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Short communication

Transcriptome analysis of spleen reveals the signal transduction of toll-like receptors after *Aeromonas hydrophila* infection in *Schizothorax prenanti*

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ABSTRACT

Schizothorax prenanti (*S. prenanti*), an important species of economical fish in Southwest China, is susceptible to *Aeromonas hydrophila* (Ah). To understand the immune response to Ah, the transcriptome profiling of spleen of *S. prenanti* was analyzed after Ah infection. A total of 6, 213 different expression genes (DEGs) were obtained, including 3, 066 up-regulated DEGs and 3, 147 down-regulated DEGs. These DEGs were annotated by KEGG and GO databases, so that the immune-related DEGs (IRDs) can be identified and classified. Then, the interesting IRDs were screened to build heat map, and the reliability of the transcriptome data was validated by qPCR. In order to clarify the mechanism of signal transduction in the anti-bacterial immunity, the signaling pathway initiated by TLRs was predicted. In this pathway, TLR25 and TLR5 mediate the NF-κB and AP-1 signals via MyD88-dependent pathway. Meanwhile, the type I IFN (IFNα/β) induced by IRF1 and IRF3/7 may play an important role in the anti-bacterial immunity. In conclusion, this study preliminarily provides insights into the mechanism of signal transduction after Ah infection in *S. prenanti*, which contributes to exploring the complex anti-bacterial immunity.

1. Introduction

The complex immune system that surveilles the invasion of pathogens consists of two diacritical systems, the innate immune system and the adaptive immune system. In mammals, the innate immune system is commonly regarded as "vanguard" to recognize and resist pathogens, while the adaptive immune system can produce specific antibody to eliminate thoroughly the invasive pathogens. According to the prevailing view, the adaptive immune system predominately contributes to the final elimination of pathogens in mammals. However, the adaptive immune system fails to dominate in fish, because the fish is poikilothermic organism and some characteristics of the adaptive immune response rely on the environmental temperature [1]. Therefore, the innate immune system in fish acts as the "protagonist" in host defense, and transmits the invasive signals to whole host immune system, which may be initiated by the interaction between the Pattern Recognition Receptors (PRRs) and the Pathogen Associated Molecular Pattern (PAMPs) [2,3].

Toll-like receptors (TLRs) are major PRRs. The complex of TLR immunobiology has rapidly increased after the discovery of TLR molecules in different species. In human, 10 TLRs were found, including TLR1-10. In mice, 11 TLRs were identified, which consists of 9 homologues of human (TLR1-9) and two distinct TLRs (TLR12 and 13). The TLR1, 2, 3, 4, 5 and 6 localize to the cell surface and recognize the bacterial, viral or parasite components, whereas the TLR 3, 7, 8 and 9 are distributed in endosome, and recognize viral genetic materials [4,5]. However, the TLR10, 12 and 13 have not yet been characterized [6]. By contrast, a total of 22 TLRs (TLR1-4, 5 M, 5S, 7–9, 13, 14, 18–28) were identified in fish [2,3,7–10], some of which were teleost-specific TLRs, such as TLR18-28 [3,9–12]. Among the teleost-specific TLRs, TLR18 and TLR25 are related to the anti-bacterial response, while TLR19 and TLR22 can recognize viral components [13–16]. In addition,

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fish's TLR5 is divided into two forms, TLR5M and TLR5S, both of which play a key role in the recognition of flagellin and the transduction of downstream signals [17,18].

TLR-mediated signaling pathway(s) is initiated by PAMPs and therefore activates downstream transcriptional factors, such as NF-κB, AP-1 and interferon regulatory factors (IRFs), leading to the enhancement of the secretion of inflammatory factors, chemokines, interferons and cluster of differentiation (CD) molecules [4]. For the transduction of TLR signal, adaptor molecule(s) is recruited by the Toll/IL-1 receptor (TIR) domain of TLR, which leads the signal to different pathways, including the myeloid differentiation primary response gene 88 (MyD88) -dependent and -independent pathways. There are four major adaptor molecules identified in mammals, including MyD88, TIR domain-containing adaptor-inducing IFN-β (TRIF), TIR domain–containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM). Interestingly, the TRAM gene is lost in fish so that some functions of TLR-mediated pathway(s) are distinct comparing to mammals [19]. This event of diversity may indicate a huge difference between mammals and fish in signal transduction after pathogens' invasion.

Schizothorax prenanti (*S. prenanti*) is a cold-water species of cyprinid that is widely farmed in Southwest China, but the intensive feeding could easily cause the outbreak of diseases, such as haemorrhagic septicaemia induced by *Aeromonas hydrophila* (Ah) [20–22]. In this study, we selected this disease model to investigate the gene expression by transcriptomics, which may reveal the mechanism of signal transduction after bacterial infection in *S. prenanti*.

2. Materials and methods

2.1. Fish and Ah

S. prenanti (mean weight of 400 g) were purchased from Sichuan Yafish Company (Ya'an, china). The fishes were fed in fiberglass tanks (70 cm × 50 cm × 46 cm) with adaptive conditions (chlorine-free water at 20 °C and natural photoperiod), and were accommodated for at least two weeks before the challenge experiment. Ah was provided by Key Laboratory of Sichuan Province for Fishes (Sichuan, China). It was incubated in Luria Bertani (LB) medium at 37 °C with overnight shaking. After overnight cultivation, Ah was injected intraperitoneally into *S. prenanti* with the density of about 10^8 CFU per fish (screened by pre-experiment), as the infection group (n = 10). The control group was performed by injecting isopyknic phosphoric acid buffer solution (PBS) (n = 10). Each group has three non-interfering repeat groups that can increase the reliability of transcriptome analysis. After 12 h infection, all the fishes were euthanized by using 200 mg/L MS222 (yuanye Bio. Co. Ltd., Shanghai, China). Then, the spleen was rapidly removed and stored under the temperature of – 80 °C until RNA extraction.

In addition, more than 50 fishes were infected by Ah with the dose of 10^8 CFU per fish, and more than 50 fishes were injected with isopyknic PBS as control. The fishes of Ah and control group were respectively divided into 5 groups (n = 10), which were euthanized after disposing for different period (3, 6, 12, 24 or 48 h). The spleen were rapidly removed and stored under the temperature of – 80 °C until analyzing the expression of alternative genes by qPCR.

2.2. Total RNA extraction, library construction and sequencing

Total RNA was extracted from spleen tissues by RNAiso plus reagent (Takara Bio, Co. Ltd., Dalian, China) according to the manufacturer's instructions, and the concentration and purity were evaluated by NanoDrop 2000 spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. The integrality of total RNA was detected by Agilent 2100 (Agilent Technologies, Co. Ltd., USA). The mRNA was enriched from total RNA by magnetic beads with Oligo (dt) after DNase I treatment. Then, the mRNA was randomly broken into small pieces in fragmentation buffer. The first-strand cDNA was synthesized by random hexamers as primers and the pieces as templates, while the complementary strand was synthesized by adding response buffer, dNTPs, DNA polymerase I and RNase H. The double-stand cDNA was purified by AMPure XP beads and added with "A" tail and adaptors. Finally, the intact cDNA was amplificated, and the product was purified by AMPure XP beads for acquiring final cNDA library, following sequencing by Illumina Hiseq 2500 platform.

Table 1
The primers used in this study.

Gene name	Primer sequence (5′ → 3′)		Length of product (bp)
TLR25	Forward	GTTCTGTCGGTGTTATCTGTG	123
	Reverse	CCTCTTGTAGCCTCTCCTTTT	
TLR22-2	Forward	CTGGGAAATCTCACTGTGTTGC	144
	Reverse	AAAGAGCGGCTGAACTTTGC	
TLR21	Forward	ATAAAGTACCTGCGCTTGCG	134
	Reverse	TGAAAGCACAGCGAAGTCAG	
TLR18	Forward	ACAGACTAAATGGCCAGGGAAG	118
	Reverse	AACCACAAGCAAGGGCAAAG	
TLR5-1	Forward	TATCGTGTAGAGCTCTGAC	139
	Reverse	CCTACCAAGCCTAGAGAAAC	
TLR5-2	Forward	ACTAGACTACAACCGCTTCCTG	148
	Reverse	CAGCATCTCTAGAGACACCAGA	
IFNr2β	Forward	TGCAGAGCTCAAGACGTTTG	84
	Reverse	AGCTCGAACAATGCTTTGCG	
IL-10	Forward	TCGCTTGCACATCACCATTC	77
	Reverse	TCCAGAAACTGAAGGTGAAGGG	
IL-12p40a	Forward	CTCTCACACACACACACATC	178
	Reverse	GAGGTCAGATATCCACACTC	
IL-12p40b	Forward	TGGAAATGGCATCAGGAACG	88
	Reverse	AGTCCAGAGTGCAGTTGATGG	
IL-12p40c	Forward	GTTACCCTGACCTGTAGAAC	199
	Reverse	GCAGGAGATAGGTGTAGTCT	
IRF1	Forward	ATCTCACGGCAATTGGAACG	91
	Reverse	CTGTTGTGGCTACTTCATTGGC	
IRF2	Forward	AGACGACCACCTGATCATCAAC	148
	Reverse	TGTATCGCTCTCTGCTCCATAC	
IRF4a	Forward	AGCTGGACATCTCAGATCCT	147
	Reverse	GCTGTAGAGGAGGATAAGAC	
IRF4b	Forward	ACTGCTGGACACTCATCTGTTC	109
	Reverse	ATCGGTGCTCTCATCCTCAAAG	
IRF9	Forward	CAAACAGGAGTTGGACGCTTTC	79
	Reverse	CAGCTCTTCTCCAAAACACAGC	
β-actin	Forward	CTGGTATTGTGATGGACTCTGG	180
	Reverse	CAATTTCTCTTTCGGCTGTGG	
40S	Forward	GTCATCAGACGGGACTACTTG	83
	Reverse	GAGAGGTGGACAGACATGTTCT	

Table 2
The quality and mapped ratio of clean reads.

Sample	Clean Reads	GC content	Q30	Mapped Reads	Mapped Ratio
Control_1	23,806,883	47.75%	94.21%	18,180,171	76.37%
Control_2	29,945,684	47.68%	94.02%	22,972,792	76.71%
Control_3	24,326,261	47.71%	93.82%	18,416,209	75.71%
Ah_1	24,645,292	48.31%	93.89%	19,192,686	77.88%
Ah_2	23,910,759	48.33%	93.97%	18,651,919	78.01%
Ah_3	24,138,276	47.98%	94.20%	18,829,141	78.01%

2.3. Transcripts assembly and annotation

Transcripts assembly was done by Trinity software [23], which was described in our previous report [24]. Briefly, all transcripts were assembled by three components: Inchworm, Chrysalis and Butterfly. Inchworm contributed to the formation of contigs; Chrysalis was used to establish de Bruijn graph by contigs; Each de Bruijn graph of the components was simplified by Butterfly, resulting in acquiring the transcripts. Finally, the longest transcript of each gene was regarded as Unigene. All the unigene sequences were annotated by BLAST software

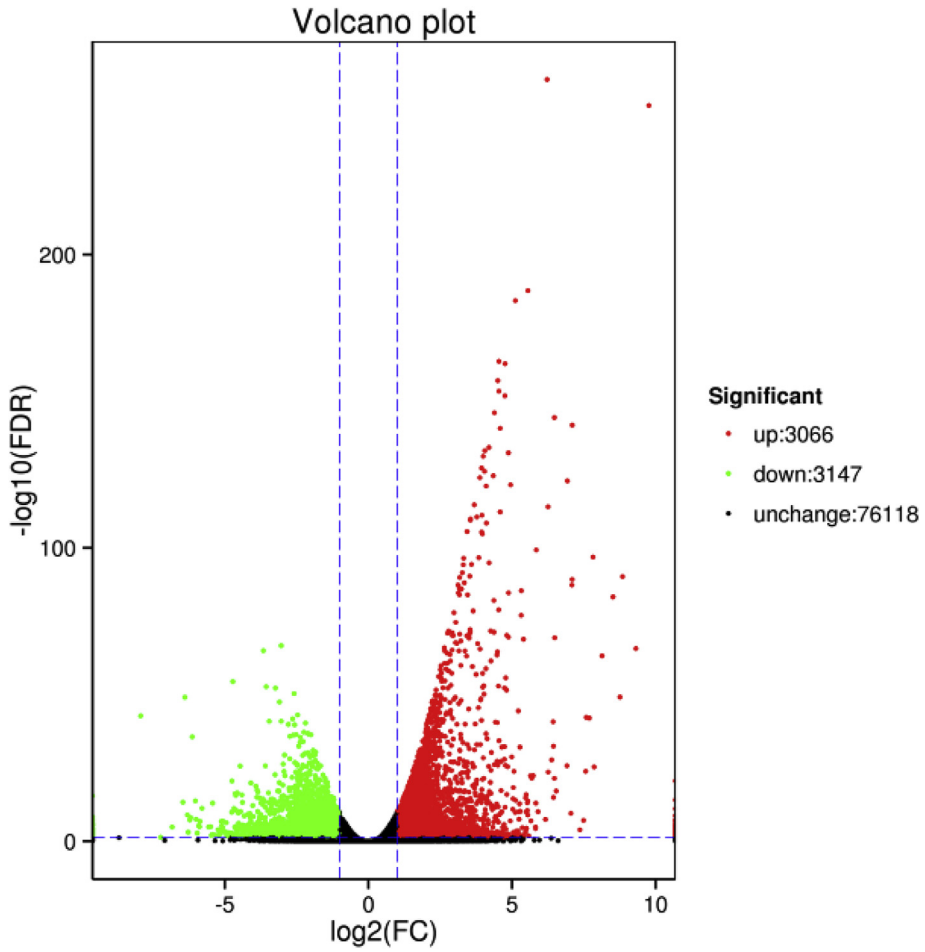

Fig. 1. Volcano plot of DEGs. The X-axis indicates the fold change of expression level of DEGs, and the Y-axis presents significance of differential expression. The blue pots mean no significantly change genes, while the red pots and the green pots indicate up- and down-regulated genes, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
The annotated ratio of DEGs.

	Number of Unigenes	Percentage (%)
Total DEGs	6213	100
Annotated in COG	938	15.1
Annotated in GO	2311	37.2
Annotated in KEGG	1852	29.8
Annotated in KOG	2543	40.9
Annotated in Pfam	3260	52.5
Annotated in Swiss-Prot	2558	41.2
Annotated in rggNOG	3746	60.3
Annotated in NR	4020	64.7
Annotated at least in one database	4036	65

[25] with using NR (NCBI non-redundant protein sequences) [26], Swiss-Prot (A manually annotated and reviewed protein sequence database) [27], GO (Gene Ontology) [28], KOG/COG/eggNOG (Clusters of Orthologous Groups of proteins) [29–31], KEGG (Kyoto Encyclopedia of Genes and Genomes) [32] and Pfam (Protein family) [33] databases.

2.4. Screening immune-related differentially expressed genes (IRDs), within GO and KEGG enrichment analysis

IRDs were screened from differentially expressed genes (DEGs). Firstly, DEGs of control group and Ah group were identified by DEseq R packages [34] within q-Value < 0.005, |log2 (foldchange)| > 1. Secondly, GO enrichment analysis of DEGs was implemented by the topGO R packages (http://www.bioconductor.org/packages/release/bioc/html/topGO.html) based Kolmog- orov-Smirnov test, while KEGG enrichment analysis of DEGs was performed by the KOBAS software [35]. Finally, the IRDs were screened from the DEGs that are involved in immune-related functions and pathways according to the results of GO and KEGG enrichment analysis.

2.5. Screening interesting IRDs and heat map

After acquiring the IRDs, we further screened several types of interesting IRDs, including TLRs, complements, cytokines and other components of signal transduction. The different expression of these genes was visualized by heat map, using HemI software (http://hemi.biocuckoo.org/) [36].

2.6. qPCR

To validate the reliability of the transcriptome data, 16 interesting IRDs were selected for qPCR analysis. Briefly, total RNA was extracted from spleen of *S. prenanti* after 12 h challenge. PrimeScripy® RT reagent kit with gDNA Eraser (Takara Bio, Co. Ltd., Dalian, China) was used to synthesize the cDNA. Then, the qPCR was performed by using SYBR® green II (Takara Bio Co. Ltd., Dalian, China) with the cDNA as template, following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 5s and several annealing temperature for 30s; 95 °C for 10s; melt curve detection of 65 °C for 5s to 95 °C increment 0.5 °C. The used primers were

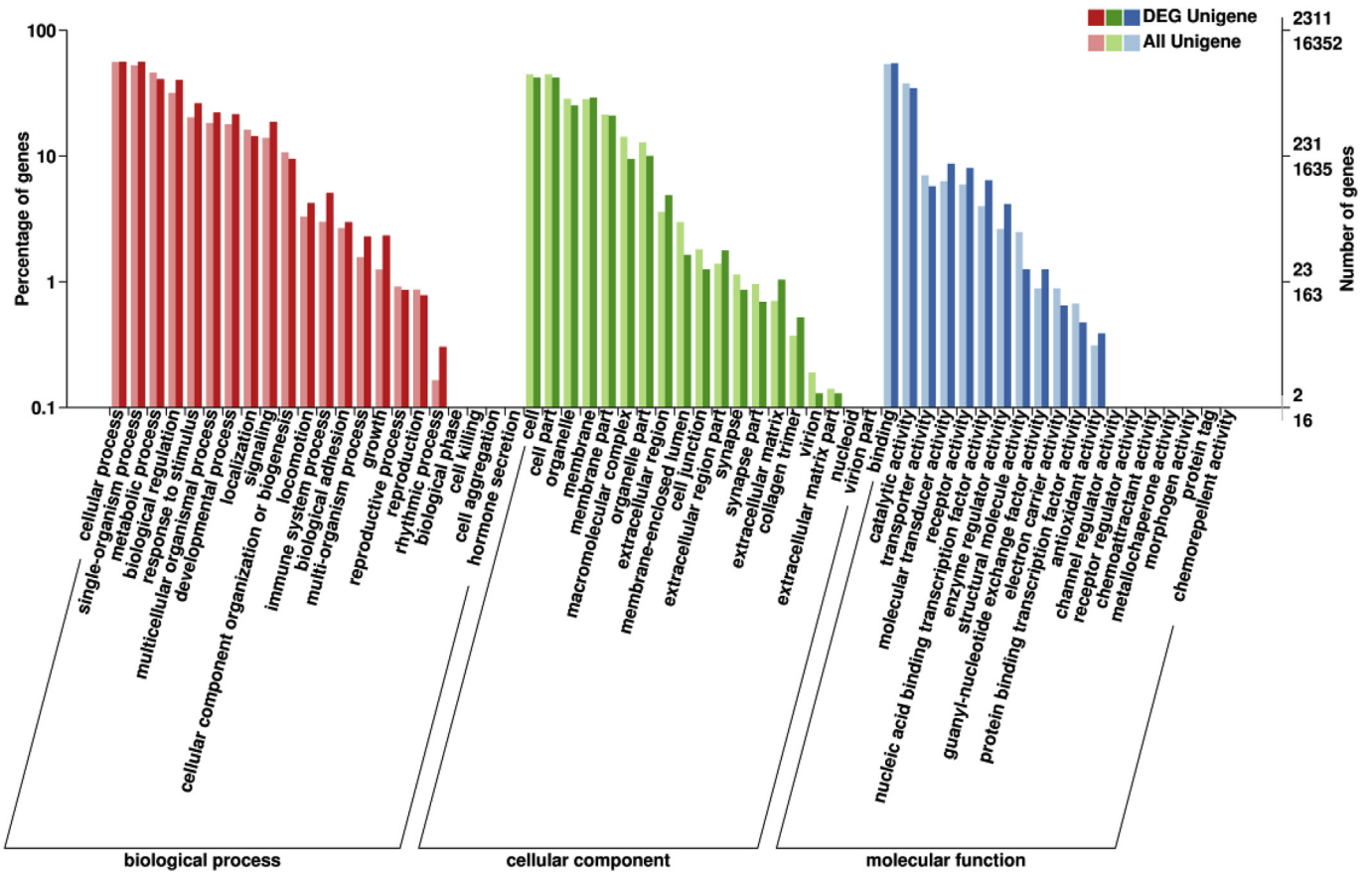

Fig. 2. Histogram description of Gene Ontology annotation analysis of DEGs. The DEGs and Unigenes were annotated into three gene ontology categories: biological process, molecular function and cellular component.

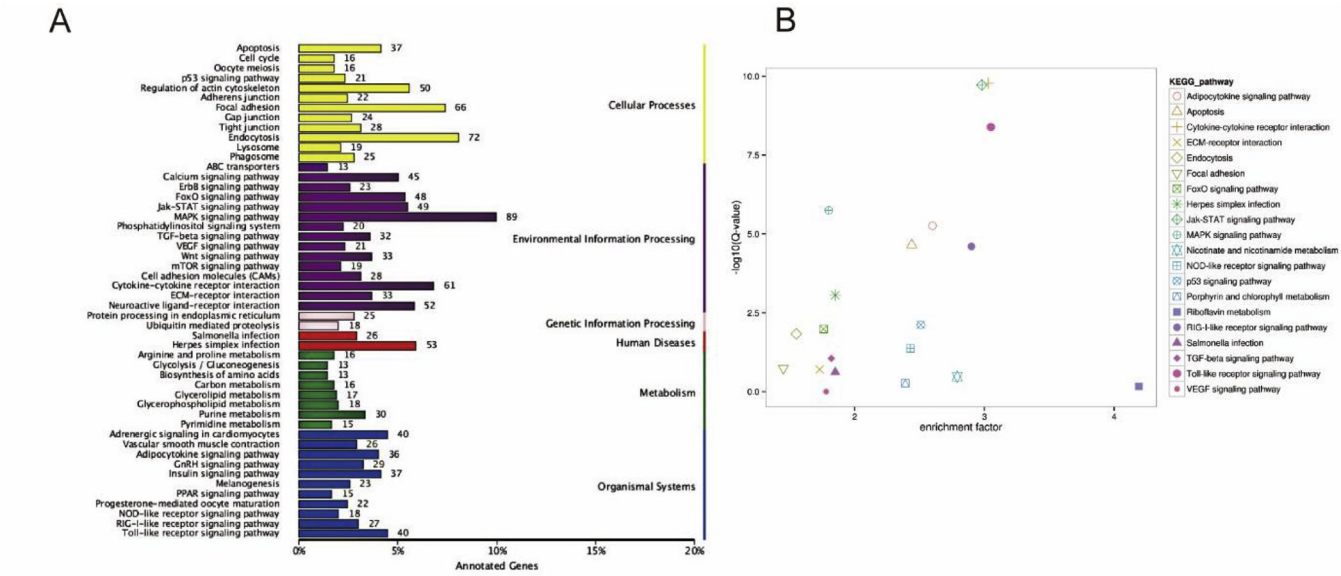

Fig. 3. Classification chart of KEGG annotation analysis of DEGs (A). Scatter diagram of KEGG enrichment analysis of DEGs (B). X-axis (enrichment factor) indicates the degree of enrichment of DEGs in each pathway, while Y-axis represents the reliability of enrichment analysis of each pathway.

listed in Table 1, and the relative mRNA expression levels were calculated by normalizing to the reference genes (β-actin and 40s). In addition, several genes were selected to analyze the expression pattern after the challenge with Ah at different time points (3, 6, 12, 24 and 48 h). The process of qPCR was performed with reference to the above.

2.7. Statistical analysis

GraphPad Prism 5 was used to graphics and differences analysis, with the p value less 0.05 as significance.

Table 4
The statistics of immune-related pathways.

Pathway	ko ID	DEG in pathway	All gene in pathway	Ratio	Corrected P-value
Cytokine-cytokine receptor interaction	ko04060	61	169	36.09%	1.69E-10
Jak-STAT signaling pathway	ko04630	49	138	35.51%	1.91E-10
Toll-like receptor signaling pathway	ko04620	40	110	36.36%	4.11E-09
MAPK signaling pathway	ko04010	89	414	21.50%	1.79E-06
RIG-I-like receptor signaling pathway	ko04622	27	78	34.62%	2.50E-05
Herpes simplex infection	ko05168	53	240	22.08%	0.000882,149
NOD-like receptor signaling pathway	ko04621	18	62	29.03%	0.042310834

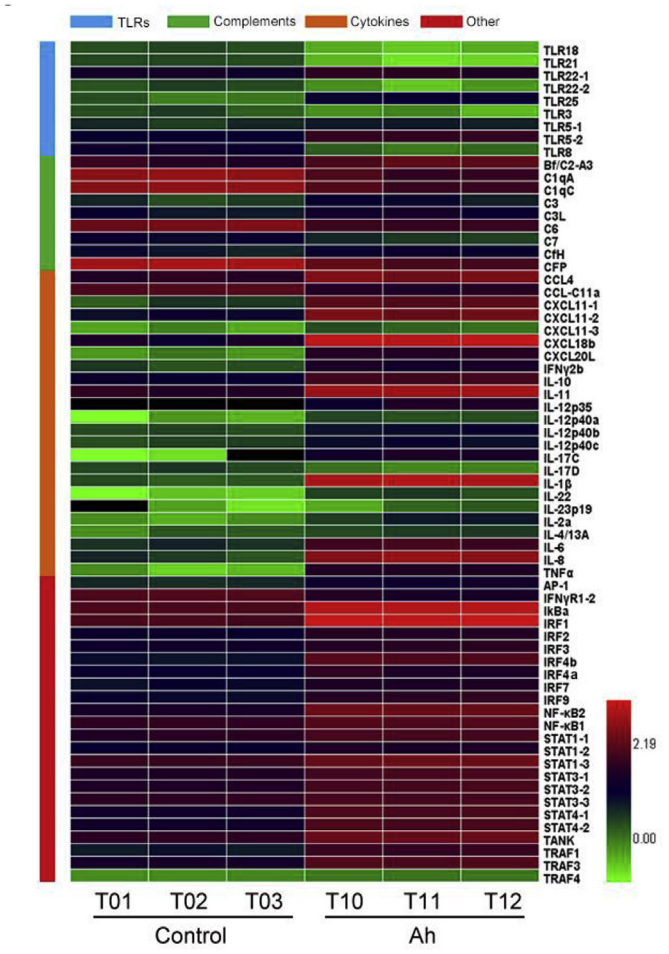

Fig. 4. Heat map of the interesting IRDs. Base on the FPKM-values of unigenes, the expression abundances of the interesting IRDs were presented, and the interesting IRDs were divided into four parts, including TLRs, complements, cytokines and other key molecules of immune-related pathways.

3. Results and discussion

After sequencing, we acquired the Raw Data of six transcriptome libraries, and the clean reads were subsequently quality-filtered from the Raw Data. About 24 million clean reads of each library were obtained with the G/C content of approximately 48% per library (Table 2). Q30 is commonly regarded as a quality index that represents the percentage of high-quality clean reads. In this study, Q30 of each library is higher than 94%, indicating that the sequencing data is high-quality (Table 2). In addition, more than 75% clean reads of each library were mapped to corresponding Unigenes (Table 2).

A total of 6, 213 genes were identified as DEGs, including 3, 066 up-regulated genes and 3, 147 down-regulated genes (Fig. 1). About 65% of DEGs (4, 036) were successfully annotated at least in one database, while 37.2% (2, 311) and 29.8% (1, 852) of DEGs were mapped to GO and KEGG database, respectively (Table 3). In the GO annotation, the DEGs and total Unigenes were annotated into three gene ontology categories: biological process, molecular function and cellular component (Fig. 2). Among the biological process, “response to stimulus” and “immune system process” have higher annotative ratios, suggesting that many DEGs are involved in immune response sparked by Ah stimulation. In the KEGG annotation, the annotation into various pathways consists of abundant immune-related pathways (Fig. 3A). Notably, “Toll-like receptors signaling pathway”, “NOD-like receptor signaling pathway”, “Jak-STAT signaling pathway”, “Cytokine-cytokine receptor interaction” and “MAPK signaling pathway” were highlighted, and the number of annotated genes of each pathway was 40, 18, 49, 61 and 89, respectively. The top 20 of high-annotated pathways were listed (Fig. 3B), which showed the high reliability and significance of these pathways in the KEGG annotation analysis. In addition, the immune-related pathways were counted with the corrected *P*-value less than 0.05 (Table 4), which consists of foregoing pathways. These results reveal that Ah infection may activate various immune-related pathways. In other word, the activated immune-related pathways are involved in the process of host defense and contribute to the elimination of Ah.

The interesting IRDs, including TLRs, complements, cytokines and other key molecules of immune-related pathways, were visualized by heat map (Fig. 4). Similar to mammals, TLRs are curial components of the innate immune system that were induced by corresponding pathogen that can regulate the production of pro- and anti-inflammatory factors in aquatic animals [4,37,38]. In the current study, TLR25, 22–1, 5-1and 5–2 were up-regulated, and the fold-change is 13.2, 3, 2.2 and 7.6, respectively. Meanwhile, a series of pro- and anti-inflammatory factors were induced after Ah infection, which not only proves the strong inflammatory response induced by Ah, but also reveals the relationship between the up-regulated TLRs and these cytokines. These results suggest that the up-regulated TLRs may play important roles in anti-bacteria immunity of *S. prenanti*.

The complement system plays an essential role in distinguishing “self” and “non-self”, as well as in clearing the “non-self”. It has been identified in reptiles, birds, amphibians and fish [39]. In teleosts, there are three pathways, including the alternative, lectin and classical pathway, which initiate the complement-mediated immune response [40,41]. The complement molecule 1–9 and their subtypes exert their function of immunity by one or three of these pathways, and these complement molecules play an indispensable role in host defense against bacterial infection [42,43]. However, our results showed that only the complement 3 (C3) and complement 3-like (C3L) were up-regulated. By contrast, the complement 1, 6 and 7 (C1, 6 and 7) were down-regulated (Fig. 4). These results reveal a weaker complement system in *S. prenanti* after bacterial infection. Nevertheless, according to previous report [44], the cross-take pathway between TLRs and complement system may occur in this study, suggesting that the weak complement system may exert its function by interacting with the TLR-mediated pathway(s).

In this study, interferon regulatory factors (IRFs) were induced by Ah stimulation. IRFs are usually believed to induce the production of

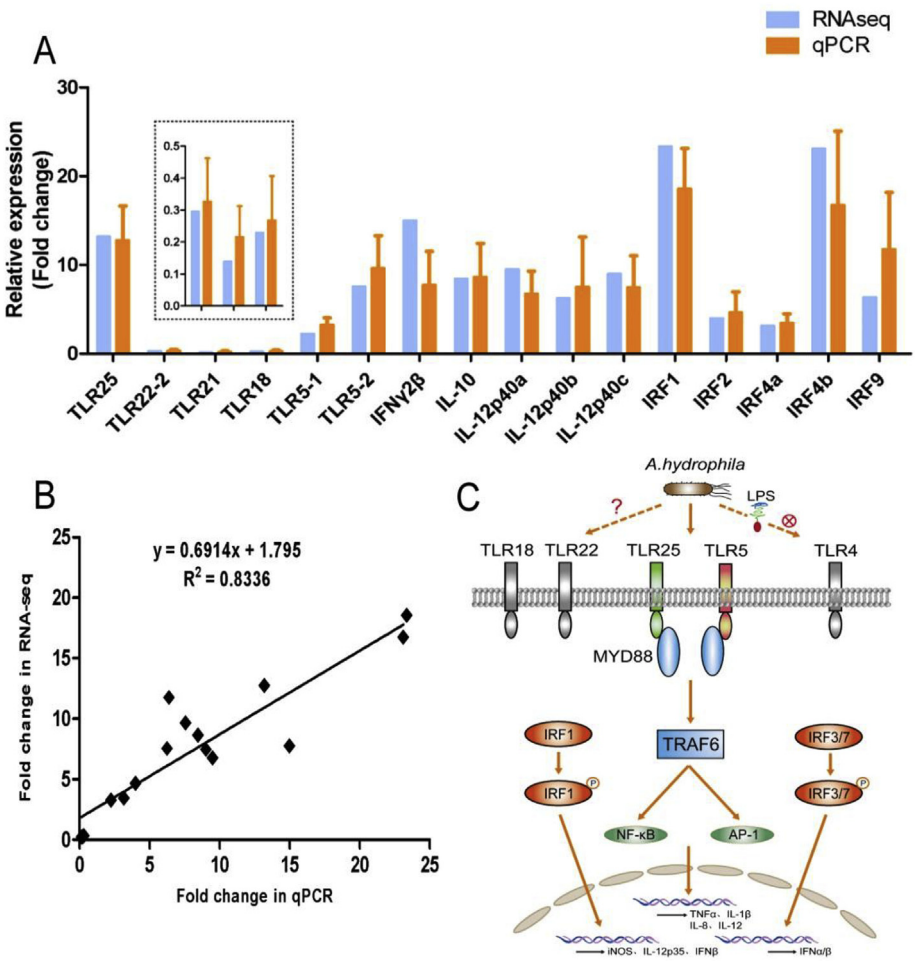

Fig. 5. Comparison of the fold change expression of 16 selected DEGs as determined by RNA-seq and qPCR (A). The results of qPCR were evaluated by normalizing to the reference genes (β-actin and 40s), Mean ± SD (n = 10). Correlation of RNA-seq and qPCR was analyzed (B). The prediction of signaling pathways of anti-bacterial immune response in *S. prenanti* (C).

interferons (IFNs) and therefore to interfere the replication of virus [45–47]. IRF1 and their analogue (named IRF2) are both expressed in a variety of cell types, and IRF1 induced by viral infection can activate type I IFNs (IFN-α/β) promoters [48]. By contrast, IRF2 fails to trigger IFN-α/β and even suppresses the production of IFN-α/β [45]. IRF3 and IRF7 are typical transcription factors for inducing IFN-α/β, and the induced IFN-α/β, together with IRF9 triggers IFN-stimulated genes (ISGs) to suppress virus via Jak-STAT signaling pathway [49–52]. Taken together, these IRFs primarily activate IFN-α/β and their downstream pathway in the host defense against viral invasion. However, recent reports reveal that the type I IFNs induced by IRFs is also involved in the anti-bacteria immunity [53,54]. Indeed, in this study, a series of IRFs were evoked by Ah infection. Among the IRFs, IRF1 gene presented the highest expression level with 23 fold-change, which highlighted the importance of IRF1 in the anti-bacteria immune response. These results suggest that IRFs, especially for IRF1, may play an important role in the anti-bacterial immunity of fish.

To validate the reliability of the transcriptome data, 16 interesting IRDs were selected to detect their expression levels by qPCR. The expression levels of these genes showed similar fold-change to the transcriptome data (Fig. 5A). And the correlation between the transcriptome data and qPCR data was high ($R^2 = 0.8336$) (Fig. 5B), indicating that our transcriptome data is reliable.

According to the transcriptome data and as-known reports, the possible signaling pathway related to the host defense of *S. prenanti* against Ah infection was deduced (Fig. 5C). After Ah infection, TLR25 and TLR5 (TLR5-1 and TLR5-2) initiate the signaling cascade by recognizing the components of Ah. Then, TLR25 and TLR5 recruit the adapter molecule MyD88 to activate two downstream transcription factors, NF-κB and AP-1, resulting in the generation of pro-/anti-inflammatory factors, such as TNFα, IL-1β, IL-8 and IL-12. Meanwhile, the burst IRF1 induces the inducible genes, including inducible nitric oxide synthase (iNOS), IL-12p35 and IFNβ [55–57]. And, the IFNα/β induced by IRF3 and IRF7 participates in the complex anti-bacterial immunity [58]. However, whether the other TLRs, such TLR18 and TLR22, regulate the signaling transduction remains to be proved, but it can be confirmed that the TLR4 fails to recognize the lipopolysaccharide (LPS) of Ah [59].

Given that the expression levels of TLRs may rely on the length of infection period, we analyzed the expression levels of such TLRs (except for the low abundance of TLR22) at different time points after Ah infection. As shown in Fig. 6, TLR25 and TLR5-2 were significantly up-regulated at 6, 12 and 24 h (Fig. 6A and C), while TLR5-1 was weakly increased at 6 and 12 h (Fig. 6B). Furthermore, TLR18 was not induced after Ah infection (Fig. 6D), and the burst of IRF1 occurred behind 6 h (Fig. 6E). TLR5 is a well-known receptor for recognizing bacterial flagellin both in fish and mammals [17,18,60]. TLR25 is believed to mediate the anti-bacterial immunity [14,61], and to activate possibly NF-κB signal via MyD88-independent pathway [62]. In this study, our results provide a new evidence that TLR25 and TLR5 directly participate in the anti-bacterial immunity of fish. Moreover, there are three types of TLR22 (named TLR22-1, TLR22-2 and TLR22-3) found in the

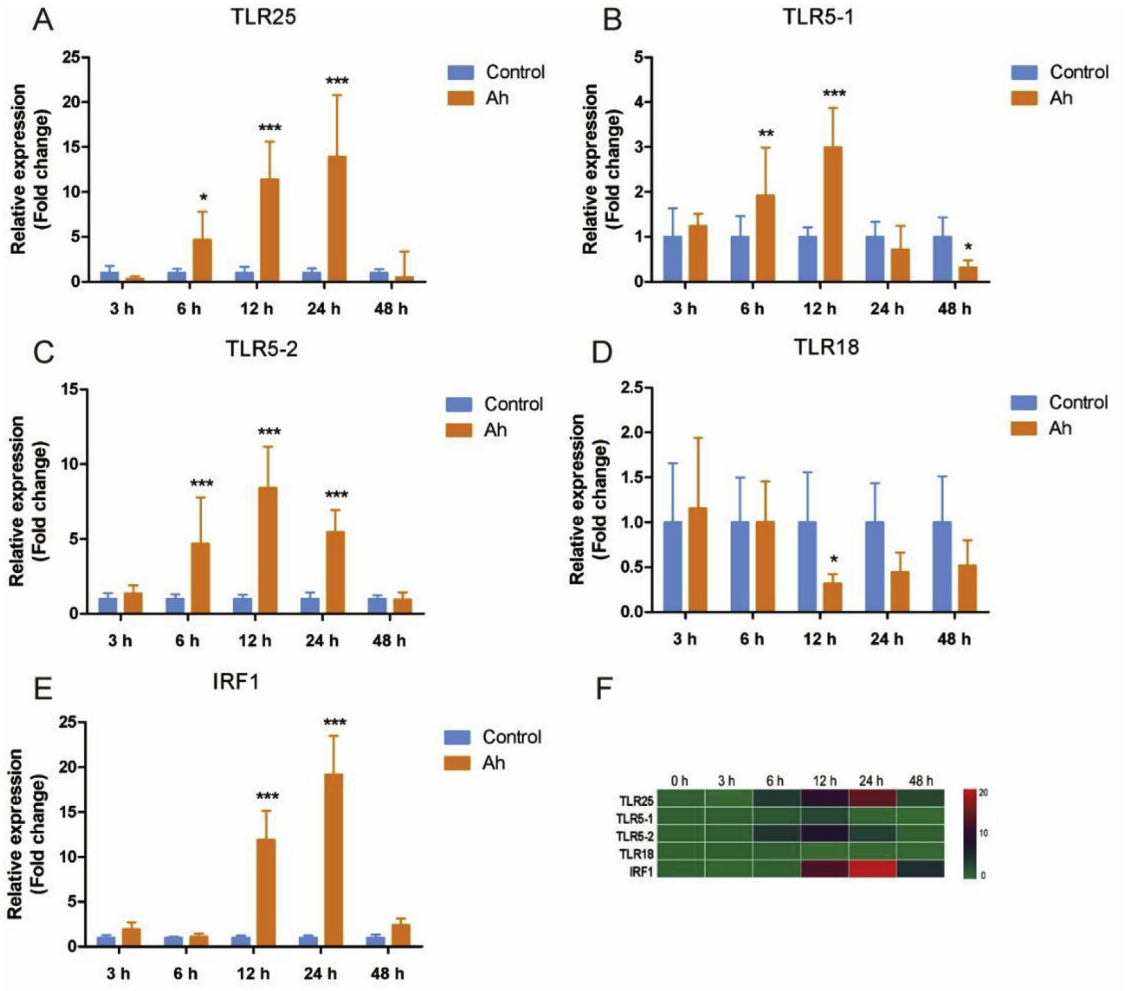

Fig. 6. The mRNA expression levels of TLR25 (A), TLR5-1 (B), TLR5-2 (C), TLR18 (D) and IRF1 (E) were analyzed by qPCR. The expression levels of these genes were visualized by heat map (F). All the results were evaluated by normalizing to the reference genes (β-actin and 40s). Mean ± SD (n = 10), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. corresponding time point of control group (Fold-change as 1); two-way ANOVA plus Bonferroni post-tests.

transcriptome data, but their functions have not yet compared. As shown in Fig. 5A, TLR22-1 and TLR22-2 present different changes after Ah infection, suggesting that three types of TLR22 may be quite different in immune function. Usually, fish TLR22 is regarded as the receptor of Poly(I:C) and mediates the anti-viral immunity [16,63]. However, whether fish TLR22 contributes to the anti-bacterial immunity remains to be investigated. In addition, carp TLR18 can recognize the bacterial components and activate NF-κB signaling pathway [64], but this phenomenon was not found in this study, suggesting that the function of TLR18 in different fish species may be different. In conclusion, the anti-bacterial immunity of fish is a complex process that evokes a series of signal transductions, and this study preliminarily provides insights into the mechanism of signal transduction of TLRs after Ah infection in fish.

Acknowledgements

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