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Molecular insights of a novel cephalopod toll-like receptor homologue in *Sepiella japonica*, revealing its function under the stress of aquatic pathogenic bacteria

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ABSTRACT

Toll-like receptors (TLRs) play an important role in defense response to pathogens in mollusk. In this study the first TLR from *Sepiella japonica* (named as *Sj*TLR) was functionally characterized, and its full-length cDNA consisted of 3914bp (GenBank accession no. AQY56780.1) including an open reading frame of 3582bp, encoding a putative protein of 1193 amino acids. Its theoretical molecular weight was 137.87 KDa and the predicted isoelectric point was 3.69. The derived amino acids sequence comprised of an extracellular domain including 26 amino acids signal peptide and eleven leucine-rich repeats (LRR), capped with LRRCT and LRRNT followed by transmembrane domain and cytoplasmic Toll/IL-1R domain (TIR). In addition, 12 potential *N*-linked glycosylation sites were present in the ectodomain to influence protein trafficking, surface presentation and ligand recognition. Multiple sequence alignment and phylogenetic analysis revealed that *Sj*TLR shared the highest similarity to that of *Euprymna scolopes* and they fell into the same clade. Real-time PCR showed *Sj*TLR expressed constitutively in all tested tissues, including gill, liver, brain, muscle, intestine, heart, lobus opticus and stomach, but showed different expression levels with genders. The highest expression was in the liver, and the lowest was in stomach for both genders. The functional domain region sequences encoding LRRs domain protein and TIR domain containing protein (TcpB) were expressed in BL21(DE3) respectively and purified with Ni-NAT Superflow resin conforming to the expected molecular weight. The cellular localization of *Sj*TLR in HEK293 cells was conducted and plasma membrane localization was detected. *Sj*LRRs internalization upon the activation of LPS was also observed, and dramatic redistribution of *Sj*LRRs in the cytoplasm with distinct perinuclear accumulation was found. After *Sj*TLR transfection Toll/NF-κB signaling pathway was active in HEK293 treated with LPS and TNFα. The nuclear related genes may also be activated by NF-κB in the nucleus, and the corresponding mRNA was transferred through the intracellular signal transduction pathway, so that IL-6 cytokines could be synthesized and released. After infection by *Vibrio parahemolyticus* and *Aeromonas hydrophila* the expression of *Sj*TLR were upregulated with time-dependent manner. These findings might be valuable for understanding the innate immune signaling pathways of *S.japonica* and enabling future studies on host-pathogen interactions.

1. Introduction

Animal immunity comprises innate immunity and adaptive immunity. The innate immune system is the first line for all multicellular animals and almost the only defense mechanism for invertebrates that protects host from invading microbial pathogens [1]. This efficient and complex system is based on a set of germ cell encoded receptors termed innate pattern recognition receptors (PRRs) that function as inductors of the host defense system and recognize the conserved microbial structures of pathogen-associated molecular patterns (PAMPs) [2]. Toll-like receptor (TLRs), an ancient family of PRRs, play an essential role in the activation of innate immunity, and they are first found in the *Drosophila melanogaster* [3]. Subsequently, mammalian homologs of TLR were identified one after another. Typical TLRs are escribed as type-I transmembrane proteins with many extracellular domains containing leucine-rich repeats (LRRs) domain necessary for the recognition of PAMPs, a transmembrane (TM) domain, and a cytoplasmic Toll/Interleukin-1 receptor homology domain (TIR) required for the downstream

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signal transduction as well as in the localization of the TLRs [4]. According to the organization of extracellular arrays of LRRs domains, TLR families can be divided into vertebrate-like TLRs (V-TLRs) and protostome-like TLRs (P-TLRs). V-TLRs have an array of LRRs capped by cysteine-rich domains located at the *N*- and C-terminal of LRR domains (LRRNT and LRRCT, respectively). P-TLRs also contain LRRNT and LRRCT domains instead of capping the LRR array, they are found within the array in a tandem orientation [5,6]. According to their respective PAMP ligands TLRs also can be divided into several subfamilies, such as the subfamilies of TLR1, TLR2, TLR5, TLR6 and TLR10, which recognize microbial lipids, sugars and proteomes, whereas the highly related subfamilies of TLR3, TLR7, TLR8 and TLR9 recognize nucleotide derivatives of a viral or bacterial origin [7]. After PAMP recognition, the intracellular TIR domain of TLRs recruits the adapter molecule myeloid differentiation factor 88 (MyD88) and triggers a multistep cascade of activation to eliminate invading microbes [8]. MyD88 consists of a carboxyl terminal TIR domain interacted between TLRs and MyD88 with a short intervening linker segment at the *N*-terminal region and an amino terminal death domain at its C-terminus, which is associated with the DD of IRAK family members [9]. Accompanying the activation of a series of downstream signaling molecules the transcription factor nuclear factor-kappaB is activated, which translocates into the nucleus to bind specific promoter motifs and activate multiple genes that are proinflammatory cytokines including interleukins, interferon, and TNF, and they are responsible for immediate innate response and for triggering adaptive immune cells [10].

TLRs are widely investigated from vertebrates to invertebrates. Until now about 10 functional TLRs have been identified in humans and 12 in mice, and TLR1–TLR9 are highly conserved [7]. Seventeen different TLRs have also been found in fish [11], and up to 16 TLRs have been identified in the lamprey genome [12]. In invertebrates, about 22 TLRs have been implicated in the sea urchin genome [13,14], nine Toll genes have been found in Drosophila [15], and several shrimp TLRs have also been reported [16], but only one Toll has been found in the *Caenorhabditis elegans* genome [13]. In mollusks, Toll receptors from *Chlamys farreri* [17,18] and *Crassostrea gigas* have been reported [19]. A primitive MyD88-dependent TLR signaling pathway has been characterized in the mollusk *C.farreri* and this TLR signaling pathway is important in immune defense against *Listonella anguillara* [16]. However, few information is available on TLR of cephalopods and its function transcriptional level under aquatic pathogenic bacterial infection [20].

Sepiella japonica is an important economic cephalopod in the East Sea of China, and emerges gradually as an economically important delicacy of the commercial invertebrate aquaculture industry [21]. However, the marine cephalopod is sensitive to a wide range of environmental conditions, some of which cause grievous impact on its survival and growth, for example pathogenic infections have emerged as prominent threats to mollusca integrity as a human food source. In view of few related reports of *S.japonica* about its immunity, this cephalopod was regarded as our experimental animals, and TLR was the candidate gene (named as *Sj*TLR) for its immunoregulatory functions in aquatic invertebrates. All the results will contribute to better understanding of *Sj*TLR in mollusca and the biochemical resistance mechanisms used by marine consumers to cope with their allelochemically defended prey.

2. Material and methods

2.1. Experimental animals

Adult *Sepiella japonica* were obtained from CangNan breeding farm in WenZhou, Zhejiang province, P. R. China. Different tissues including brain, liver, muscle, gill, heart, intestine, pancreas, optic lobe, ovary, nidamental gland of females in the late vitellogenic stage and testis in the spermiation stage of males were collected for further gene expression analysis. Total RNA was isolated from different tissues with Trizol reagent (TaKaRa, China) and the ratio of A_{260}/A_{280} was determined. The cDNA synthesis was carried out with M-MLV RTase cDNA Synthesis Kit (TaKaRa, China).

Table 1
PCR primer sequences.

Primer	Sequences
For cDNA clone	
TLR –F	5′-GTCCCAAACAGTGCGAAT -3′
TLR –R	5′-GCGACAAGTCCAATACCG -3′
For 5′ and 3′ RACE	
5′-1	5′- GGACAGTGGTGTAACG-3′
5′-2	5′-GCGAGAAGACGCTGAAGTTG-3′
5′-3	5′-GAGCCATGCTGTCGTTGA-3′
3′ −1	5′-TGGAAGCAGATCAACTTGAGTCAGGT-3′
3′ −2	5′-CTTAATTGAAGAAATCATGGCCTCGAAC-3′
For qRT-PCR	
SJTLR –F	5′-TCCGTTCCGATGTGACTG-3′
SJTLR-R	5′-TCCGACTCGCTTATGTGAT-3′
β-actin-F	5′-GCCAGTTGCTCGTTACAG-3′
β-actin-R	5′-GCCAACAATAGATGGGAAT-3′
GAPDH-F	5′-TGGTTCCTTGGCTTTTGCT-3′
GAPDH-R	5′-GGTGGTGGTGCGGGTAGT-3′
TUBA-F	5′-GATGCTGCCAACAACTACGCC-3′
TUBA-R	5′-AAGCCACTTCCTGTGCCTCCA-3′
For pET28a	
LRRs-F	5′-CGGGATCCCTCTTCTACCTCG-3′
LRRs-R	5′-CCCAAGCTTCGCCATGAAACTTG-3′
TcpB-F	5′-CGGGATCCAAATATAATGTCTTTGTCG-3′
TcpB-R	5′-CCCAAGCTTTCCCCGTACTTTTGGT-3′
For Eukaryotic	
SjTLR-EGFP-F	5′-CCCAAGCATGTCGTCTTTGTCGTC-3′
SjTLR-EGFP-R	5′-CGGGATCCCATTTGCAGTTTGTTT-3′
SjLRRs-pmC-F	5′-CCCAAGCCTCTTCTACCTCG -3′
SjLRRs-pmC-R	5′-CGGGATCCCGCCATGAAACTTG -3′

2.2. TLR identification and full-length amplification of S.japonica

The total RNA from liver of *S. japonica* was used as template after reverse transcription and the ratio of A260/A280 was 1.90. A partial sequence of TLR was cloned from *S.japonica* by specific primer pairs of TLR-F and TLR-R (Table .1) which were derived from transcriptome data. The reaction system was performed in 20 μL volume, including 10 × PCR Buffer 2 μL, $MgCl_2$ 2 μl (25 mmol/L), dNTPs 0.4 μL (2.5 mmol/L), TLR-F 0.8 μL (10 mol/L), TLR-R 0.8 μL (10 μmol/L), template cDNA 0.6 μL, Taq DNA polymerase (TaKaRa, China) 0.4 μL (1U) and 13.0 μl of PCR-Grade water. The PCR amplification was conducted on a Thermal Cycler (Bio-Rad, USA), and amplification conditions were: 4 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 30 s at 56.5 °C, and 60 s at 72 °C, with a final extension of 10 min at 72 °C. The PCR products were gel-purified and sequenced at Shanghai Invitrogen Biological Technology Company (P.R. China). Gene specific primers for rapid-amplification of cDNA ends including 5′-RACE (5′-1, 5′-2 and 5′-3) and 3′-RACE (3′ −1 and 3′ −2, Table .1), were designed based on the known partial sequence. Full-length cDNA sequence of TLR was performed with the specific primers (shown in Table .1) and the primers in the Smart RACE cDNA amplification kit (Clontech, USA). Both 5′-RACE and 3′-RACE were carried out according to the manufacturer's instructions. The PCR products were cloned into the PMD18-T simple vector (TaKaRa, China) and sequenced from both directions. The full-length cDNA was obtained by overlapping the forward and reverse strand sequences. The resulting sequences were verified by the amplification of the whole full length and further subjected to cluster analysis.

2.3. Sequence and phylogenetic analysis

Identity analysis of the cDNA sequence with known sequences

published in GenBank was performed using BLASTn (https://blast.ncbi.nlm.nih.gov), and The full length of it was translated into the amino acids sequence with DNAMAN 8.0. The prediction of *N*-glycosylation sites was performed with the NetNGlyc1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The analysis of transmembrane proteins was achieved by Tmpred (http://www.ch.embnet.org/software/TMPRED form.html). Signal P 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Protein domains were predicted using SMART (http://smart.embl-heidelberg.de/). The draft EST sequence of *S. japonica* and the corresponding gene predictions, annotation, searching, and downloading services can be accessed through the website http://genome.jgi-psf.org/Brafl1/Brafl1.home.html. All other sequences were downloaded from NCBI websites. A phylogenetic tree was constructed based on the neighbor-joining (NJ) method of Molecular Evolutionary Genetics Analysis (MEGA 5.0). Bootstrap analysis was repeated 1000 times to compute a confidence interval.

2.4. Tissues expression profiles analysis of TLR in S.japonica

Total RNA from several tissues (brain, liver, muscle, gill, heart, ovary, nidamental gland, intestine, optic lobe and pancreas) was extracted from adult *S. japonica* (every tissue was pooled from 5 individuals). Two micrograms of total RNA were reverse transcribed in a final 40 μL with PrimeScript™ RT reagent kit (Tli RNaseH Plus, TaKaRa, China) according to the manufacturer's instructions. For each sample, the test and control reactions were run in triplicate. The qRT-PCR was performed in a reaction mixture of 20 μL, containing primer-F 0.8 μL, primer-R 0.8 μL, 2 × SYBR® Premix Ex TaqTM II 10 μL, cDNA template (100 ng/μL) 0.8 μL, ROX II 0.4 μL, ddH_2O 7.2 μL. The standard cycling conditions were: 95 °C for 1 min (initial polymerase activation), followed by 40 cycles of 10 s at 95 °C, 45 s at 59 °C. PCR specificity was checked with dissociation curve analysis from 55 to 95 °C, β-actin, GAPDH and TUBA of *S. japonica* were used as the internal standard, and the specific primers were designed according to the transcriptome data, which had been sequenced and certified. All qRT-PCR primers were shown in Table 1.

2.5. Expression and purification of recombinant LRRs and TcpB

The functional domain region sequences encoding LRRs domain protein and TIR domain containing protein (TcpB) were amplified by PCR with specific primers containing restriction enzyme sites (underline), which contained a recognition sequence at the 5' end to facilitate cloning of PCR product (LRRs-F, LRRs-R, TcpB-F and TcpB-R were shown in Table 1). The DNA fragments of LRRs and TcpB sequences with cohesive ends were obtained by BamH I and Hind III digestion from the PCR product and then ligated into the multiple cloning sites of the vector pET28a (EMD biosciences). The recombinant (pET28a/LRRs/TcpB) were reclaimed and purified by excision from a low melt point agarose gel. After sequencing the plasmid was transformed into *Escherichia coli* BL21 (DE3) (BD Biosciences, San Jose, CA) competent cells. The single colony of *E. coli* cells harboring the pET28a/*Sj*LRRs/*Sj*TcpB was picked and initially grown in 5 mL of LB medium with 50 μg/mL kanamycin, 200 rpm overnight at 37 °C. To induce the expression of the recombinant protein, IPTG(1 mM) was added at the point that the OD_{600} of primary culture had reached 0.8. During the induction stage, the cells were maintained at 20 °C and 200rmp for 3–12 h, and 1 mL bacterium solution was sampled at 3, 6, 8 and 12 h for optimization analysis. The controls were disposed similarly without IPTG.

The purification of histidine-tagged recombinant protein from the crude extract was performed with 3 mL of Ni-NTA Superflow resin (Qiagen Inc., CA, USA), which was equilibrated by 5 mL of pH7.0, 50 mM Tris buffer with 0.3 M NaCl, 1.0 m M β-mercaptoethanol (BME), 1%Triton and 12%glycerol. 50 mL of crude extract was added to the affinity resin (3 ml), and gently mixed for an hour at room temperature. Unbound proteins were removed by centrifugation (2000rmp, 5min, 4 °C) and bound proteins were washed with Tris buffer. The recombinant protein was eluted from the Ni-NTA resin with 0.25 M imidazole in the wash buffer and stored in −80 °C after being freeze-dried for further analysis. The samples were run on 8% and 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with protein markers (Solarbio, CN). After being rinsed twice with distilled water (3min each time wash), the gel was added Coomassie Blue working solution (Beyotime, CN), stained for about 2 h at room temperature and then de-stained with water until the back ground became clear and bands were clearly visible to photograph and analyzed with image acquisition and analysis system (Bio-rad, USA).

2.6. The location of SjTLR in the human embryonic kidney cell line

To examine the *Sj*TLR expression protein location and the effect on NF-κB Signaling pathway activity, eukaryotic expression plasmid of *Sj*TLR and *Sj*LRRs were constructed. Complete ORFs of *Sj*TLR and the segments of LRRs were amplified and inserted respectively into pEGFP-N1 and pmCherry-C1. The primers for the above expression vectors were listed in Table 1, and the recombinant plasmids were named *Sj*TLR-EGFP and *Sj*LRRs-pmCherry severally. The constructed vectors were sequenced to verify the encoding sequence, its orientation and the correct reading frame. The human embryonic kidney cell line (HEK293) was cultured in Dulbecco's modifed Eagle medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 4 mM Lglutamine (Invitrogen, Madison, WI, USA) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. *Sj*TLR -EGFP and *Sj*LRRs-pmCherry were transfected into an HEK293 cell using Lipofectamine 2000 (Invitrogen, Madison, WI, USA) according to the manufacturer's instructions.

After transfection, HEK-293 cells were grown overnight and seeded onto glass coverslips coated with 0.1 mg/mL of polyL-lysine. After 24 h, HEK293 cells were starved for 60 min in serum-free medium to eliminate the effects of medium change. For the *Sj*TLR expression analysis, cells were stained with the membrane probe DiI (sigma, USA) at 37 °C for 5–10 min, fixed with 4% paraformaldehyde for 15 min, and finally incubated with 2-(4-Amidinophenyl)-6-indolecarbamidine (DAPI) (Beyotime, Haimen, China) for 10 min. For the internalization assay, cells expressing *Sj*LRRs-pmCherry were treated with 1 μM FITC-LPS (Sigma, USA) for 5, 30 min at 37 °C, and then fixed with 4% paraformaldehyde for 15 min. Cells were visualized by fluorescence microscopy on a Leica TCS SP5II laser scanning confocal microscope using a HCX PL APO lambda blue 63 × 1.4 oil immersion lens.

2.7. Expression in response to LPS with western blotting and ELISA

After transfection 24 h, HEK293 cells were inoculated in 24 aperture plank, the cells were transfected in serum-free culture medium for 2 h after adhering the wall, and then treated with LPS (0.5, 1 and 2 ng/ml) which was diluted for 5min. Total proteins were extracted from each sample using RIPA Lysis Buffer and quantified by the Bradford method, and then the nuclear proteins of each sample were extracted by EpiQuik Nuclear Extraction Kit (Solarbio, China). 20 μg of each sample was subjected to fractionation by SDS- PAGE and transferred to PVDF membranes, and the membrane was blocked with 5% non-fat powdered milk (Oxoid, UK) for 1 h at room temperature. Mouse monoclonal NF-κB, NF-κB p65, IκB antibody were diluted 1:400 and GAPDH antibody was diluted 1:500 with 5%BSA and incubated with the membrane overnight at 4 °C. The membrane was then washed with TBST five times (5 × 10 min), and incubated with m-IgGκBP-HRP (santa cruz, US) antibody for 1 h, followed by washing with TBST three times (3 × 10 min) and TBS twice (2 × 5 min). The reactive protein bands on the membrane were visualized using the ECL reagent (Transgen) and exposed to an x-ray film in the darkroom. Image gray scale analysis was used to

compare the density of bands on the Western blot. The expression trend of NF-κB, NF-κB p65 and IκB in response to increasing LPS stimulation concentration was assayed by Western blotting, with GAPDH as the control. After transfection cells were stimulated by LPS and TNFα (2 μg/ml) for 24 h, the concentration of IL6 cytokines were measured by cell culture supernatant. Concentrations of IL6 cytokines in the supernatants of HEK293 were measured using commercially available ELISA kits following the manufacturers' instructions (R&D systems).

2.8. Expression analysis of SjTLR upon bacterial stimulation

Liver and gill were selected as candidate tissues for investigating the temporal expression profiles of *Sj*TLR challenged by pathogenic bacteria. The live *V.parahemolyticus* and *A.hydrophila* that were isolated from marine shellfish before, were injected into the muscles of *S. japonica* respectively of 100 μL diluted with PBS (pH 7.4, final concentration of O.D 600 = 0.4). Forty-two individuals of the challenged squids by different Vibrio were randomly collected at 0, 2, 4, 8, 12, 24 and 48 h post-challenge. The squids that were injected with the same amount of PBS, were used as the blank group. The tissues from three squids of the same time were pooled together as one sample for total RNA extracting. Two micrograms of total RNA from each group (n = 3) were reverse transcribed in the final volume of 40 μL with a PrimeScript TM RT reagent kit (Perfect Real Time) (TaKaRa) following the manufacturer's instructions. The methods of tissues collection, total RNA extraction, cDNA synthesis and real-time PCR analysis were performed as described above. The relative mRNA level of *Sj*TLR, was calculated using the $2^{-\Delta\Delta CT}$ method. All data are given as mean ± SD (standard deviation). Differences between experimental and control groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with PASW Statistics 22.00 (SPSS, Chicago, IL, USA). The significance level was set at 0.05.

3. Results

3.1. Isolation and characterization of the full-length SjTLR cDNA

In this study the full-length TLR cDNA sequence of 3914bp was cloned from *S. japonica* (GenBank accession no. AQY56780.1), which contained an open reading frame of 3582bp, encoding 1193 amino acids. The 5′ UTR was 185bp and the 3′-UTR was composed of 147bp with a polyadenylation signal (AATAAA) appearing at position 13bp downstream of stop codon (TAA) (Fig. 1). The putative protein, which shared 89% identity with *Euprymna scolopes* counterpart, had a theoretical molecular weight of 137.87 KDa and the predicted isoelectric point was 3.69. The deduced amino acids sequence of *Sj*TLR had a typical organization that was characteristic of Toll proteins: 1 LRRNT, 11 LRR and 1LRRCT motifs in the ectodomain and 1 TIR domain in the cytoplasmic region (Fig. 2). In addition, 12 potential *N*-linked glycosylation sites were present in the ectodomain, which may influence protein trafficking, surface presentation and ligand recognition. A signal peptide of 26 amino acids was predicted by the SignalP program. According to the predicated LRRs, TM and TIR domains of this putative amino acid sequence, *Sj*TLR was classified as TLR family.

3.2. Sequence and phylogenetic analyses of SjTLR

The deduced amino acids sequence of *Sj*TLR was aligned with TLR sequences of protostome, deuterostome invertebrates and vertebrates. In the resulting tree. The predicted *S. japonica* TLR amino acids sequence showed a high degree of similarity with the sequence of Mollusk, especially with Cephalopoda (Fig. 3). *Sj*TLR formed one common clade with TLR of *E.scolopes*, and then *Octopus bimaculoides*. *Sj*TLR sequence were aligned in ClustalW program, which showed their high homology and conservation, especially in TIR, TM and some crucial regions (Fig. 4).

3.3. Tissue expression profiles of SjTLR

To investigate the tissue profiles of *Sj*TLR, real-time PCR was performed on various tissue in different gender. As shown in Fig. 5, *Sj*TLR expressed constitutively in all tested tissues, including gill, liver, brain, muscle, intestine, heart, lobus opticus and stomach, but showed different expression levels with genders, for example, lobus opticus and nidamental gland had higher expression in females. However, the highest expression was in the liver, and the lowest level was in stomach for both genders.

3.4. Expression and purification of recombinant LRRs and TIR

The pET-LRRs and pET-TcpB plasmids were successfully constructed and expressed in BL21(DE3). IPTG induction on the expression of recombinant LRRs and TcpB were carried out with different duration to optimize the optimal induced conditions. The most suitable condition was 20 °C at 16 h. After IPTG induction, the whole-cell lysate was separated by SDS-PAGE, and a distinct band of LRRs was revealed with molecular mass of 85 kDa and TcpB was approximately 17 kDa. The expression recombinant LRRs and TcpB were purified with Ni-NAT Superflow resin. The electrophoretogram showed the bands of the reclaimed protein (Fig. 6).

3.5. Location of the SjTLR protein and its interaction with LPS

The *Sj*TLR-EGFP and *Sj*LRRs-pmCherry plasmids were successfully constructed. The fusion expression, enhanced green fluorescent protein (EGFP) tagged at the C-terminal, of *Sj*TLR-EGFP in HEK293 cells was conducted and plasma membrane localization was detected by fluorescent microscopy with minimal intracellular accumulation (Fig. 7A). *Sj*LRRs-pmCherry internalization upon the activation of 1 μM FITC-LPS in HEK293 cells was also detected, and dramatic redistribution of *Sj*LRRs in the cytoplasm with distinct perinuclear accumulation was observed (Fig. 7B).

3.6. Activation of NF-κB signaling pathway by LPS stimulation

The cytoplasmic protein level of IκBα, NF-κB and the nuclear protein NF-κB p65 in HEK293 stimulated with LPS were measured for 0.5, 1 and 2 ng/ml by Western blotting. In the cytoplasm, the protein levels of IκBα and NF-κB were gradually increased, but the protein levels of NF-κB were not obvious (Fig. 8A). In the nucleus, the protein levels of NF-κB p65 were increased with different concentrations. It was predicted that Toll/NF-κB signaling pathway was active in HEK293 treated with LPS. After stimulation by LPS and TNFα, the nuclear related genes were activated by activated NF-κB in the nucleus, and the corresponding mRNA was transferred through the intracellular signal transduction pathway, so that IL-6 cytokines could be synthesized and released (Fig. 8B).

3.7. Expression of SjTLR selectively responded to acute immune challenge

According to relative researches and the results of different tissues distribution, liver and gill were selected as the candidate tissues for investigating the temporal expression profiles of *Sj*TLR after challenge. The temporal expression profiles witnessed explicit expression of *Sj*TLR in gill from 0 to 48 h after *V.parahemolyticus* and *A.hydrophila* challenge (as shown in Fig. 9). The expression of *Sj*TLR for *V.parahemolyticus* infection appeared the highest expression at 12 h with 12.4-fold in liver and 7-fold in gill, and for *A.hydrophila* challenge the highest point appeared at 8 h after injection with 5.4-fold in gill and 8.3-fold in liver compared with the PBS group, then declined slowly in the following hours until 48 h. *Sj*TLR expression in *A.hydrophila* was always different from the expression in *V. parahemolyticus* from 0 h to 48 h ($P < 0.05$).

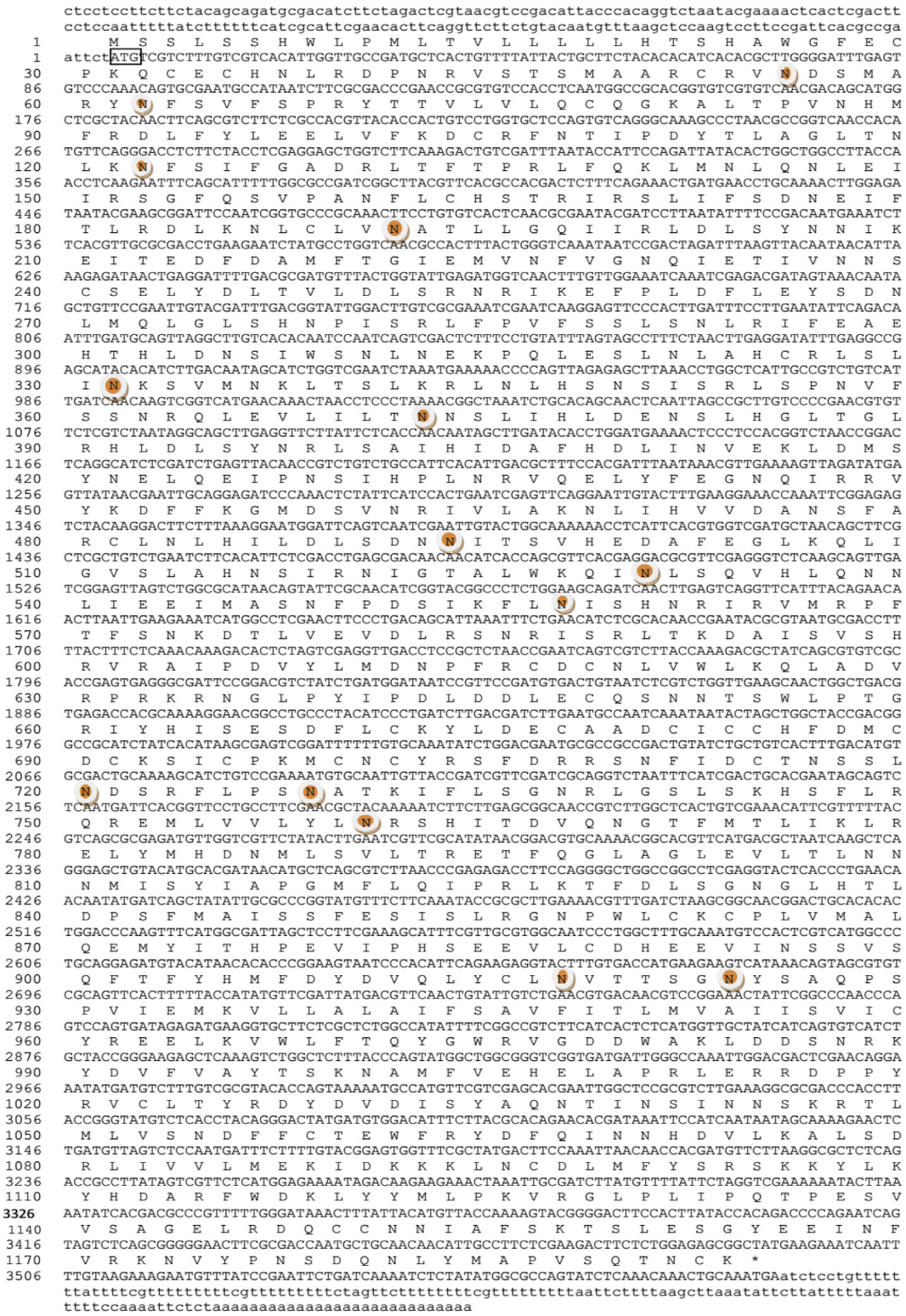

Fig. 1. Nucleotide sequence of *S. japonica* TLR cDNA. The predicted amino acids sequence was shown below the nucleotide sequence. The start codon and stop codon were marked by ☐ and ∗respectively. The *N*-glycosylated sites were marked with. Ⓝ.

4. Discussion

The family of Toll-like receptors (TLRs) are important PRRs, which mediate specific recognition of pathogens by detecting conserved microbial components known as pathogen-associated molecular patterns (PAMPs), and initiate a series of signaling cascades that culminate in the expression of antimicrobial products, inflammatory cytokines and chemokines [22]. This study reported for the first time about molecular clone of TLR in *S. japonica* (*Sj*TLR), whose cDNA sequence contained 1193 amino acids residues including 12 potential *N*-linked glycosylation sites and shared conserved domains with other counterparts in TLR family, for example the tandem LRRs flanking LRR-N/CT for PAMP binding or recognition and the intracellular TIR domain for signal transduction. However, the number of LRR domains varied among

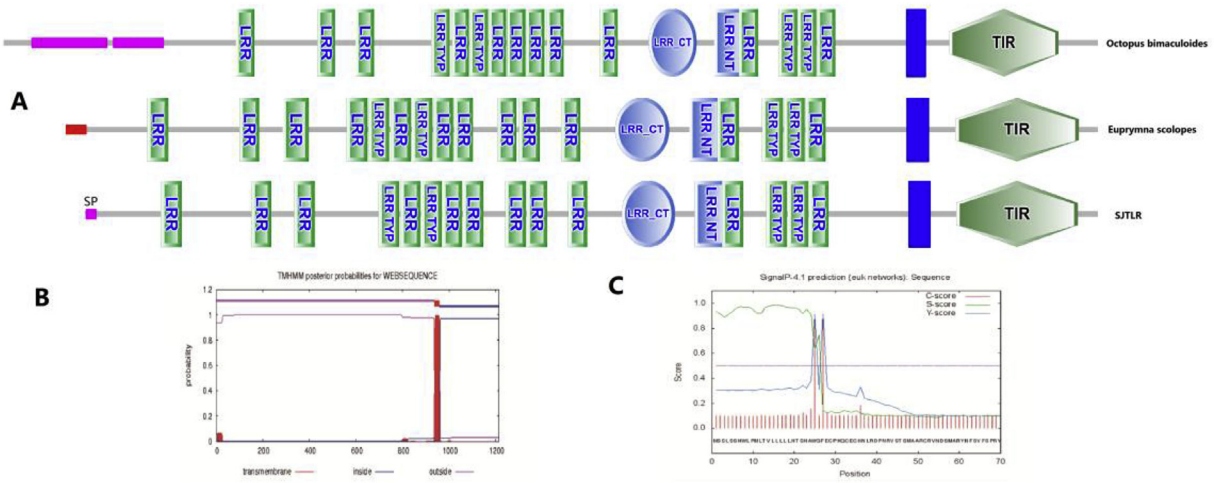

Fig. 2. A: Schematic representation of typical TLRs (*Euprymna scolopes* and Prediction: *Octopus bimaculoides*) and *Sj*TLR: SP (signal peptide), LRR-NT (leucine-rich repeat *N*-terminal domain), LRR-CT (leucine-rich repeat C-terminal domain), TM (transmembrane domain) and TIR (Toll/Interleukin-1 receptor homology domain). B: The analysis of transmembrane proteins was achieved by Tmpred. C: The signal peptide was predicted by Signal P 4.1.

species and it remained unclear how this variability impacted functionality [23]. Some large insertions were also present between LRR1-LRR4 and LRR10-LRR11, which may confer ligand binding specificity [24]. A 26-amino long signal peptide was predicted in *Sj*TLR sequence, which was not always predicted in invertebrate TLRs, despite their localization on endoplasmic or cytoplasmic membranes [25]. Based on the comparison of Toll-like sequences a phylogenetic tree was conducted, the results showed that *Sj*TLR shared 100% sequence homology with TLR of its recently divergent parent *E.scolopes*, then with *O.bimaculoides* and *C.gigas* than other Toll-like sequences, and they fell into the

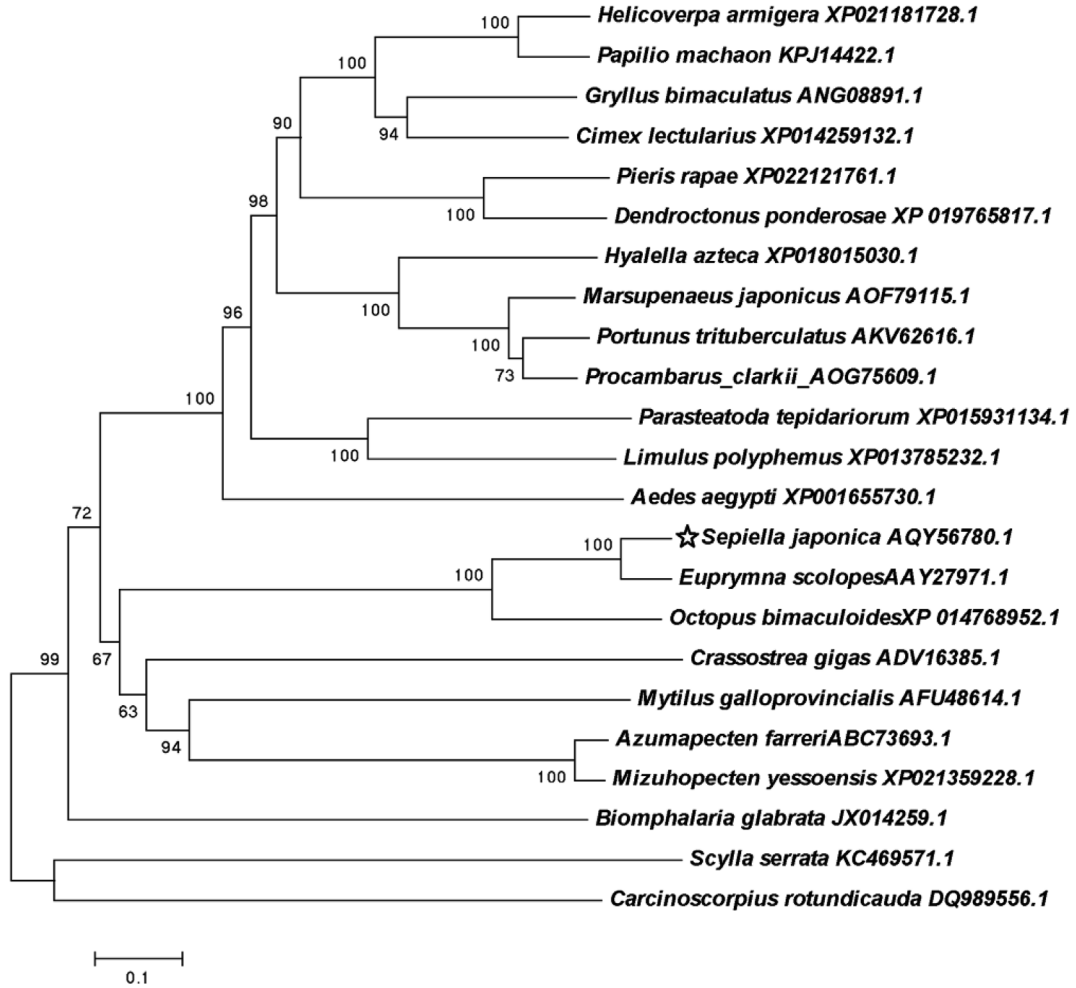

Fig. 3. Phylogenetic tree of *Sj*TLR(⋆) with 22 other TLRs amino acid sequences. The tree was generated based on NJ algorithms using MEGA 5.0. The topological stability of the NJ tree was achieved by running 1000 bootstrap ping replications.

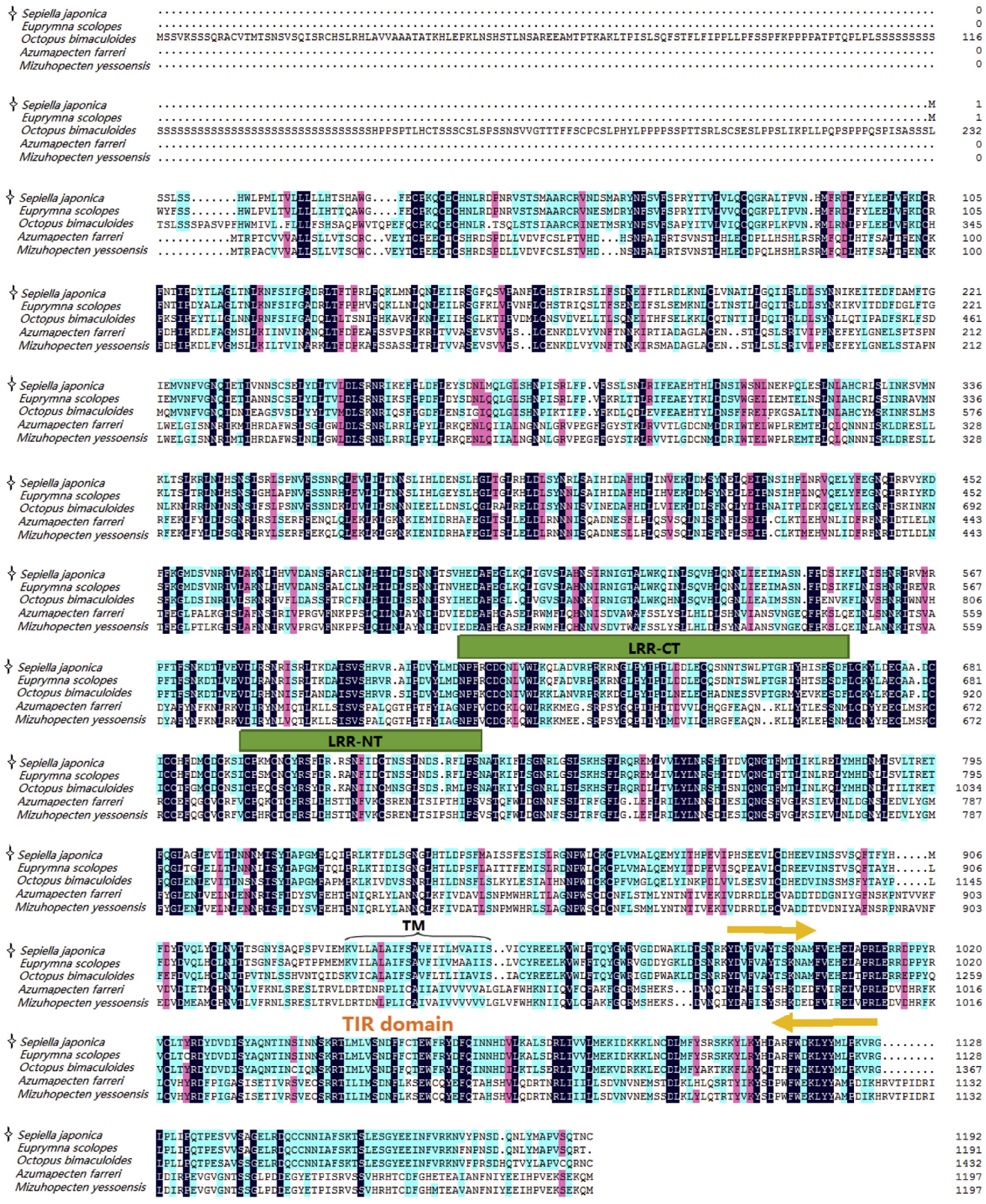

Fig. 4. Multiple alignment of the deduced amino acid sequences of *Sj*TLR with other species. The GenBank accession no. for the sequences were as followed: *Euprymna scolopes* (AAY27971), *Azumapecten farreri* (ABC73693), *Mizuhopecten yessoensis* (XP021359228) and *Octopus bimaculoides* (XP014768952). The dark shade represented 100% identity, red represented 80% identity, and light blue represented 60% identity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

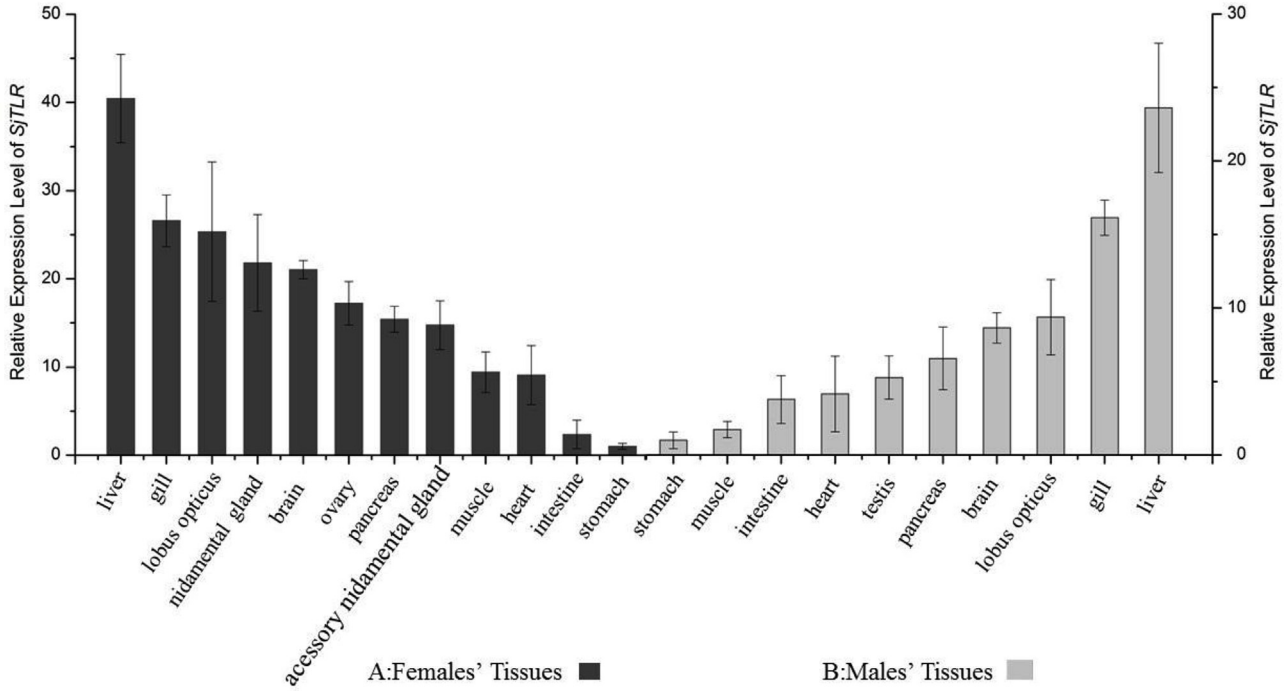

Fig. 5. Gene expression of *Sj*TLR determined by real-time PCR and normalized for the housekeeping gene *β*-actin, GAPDH and TUBA. The values were means (± SD) of three independent experiments performed in duplicate ($P < 0.05$).

common clade. The homology molecules of these species represented the V-type TLRs, which occurred originally after the divergence of cnidaria and bilateria, and appeared extensively in invertebrates [26]. Genomes of two invertebrates, echinoderm *S. purpuratus* and annelid *C. capitata*, contained high numbers of both P-type and V-type TLR genes [27], and their homologue also appeared in more homologous species such as *E.scolopes* and *O.bimaculoides*. Therefore, *Sj*TLR belonged to TLRs family as evidenced by its similarity to other TLRs in molecular structure and genetic relationship and it might have similar intracellular signal transduction pathway mechanism as in other mollusca.

In innate immune defense, TLRs will form dimerization after the PAMPs on the surface of pathogenic microorganisms being recognized by their extracellular LRR domains, and their intracellular TIR domains will combine with MyD88 adaptor protein to activate downstream NF-κB signal pathway to regulate cellular and humoral immune response [28]. The expansion of TLRs has been reported in invertebrate, and it is an intriguing question about the characters and immune function of these large amounts of TLRs in invertebrates which lack adaptive immunity. To verify the proteolytic activities of the predicted *Sj*TLR and PAMP binding activity of recombinant LRRs protein, the LRRs and TIR region were recombinantly expressed in *E. coli* BL21(DE3) and the expression recombinant LRRs and TcpB were purified with Ni-NAT Superflow resin. The electrophoretogram showed the bands of the expressed bands and the reclaimed protein were consistent with the predicted molecular mass. TIR segments encoded a TIR domain containing protein, TcpB, which suppressed NF-κB activation as well as proinflammatory cytokine secretion, was mediated by TLR receptors [29]. LRRs were short sequence motifs corresponding to β-αstructural unit which could form a parallel β-sheet with one surface exposed to solvent, and the proteins with LRRs usually acquired an unusual, non-globular shape [29]. These features endowed diverse proteins

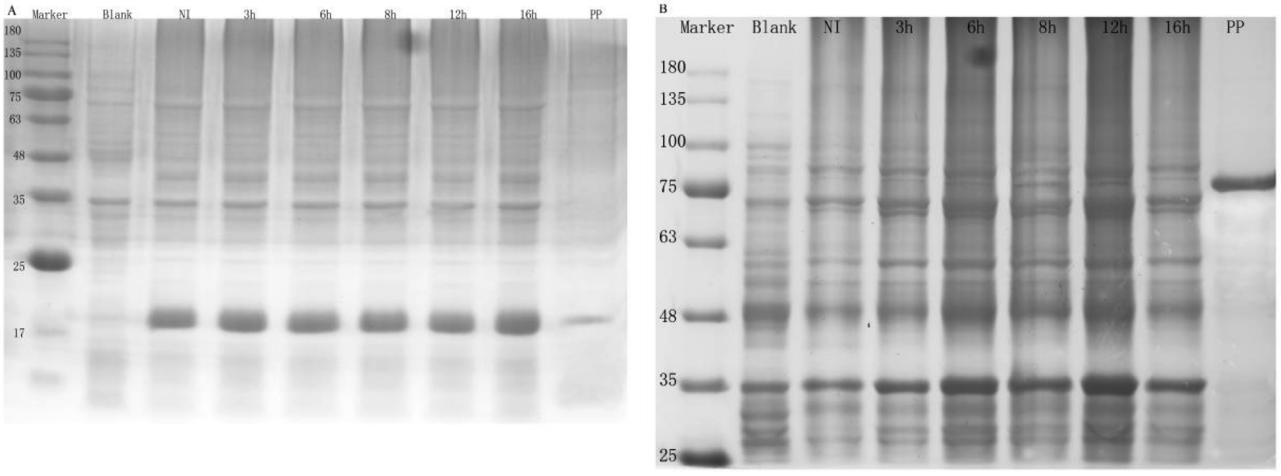

Fig. 6. Electrophoretogram of recombinant TcpB protein (A) and LRRs protein (B). *Sj*LRRs was revealed with molecular mass of 85 kDa and *Sj*TcpB was approximately 17 kDa. Blank-pET 28 induction, PP-purified protein and NI-No IPTG induction were as the control.

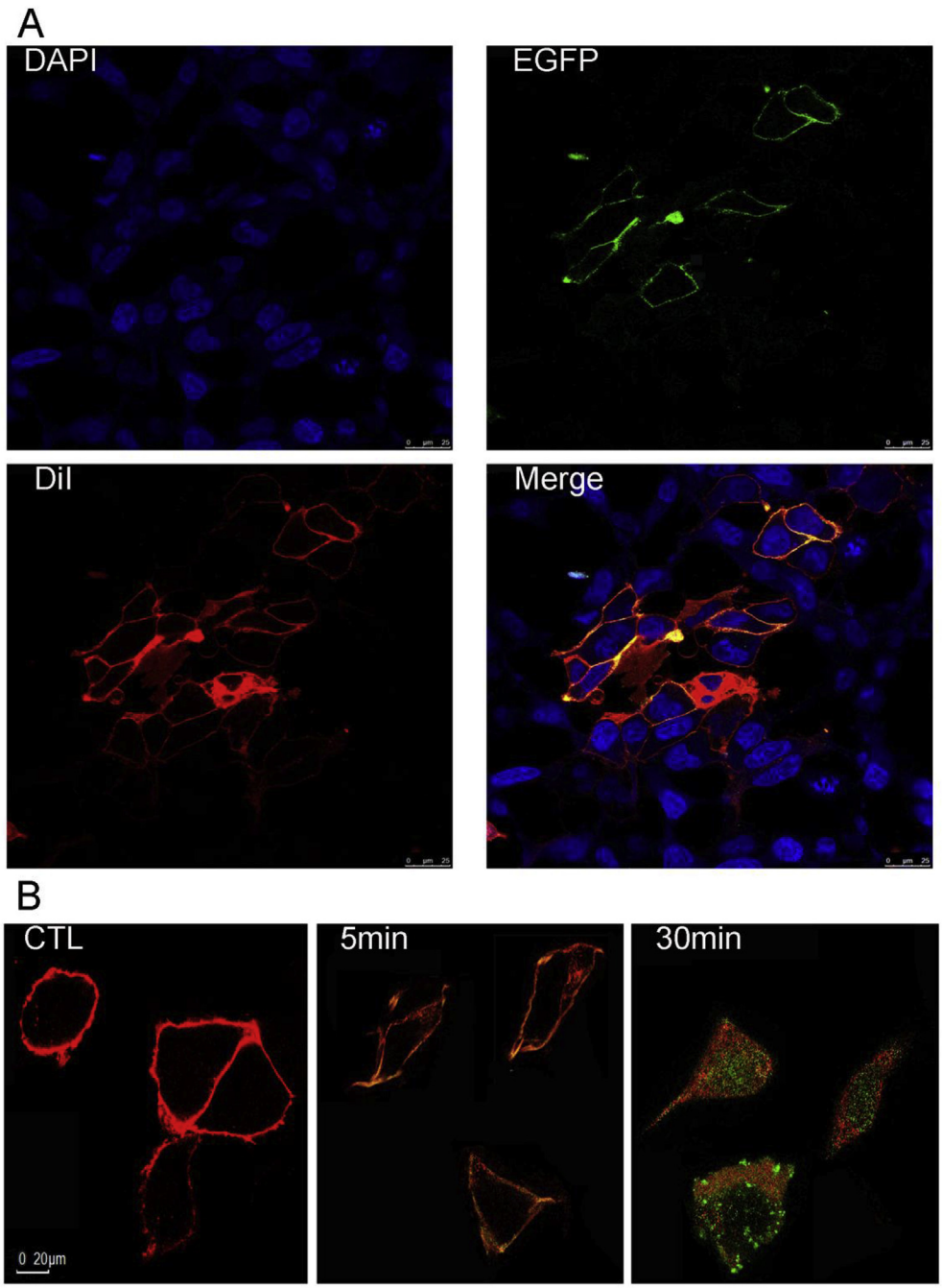

Fig. 7. Confocal microscopy of HEK293 cells expressing the *Sj*TLR-EGFP fusion protein. (A) *Sj*TLR distribution in HEK293 cells. Cells were stained with a membrane plasma probe (DiI) and a nuclei probe (DAPI). Cells stably expressing *Sj*TLR–EGFP were seeded on glass bottom six-well plates overnight, incubated with DiI (10 μM) and DAPI, and examined by confocal microscopy as described in the section Materials and Methods. (B) Internalization of *Sj*LRRs-pmCherry-C1 expressing cells. HEK293 cells transfected with *Sj*LRRs-pmCherry were activated by treatment with 1 μM FITC-LPS during 5min, 30 min and detected by confocal microscopy. The "CTL" referred to control without FITC-LPS stimulation. All images represented at least three independent experiments.

containing LRR with the function of protein-protein interactions, such as TLR proteins in various organisms. The LRRs and TIR domain in *Sj*TLR might play similar roles in *S. japonica*.

To further assess the *Sj*TLR functional activity as a transmembrane receptor, the EGFP was tagged at the C-terminal of *Sj*TLR and stably expressed in HEK293 cells. Significant cell surface expression was observed under fluorescence microscopy, suggesting that the C-terminal EGFP tag didn't affect *Sj*TLR expression and orientation in the cell membrane of HEK293 cells. Meanwhile, the fluorescence of *Sj*LRRs-pmCherry was dramatically and rapidly internalized into the cytoplasm in response to the incorporation of 1 μM FITC-LPS in HEK293 cells being observed with confocal microscopy. These results revealed the interaction between *Sj*LRRs and LPS and the recombination of PAMP binding activity of recombinant LRRs protein. Once bound with PAMPs, the intracellular TIR domain of TLRs would recruit several downstream signaling adaptors through TIR dimerization and activate nuclear factor-κB (NF-κB) consequently [30,31], LPS-induced NF-κB signaling pathway activation in HEK293 was also observed after transfecting *Sj*TLR-EGFP [4,32]. The experimental results also showed TLRs played an important role in the host cell immune response induced by pathogenic bacteria, and contributed to maintaining a healthy and dynamic balance between the host and its intestinal bacteria.

*Sj*TLR mRNA was detected in all tested tissues and the highest expression level was found in the liver, which was consistent with Toll2 and Toll3 in *Hyriopsis cumingii* [33]. Two other molluscan Toll receptors from *C.farreri* and *C. gigas* were also widely distributed but mainly expressed in hemocytes [17,19]. The broad tissues distribution of *Sj*TLR suggested this molecule might be involved in adverse physiological processes. High expression of *Sj*TLR in immune-related tissues, for example in liver, revealed its important role in immune defense against pathogen infection. Meanwhile, liver was also considered to be a crucial site of toxic metals and bacteria accumulation among filter animals

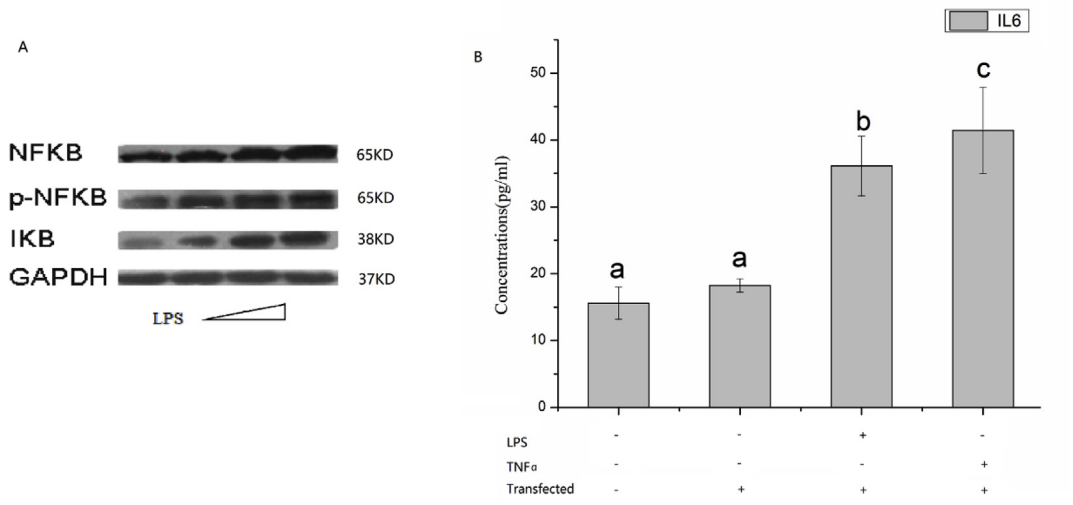

Fig. 8. The protein levels of NF-κB, pNF-κB (NF-κB p65) and IκB in HEK293 stimulated with LPS for different concentrations (A). The protein level of NF-κB, IκB and GAPDH in cytoplasm and the protein levels of NF-κB p65 in nucleus were detected by Western blotting. GAPDH was used as loading control in cytoplasm. The data presented here were results from one experiment of three western blotting experiments. Concentrations of cytokines IL6. (B) were detected by ELISA. The values were means (± SD) of three independent experiments performed in duplicate ($P < 0.05$).

[34], and most of complement proteins were produced in the liver and then released into the circulation of blood in vertebrates to play the role of immune defense [35].

Many studies showed that TLRs can be induced by lipoprotein, double-stranded RNA (viral or damaged tissue), Gram-negative bacteria and so on [36]. Meanwhile, several laboratories had used different techniques to study the up or down regulation of various TLR genes in mollusca and appeared to be somehow similar and contradictor, for example up-regulation of MgTLR-i in *M. galloprovincialis* with Gram-negative bacteria infection [37], TLR in hemocyte of *C. farreri* for LPS challenge [17] and Toll-like in hemocytes of the *C. gigas* after injection of *V. anguillarum* [19]. However HcToll3 in (*H.cumingii*) transcript level was significantly up-regulated by challenge with gram-negative bacteria *V.parahaemolyticus* or lipopolysaccharide, but not gram-positive *Staphylococcus aureus* or peptidoglycan [33]. In this study after *A. hydrophila* and *V. parahaemolyticus* challenges, a dramatic increase for the expression of the *Sj*TLR genes were detected by RT-PCR with time-dependent manner, which were similar to previous studies that demonstrated up-regulation of TLR in the *Eisenia andrei* earthworm [38], and suggested that *Sj*TLR functioned as a PRR in the innate immune system of *S. japonica*, with specificity for gram-negative bacteria. This was intriguing because Toll-like receptors were generally accepted as responding to gram-positive bacteria or fungi in invertebrate immunity [30].

In conclusion, a new cephalopod TLR member containing eleven leucine-rich repeats (LRR) was identified from *S.japonica* (*Sj*TLR), whose cellular localization was plasma membrane, *Sj*LRRs internalization upon the activation of LPS was found, and dramatic redistribution of *Sj*LRRs in the cytoplasm with distinct perinuclear accumulation was observed. After *Sj*TLR transfection Toll/NF-κB signaling pathway was active in HEK293 treated with LPS and TNFα. The nuclear related genes may also activated by NF-κB in the nucleus, and the corresponding mRNA was transferred through the intracellular signal transduction pathway, so that IL-6 cytokines could be synthesized and released. After infection by *V.parahemolyticus* and *A.hydrophila* the expression of *Sj*TLR were upregulated with time-dependent manner. All the results showed that *Sj*TLR represented a conserved and basic route leading to the activation of NF-κB pathway for its extraordinary complexity and novelty,

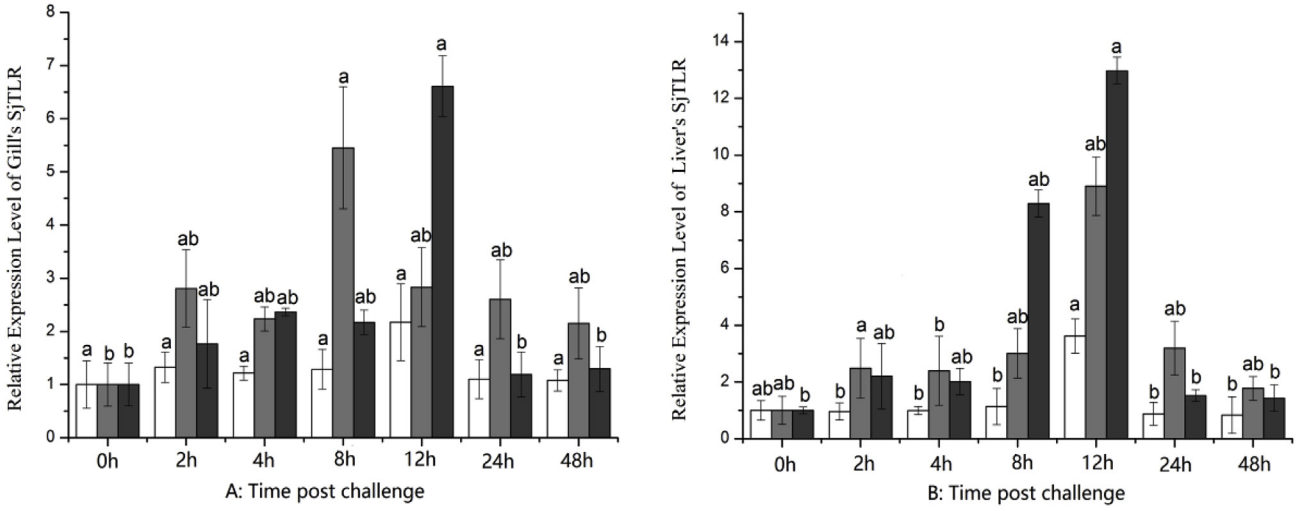

Fig. 9. Gene expression levels of *Sj*TLR in gill (A) and liver (B) were stimulated by PBS, *V. parahemolyticus* and *A.hydrophila*, determined by real-time PCR and normalized with the housekeeping gene *β*-actin, GAPDH and TUBA. Fold changes in gene expression were relative to *Sj*TLR expression in PBS (PBS *A.hydrophila* *V. parahemolyticus*). The values were means (± SD) of three independent experiments performed in duplicate ($P < 0.05$).

which established a fundamental pathway for the expanded TLRs in *S. japonica* and provided the basic framework for further study of the cephalopod TLR system.

Acknowledgements

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