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# Clone, identification and functional character of two toll-like receptor 5 molecules in *Schizothorax prenanti*

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ABSTRACT

Mammal Toll-like receptor 5 (TLR5) can directly recognize bacterial flagellin, initiate the inflammatory signaling cascades and trigger body immune system to clear the "non-self" substances. In teleosts, TLR5 has presented more complexes not only in increasing the molecular types, but also in elevating the functional diversity. In this study, we identified two TLR5 family members in *Schizothorax prenanti*, named as spTLR5-1 and spTLR5-2. The complete coding sequence (CDS) of *spTLR5-1* is 2622 bp, encoding 873 amino acids, while the complete CDS of *spTLR5-2* is 2640 bp, encoding 879 amino acids. Phylogenetic analysis showed that spTLR5-1 and spTLR5-2 were clustered to the TLR5 of *schizothorax richardsonii* and *Cyprinus carpio* respectively. The 3D structure analysis exhibited that the α-helix, β-sheet, and the ligand binding site of spTLR5-1, spTLR5-2 and human TLR5 have large differences. The *spTLR5-1* and *spTLR5-2* had extensively expressed in various tissues, including the higher expression in liver, spleen and head kidney. Both the expression levels of *spTLR5-1* and *spTLR5-2* were significantly up-regulated after *Aeromonas hydrophila* (*A. hydrophila*) challenge. And, the downstream genes, such as *AP-1*, *IKK-α*, *NF-kB*, *IL-1β*, *IL-8* and *TNF-α*, were also significantly up-regulated after *A. hydrophila* challenge. Apart from that, the luciferase reporter assay demonstrated that the co-transfection of spTLR5-1 or spTLR5-2 into HEK293T cells showed the significantly increased NF-kB luciferase activity after flagellin stimulation. In conclusion, our results reveal that both two molecular types of fish TLR5 may commonly mediate the recognition of flagellin and the activation of the downstream inflammatory signaling molecules.

## 1. Introduction

*Schizothorax prenanti* (*S. prenanti*), also known as ya fish, is a freshwater fish distributed in the upper reaches of the Yangtze river and identified as the cultural symbols of Ya'an city in China. However, with the intensive high-density feeding, it is very easy to fall into the abyss of bacterial diseases, such as haemorrhagic septicaemia caused by *Aeromonas hydrophila* (*A. hydrophila*) that seriously impedes the development of the fish feeding industry [1–3]. Therefore, understanding the mechanism of anti-bacterial immunity mediated by some key genes has very important significance for the disease prevention and healthy feeding in *S. prenanti*.

Toll-like receptors (TLRs) are a class of evolutionarily highly conserved membrane-bound receptors that recognize pathogen-associated molecular patterns (PAMPs) and initiate activation of the immune system [4]. TLRs are composed of a leucine-rich repeat (LRR) domain, a transmembrane region, and a Toll/IL-1 receptor (TIR) segment [5,6]. The LRR domain of TLRs binds to corresponding ligand and activates the TIR domain, ultimately leading to the recruitment of downstream signaling molecules and completing signal transmission [7]. Fish TLRs is diversified that may elevate the recognition range for and potential of PAMPs [8]. For instance, TLR1, TLR2 and TLR14 can recognize lipoproteins [9–11]; TLR3, TLR19 and TLR22 can recognize dsRNA [12–15]; TLR7, TLR8, and TLR9 can recognize ssRNA or CpG-DNA [16,17], and TLR15 responds to microbial proteases [18]. Among the TLRs, TLR5 can recognize flagellin and recruit MyD88 through the

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**Table 1**
Primers used in this study.

| Primer | Sequence (5′–3′) | Application |
|---|---|---|
| TLR5-1 F1 | ATGGAATATATATTTATACTGATCCTTTTTGG | Cloning of *spTLR5-1* |
| TLR5-1 R1 | TTATACAGCTGTGTTTGCATGGACA | |
| TLR5-1 F2 | TGTCCAACAACTTCCTGGGTAG | Real-time PCR |
| TLR5-1 R2 | AGAGCATTTCCTGTTAGATTTA | |
| TLR5-1 F3 | tagtccagtgtggtggaattcATGGAATATATATTTATACTGATCCTTTTTGG | Construction of pCDNA3.1-spTLR5-1 plasmid |
| TLR5-1 R3 | ggtttaaacgggccctctagaTTATACAGCTGTGTTTGCATGGACA | |
| TLR5-1 F4 | cttggtaccgagctcggatccGCCACCATGGAATATATATTTATACTGA | Construction of pCDNA3.1-HA-N-spTLR5-1 plasmid |
| TLR5-1 R4 | tgctggatatctgcagaattcTACAGCTGTGTTTGCATGGACAT | |
| TLR5-2 F1 | ATGGCAGTAAGACAAACACTATCTCTG | Cloning of *spTLR5-2* |
| TLR5-2 R1 | TTACACTGCTGTGTCTGCATGAAC | |
| TLR5-2 F2 | TCAGTATTTAGTTATTTGCCAG | Real-time PCR |
| TLR5-2 R2 | GAAGTTTACGGACAAGTTTAGC | |
| TLR5-2 F3 | tagtccagtgtggtggaattcATGGCAGTAAGACAAACACTATCTCTG | Construction of pCDNA3.1-spTLR5-2 plasmid |
| TLR5-2 R3 | ggtttaaacgggccctctagaTTACACTGCTGTGTCTGCATGAAC | |
| TLR5-2 F4 | cttggtaccgagctcggatccGCCACCATGGCAGTAAGACAA | Construction of pCDNA3.1-HA-N-spTLR5-2 plasmid |
| TLR5-2 R4 | tgctggatatctgcagaattcCACTGCTGTGTCTGCATGAACA | |
| MyD88 F | TTGGAAAGACAACTGAGGAAGC | Real-time PCR |
| MyD88 R | TGACAATAGCAGATGAAAGCAT | |
| AP-1 F | TACAACCCAAACCCTTACCCCA | Real-time PCR |
| AP-1 R | CGGACTCTTTTCTTCTCATTCT | |
| IKK-α F | ACTCATTTCTGCTTAGCCCCGA | Real-time PCR |
| IKK-α R | GTCCCATCCTCTTACTCCATCT | |
| NF-κB F | TGCCTGTTGCTGGAGGGTAATG | Real-time PCR |
| NF-κB R | CAGTTTGGTTGAGTCCCGTCCT | |
| IL-1β F | CAGAGCAACAATCTAACCAACG | Real-time PCR |
| IL-1β R | ATTCCCAGGCACACAGGCAGGC | |
| IL-8 F | TGTGAAGTTGAGCCTGAAGATA | Real-time PCR |
| IL-8 R | AGTAGGACTGTGCCCATCGGTA | |
| TNF-α F | CACCAAAACCACCACAGAACTC | Real-time PCR |
| TNF-α R | TAAAGCAAACACTCCAAAAAAG | |
| β-actin F | CTGGTATTGTGATGGACTCTGG | Real-time PCR |
| β-actin R | CAATTTCTCTTTCGGCTGTGG | |

TIR domain, thereby activating the transcription factor NF-κB and AP-1 [19]. Fish TLR5 exists two molecular types, containing the membrane-type TLR5M and the soluble-type TLR5S [20]. TLR5S recognizes flagellin in fluid phase and subsequently amplifies the signal by combining with TLR5M [21]. However, not all fishes contain TLR5M and TLR5S. In zebrafish and grass carp, only membrane-type TLR5a and TLR5b are existent [8,22]. In grass carp, TLR5a and TLR5b can strongly response to viral infection, suggesting that TLR5 may have other ligands in fish [22]. However, the type of TLR5, whether to recognize flagellin, and the signal transduction in *S. prenanti* are still unclear.

In this study, we provide evidence that *S. prenanti* contains two membrane-type TLR5, namely spTLR5-1 and spTLR5-2. We analyzed the molecular and transcriptional characterization of spTLR5-1 and spTLR5-2 by sequence alignment, structural prediction, tissue distribution and qRT-PCR. We also present evidence for the NF-kB activation by spTLR5-1 and spTLR5-2 upon flagellin treatment using a luciferase reporter assay.

## 2. Materials and methods

### 2.1. Fish breeding, bacterial infection and sample collection

*S. prenanti* were purchased from Sichuan Ya-fish Company (Ya'an, China) and bred in fiberglass tanks. Each fish with an average weight of approximately 300 g was kept at 20 °C and maintained under 12 h light/dark cycle in chlorine-free freshwater. Fish were randomly divided into two groups: control group and infection groups (n = 50 per group). After two weeks of habituation, the fish were challenged with *A. hydrophila* which was incubated in Luria Bertani (LB) medium at 37 °C with overnight shaking before the challenge. For the bacterial challenge, fish were injected intraperitoneally with 0.5 mL suspension of *A. hydrophila* (approximately $1 \times 10^8$ CFU per fish), and injecting with the equal volume of PBS as the control group. All the fish were euthanized by using 300 mg/L MS222 (yuanye Bio. Co. Ltd., Shanghai, China) after disposing for different period (3, 6, 12, 24 and 48 h) (n = 10). Then, the spleen, head kidney and liver tissues of each fish were removed and stored in −80 °C until for subsequent RNA extraction. The handling of animals was performed in accordance with the protocols approved by the Animal Protection Committee of Sichuan Agricultural University. All treatments were performed under gentle care and all efforts were made to minimize suffering of the animals.

### 2.2. RNA extraction and cDNA synthesis

Total RNA of each tissue was extracted by RNA pure kit (Aidlab Biotech, Beijing, China), and its concentration and purity were examined by Nano-Drop (2000) spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. First-strand complementary DNA (cDNA) was synthesized using PrimeScript® RT reagent kit with gDNA Eraser (Takara Bio, Co. Ltd., Dalian, China) and used as template for subsequent quantitative PCR reaction. All the operations strictly followed the manufacturer's instruction.

### 2.3. Genes clone and plasmids construction

To clone the complete CDS of *spTLR5-1* and *spTLR5-2,* the primers (Table 1) were designed according to the previous transcriptome database [23]. In addition, the homologous sequences of pcDNA3.1 and pCDNA3.1-HA-N were added to both ends of *spTLR5-1* and *spTLR5-2* of intact CDS, and a fusion plasmid was constructed by homologous recombination according to the manufacturer's instructions.

### 2.4. Sequence analysis, phylogenetic analysis and protein structural model

The amino acid sequences of spTLR5-1 and spTLR5-2 were predicted using the ExPASy translate tool (https://web.expasy.org/cgi-

**Table 2**
Genbank accession numbers of the sequences used in phylogenetic analysis.

| Toll-like receptor | Species | GenBank accession number |
|---|---|---|
| TLR5M | *Homo sapiens* | AAI09119 |
| TLR5M | *Cervus elaphus hippelaphus* | OWK08509 |
| TLR5M | *Tupaia chinensis* | AMN88577 |
| TLR5M | *Mus musculus* | AAI25241 |
| TLR5M | *Rattus norvegicus* | NP_001139300 |
| TLR5M | *Sus scrofa* | NP_001335700 |
| TLR5M | *Crotalus adamanteus* | AFJ51724 |
| TLR5M | *Anolis carolinensis* | ALT10445 |
| TLR5M | *Notechis scutatus* | XP_026538097 |
| TLR5M | *Pogona vitticeps* | XP_020642290 |
| TLR5M | *Python bivittatus* | XP_025026415 |
| TLR5M | *Terrapene mexicana triunguis* | XP_024055424 |
| TLR5M | *Anas platyrhynchos* | AGT21571 |
| TLR5M | *Gallus gallus* | ABW07794 |
| TLR5M | *Meleagris gallopavo* | ADX33343 |
| TLR5M | *Pavo cristatus* | ANC67625 |
| TLR5M | *Xenopus laevis* | AAI70108 |
| TLR5M | *Xenopus tropicalis* | AAI21459 |
| TLR5M | *Carassius auratus* | AQX43081 |
| TLR5M | *Cyprinus carpio* | AGH15501 |
| TLR5M | *Lateolabrax japonicus* | ASW35149 |
| TLR5M | *Lutjanus peru* | ANZ54249 |
| TLR5M | *Oncorhynchus mykiss* | BAC65467 |
| TLR5M | *Takifugu rubripes* | AAW69374 |
| TLR5M | *Cirrhinus mrigala* | AHI59128 |
| TLR5M | *Megalobrama amblycephala* | APT35502 |
| TLR5M | *Salvelinus malma* | AYA57858 |
| TLR5M | *Schizothorax richardsonii* | AIB53747 |
| TLR5M | *Plecoglossus altivelis altivelis* | BAI68384 |
| TLR5a | *Danio rerio* | AVQ55079 |
| TLR5b | *Danio rerio* | AAI63198 |
| TLR5a | *grass carp* | KF736231 |
| TLR5b | *grass carp* | KF736232 |
| TLR5S | *Epinephelus coioides* | KR005612 |
| TLR5S | *Paralichthys olivaceus* | AB562154 |
| TLR5S | *Miichthys miiuy* | KR709249 |
| TLR5-1 | *Schizothorax prenanti* | MK474077 |
| TLR5-2 | *Schizothorax prenanti* | MK474078 |

bin/translate/dna2aa.cgi). The multiple sequence alignment of spTLR5-1 and spTLR5-2 was performed by ClustalW (https://www.genome.jp/tools-bin/clustalw). The predicted amino acid sequences were used to analyse the signal peptide sequences by using the SignalP software (http://www.cbs.dtu.dk/services/SignalP/). Furthermore, spTLR5-1 and spTLR5-2 protein domains were predicted by the SMART program (http://smart.embl-heidelberg.de/) and LRRfinder (http://www.lrrfinder.com/index.php). Individual LRR was identified manually according to previous descriptions [24,25]. The subcellular localization was predicted using PSORT (https://psort.hgc.jp/). Subsequently, the phylogenetic tree was established by MEGA 7.0 software by using the Neighbor-joining method. The GenBank accession numbers of selected proteins were shown in Table 2. In addition, protein structures were analyzed by I-TASSER program (http://zhanglab.ccmb.med.umich.edu/) and modified by PyMOL.

### 2.5. Real-time PCR assay

The expression levels of *spTLR5-1, spTLR5-2* and downstream related genes were evaluated by real-time PCR with SYBR green II (Takara Bio, Co. Ltd., Dalian, China), following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 5 s and several annealing temperature for 30 s; 95 °C for 10 s; melt curve detection of 65 °C for 5 s to 95 °C increment 0.5 °C. The specific primers of *spTLR5-1* and *spTLR5-2* were designed by Primer 5.0 software and shown in Table 1. The relative expression levels were calculated by the $2^{-\triangle\triangle Ct}$ method with *β-actin* as the reference gene.

### 2.6. Cell culture, transfection and NF-kB luciferase assay

Adherent Human Embryonic Kidney 293T (HEK293T) cells were cultured in DMEM medium (Hyclone, UK) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) and maintained at 37 °C in $CO_2$ (5%) humidified incubator. For transfection, when cellular density reached to about 80% confluence in 24-well plate, the cells were transfected with 200 ng of pCDNA3.1-spTLR5-1, pCDNA3.1-spTLR5-2, or empty pCDNA3.1 vectors together with NF-kB (200 ng) luciferase reporter plasmid and renilla reporter plasmid (20 ng) using jetPRIME transfection reagent (Polyplus-transfection Inc., USA). In addition, the cells were transfected with pCDNA3.1-spTLR5-1 (200 ng) or pCDNA3.1-spTLR5-2 (200 ng) together with empty pCDNA3.1 vectors (20 ng) or pCDNA3.1-spMyD88 (20 ng) and co-transfected along with the NF-kB (200 ng) luciferase reporter plasmid and renilla reporter plasmid (20 ng). After 4 h of incubation, the medium was replaced with fresh complete medium to maintain the normal state of the cells. After transfection at 24 h, cells were stimulated with ultrapure flagellin (InvivoGen, USA) with the final concentration of 50 ng/mL, and sequentially incubate for 12 h. After 36 h of transfection, the luciferase activity was measured by the dual-luciferase reporter assay kit (Promega, USA). The luciferase activity was calculated as fold change comparing to the control group after normalizing to renilla luciferase activity.

### 2.7. Confocal fluorescence microscopy

The spTLR5-1-HA and spTLR5-2-HA plasmids were transiently transfected into HEK293T cells which density reached to about 80% confluence in 24-well plates. After transient transfection for 24 h, the cells were removed into 6 cm glass bottom cell culture dish. After adherence into the dish, the cells were gently washed twice with Hank's Balanced Salt Solution (HBSS, Solarbio Science & Technology Co., Ltd., Beijing, China). Subsequently, the cells were fixed with 4% of formaldehyde for 30 min at room temperature, and then washed three times with HBSS. Blocking buffer (BSA) covered at 37 °C for 1 h. The cells were permeated using 0.05% of Triton X-100 (Sigma, USA) and incubated with anti-HA tag mouse monoclonal antibody (1:400) (Abbkine, USA) overnight at 4 °C. Then, the cytomembrane was dyed by 5 μg/mL Wheat Germ Agglutinin (WGA) for 15 min after the cells were washed three times by HBSS and the nucleus were dyed by using 5 μg/mL Hoechst 33342 for 10 min after the cells were washed three times by HBSS. Finally, after washing the cells twice with HBSS, the location of spTLR5-1 and spTLR5-2 in HEK293T cells was observed by the confocal fluorescence microscopy.

### 2.8. Statistical analysis

Statistical analysis and presentation graphics were carried out by the SPSS 22.0 software and GraphPad Prism 6.0 software, respectively. The significance of the gene expression levels in liver, spleen and head kidney was evaluated by one-way ANOVA plus General Linear Models (GLM) procedure. Where applicable, multiple comparisons were performed by Duncan's method. Results were presented as mean ± SEM. $P$ values $< 0.05$ were considered statistically as significance change.

## 3. Results

### 3.1. Cloning and characterization of spTLR5-1 and spTLR5-2

The complete CDS of two *S. prenanti TLR5* genes, named *spTLR5-1* and *spTLR5-2*, were obtained by PCR and sequencing. The sequences were uploaded to NCBI database with the GenBank accession Number (MK474077 and MK474078, respectively). The CDS of spTLR5-1 contains 2622 bp bases and encodes 873 amino acids, while the CDS of TLR5-2 contains 2640 bp bases and encodes 879 amino acids. The


**Fig. 1. Multiple sequence alignment of TLR5-1 with other known TLR5**. The signal peptide, LRR domains, transmembrane (TM) region and TIR domain are indicated as underline and boxes. Among them, the signal peptide is marked with a red line, the LRR domains are indicated by gray boxes, the TM region is indicated by yellow boxes, and the TIR domain is indicated by green boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)


**Fig. 2. Multiple sequence alignment of TLR5-2 with other known TLR5**. The signal peptide, LRR domains, transmembrane (TM) region and TIR domain are indicated as underline and boxes. Among them, the signal peptide is marked with a red line, the LRR domains are indicated by gray boxes, the TM region is indicated by yellow boxes, and the TIR domain is indicated by green boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

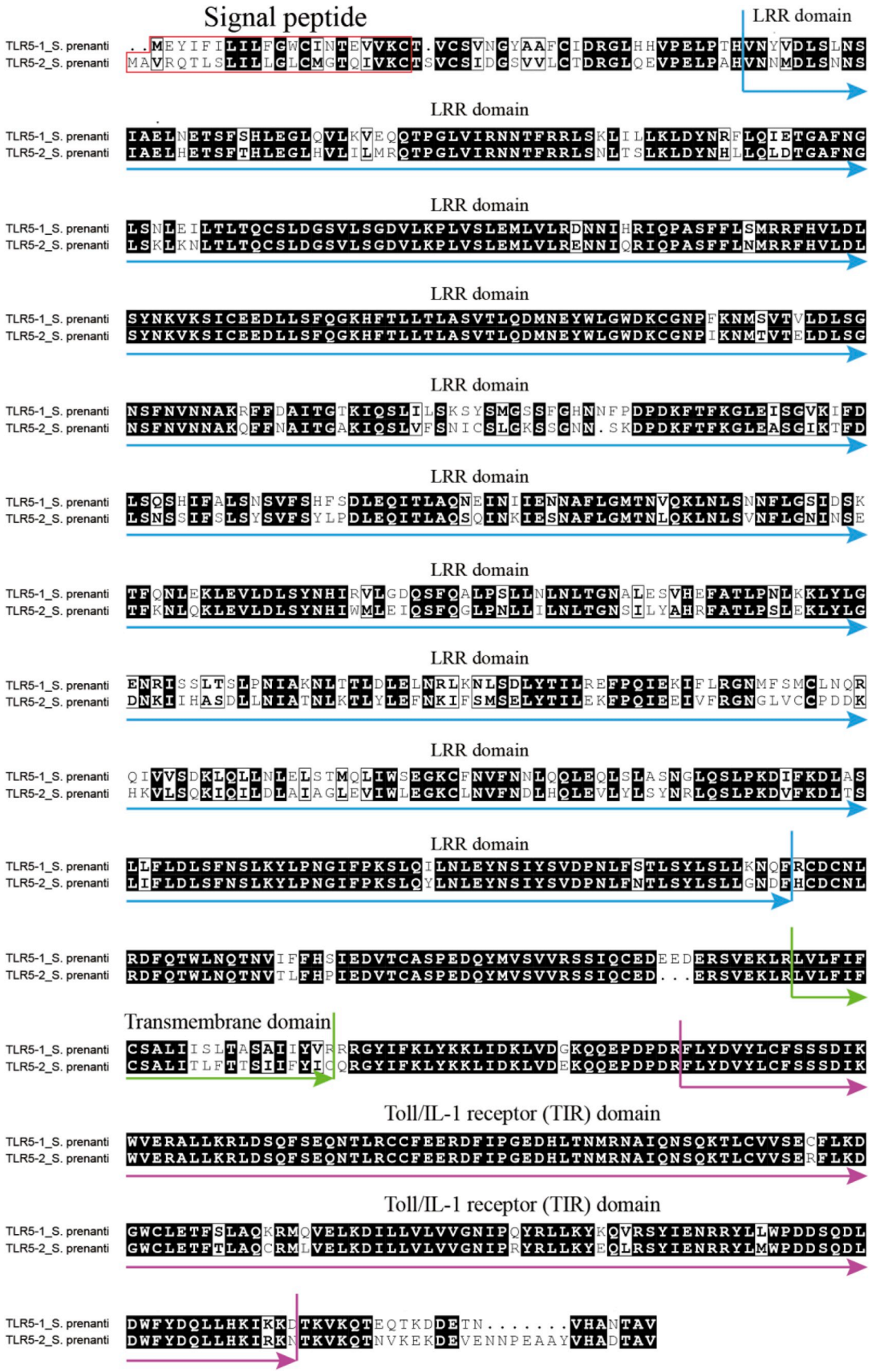


**Fig. 3. Sequence alignment and analyse of TLR5-1 and TLR5-2.** The signal peptide, LRR domain, transmembrane (TM) region and TIR domain are indicated as red box and arrows. Among them, the signal peptide is marked with a red box, the LRR domain is indicated by blue arrow, the TM region is indicated by green arrow, and the TIR domain is indicated by magenta arrow. The black shaded areas present the exact amino acid sequnence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sequences analysis of spTLR5-1 and spTLR5-2 showed that both of them were membrane-type of TLR5. The spTLR5-1 is consisted of a signal peptide of 21 aa, 22 LRR domains, a TM domain at positions 652–674 aa and an intracellular TIR domain at positions 703–851 aa (Fig. 1). The spTLR5-2 is composed of a signal peptide of 23 aa, 22 LRR domains, a TM domain at positions 651–673 aa and an intracellular TIR domain at positions 702–850 aa (Fig. 2).

### 3.2. Sequence alignment between spTLR5-1 and spTLR5-2

To elucidate the similarities and differences of sequences between spTLR5-1 and spTLR5-2, we performed sequence alignment of the signal peptides and domains of spTLR5-1 and spTLR5-2. AS shown in Fig. 3. The signal peptide of spTLR5-1 and spTLR5-2 had 43.5% similarity. The LRR domain and TM domain of spTLR5-1 and spTLR5-2 had 74.8% and 65.2% similarity respectively. In particular, The similarity of TIR domain of spTLR5-1 and spTLR5-2 reached 93.3%, indicating that the domain is very conserved.

### 3.3. Phylogenetic analysis and protein structural model

To investigate the genetic relationship between spTLR5-1, spTLR5-2 and other species TLR5, including the TLR5 of mammals, reptiles, aves, amphibian and teleosts, the phylogenetic trees were established by their amino acid sequences (Fig. 4). Phylogenetic analysis showed that spTLR5-1 had 98.5% similarity with *schizothorax richardsonii* TLR5 and clustered into one branch, while spTLR5-2 had 85% similarity with *cyprinus carpio* TLR5 and clustered into one branch. The spTLR5-1 and spTLR5-2 did not cluster together, indicating that the spTLR5-1 and spTLR5-2 belong to different molecules but still have 77.1% similarity. After that, in order to reveal the relationship between structure and function, the 3D protein structural model of spTLR5-1, spTLR5-2 and


**Fig. 4. Phylogenetic tree of TLR5-1 and TLR5-2 homologs.** Analysis was performed according to the neighbor-joining method using MEGA 7, and bootstrap values were calculated from 1000 replicates. Accession numbers used in this study are given in Table 2.

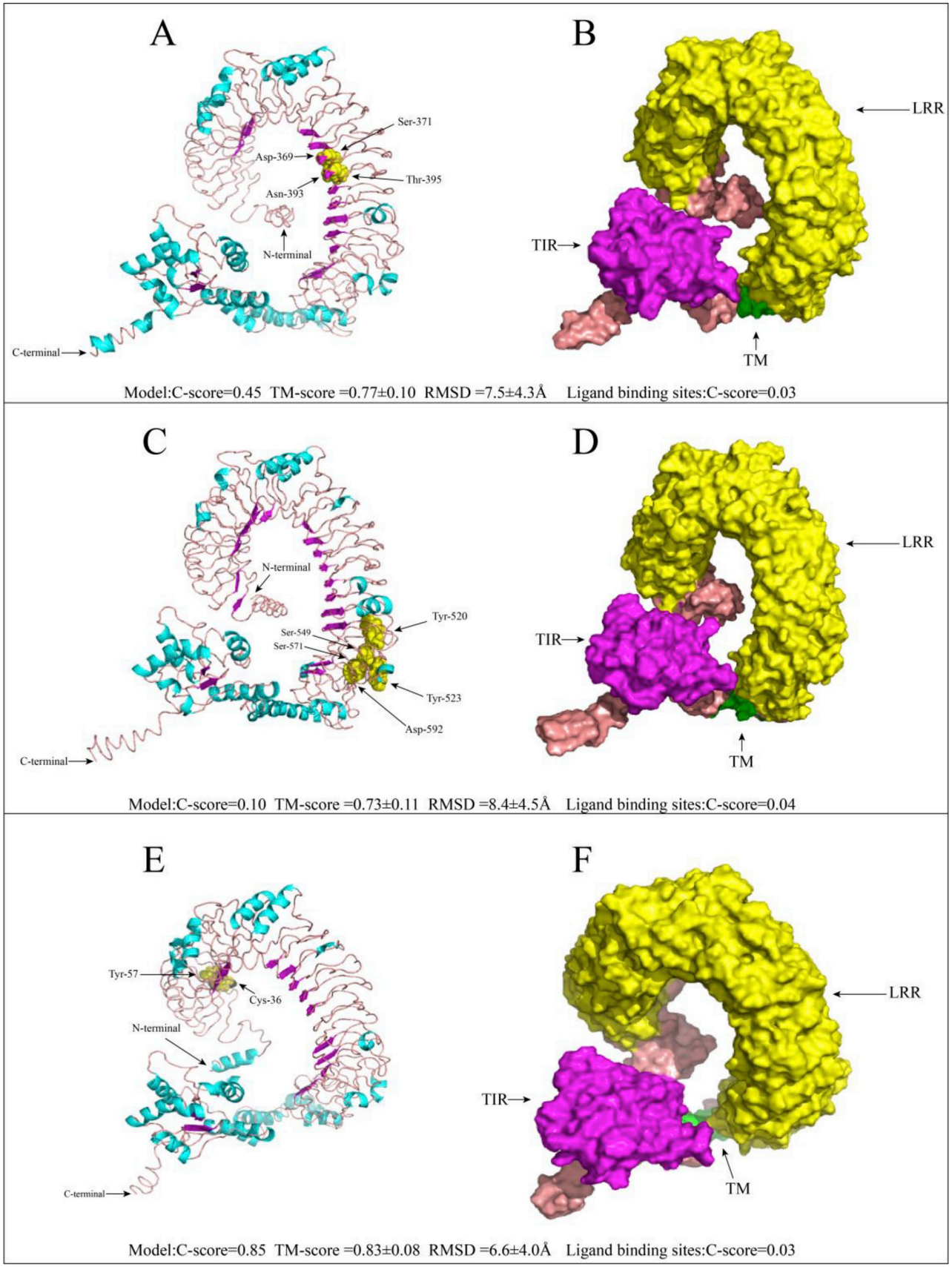


**Fig. 5. The Schematic representation of predicted protein 3D model of TLR5-1, TLR5-2 and human TLR5 created using I-TASSER online server.** Cartoon diagrams of TLR5-1 (A), TLR5-2 (C) and human TLR5 (E), and 3D surface diagrams of TLR5-1(B), TLR5-2 (D) and human TLR5(F), were analyzed by using PyMOL. TLR5-1, TLR5-2 and human TLR5 ligand binding sites as well as N-terminal and C-terminal are marked with arrows in Cartoon diagrams (A, C, E). The 3D surface diagrams of TLR5-1, TLR5-2 and human TLR5 are labeled with LRR motifs (yellow), TM domain (green) and TIR region (magenta) respectively (B, D, F). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

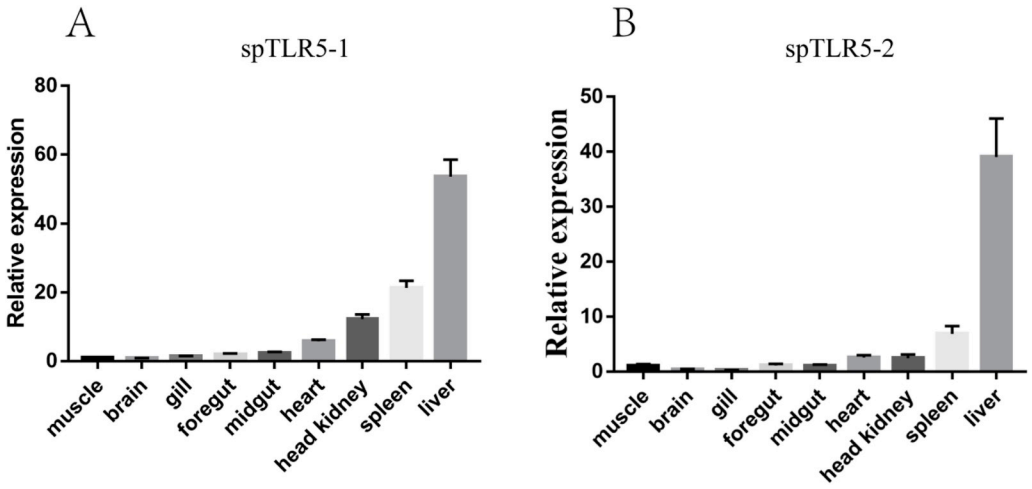


**Fig. 6. qPCR analysis of *TLR5-1* and *TLR5-2* mRNA expression in various tissues.** (A) *TLR5-1* mRNA expression in healthy *S. prenanti* and the results were normalized to *β-actin*. The *TLR5-1* expression level of muscle was used as a control. (B) *TLR5-2* mRNA expression in healthy *S. prenanti* and the results were normalized to *β-actin*. The *TLR5-2* expression level of muscle was used as a control. Data were represented as the mean ± SEM (n = 8).

human TLR5 (hTLR5) were predicted by I-TASSER and modified by PyMOL (Fig. 5). As shown in Fig. 5A, spTLR5-1 protein contains 22 α-helices and 16 β-strands, and there are 4 predicted binding site residues (Asp-369, Ser-371, Asn-393 and Thr-395). spTLR5-2 contains 19 α-helices and 19 β-strands, and there are 5 predicted binding site residues (Tyr-520, Tyr-523, Ser-549, Ser-571and Asp-592) (Fig. 5C). Human TLR5 contains 20 α-helices, 15 β-strands and 2 predicted binding site residues (Tyr-57, Cys-36) (Fig. 5E). Finally, the 3D surface diagrams were presented, which showed greater similarity between spTLR5-1, spTLR5-2 and human TLR5 (Fig. 5B, D and 5F).

### 3.4. Tissue distribution of spTLR5-1 and spTLR5-2

In healthy fish, *spTLR5-1* and *spTLR5-2* transcripts were detected in all tested tissues, including muscle, brain, gill, foregut, midgut, heart, head kidney, spleen and liver. Both *spTLR5-1* and *spTLR5-2* ubiquitously expressed in all tested tissues. Interestingly, both *spTLR5-1* and *spTLR5-2* were strongly expressed in liver tissue among the tested tissues (Fig. 6A and B).

### 3.5. Expression of spTLR5-1, spTLR5-2 and downstream genes after A. hydrophila stimulation

In order to understand the response of *spTLR5-1*, *spTLR5-2*, and their downstream genes after *A. hydrophila* stimulation, *in vivo* experiments were performed. Based on the results of tissue expression distribution, we firstly investigated the expression levels of these genes in the liver. As shown in Fig. 7A, the tested genes, except *IL-8* and *MyD88*, had the higher expression levels after 12 h stimulation, comparing to the control group. The expression levels of *IL-8* peaked at 24 h. The expression levels of *MyD88* showed an increasing trend until 12 h, while *MyD88* expression levels was not significant at 12 h.

Considering that the spleen and head kidney are very important in the antibacterial process of the *S. prenanti*, and *spTLR5-1* and *spTLR5-2* showed the higher expression levels in these tissues. We analyzed the gene expression levels in the spleen and the head kidney after *A. hydrophila* stimulation. In the spleen, *spTLR5-1* mRNA expression levels were significantly up-regulated at 3, 6, and 12 h ($p < 0.05$), but returned to the control levels at 48 h. Moreover, the expression levels of *spTLR5-2* mRNA reached to the peak at 24 h, and the expression levels remained high at 48 h (Fig. 7B). In the head kidney, the expression levels of *spTLR5-1* were gradually increased with the growth of stimulation time except for the 48 h, while the expression levels of *spTLR5-2* maintained equably high expression at the 3, 6, 12, 24 h, but not at the 48 h (Fig. 7C). The downstream genes of TLR5, such as *MyD88*, *AP-1*, *IKK-α*, *NF-κB*, *IL-1β*, *IL-8* and *TNF-α*, showed the different degrees of increase after *A. hydrophila* stimulation both in spleen and head kidney (Fig. 7B and C).

### 3.6. spTLR5-1 and spTLR5-2 are localized on the cell membrane

The subcellular localization of spTLR5-1 and spTLR5-2 was predicted by online software. The predicted results showed that both spTLR5-1 and spTLR5-2 were localized on the cell membrane. For precise subcellular localization, we transfected spTLR5-1-HA and spTLR5-2-HA fusion vector into HEK293T cells, following by staining with WGA red dye and Hoechst33342 blue dye. Then, localization was performed under a confocal microscopy. As shown in Fig. 8, the green fluorescence was coincided with red fluorescence to form yellow fluorescence, which indicates that spTLR5-1 and spTLR5-2 are localized on the cell membrane.

### 3.7. Overexpression of spTLR5-1, spTLR5-2 and spMyD88 in HEK293T cells leads to activation of the NF-kB upon flagellin stimulation

To study the effects of spTLR5-1, spTLR5-2 and spMyD88 on transcription factor NF-kB activation, we performed the luciferase reporter assay using HEK293T cells transiently cotransfected with pCDNA3.1-spTLR5-1, pCDNA3.1-spTLR5-2, or empty pCDNA3.1 vectors together with NF-κB luciferase reporter plasmid and renilla reporter plasmid. In addition, the cells were transfected with pCDNA3.1-spTLR5-1 or pCDNA3.1-spTLR5-2 together with empty pCDNA3.1 vectors or pCDNA3.1-spMyD88 and co-transfected along with the NF-kB luciferase reporter plasmid and renilla reporter plasmid (Fig. 9). spTLR5-1 could significantly activated NF-κB without flagellin stimulation, whereas spTLR5-2 could not (Fig. 9A). In addition, both spTLR5-1 and spTLR5-2 could significantly activate NF-κB signaling pathway upon flagellin stimulation (Fig. 9B). Furthermore, co-transfection of spTLR5-1 and spMyD88 (Fig. 9C) or co-transfection of spTLR5-2 and spMyD88 (Fig. 9E) could significantly activate NF-κB signaling pathway, and NF-κB activity were enhanced upon flagellin stimulation (Fig. 9D and F). The results of the present study imply that spTLR5-1 and spTLR5-2 are involved in the response of flagellin stimulation and synergy with spMyD88.

## 4. Discussion

The recognition of flagellin by TLR5 to initiate immune response is of major significance against pathogenic bacteria [26]. Vertebrates, including mammals, reptiles, and birds, have only one membrane type TLR5 [27–29]. However, teleost fish harbor duplications throughout the genome for many genes, which allows them to possess multiple

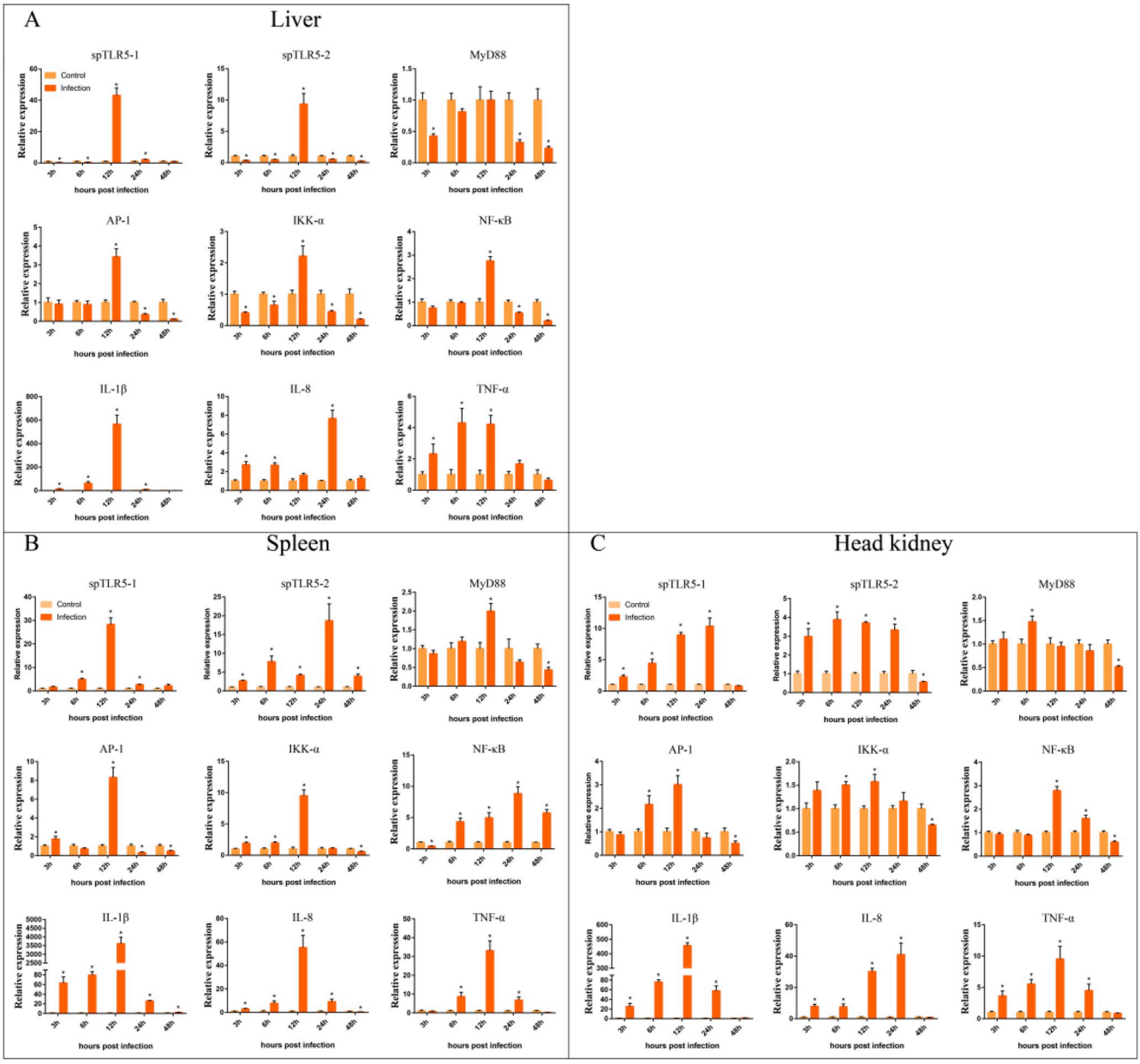


**Fig. 7. Expression levels of *TLR5-1*, *TLR5-2*, and downstream genes after *A. hydrophila* stimulation in liver, spleen and head kidney**. (A, B, C) Fish were injected intraperitoneally with *A. hydrophila* for 3, 6, 12, 24 and 48 hpi. The expression levels of *TLR5-1*, *TLR5-2*, *MyD88*, *AP-1*, *IKK-α*, *NF-κB*, *IL-1β*, *IL-8* and *TNF-α* were determined by qPCR. All results were evaluated by normalizing to the *β-actin* reference gene and gene expression level of control at 5 hpi was set to 1 in every graph. Data were expressed as the mean ± SEM. n = 8. *P < 0.05.

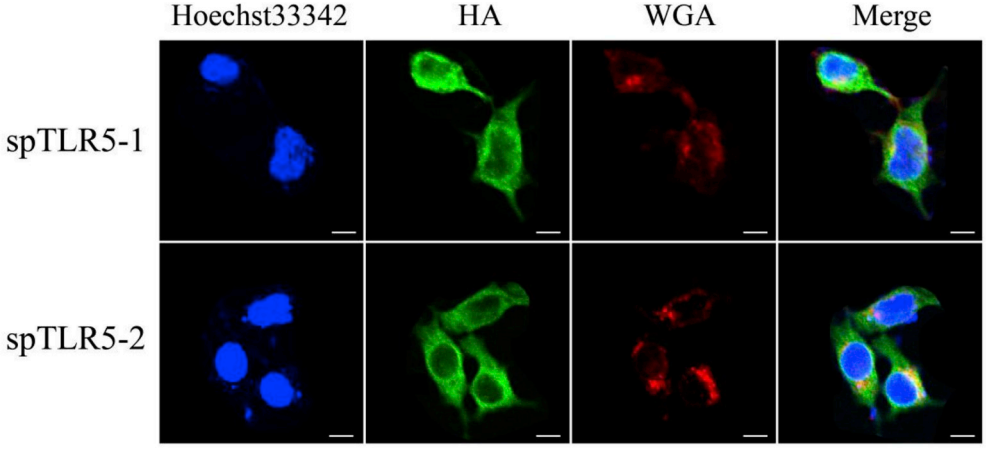


**Fig. 8. TLR5-1 and TLR5-2 localized on cell membrane.** HEK293T cells transiently transfected with TLR5-1-HA or TLR5-2-HA in 24-well plates. After 24 h, the cells were moved to 6 cm glass bottom cell culture dish. The following day, the cells were fixed with 4% paraformaldehyde and permeated use 0.05% of Triton X-100 and incubated with anti-HA tag mouse monoclonal antibody (1:400) overnight at 4 °C. Finally, the membrane was dyed by WGA (5 μg/mL), and the nucleus was labeled by Hoechst 33342 (5 μg/mL).

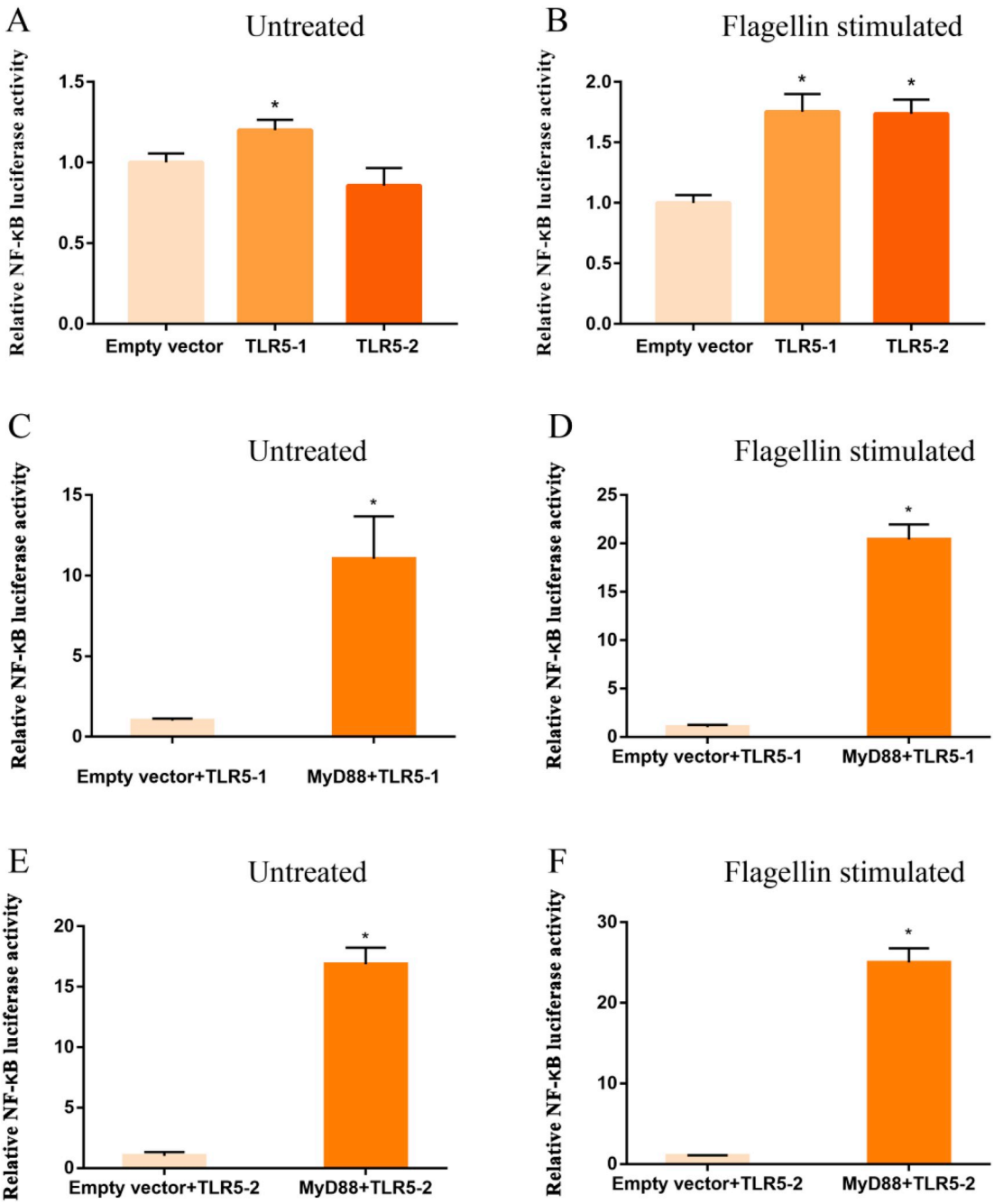


**Fig. 9. Effects of *spTLR5-1*, *spTLR5-2* and *MyD88* overexpression and/or flagellin stimulation on NF-κB luciferase activity in HEK293T cells.** (A, B) HEK293T cells were respectively transfected empty vector (200 ng), TLR5-1 (200 ng) or TLR5-2 (200 ng) together with NF-κB and renilla reporter plasmids. (C, D) HEK293T cells were respectively transfected with TLR5-1 (200 ng) + empty vector (20 ng) or TLR5-1 (200 ng) + MyD88 (20 ng) together with NF-κB and renilla reporter plasmids. (E, F) HEK293T cells were respectively transfected with TLR5-2 (200 ng) + empty vector (20 ng) or TLR5-2 (200 ng) + MyD88 (20 ng) together with NF-κB and renilla reporter plasmids. After 24 h transfection, HEK293T cells were stimulated with ultrapure flagellin of Ecoli at a final concentration of 50 ng/mL in a 24-well plate and continued to incubate at 37 ˚C. After 36 h of transfection, the luciferase activity was measured by the dual-luciferase reporter assay kit and all of luciferase activity were calculated by normalizing to renilla luciferase activity. Data were represented as the mean ± SEM (n = 3). $^{*}P < 0.05$.

types of TLR5, including membrane type TLR5M and secretory TLR5S, thereby conferring a functional diversity of TLR5 in fish [8]. Studies exploring type and function of TLR5 in *S. prenanti* are lacking. Here, we cloned two *S. prenanti* genotypes of *TLR5*, *spTLR5-1* and *spTLR5-2*, respectively. Sequence alignment revealed that both spTLR5-1 and spTLR5-2 contained one signal peptide, 22 LRR motifs, a transmembrane domain, and a TIR domain. The number of LRR motifs are identical to human TLR5 [24], suggesting that the extracellular leucine-rich repeats of spTLR5-1, spTLR5-2, and human TLR5 may have a similar structures. However, the tertiary structure of the protein sequences showed that the amino acid residues at the binding positions of spTLR5-1, spTLR5-2, and human TLR5 ligandswere different. This may indicate that the species difference causes different strengths of TLR5–flagellin interaction. Furthermore, we performed a subcellular localization test and demonstrated that spTLR5-1 and spTLR5-2 localized on the cell membrane, which demonstrates that both spTLR5-1 and spTLR5-2 are membrane receptors. In a previous study, Carlos et al. [6] demonstrated that the TLR5a and TLR5b of zebrafish must form heterodimers for a functional TLR5. In this study, we found that both spTLR5-1 and spTLR5-2 were individually activated by flagellin,

suggesting that spTLR5-1 and spTLR5-2 could be activated without heterodimer formation.

In *Pampus argenteus* and *Pelteobagrus vachellii*, TLR5 can respond to *A. hydrophila* and activate downstream gene expression [30,31]. To verify a similar function in spTLR5-1 and spTLR5-2, we performed a stimulation test *in vivo*. Based on the results of tissue expression, we selected tissues from three organs, the liver, kidney head, and spleen for detection. Consistent with the above results, *spTLR5-1* and *spTLR5-2* were highly expressed in the liver, kidney head, and spleen after *A. hydrophila* infection. In addition, *spTLR5-1* and *spTLR5-2* downstream-related genes, such as *AP-1*, *IKK-α*, *NF-kB*, *IL-1β*, *IL-8,* and *TNF-α* exhibited significant expression. However, *TLR5* expression was very low in the liver of *Ctenopharyngodon idellus*, *Pelteobagrus vachellii* and *Scophthalmus maximus* [22,31,32], which could be due to species differences. Collectively, we speculate that spTLR5-1 and spTLR5-2 respond to *A. hydrophila* primarily through immune-related tissues.

Previous studies have found that a chimera composed of TLR5S from rainbow trout and human intracellular TIR from TLR5 recognizes flagellin and activates NF-κB in HeLa cells [33]. This suggests that fish TLR5S can serve as an adjuvant amplifying membrane TLR5 signaling even across species. Similarly, since spTLR5-1 and spTLR5-2 could respond to bacteria, the next question was whether they recognize flagellin like human TLR5. Therefore, we performed a dual luciferase reporter test. As expected, spTLR5-1 and spTLR5-2 significantly activated NF-κB signaling pathway, when HEK293T cells were stimulated with ultrapure flagellin. In addition, spTLR5-1 or spTLR5-2 co-transformed with MyD88 significantly activated NF-κB signaling and this signal could be strengthened upon flagellin stimulation. These results are similar to that of previous studies [22,33]. To the best of our knowledge, only *Cyprinidae* exhibit two membrane-type TLR5, with significant differences within genera and species [8,22]. For example, in zebrafish, TLR5a and TLR5b cannot individually activate NF-κB signaling, and formation of heterodimers is necessary for the recognition of flagella and activation of NF-κB signaling pathway [8]. Further studies are needed to explore the significance of these differences in the *Cyprinidae*.

In conclusion, both of *spTLR5-1* and *spTLR5-2* have similar structures and localize on the cell membrane. Both have extensive tissue expression and can be activated after *A. hydrophila* infection. Both spTLR5-1 and spTLR5-2 activate NF-κB signaling pathway upon flagellin challenge and synergy with spMyD88. These results enrich our understanding of the TLR5 family.

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