fish, and the immune responses of gills were investigated at the mRNA and protein levels [2,5–7]. The evidences indicated that fish gill tissue provides major point of entry for pathogens and attachment sites [8]. And a great deal of immune-relevant genes participate in the gill local immune response, adding its most important function for immune response and survival in fish [6,9,10]. Previous studies demonstrated that several key immune-related genes such as tumor necrosis factor [6], interleukins [11] , heat shock proteins [12], major histocompatibility complex [9] and complements [10,13,14] were identified with respect to the involvement in immune defenses of gills. Nevertheless, the precise mechanisms of gill immune response to bacterial-infection were still unclear in fish, and applying in-depth research and analysis to achieve better outcomes were required for the immunological system of the cyprinid species, and signaling pathways implicated in its immune response following exposure to the bacteria [8].

In recent years, a variety of molecular techniques have developed rapidly, in which the multi-omics (e.g., genomics, transcriptomics, proteomics, metabolomics) were extensively used to explore the mucosal immune response in fish species upon different infections [13,15,16]. Recently, the sequencing-based high-throughput analyses (eg.,

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RNA-seq, iTRAQ) were massively applied to characterize gene expression profiling in detail and its involvment in immune response in fish, and allow an in-depth understanding of gill mucosal immune processes [2,7,17]. Recently, several studies have reported abundant transcriptome analyses of gills from flounder *Paralichthys olivaceus* [2], rainbow trout *Oncorhynchus mykiss* [13], atlantic salmon *Salmo salar* [17] and yellow croaker *Larimichthys crocea* [18]. Notably, the most researches were mainly focused on identification of immunologically relevant genes and significantly contributed to better understanding the mechanisms of the host systemic immunity to pathogen infection [16], and a few descriptions at proteomic level was illustrated for the mucosal local immune mechanism [15,19]. However, the lack of proteomic techniques was applied for mucosal immunity, recently with an increased interest in fish [5,20]. To date, proteomic approaches (eg., iTRAQ, LC-MS/MS) were used to investigate changes in differentially expressed proteins in fish gills [20–22]. In our previous studies, the comparative proteomic profiling in the gills of zebrafish infected with *A. hydrophila* infection was investigated with iTRAQ and LC-MS/MS analysis [5]. To date, the mucosal immune mechanism in the gills of *C. auratus* upon bacterial infection has not been identified whether in transcript or protein levels.

Crucian carp (*Carassius auratus*) is a primary freshwater fish species in aquaculture around the world, owing to its good disease resistance, high reproduction and strong survivability [23,24]. In recent years, the outbreak cases of infectious disease were becoming more frequent in crucian carp *C. auratus* in China [23,25]. In fish, the Gram-negative bacterium *A. hydrophila* is considered to be one of the important pathogens that causes septicaemia and high mortality in *C. auratus* [26]. As an opportunistic pathogen of fish and terrestrial animals, it is thereby bringing tremendous economic losses [5,27]. Although *C. auratus* is highly susceptible to bacterial pathogenes, the exploration of immunity at the molecular level is little investigated by multi-omic approaches [28]. Recently, our group studies have reported the gene differential expression profiling in skin immune response of *C. auratus* during *A. hydrophila* infection [29,30]. However, the mucosal immune mechanism of gills at transcriptomic and proteomic levels in fish against *A. hydrophila* infection, are still poorly understood so far.

2. Materials and methods

2.1. Experimental challenge and samplings

Crucian carp (*C. auratus*, average weight 50 g) were cultured and acclimated 14 days under the laboratory conditions for artificial infection, and maintained at 25 ± 2 °C in a flowthrough water system. Fishes were challenged with *A. hydrophila* by immersion as our previously reported by Wang et al. [30], and fed with commercial dried pelleted feed (Tongwei, China) twice daily. Briefly, sixty fish were randomly chosen and averaged to two groups namely the control group and infected group, respectively. For the challenge, the fish were immersed in 1.0×10^8 colony forming unit (cfu) per milliliter of *A. hydrophila* for three hours, and then the inoculated fish were transferred to freshwater culture for the infection. Control fish were incubated in freshwater. Nine control and infected fishes were randomly selected, and gill tissues samples were aseptically excised and collected, frozen immediately in liquid nitrogen and finally stored at -80 °C in order to extract total RNA. All procedures involving the handling and treatment of fish used during this study were approved by the Tianjin Agricultural University Institutional Animal Care and Use Committee (TJAU-IACUC) prior to initiation.

2.2. Histopathologic examination

Fishes were randomly selected and dissected for histological examination. After deep anesthesia with MS-222 (200 mg/L), the gill tissues samples were aseptically collected and fixed in 10% formalin for 24 h, respectively. The tissue samples were dehydrated by using ascending concentrations of 70-100% ethanol, cleared in xylene and embedded in paraffin wax. The 5 μm thickness sections were cut, then stained with hematoxylin and eosin (H&E) and examined with a light microscope (Leica DM 5000, Germany).

2.3. RNA extraction, library construction and sequencing

RNA extraction and library construction were performed as our previously described by Bai et al. [31]. Briefly, total RNA of gill samples was extracted by using Trizol reagent and further purified with the RNeasy Mini kit (Qiagen). RNA degradation, purity and integrity were checked by using the Agarose electrophoresis, NanoPhotometer® spectrophotometer (NanoDrop). Two cDNA libraries (tGC and tGT) from gill samples of the control group and infected group (6 and 12 hpi) were subsequently sequenced on the Illumina Hiseq 2000 platform. To minimize the differences within group, three RNA samples of each treatment were pooled together as one replicate for library construction.

2.4. Transcriptome assembly and annotation, and DEGs analysis

After library construction, the transcriptome, *de novo* transcriptome assembly were carried out prior to quantification, and generated by BGI Genomics Co., Ltd (Shenzhen, China). The annotation of general unigene sequences were searched and obtained with KEGG, GO, Nr, Swiss-Prot and COG online databases. The expression data of each transcript were obtained, and FDR ≤ 0.001 and the absolute value of $\text{Log}_2\text{Ratio} \geq 1$ were used as a threshold to screen the significant differentially-expressed genes (DEGs).

2.5. Protein extraction, iTRAQ and screening of DEPs

Gill samples of control and infected fishes were used to extract protein. Protein extraction and isobaric tags for relative and absolute quantification (iTRAQ) labeling were performed as previously described [5,32]. The protein concentration was determined using the BCA protein assay kit (Sangon Biotech, China) and the proteins were visualized by SDS-PAGE to determine their quality. The iTRAQ analysis, protein identification and quantification were performed at Genomics Co., Ltd (Shenzhen, China). For protein quantitation, the ratios with p-values < 0.05 and fold changes >1.2 were considered significant differentially expressed proteins (DEPs).

2.6. GO and KEGG enrichment analysis

To better understand the functional relevance of the DEGs and DEPs, these were mapped to Gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) database, respectively. GO and KEGG functional enrichment analyses were performed for DEGs and DEPs with differential expression, according to our recently reported by Bai et al. [29].

2.7. qRT-PCR and statistical analysis

Quantitative real-time PCR (qRT-PCR) analysis was used to verify the gene expression from the transcriptomic analysis, according to our recently reported by Liu et al. [32]. Primer sequences of the selected gene were designed using the Primer Premier 6 software (Supplemented Table 1). According to our previous studies [31], because of the stable expression of *GAPDH* in *C. auratus,* it was chosen as an internal reference. The qRT-PCR primers were synthesized by Shenggong Bioengineering Technology Limited (Shanghai, China). All qRT-PCR were run with three biological replicates on a CFX 96™ Real-Time PCR Detection System (Bio-Rad, USA) in 96-microwell plates. The thermal profile for the qRT-PCR was 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C and 1 min at 60 °C. Relative gene expression was calculated using the

formula $2^{-\Delta\Delta Ct}$ [33]. Data analysis was performed using the SPSS 22.0 software (Chicago, USA), and Pearsons analysis was adopted for inter-factor correlations.

3. Results

3.1. Pathological changes of gills in A. hydrophila-infected C. auratus

The moribund crucian carp (*C. auratus*) infected with *A. hydrophila* displayed the hyperemia, hemorrhage, and inflammatory lesion of mucosal tissues (gills, skin and intestine), and congestion of visceral organs. Pathologic examination revealed a large number of inflammatory cell infiltration in the base of gill lamellae, and the respiratory epithelial cells of secondary gill lamellae were hyperplasia, sloughing and necrosis in the infected fish, and no obvious pathological changes were observed in the control group fish (Fig. 1).

3.2. Gill transcriptome, and identification of differentially expressed genes (DEGs)

Transcriptome analysis in the gills of crucian carp infected with *A. hydrophila* was performed by using RNA-seq technique. The results showed a total of 104,213 unigenes, with the N50 obtained for 928 bp and 1798 bp, respectively. The total annotation unigenes of 84259 were identified, of which 81029, 55283, 46029, 33388, 16331 and 39303 unigenes were respectively annotated to the NT, NR, Swiss-Prot, GO, COG and KEGG database. Moreover, the expression levels of DEGs in gill tissues after the bacterial challenges were compared with the control fish. Among these unigenes, lots of genes were found to be differently expressed following *A. hydrophila* infection (Fig. 2A-B). Overall, in the gill of *C. auratus*, the detected 3842 DEGs were 1829 up- and 2013 down-regulated genes compared with the control fish. Meanwhile, the differentially expressed proteins (DEPs) between control and infected-fish were also presented in Fig. 2C-D, respectively.

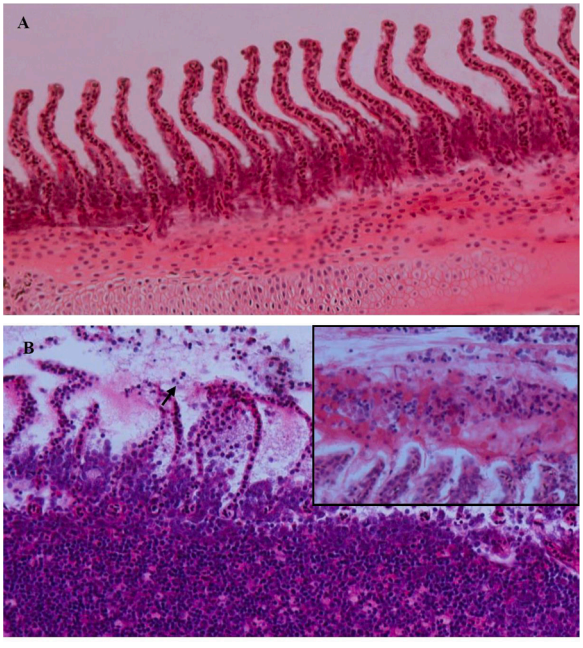

Fig. 1. Pathological changes of *C. auratus* infected with *A. hydrophila*. (A) Control fish gills. (B) Infected fish. Exfoliation and necrosis of gill respiratory epithelial cells (black arrow), hyperemia and extensive inflammatory cell infiltration (rectangle).

3.3. GO and KEGG enrichment analysis of the DEGs

GO analysis was used to classify the functions of DEGs, and enrichment annotated in the GO database showed that most of DEGs were divided into three major functional grouping terms, such as biological process, cellular component and molecular function (Supplemented Fig. 1). Among them, biological regulation, metabolic, cellular process, response to stimulus and regulation of biological process were dominant in biological process terms. In the cellular composition category, the dominant terms were distributed in the membrane, organelle part, organelle, cell part and cell. Beside, for the molecular functional annotation they were predominantly mapped for molecular binding and catalytic activity.

In order to further prove the biological pathways involved in the gills of crucian carp challenged by *A. hydrophila* infection, the DEGs were mapped to the KEGG pathway database. Based on the KEGG signaling pathway enrichment analysis, it indicated that 1397 DEGs were identified in 236 known KEGG pathways. A total of 27 immune-related pathways were enriched in the gill local immune responses including cell adhesion molecules (102 genes; 7.30%), phagosome (99 genes; 7.10%), natural killer cell mediated cytotoxicity (88 genes, 6.30%), graft-versus-host disease (83 genes, 5.94%), autoimmune thyroid disease (83 genes, 5.94%), antigen processing and presentation (82 genes, 5.87%), NLR signaling pathway (55genes, 3.93%), cytokine-cytokine receptor interaction (49 genes, 3.51%), NF-kappa B signaling pathway (44 genes, 3.15%), chemokine signaling pathway (43 genes, 3.08%) and Toll-like receptor signaling pathway (31 genes, 2.22%), of which were prominently presented in Table 1.

3.4. DEPs identified in the gills of C. auratus infected with A. hydrophila

A total of 423,057 peptide spectra were measured by using the iTRAQ coupled with LC-MS/MS analysis, and then of these 65,186 MS/MS spectra were matched against NCBI protein sequence database. In addition, 8587 peptides and 7239 unique peptides were availably identified, and ultimately a total of 3082 proteins were validated in the tested gills of fish. Identification of the significant DEPs between the detected control and infected-fish gill samples will provide insight into the biological function of the gills in mucosal immunity. In gill protein significantly expression change after the infection of *A. hydrophila*, we found that 430 DEPs reliably quantified and included 177 up-regulated and 253 down-regulated proteins by iTRAQ identification. The data produced by LC-MS/MS were analyzed to identify significantly differentially expressed related proteins, more than 50 immune-related proteins were obtained, mainly including Caln, CaM, CALR, C3, C7, C9, A-2M, parvalbumin 6, Hsp90, RNA-binding protein, splicing factor 3A, ITLN, Psme2, small nuclear ribonucleoprotein, Lys C and so on (Table 2). For example, most immune-relevant DEPs were involved in the complement activation and coagulation cascades (ko04610), antigen processing and presentation (ko04612), regulation of actin cytoskeleton (ko04810) and focal adhesion (ko04510) signaling pathways. Among them, several representative immune response pathways were the significantly enriched signaling pathways of the crucian carp gills identified by KEGG analysis (Fig. 3).

3.5. Functional annotation and classification of DEPs

The DEPs were classified into three main groups by using GO enrichment analysis, such as cellular components, molecular functions and biological processes. The top 32 GO enrichment subclasses were presented in Fig. 4. The DEPs proteins were primarily involved in the GO biological processes including single-organism, multicellular-organism, cellular process, organic substance, regulation of biological processes and metabolic process (Fig. 4A). For the cellular components, cells and cell parts were the two largest subcategories of cell components, followed by cytoplasm, organelle, intracellular organelle and membrane-

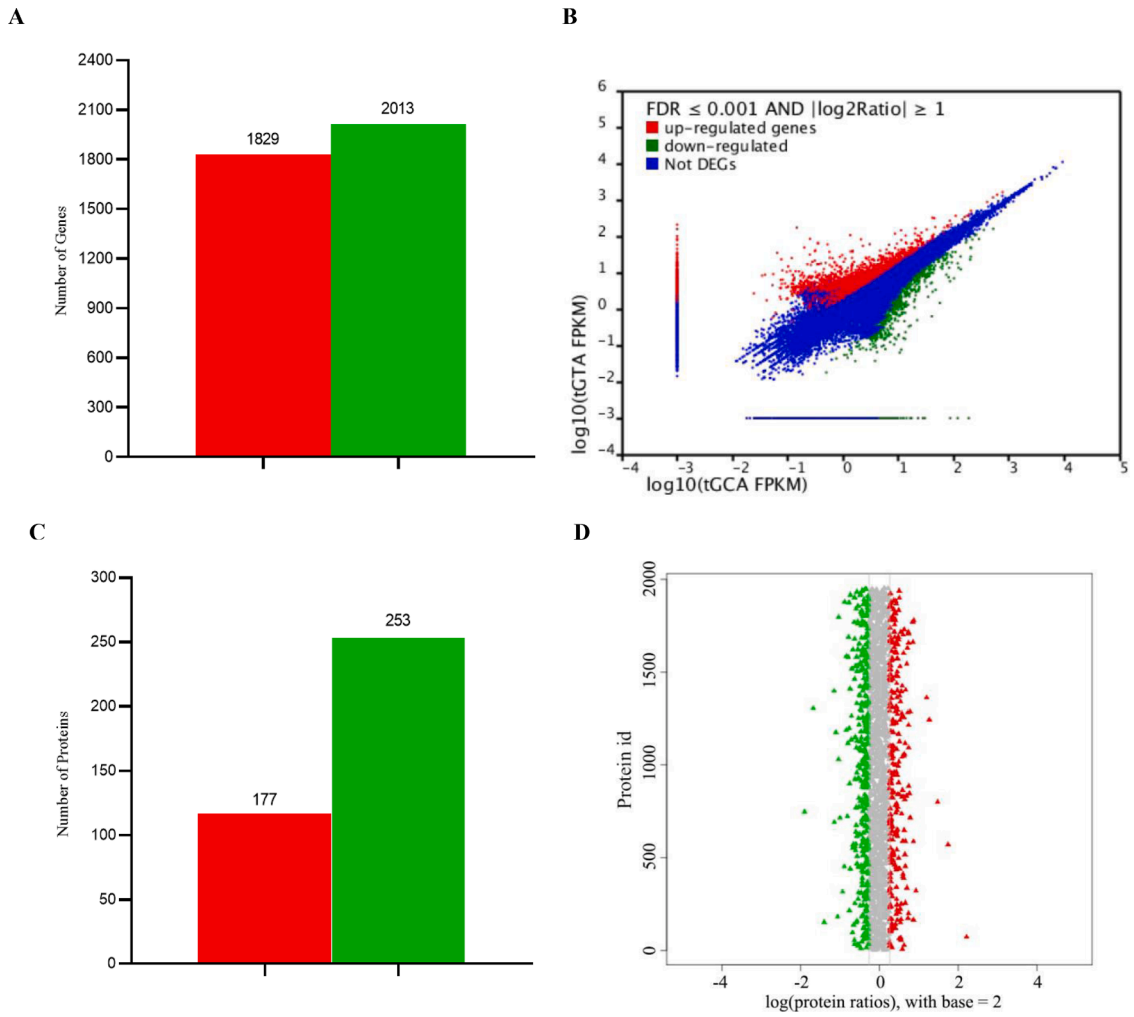

Fig. 2. Differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) between control and *A. hydrophila* -infected *C. auratus*. (A) The numbers of DEGs. (B) Scattered plot of DEGs. (C) The numbers of DEPs. (D) Scattered plot of DEPs. Genes that were significantly differentially expressed are represented by red dots (up-regulated) and green dots (down-regulated), while insignificantly differentially expressed genes are represented by blue dots. Proteins that were significantly differentially expressed are represented by red dots (up-regulated) and green dots (down-regulated), while insignificantly differentially expressed genes are represented by gray dots, FDR corrected P-value < 0.05.

bounded organelle (Fig. 4B). Binding was the most abundant GO terms for the molecular function category, followed by protein binding, catalytic activity, ion binding and organic cyclic compound binding (Fig. 4C).

In order to identify the biological pathways that play key roles in response to the *A. hydrophila* infection, we performed an enrichment analysis of these DEPs against the KEGG database. A total of 29 different immune pathways were found, including metabolic pathways of 390 proteins, regulation of actin cytoskeleton of 116 proteins, focal adhesion of 109 proteins, endocytosis of 103 proteins, spliceosome of 97 proteins, complement and coagulation cascades of 64 proteins, antigen processing and presentation of 53 proteins and salivary secretion of 17 proteins (Supplemented Table 2).

3.6. Validation of DEGs by qRT-PCR analysis

To confirm the accuracy and reproducibility of the transcriptome of gills, a total of 21 target genes representing a good coverage of both up- or down-regulated genes in *A. hydrophila* infection were randomly selected for qRT-PCR confirmation (Fig. 5). The 21 representative genes encoded Calmodulin (calm), Novel protein similar to vertebrate nuclear transcription factor Y (nfyal), Small nuclear ribonucleoprotein F (snrpf), Apoptotic chromatin condensation inducer in the nucleus (acin1b), Proteasome activator subunit 2 (psme2), Splicing factor 3B subunit 10 (sf3b10), RNA-binding protein 8A (rbm8a), RNA-binding protein 25-like (rbm25), Pre-mRNA-splicing factor 18 (prpf18), GTPase-activating protein-binding protein 2 (g3bp2), Small nuclear ribonucleoprotein D3 (snrpd3l), Membrane-bound complement regulatory protein (gTecrem-2), Complement factor I-A (CfI-A), Complement 7 (C7), Lysozyme C (LysC), ATP-dependent RNA helicase DDX5 (ddx5), Heat shock protein 90 (hsp90), Alpha-2-macroglobulin (α-2M), Complement 9 (C9), Complement 3-like (C3), Solute carrier family 4, anion exchanger, member 1 (slc4a1a), which are key proteins or enzymes in immune-related signaling pathways, such as salivary secretion, complement activation and coagulation system, antigen processing and presentation, and spliceosome. As demonstrated in Fig. 5A, for the all selected 21 host immune-related genes, the qRT-PCR data for the detected candidate genes had similar expression trends as in the iTRAQ proteins results, suggesting that our transcriptomic data were reliably quantified. As shown in Fig. 5B, the correlation analysis indicated that Pearson's correlation between the iTRAQ and qRT-PCR results was high and significant ($r = 0.70$, $p < 0.01$).

3.7. Protein-protein interaction networks in A. hydrophila infection

The protein-protein interactions (PPI) for the prediction of their

Table 1
KEGG pathway analysis for differentially expressed genes in *C. auratus* gills.

Pathway ID	Pathways	DEGs	p value
ko04514	Cell adhesion molecules, CAMs	102	8.33e-30
ko04145	Phagosome	99	2.88e-22
ko04650	Natural killer cell mediated cytotoxicity	88	2.56e-25
ko05332	Graft-versus-host disease	83	3.06e-58
ko05320	Autoimmune thyroid disease	83	9.96e-53
ko04612	Antigen processing and presentation	82	5.21e-36
ko04621	NOD-like receptor signaling pathway	55	1.69e-08
ko04060	Cytokine-cytokine receptor interaction	49	1.15e-04
ko04064	NF-kappa B signaling pathway	44	9.97e-05
ko04062	Chemokine signaling pathway	43	0.23
ko05110	Vibrio cholerae infection	32	2.06e-03
ko04620	Toll-like receptor signaling pathway	31	6.91e-04
ko04660	T cell receptor signaling pathway	29	0.30
ko04110	Cell cycle	28	0.25
ko04512	ECM-receptor interaction	26	0.24
ko04210	Apoptosis	23	5.37e-02
ko04662	B cell receptor signaling pathway	23	0.28
ko04630	Jak-STAT signaling pathway	23	0.57
ko05340	Primary immunodeficiency	21	4.01e-04
ko04610	Complement and coagulation cascades	19	2.08e-03
ko04370	VEGF signaling pathway	18	0.30
ko04623	Cytosolic DNA-sensing pathway	15	4.77e-03
ko04115	p53 signaling pathway	12	0.74
ko04310	Wnt signaling pathway	12	1.00
ko04150	mTOR signaling pathway	10	0.84
ko04330	Notch signaling pathway	7	0.97
ko04350	TGF-beta signaling pathway	7	1.00

function were essential in immune processes of fish gills. In order to explore the interaction relationship in *C. auratus* gill after *A. hydrophila* infection, some selected DEPs involved in complement activation, coagulation system, and antigen presentation were submitted to the STRING database for further analyzing the PPI networks, and then mapped against the proteins database of zebrafish *Danio rerio* showed that the high-order interactions among 51 network edges and 26 nodes were observed (Fig. 6; p-value < 1.95e-12). The interactions of DEPs in gills that were correlated with antigen processing and presentation and complement system were observed for part of the signaling process. According to the STRING network, cluster one revealed strong interactions between 13 DEPs of mainly complement and coagulation cascades pathway and included Kininogen (KNG), Alpha-2-macroglobulin (A2M), Complement decay-accelerating factor (DAF), C7, Complement receptor type 1 (CR1), Complement factor I (IF), Complement factor H (FH), Complement factor D (CFD), C3, C9, Lysozyme (LYZ), Prolyl 4-hydroxylase subunit beta (P4HB) and Intelectin (ITLN). Cluster two revealed multiple strong interactions between 13 DEPs of mainly antigen processing and presentation pathway and included Calreticulin (CLAR), Calnexin (CANX), Heat shock protein HSP 90 (HSP 90), HSP 90B1, Nuclear transcription factor Y (NF-Y), NFYB, MHC class II transactivator (MHC II), Serum protease inhibitor, member 1 (SERPINA1), Cell division cycle 37 (CDC37), Protein disulfide-isomerase A3 (PDIA3), Proteasome activator subunit 1 (PSME 1), PSME 2 and PSME 3. The interaction networks for these DEPs of gills were predicted successfully, which will provide a foundation for PPI in *C. auratus* responding to *A. hydrophila* for further investigations.

4. Discussion

As is well known, the gills fish represent a critical organ for the continuously contact with the water, and frequently exposed to the pathogenic-enriched aquatic environment [2]. Despite the importance of the gills in fish immunity to waterborne pathogens, not many studies have addressed the immune responses elicited in this tissue by bacterial infections at the molecular level [5]. High-throughput genomic and transcriptome sequencing were used to check the transcriptional variations in gene expression, while the posttranscriptional regulation and posttranslational modification could not be detected [4,7,13]. Recently, the isobaric tags for relative and absolute quantitation (iTRAQ) method was an innovatively developed technique for the characterization of proteins and was mainly applied for quantitative analysis of changes in immune-related proteins in fish [1,5,34]. To better understand the mucosal local molecular mechanisms of *C. auratus* upon bacterial infection, in this research we used multi-omics combined methods of RNA-seq transcriptomics and iTRAQ proteomics to explore the expression profiles for the changes at gene and protein levels in the gills of *C. auratus* during *A. hydrophila* infection. RNA-seq transcriptomic analysis showed that a large number of differentially expressed genes (DEGs) were significantly observed for 3842 in gills, while the iTRAQ proteome revealed 430 differentially expressed proteins (DEPs) that were significantly expressed after the infection. Furtherly, the DEGs and DEPs were identified and analyzed by the enrichment analysis of both KEGG and GO annotations. In this study, we focused on the immune-related signaling pathway induced by *A. hydrophila* infection, in which a large number of genes and proteins were identified to be involved in different immune responses, including antigen presentation process, complement activation, coagulation system, phagosome, NLR and NF-κB inflammatory signaling pathways. The results and findings could be the foundation for future experimental exploration of the mucosal immune system in teleost fishes.

Crucian carp (*C. auratus*) is a main fish species with the most stunning production, which was cultured in different freshwater areas in China [24]. As a common opportunistic bacterium in fish, pathogenic *A. hydrophila* could cause *Aeromonas* septicemia and hemorrhagic septicemia, resulting in significant economic losses [35]. In the present study, the indicating that the pathogenic *A. hydrophila* infected-fish displayed obviously clinical symptoms during early infection at about 12 hpi, which then was quickly developed a typical signs of this disease. The pathological changes were basically consistent with inflammatory cell infiltration, hemorrhage and necrosis accorded with previous *A. hydrophila* infection reports [27,30,36]. Recently, the transcriptomics analysis in various organs (eg., kidney, skin and pharyngeal tissue) of *C. auratus* infected with different pathogens were successively reported [30,37,38]. Notably, many immunes signaling pathways were identified in different tissues of *C. auratus*, including MAPK, TLR, NLR, RLR, p53, necroptosis, antigen presentation process, complement activation, coagulation system, phagosome, JAK-STAT and NF-κB immune-related signaling pathways [13,38,39]. However, there are few reports on the immune responses of gill mucosal immune in *C. auratus* during bacterial infection. Previous evidences revealed that the gill immune response was actively induced by the pathogen infection in fish. For example, Zhang et al. [39] demonstrated that the complements, apoptosis, endocytosis, coagulation factors, cytokine, TNF and RLR signalings showed significant transcriptional changes in the gills of amphioxus *Branchiostoma belcheri*. A parasite infection study revealed that the chemokine, antigen presentation, TLR and NLR signaling pathway in the gill were proposed to be involved in the recognition of parasite and subsequent inflammatory induction [13]. In the gills of teleost fish to hypoxia stress found various innate immune-relevant genes were significantly down-regulated, such as complements, coagulation factors and chemokines [18]. In our previous study in the zebrafish gills upon *A. hydrophila* infection showed that the complements, coagulation factors and phagosome pathways were also the significant differentially expressed [5]. These results indicated that the immunological pathways such as complements activation, coagulation systems, antigen presentation, TLR, NLR, RLR, chemokine and phagosome may be involved in the gill local mucosal immune network in fish. In the present study of the transcriptome and proteome in the gills in *A. hydrophila*-infected *C. auratus*, our data suggest that many DEGs and DEPs were involved in the same immune defenses, including the antigen presentation, chemokine, complements, phagosome and inflammatory pathway. Among these pathways, multiple immunity genes were significantly altered in the infected *C. auratus*, suggesting that these genes might be important

Table 2
Differentially expressed proteins associated with immune response in the gills of *C. auratus* infected by *A. hydrophilis*.

NCBInr Accession	Description	Hit Number	Score	Mass	Coverage	Fold-change
gi|55741912	Acidic leucine-rich nuclear phosphoprotein 32 family member E	1042	186	32852	4.80	1.49
gi|439153	Alpha-1 antiproteinase antitrypsi	1253	133	17826	16.70	0.65
gi|6009731	Alpha-2-macroglobulin-3	311	836	102291	12.70	1.59
gi|326674178	Alpha-2-macroglobulin-like	415	645	183402	4.50	1.68
gi|189521150	Apoptotic chromatin condensation inducer in the nucleus	1082	174	167922	3.20	0.67
gi|45709504	ATP-dependent RNA helicase DDX5	378	710	80972	25.10	1.48
gi|345312071	Calmodulin	224	1150	19565	45.60	0.38
gi|47087435	Calnexin 1	698	334	83451	6.20	1.73
gi|41054373	Calreticulin	985	201	63383	11.80	1.32
gi|46329655	Calreticulin	574	431	63356	18.40	1.21
gi|50355968	Calreticulin 3b	462	571	62118	16.50	1.31
gi|4126589	Complement C3-H1	126	2026	227247	19.20	1.42
gi|4126587	Complement C3-H1	410	657	225727	5.70	1.66
gi|4126597	Complement C3-H2	261	990	221814	6.20	1.54
gi|4126593	Complement C3-S	271	950	224804	10.40	1.25
gi|66773068	Complement C9	654	371	88067	5.10	1.63
gi|383282283	Complement component C7-1	1724	74	20270	8.50	1.24
gi|20142081	Complement control protein factor I-A	1779	70	78677	2.50	1.22
gi|66793372	Complement factor D-like	1845	64	33118	6.00	0.82
gi|148744428	Complement factor H like 4	1174	151	13044	12.50	1.91
gi|41054055	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	2040	54	121928	1.40	1.36
gi|326664533	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like	1543	90	68568	2.20	1.47
gi|324388047	Heat shock protein 90	427	629	100937	19.50	1.58
gi|49402291	Heterogeneous nuclear ribonucleoprotein A1b	674	355	46151	11.90	1.21
gi|226358635	Intelectin	521	484	39515	22.9	0.48
gi|54400464	Kininogen 1	832	260	28357	16.80	0.82
gi|7939556	Lysozyme C	793	277	22068	11.70	1.32
gi|425874851	Membrane-bound complement regulatory protein	1539	90	42656	4.60	1.21
gi|313762294	MHC class II antigen beta chain	1399	107	12779	20.70	0.27
gi|50540532	nuclear transcription factor Y	2087	51	42001	5.30	0.59
gi|45387575	parvalbumin 6	1236	137	16778	14.70	0.59
gi|50345066	Pre-mRNA-splicing factor 18	2229	45	50018	2.30	0.79
gi|91798526	Proteasome activator PA28 subunit	772	289	38313	28.10	0.70
gi|18859279	Proteasome activator subunit 1	681	349	38519	19.00	0.70
gi|160964416	Proteasome activator subunit 2	547	458	36686	23.80	0.69
gi|123703665	Ras GTPase-activating protein-binding protein 2	727	314	64813	6.30	0.81
gi|41054239	RNA-binding protein 25-like	2031	54	107956	1.50	0.79
gi|61651846	RNA-binding protein 8A	818	266	23880	17.20	0.78
gi|157422754	Serpin peptidase inhibitor 1A	2161	48	27403	11.40	0.82
gi|62955141	Small nuclear ribonucleoprotein D2 polypeptide	584	425	19138	53.40	0.81
gi|41054297	Small nuclear ribonucleoprotein D3 polypeptide	1105	168	17448	14.80	0.82
gi|51468014	Small nuclear ribonucleoprotein F	1088	172	11571	15.10	0.67
gi|7208223	Small nuclear ribonucleoprotein-associated protein	500	505	27964	15.50	0.69
gi|26984623	Solute carrier family 4, anion exchanger, member 1	1875	63	111635	2.70	1.68
gi|51972162	Splicing factor 3A subunit 3	1623	82	73005	10.20	1.56
gi|50540016	Splicing factor 3B subunit 10	1780	69	12026	10.50	0.75
gi|157426951	Splicing factor 3B subunit 2	1023	191	120201	5.20	0.83
gi|326665256	CD59 glycoprotein-like	2275	43	11115	16	1.22
gi|318104952	CD4-like protein 2 precursor		186		62	-2.93

Note: CD4-2 is identified by DEGs analysis.

in defense against *A. hydrophila*. Previous studies on the molecular immune pathways in the gill tissue of teleost fish exhibited similar expression patterns in response to various pathogens. These results in fish further support the idea of the gills serve as the important organs for physiological respiration and immune defense against pathogens.

In teleost fishes, antigen processing and presentation pathway plays a necessary role in the adaptive immune response [38]. Previous studies in the gills of rainbow trout *O. mykiss* indicated that antigen presenting cells (APCs) were activated for external infection and in response to stronger stimulation [40]. In addition, transcriptome analyses of kidney, liver and spleen tissues of Chinese sturgeon (*Acipenser sinensis*), Nile tilapia (*Oreochromis niloticus*) challenged by bacterial *Citrobacter freundii* and *Streptococcus agalactiae* has revealed that antigen presentation signalings were significantly enriched among post-infection altered genes [41,42]. In our present data, the potential role of antigen processing and presentation in mucosal immunity was supported by both transcriptomic and proteomic analyses, indicating that antigen processing and presentation may be significantly involved in innate and adaptive immunity in the gill tissue of teleost fish. Similarly, a study of the molecular mechanism in the gills of *O. mykiss* by using transcriptome analysis found that some increased immune-related genes were significantly related with antigen processing and presentation in the infected gills [13]. Several antigen processing and immune-relevant proteins included major histocompatibility complex (MHC I/II), heat shock proteins (Hsp70/90A/A1) and calreticulin (CALR/3b) were crucial for the host immune defense against bacterial infection [9]. The MHC molecules may have participated in antigen presentation for specific immunity by modulating mechanism through interactions with T-lymphocyte and Natural killer cells in animals [13,43]. In the present study on the gills in *A. hydrophila*-infected *C. auratus*, the expression level of MHC II was significantly down-regulated in expression of 0.27-fold, which were basically consistent with that reported from *Flavobacterium columnare* challenged-gills of channel catfish *Ictalurus punctatus* and catla *Catla catla* [43,44], *Vibrio alginolyticus* infected giant grouper *Epinephelus lanceolatus* [45]; and similar lower expression of MHC II was previously reported in *V. anguillarum* challenged turbot *Scophthalmus maximus* and tongue sole *Cynoglossus semilaevis* [46,47], respectively. According to those reported earlier in fish, it revealed that

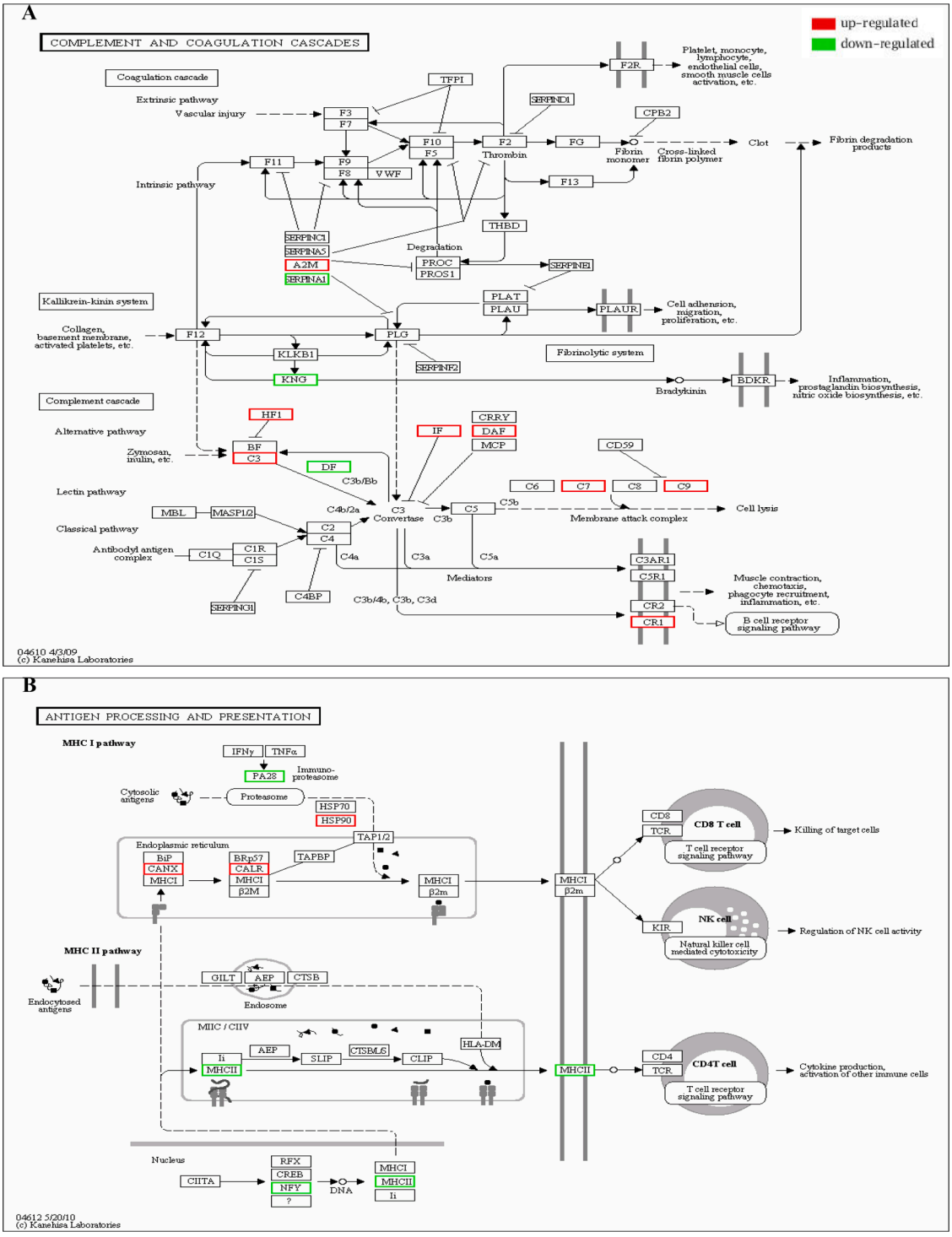

Fig. 3. Complement and coagulation cascades and antigen processing and presentation signaling pathway and DEPs involved in the gills of *C. auratus*. Proteins in red boxes were upregulated, and those in green boxes were downregulated.

antigen processing and presentation pathway plays a necessary role in immune response to pathogen infection. In addition, HSPs play a highly conserved role in immune responses and defenses against bacterial infection in fishes [41]. Based on the present observation for the protein expression level of Hsp90 was altered obviously after infection with up-regulation of 1.58-fold in the gills of *C. auratus* challenged *A. hydrophila,* and several studies in the gills of *I. punctatus* [12], Ya fish *Schizothorax prenanti* and Chinese sturgeon *Acipenser sinensis* [41,48], it demonstrating that the differentially expressed pattern of HSP90 was early induced after bacterial infection and consequently provided an important immune resistance. Moreover, calreticulin (CALR) participates in the regulation of immune responses such as the adhesion, migration, phagocytosis and multiple biological effects [49]. Several studies have found that the expressions of CALR in the spleen of *O. niloticus* and liver of *I. punctatus* were rapidly increased after the infection [49,50]. In our present study, the expression level of CALR was significantly increased 1.32-fold in the infected gills of *C. auratus*, which was basically accordant with those reported from the studies on gills in

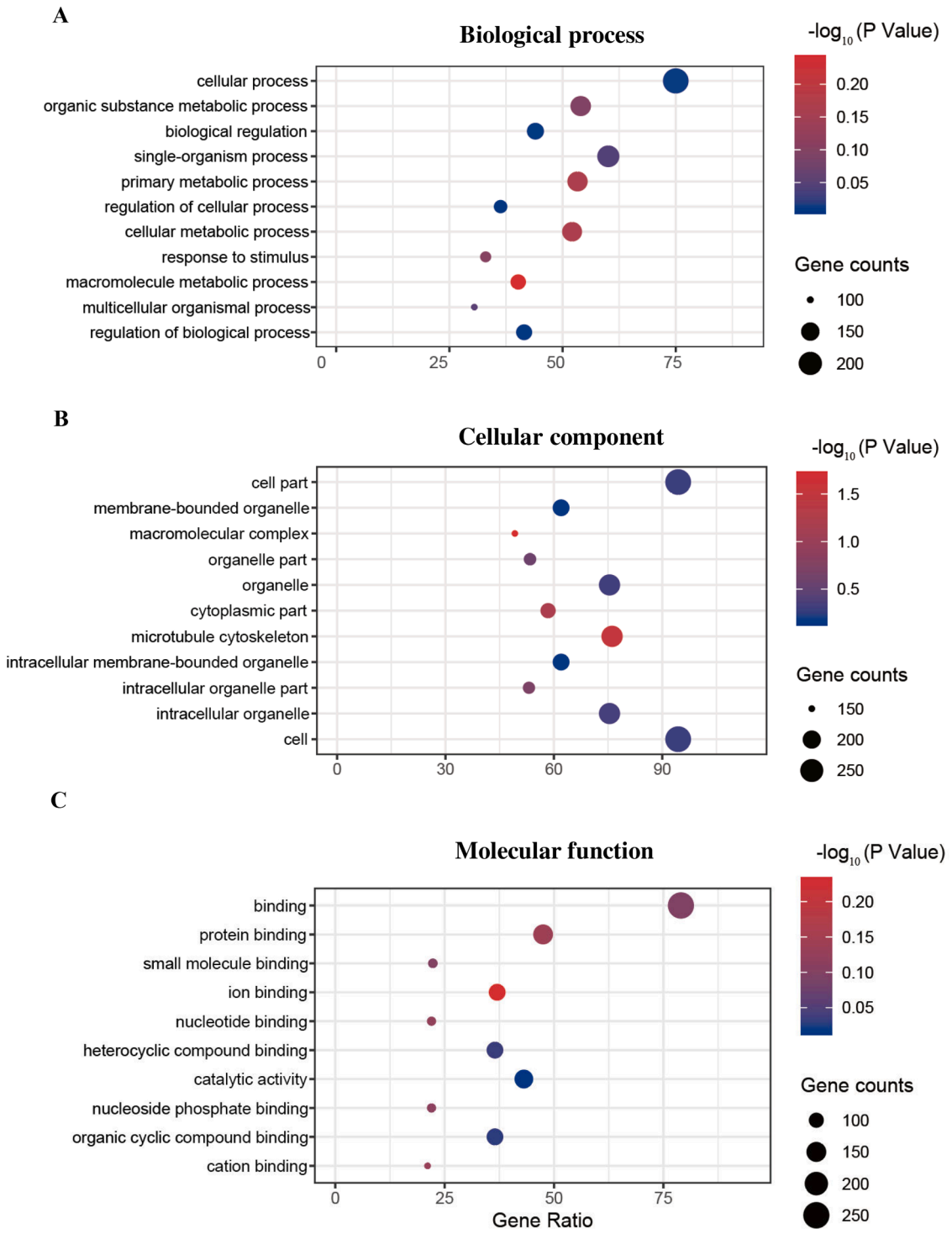

Fig. 4. GO enrichment analysis of the DEPs in gill tissues of *C. auratus* infected with *A. hydrophila*. GO analysis classified the differentially expressed genes into three groups, including (A) biological process, (B) cellular component and (C) molecular function. Y-axis represents GO terms; X-axis represents rich factor; (rich factor equals the ratio between the DEPs and all annotated proteins enriched in the GO terms); The color and size of each bubble represent enrichment significance and the number of DEPs enriched in a GO term, respectively.

other fish species [49].

The complement signaling pathway plays essential roles in killing and elimination of pathogens, modulation of immune and inflammatory responses, which be activated and mediated by three means such as the classical, lectin and the alternative pathway [51–53]. Evidences demonstrated that the expression of the several complements (ie., C3, BF2, C4, 1B, C8, C8b, C9) were mainly increased in the liver of *C. irritans*-infected Barramundi *Lates calcarifer* [54], of which has a significant influence from the liver of catfish *I. punctatus* and grouper *Epinephelus coioides* during LPS and *C. irritans* infection [11,55]. A recent study suggested that the expression levels of complement factors considerably varied and significantly expressed in gills [13]. Notably, in the present study, the complement signaling pathway was found to be enriched in both transcription and protein levels in the gill of *C. auratus* following exposure to *A. hydrophila*. Recently, the strong expression of complement pathway was reported in the skin of zebrafish injected with

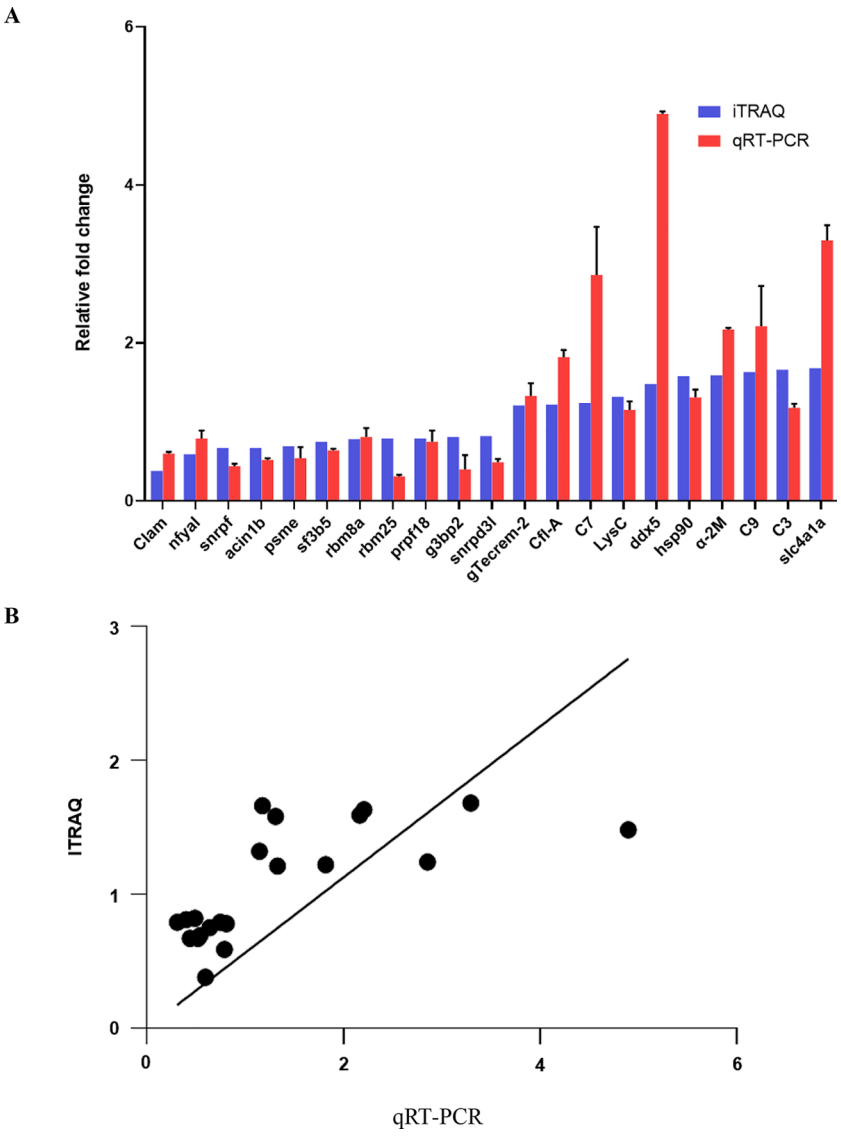

Fig. 5. Validation of 21 DEPs generated from iTRAQ results in the gills of *C. auratus* by qRT-PCR assay. (A) Gene expression patterns from the iTRAQ analysis (in blue) were validated for 21 representative genes by qRT–PCR (in red); (B) Correlation analysis between iTRAQ identified DEPs and qRT-PCR. Plot represents the mean value for selected genes in each group. Data were fitted by linear regression and adjusting quality determined. Error bars represent SE values from three biological replicates ($n = 3$).

S. agalactiae [56]. A total of 23 functional genes associated with complement and coagulation were obtained in the gills of *C. auratus* following exposure to *A. hydrophila*, in which most of up-regulated genes (eg., C3, C3H2, C3S, C7, C9, A2M, DAF, HF1, IF, CR1) were involved in the alternative pathways. Complement C3 was regarded as an essential bridges crossing innate and acquired immune responses, which was directly activated by invasive pathogenic substances [52,57]. From our observation for the transcriptomics analysis in the gills of *C. auratus*, the significantly up-regulated expression of C3 was detected in *A. hydrophila*-challenged fishes after early infection, and similar expressions were also observed in the spleen and liver of *L. crocea* challenged by *V. alginolyticus* [58]. In the expression of C3 of gills, our results were in accordance with the upregulated levels in the gill of *S. salar L.* and grass carp *Ctenopharyngodon idella* following infection with *Neoparamoeba perurans* and *A. hydrophila* [10,14]. Previous study showed that the C7 and C9 were rapidly alterable in the kidney and liver of *L. crocea* [51]. In the present study on the *A. hydrophila* challenged *C. auratus* gills, the gene expression of both C7 and C9 were significantly up-regulated, suggesting their participation in nonspecific immunity at the early stage of pathogenic invasion. In addition, Syahputra et al. [13] reported that the complement C5 had a necessary role in the mucosal immunity of *O. mykiss* during *Ichthyophthirius multifiliis* infection. However, it is not well accordant with the substantially changed expression patternof C5 observed in the gill of *C. auratus*, suggesting the different molecular mechanisms at the early stage of anti-infection in the local mucosal immune responses to various pathogens. It was speculated that most components of complement signaling pathway in *C. auratus* were involved in the gill mucosal immune response to *A. hydrophila* infection, which still need for further study on the interaction of the gills with pathogen in the future.

Protein–protein interactions (PPI) networks are useful for gene function identification and functional module analysis [2]. In addition to KEGG enrichment analysis of key immune signaling pathways in fish gills, the STRING protein interaction database was used to conduct PPI network of some hub molecules in key immune-related pathways in the present study. It showed the high connectivity degree and protein–-protein association as predicted by PPI analysis, including both complement-related proteins and antigen processing and presentation-related proteins (eg., KNG, A2M, DAF, C7, CR1, HF1, DF, C9, IF, C3, CLAR, CANX, HSP 90, NF-Y, PA28 MHC II and SERPINA1). As reported previously, the complement systems, acute-phase protein response, antigen processing and presentation signaling pathways had essential roles in gill mucosal and local immune responses in fish [41, 59]. In a study on mucosal immunity in zebrafish, many sub-networks such as TNF, MAPK, p53, phagosome, complement activation and coagulation signaling pathway, which were made by DEGs in the skins

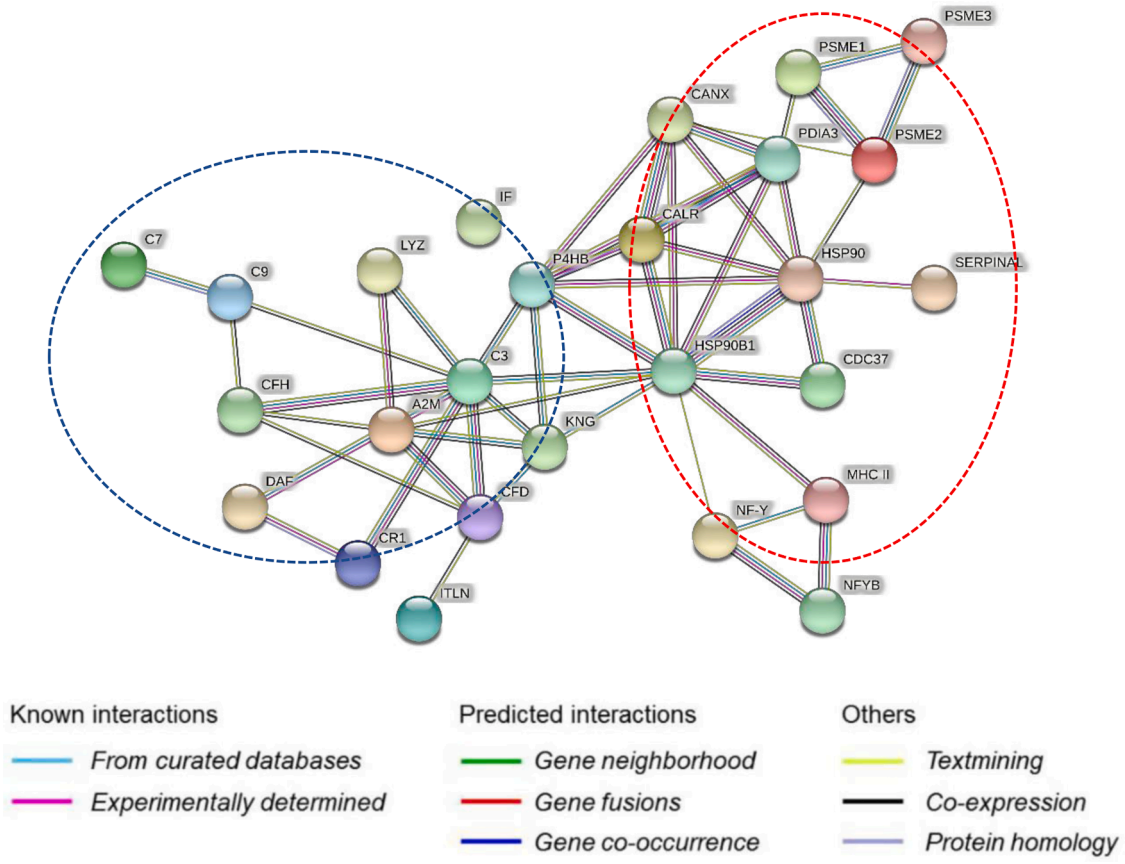

Fig. 6. Protein–protein interaction network of DEPs identified in *C. auratus* gills. Sky blue lines represent known interactions from curated databases. Purple lines represent experimentally determined known interactions. Green lines represent predicted interactions with neighborhood genes. Red lines represent gene fusions. Blue lines represent gene co-occurrence. Yellow lines represent text-mining evidence. Black lines represent co-expression. Light blue lines represent protein homology-based interactions. The colored nodes represent query proteins and first shell of interactors, whereas, white nodes represent second shell of interactors. Image generated by String software v 11.0.

and intestines via PPI analysis [56]. Then, it could be hypothesized that the complement systems, acute-phase protein response, and antigen processing pathway might play an vital role in mucosal immunity such as intestines, skins and gill of fish. Our PPI result suggested that gills might be involved in fighting microbiological infections through the activation of complement cascades and antigen presentation signaling pathways. The PPI network analysis provided insights to the interacting proteins of *C. auratus* gills and the immune signal pathways they might participate in mucosal defence against bacterial infection. However, more detailed research is needed to support our speculations on the functions of these PPIs network.

5. Conclusion

In sumarry, the gill transcriptomic and proteomic analyses revealed the mechanism of local mucosal immunity in crucian carp *C. auratus* during *A. hydrophila* infection, and we identified and screened a large number of immune-related molecules (genes and proteins) differentially expressed following the infection. Importantly, similar to previous findings in teleost fish mucosal organs (gut, skin, and gill) after pathogenic stimulation, our report on gill mucosal immunity revealed that complement and coagulation cascades, acute-phase protein response, antigen processing and presentation signaling pathway act as an the important immune response to overcome *A. hydrophila* infection. To our knowledge, the transcriptomics and proteomics of gills firstly identified by multi-omics analyses serve as a valuable reference for understanding on the molecular mechanisms of local mucosal immunity in cyprinid species.

Supplemented Fig. 1. GO enrichment analysis of the differentially expressed genes in gill tissues of *C. auratus* infected with *A. hydrophila*.

Declaration of Competing Interest

All authors do not have a conflict of interests in this manuscript.

Data availability

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Acknowledgment

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2022.100077.

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