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A novel interleukin-1 receptor-associated kinase-4 from thick shell mussel *Mytilus coruscus* is involved in inflammatory response

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ABSTRACT

Interleukin-1 receptor-associated kinase-4 (IRAK4) is considered as the most upstream kinase of IRAKs and plays a vital role in Toll-like receptor/Interleukin-1 receptor (TLR/IL-1R) signal transduction. In the present study, IRAK4 from thick shell mussel *Mytilus coruscus* (*Mc*IRAK4) was identified and characterized. *Mc*IRAK4 showed the most similarity to its counterparts in bivalves. The conserved death domain (DD) and catalytic domain of serine/threonine kinases (STKc) were predicted in all examined IRAK4s. *Mc*IRAK4 transcripts were constitutively expressed in all examined tissues with the higher expression level in immune related tissues, and were significantly induced in haemocytes upon lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly I:C) challenge. Further, the expression of *Mc*IRAK4 was obviously repressed by dsRNA mediated RNA interference (RNAi), meanwhile the proinflammatory cytokines TNF-alpha and IL17 were down-regulated while the anti-inflammatory cytokine TGF-β was up-regulated. Additionally, *Mc*IRAK4 showed a global cytoplasmic localization in HEK293T cells through fluorescence microscopy. These results collectively indicated that *Mc*IRAK4 is one member of IRAK4 subfamily and might play the potential signal transducer role in inflammatory response. The present study provides supplement for TLR-mediated signaling pathway triggered by pathogenic invasions in thick shell mussel, and contributes to the clarification of the innate immune response in molluscs.

1. Introduction

The thick shell mussel *Mytilus coruscus* represents one of the marine mussel species that mainly distributed in Chinese Yellow Sea, Korean Peninsula, and Japanese Hokkaido coastal areas. Due to its rapid growth, delicious taste and nutritional value, *M. coruscus* has developed into one of the most economically important aquaculture mussel species in China in recent years [1]. However, over the past decade, thick shell mussel aquaculture industry was suffering from seriously infectious pathogens accompanying with its rapid expansion of breeding scale as well as the deterioration of breeding environment, which was the same as the most marine breeding species facing, resulted in dramatic economic losses. Knowledge about the immune response to pathogenic invasions is helpful to develop the environmentally friendly strategies for disease prevention, such as disease-resistant breeding or development of vaccines [2]. Unfortunately, the information on immunity of thick shell mussel is still very scarce to date. This pitiful scenario has seriously hindered the sustainable development of mussel aquaculture industry.

Generally speaking, invertebrates lack of adaptive immune system, and mainly rely on the innate immune response to trigger diverse humoral and cellular activities to defense against microbial infections [3]. The innate immune system is the first defensive line against pathogens invasion, which is initiated by the recognition of a variety of pathogens via a limited number of pattern recognition receptors (PRRs) that recognize microbial components called pathogen associated molecular patterns (PAMPs) [4–6]. The Toll like receptor (TLR) family is one of the most well-studied PRRs, which can recognize various PAMPs of different microbial pathogens including bacteria, viruses, fungi and protozoa [7–11]. The mammalian TLR family consists of 13 members, sharing conserved functional characteristics containing C-terminal cytoplasmic toll-interleukin (IL)-1 receptor (TIR) domains, a transmembrane domain, and N-terminal leucine-rich repeats (LRRs) motifs [12]. Once PAMPs binding with their PRRs, most of TLRs trigger signaling cascades via applying their TIR domains to recruit downstream TIR domain-containing adaptor protein such as myeloid differentiation factor88 (MyD88) [13,14]. After that, MyD88 associate with IL-1R-associated kinase (IRAK) family members to form a complex through a homotypic interaction between their death domains (DD) [15–17], leading to the recruitment of tumor necrosis factor receptor-associated factor-6 (TRAF6). Subsequently, the complex induces the activation of downstream molecule transforming growth factor (TGF)-β-activated

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Table 1
PCR primer pairs used in the present study.

Primer pairs	Sequence (5'to 3')	Usage
IRAK4-ORF	ATGCCCACAAACAGTCGTGA CTAAATTTTATTTACAAGCT	For ORF cloning
IRAK4-5′	GCAAAATCTTCACAGAC GTTCTGCTTCAGTTAATGCCTT ATAAGTGTTTAACTCGCGAATT	For 5'RACE
IRAK4-3′	CTTAGATGAACAGCGATCAGAATGTGAC AGCAGGATCATGGGATATAGACCTTGCA	For 3′ RACE
Real-IRAK4	CCTTTTATGGCAGCAGCGTG AAAATCCAGTGCCCGATGGT	For *Mc*IRAK4 qPCR
Real-TNF-α	AACCAACCGGTGATTGTGGT TGGGATCAAGCAGCAACCAA	For *Mc*TNF-αqPCR
Real-IL-17	GGAGTTTGCGAAAATGGCGT AGCACCGATTGGAGGACTTG	For *Mc* IL-17 qPCR
Real-TGF-β	TGCGGGTAAAACCAAGACCA TCCCTGGCGGCTTCAATTAC	For *Mc*TGF-βqPCR
β-actin	GCTACGAATTACCTGACGGACAG TTCCCAAGAAAGATGGTTGTAACAT	Internal reference
Y- IRAK4	CAGAATTCATGCCCACAAACAGTCGTGA GAGGATCCAATTTTATTTACAAGCTTTT	For pEGFP-N1-*Mc*IRAK4 construction
*Mc*IRAK4-ds	TAATACGACTCACTATAGGGATGCCCACAAACAGTCGTGA TAATACGACTCACTATAGGGAATTTTATTTACAAGCTTTT	For *Mc*IRAK4 gene silencing
GFP-ds	TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA	Negative control in RNAi

kinase (TAK)1 and/or IκB kinase (IKK), which then leading to the activation of downstream signals, such as nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK), inducing the production of inflammatory cytokines and type I interferon [4,12,18].

Inflammation occurs after a pathogen infection, which is often characterized by the cell injury in host tissues [19]. In vertebrates, inflammation involves changes in local circulation and the recruitment of immune cells to injured foci to isolate or eliminate the causes of cell damage and eventually initiate tissue repair [20]. Although the complexity of the inflammatory response has increased during the differentiation of metazoan animals, its functional basis has not changed substantially during evolution [21]. Thus, similar functional events seem to occur in the inflamed tissue of molluscs as in vertebrates, though with obvious differences in some molecular features, the immune cells involved and their arrangement in injured tissues [22]. IRAK family contains four members: IRAK1, IRAK2, IRAK3 (also called IRAK-M) and IRAK4, which share general structural features containing an N-terminal DD domain and a conserved kinase domain [23]. Amongst, IRAK1 or IRAK2 are first recruited by IRAK4 and, in turn, they recruit TRAF6, and thus IRAK4 is considered as the most upstream kinase of IRAKs and plays crucial roles in TLR pathways [24,25]. Studies on mammals showed that IRAK4-deficient mice are completely resistant to lipopolysaccharide (LPS)- and CpG-induced shock, and severely impaired in responses to viral and bacterial challenges, which resulted from the impaired TLR/IL-1R-mediated induction of proinflammatory cytokines and chemokines [26,27]. Further studies have also revealed the essential role of IRAK4 in Tcell receptor (TCR) signaling [28], suggesting that IRAK4 may be involved in signal crosstalk between the innate and adaptive immune responses, although such hypotheses remain to be controversial in some other study [29].

Despite of the vital role of IRAK4 in TLR/IL-1R signaling pathway in mammals, the data about this molecule in lower vertebrates and invertebrates is still limited. Nevertheless, IRAK4 orthologs were identified in some teleost fishes, such as zebrafish [30], roughskin sculpin [31], half-smooth tongue sole [32], rainbow trout [33], grouper [34], rock bream [35], large yellow croaker [36] and golden pompano [2], as well as in some invertebrates, such as soft-shell clams [37], small abalone [38], pacific oyster [39] and brine shrimp [40]. In the present study, we focused on the molecular identification as well as its involvement in inflammatory response of IRAK4 in thick shell mussel (*Mc*IRAK4), in addition, its phylogenetic status, spatial and temporal expression profiles and subcellular localization were also assessed. The present research shed a new light on the functional role of IRAK4 in innate immunity of molluscs.

2. Material and methods

2.1. Animals

Thick shell mussel *Mytilus coruscus* healthy adults (shell length, 7.61 ± 0.43 cm; shell width, 3.53 ± 0.36 cm; wet weight, 60.3 ± 2.3 g) were bought from Donghe fish market, Zhoushan City, Zhejiang Province, China. These mussels were acclimated in 300 L aquaria under a laboratory condition with temperature of 24 ± 0.5 °C, salinity 28 ± 1‰ for one week before treated. Mussels were fed daily with spirulina powder, and filtered seawater was changed for half aquaria every day.

2.2. Challenge experiment

The LPS or polyinosinic-polycytidylic acid (poly I:C) challenge experiment was performed as described previously [41] with little modification. Briefly, 270 mussels were randomly divided into three groups, control group, LPS and poly I:C challenge groups, each group consisted of three biological duplications and 30 individuals in each duplication.

Ninety individuals of control group were adductor injected with 500 μL PBS (pH 7.4) while 90 individuals of each challenge group were injected with an equal volume of LPS (1 μg/mL) or poly I:C (1 mg/mL). These injected mussels were kept under the same conditions as above mentioned without food during the test. Haemolymph was collected at 0, 3, 6, 12, 24, and 36 h post induction (hpi) from the pericardial cavity through the adductor muscle and immediately centrifuged (700 × *g* for 10 min at 4 °C) to separate the haemocytes. Three individuals in each duplication were randomly sampled at every time point and pooled together to obtain enough blood cells and to reduce individual variation.

Seven tissues, including the gills, gonads, digestive glands, hepatopancreas, adductor, haemocytes and mantles were dissected from eight adult individuals to examine the tissue distribution of *Mc*IRAK4. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

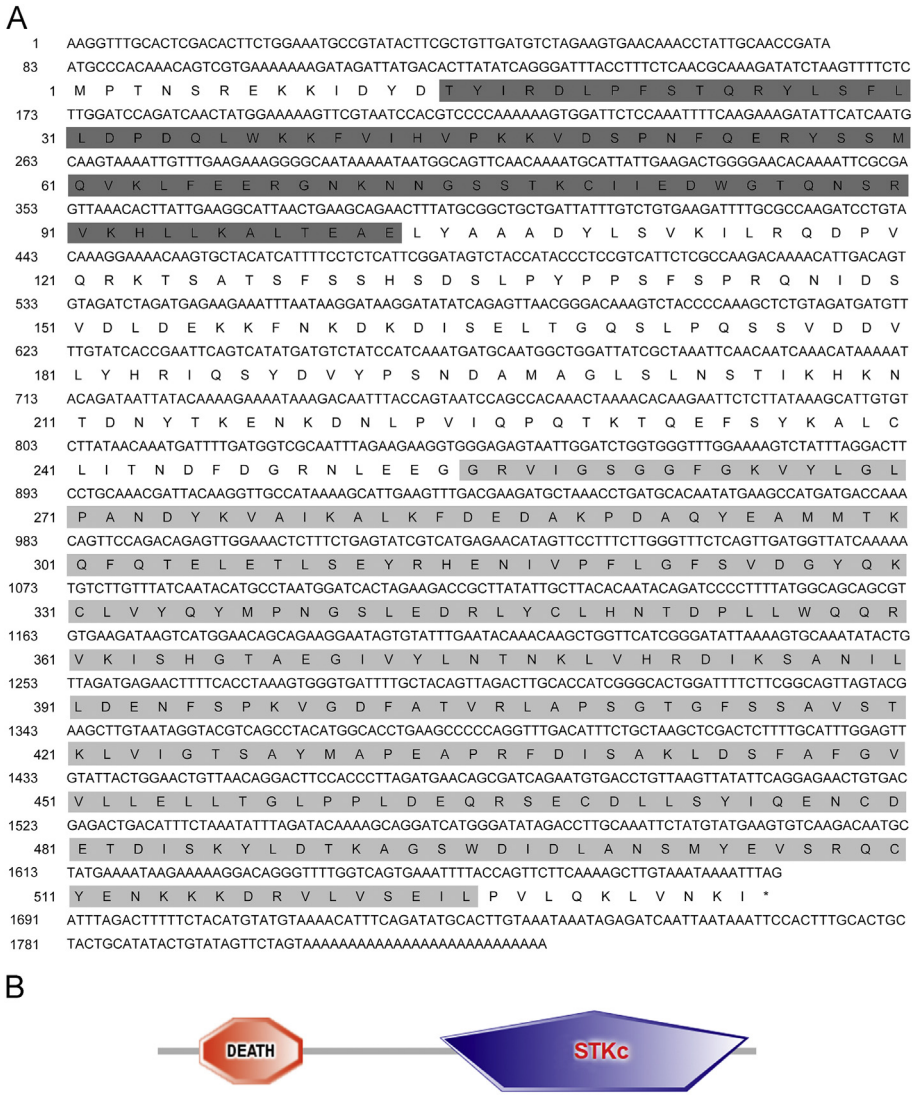

Fig. 1. Molecular characterization of*McIRAK4*. (A) The nucleotide sequences and the deduced amino acid sequences of interleukin-1receptor-associated kinase 4 (IRAK4) in thick shell mussel *Mytilus coruscus*. The complete sequence of *McIRAK4* Cdna is 1833 bp, containing an 82 bp 5'-UTR region and an 143 bp 3'-UTR region, an 1608 bp ORF region coding the protein of 535 amino acid residues. The predicted death domains (DD) and protein kinase domain (STKc) were marked with 60% and 30% gray, respectively. (B) Schematic diagram of *McIRAK4* functional domains.

2.3. Cloning of the McIRAK4cDNA

Total RNA was extracted using Total RNA Kit I according to the manufacture's protocol (OMEGA). First-strand cDNA synthesis was carried out based on Promega M-MLV reverse transcriptase using oligo (dT)-adaptor as primer. The reaction was performed at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min.

The cDNA sequence with the coverage of open reading frame (ORF) region was obtained by scanning the *M.coruscus* transcriptional database [42]. Following, one pair of primer set IRAK4-ORF (Table 1) was designed to amplify the ORF sequence, and then specific and adaptor primers (Table 1) were designed to clone the 5′ and 3′ untranslated regions (UTR) using rapid-amplification of cDNA ends (RACE). This experiment was performed using the RACE cDNA Amplification Kit (Life Technologies, USA) according to the manufacturer's protocol. The PCR products were sequenced using an ABI 3730 automated DNA sequencer. Finally, the ORF sequence and the 5′- and 3′-UTR sequences were assembled to obtain the *Mc*IRAK4 full length cDNA using CAP3 software [43].

2.4. Bioinformatic analysis of McIRAK4

The obtained *Mc*IRAK4 cDNA sequence was analysed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) website. We searched for the ORF of the target gene using ORF finder, and then the amino acid sequence was deduced. The functional domains of the deduced amino acid sequence were predicted using on line SMART (http://smart.embl-heidelberg.de/) [44]. Phylogenetic relationships were investigated by the neighbour-joining (NJ) method using MEGA 7.0 [45]. The nucleotide sequence similarity andidentity were calculated by MatGAT 2.0 [46].

2.5. Double-stranded RNA preparation and RNA interference

The double-stranded RNA (dsRNA) was generated according to our previously described method [47], briefly, specific primers linked to the T7 promoter (Table 1, *Mc*IRAK4-ds) was designed to amplify the *Mc*IRAK4 cDNA fragment, and the sequenced fragment was used as template to synthesize dsRNA of *Mc*IRAK4. The GFP gene was used as control and amplified with primers GFP-ds (Table 1) from the pEGFP-

Percents of identity

Percents of similarity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. M. coruscus		30.8	34.3	97	48.5	44.8	34.8	36.2	35.4	35.3	34.6	33.7	33.9	35.2	33	34.5	35.2	35.9	34.8	34.3	35.1	34.8	33.2
2. R. microplus	49.3		31.1	30.9	30.4	29.3	30.8	32.2	31.6	32.4	31	33.3	33.9	34.1	30.2	33.3	32.8	32.8	31.9	32.7	31.7	31.3	31.2
3. C. secundus	56.3	47.6		33.7	34.9	36	32.8	32.8	31.8	31.4	32.6	31	32.3	32.3	33	32.9	32.4	33	32.2	31.5	31.2	32.7	33.2
4. M. galloprovincialis	98.5	49.3	55.8		48	45.6	34.5	36	35.3	34.8	34.1	33.3	34	34.6	32.9	34.1	35.1	35	34.7	34.1	35.3	34.9	33.3
5. C. gigas	68.3	46.6	56.3	67.2		46.2	33.8	34.6	34.6	35.1	33.6	34.7	35.8	34.5	32.4	35.2	33.6	35.1	34.6	34.2	34.9	34.2	33.6
6. H. discus discus	64.1	49.9	57.2	64.5	66.4		36.1	34.4	34.7	34.2	36.3	33	33.7	34.5	32	33.8	34	35.4	34.3	34.4	34.6	33.5	35.2
7. M. musculus	50.1	49.2	48.7	50.7	52.4	53.2		83.7	80.3	81.5	98.3	52.1	53.2	54	48.3	53.4	68	65.3	66.2	67.5	63.9	59.6	54.1
8. H. sapiens	52	51.5	49.4	52.7	52.6	55.7	91.5		90	90.9	84.3	52.5	52.7	54	47.4	52.2	69.7	68.6	68.2	69.2	65.5	60.8	56.4
9. B. taurus	51.4	51.4	49.8	51.4	52.4	53.2	88.3	94.6		88.1	80.9	52.8	52.3	53.9	48.8	53.1	68.9	67.3	67.2	68.7	65.1	61.6	55.9
10. L. africana	51.6	51.3	49.6	52.3	52.2	54.2	90	95.2	93.5		82.4	51.6	52.3	53.2	47.6	53.3	69.5	69	68.2	69.6	65.9	60.1	56
11. M. caroli	50.3	49.7	49.1	51	52.1	53.4	99.1	92	88.9	90.4		52.1	53.2	53.8	49.2	53.3	68.6	66.6	66.9	68.1	64.5	59.8	54.3
12. C. semilaevis	53.1	50.4	50.7	52.7	52.1	51.1	71.5	72.5	72.7	73.2	71.5		69.1	60.1	49.7	69.3	52.5	52	52.3	52.6	51.2	52.7	52.4
13. O. niloticus	51.4	51.8	50.2	51.8	50.7	52	74.3	73.9	74	73.9	74.5	81.3		62.4	49.6	76.3	52.9	53.5	52	51.5	50.9	50.6	53.3
14. O. mykiss	52	51.1	51.1	51.2	51.1	52.4	71.7	71.7	71.7	71.1	71.9	75.5	77		50.3	63.4	51.1	52.6	49.3	50.6	51.1	51.8	51.3
15. D. rerio	50.8	49.3	50.4	50.5	52.1	50.5	67.7	68.3	67.1	67.5	67.7	68.1	67.3	69.4		49.5	47.6	48.7	47.8	46.7	46	47.7	45.2
16. A. ocellaris	51.6	51.5	51.1	50.8	52.2	52.6	72.6	74.1	74.8	75	73.7	82.8	87.3	81	67.5		51.4	53.1	52.1	52	50.7	51.3	51.2
17. A. forsteri	50.8	53	49.8	52.2	52.1	54	80.2	83.4	82.8	82.5	80.6	70.6	73.1	73	65.4	72		87.3	93.5	93.1	86.9	61.9	54.9
18. C. japonica	50.7	51.7	50.4	50.1	53	53.6	80.8	83.2	83.6	83.2	81.5	71	73.5	72.2	66.9	73.7	94.6		85.6	85.8	81.5	60.6	55.1
19. C. pugnax	51.6	50.9	50.6	52.2	53.2	53.4	78.9	82.5	82.8	81.9	79.3	69.5	72	71.9	67.1	71.6	96.8	93.3		91.2	85.7	60.9	54.2
20. F. cherrug	52	52.5	51.3	52.7	53.2	53.2	81.3	83.9	83.2	83.4	81.7	69.7	72	72.8	66.7	72.5	97.2	93.5	95.7		85.5	62	55.9
21. T. guttata	52.1	49.8	50.7	52.7	53	54	77.6	79.5	79.5	79.9	78	67	69.5	71.4	66.9	69.5	92.3	89.2	92.1	91.9		59.8	53.2
22. X. tropicalis	51	50.8	49.6	50.5	51.1	52.2	76.3	76.3	76.8	76.5	76.3	71	69.4	70	65.4	69.6	74.4	74.4	74.8	75.9	71		63.4
23. N. parkeri	48.2	48.9	49.6	47.8	48.3	51.3	72.8	73	72	72.8	73	70.2	70.4	68.8	63.4	69.6	71.3	71.8	70	71	67.4	76.4	

Fig. 2. Identity and similarity of *Mc*IRAK4 with its counterparts from other animals by MatGAT. The percents of identity are showed in upper triangle and the percents of similarity are showed in lower triangle.

N1 vector (Table 1). dsRNA was synthesized with the T7 polymerase using the HiScribe™ T7 in vitro transcription kit (NEB, USA) according to the manufacturer's protocol. The reaction system included 2 μL of 10 × transcription Buffer, 2 μL of 20 × Ribonucleotide Solution Mix (80 mM each), 0.5 μL of RNase inhibitor, 2 μL of T7 RNA polymerase (500 units/μL) and 1 μg of linear template DNA, and RNase free ddH2O was added to a total of 20 μL system. The reaction was then conducted at 16 °C overnight to obtain dsRNA. After removal of DNA template, the RNA integrity was examined by electrophoresis, and the concentration was quantified using microspectrophotometer NanoDrop 2000 (Thermo, USA) by the absorbance at 260 nm and adjusted to a final concentration of 1 mg/mL.

*Mc*IRAK4 dsRNA (100 μg per mussel) was injected into the adductor muscle of each mussel, while the control groups received an injection of EGFP dsRNA (100 μg per mussel) or PBS (100 μL per mussel). To enhance the RNA interference (RNAi) effect, a second injection was performed at 12 h after the first injection. Haemolymph was randomly isolated from four mussels at 24 h post the first dsRNA injection and pooled to reduce individual variation and to provide sufficient haemocytes for total RNA extraction. The efficiency of the gene silencing was evaluated using the quantitative real-time PCR (qPCR) with the specific primer pair Real-IRAK4 (Table 1). For the aim to understand the functional role of *Mc*IRAK4 in inflammatory response, the transcriptional expression of proinflammatory cytokines TNF-α and IL17, antiinflammatory cytokine TGF-β were assessed using qPCR with their respective primer pairs (Table 1) (Partial cDNA sequences of *Mc*TNF-α, *Mc*IL-17 and *Mc*TGF-β for primer designing were retrieved from the transcriptome database of *M. coruscus*, data was not shown here).

After the first sampling, the rest of mussels were immediately injected with LPS according to aforementioned protocols. Twelve hours after LPS challenge, haemocytes were sampled as previously mentioned, followed by RNA extraction and cDNA synthesis, then qPCR was conducted to assess the mRNA expression of *Mc*IRAK4, *Mc*TNF-α, *Mc*IL-17 and *Mc*TGF-β aiming to further understand the involvement of *Mc*IRAK4 in inflammatory response.

2.6. qPCR

qPCR was conducted on a 7500 Real Time PCR System (Applied Biosystems, USA), and in a final volume of 10 μL consisted of 0.4 μL each for F and R primers (10 μM each), 5 μL of 2 × SYBR® Premix ExTaq™ II, 0.4 μL of cDNA sample (100 ng/μL), 0.2 μL of ROX II and 3.6 μL of ddH_2O. The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference [48]. All samples were analysed in triplicate. Data were analysed by one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple range tests using SPSS 17.0 software [49]. Differences were deemed significant at $P < 0.05$.

2.7. Plasmids construction and cell culture

Aiming to investigate the subcellular localization of *Mc*IRAK4, pEGFP-N1vector was used for the construction of expression plasmid. In addition, the HEK293T cells were used in this assay due to the lack of established cell lines available for marine bivalves. Specific primer pair was designed to amplify the ORF sequence of *Mc*IRAK4 with the link of restriction enzyme cutting sites (Table 1). After digested by restriction enzyme, the PCR product was cloned into pEGFP-N1 vector to produce GFP-tagged expression plasmids. Finally, the recombinant plasmid was cleaved by restriction enzymes and verified by DNA sequencing, and designated pEGFP-N1-*Mc*IRAK4.

HEK293T cells were cultured in DMEM medium, with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 100 units/ml penicillin and 100 mg/mL streptomycin in TC plate under humidified conditions with 5% CO_2 at 37 °C [41].

2.8. Subcellular localization

HEK293T cells were seeded onto a sterile microscope cover glass and placed in a 6-well cell culture plate prior to transfection. After seed cells grown at 70–90% confluence, 1 μg of pEGFP-N1-*Mc*IRAK4 vectors were transiently transfected using Lipofectamine 3000 (Invitrogen), empty pEGFP-N1 vector was used as the control. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde for 10 min, and then stained with 4′, 6-diamidino-2-phenylindole (DAPI) (1 μg/mL) for 5 min. After washing, the cells were directly observed by a fluorescence microscopy (Leica, Germany) [41].

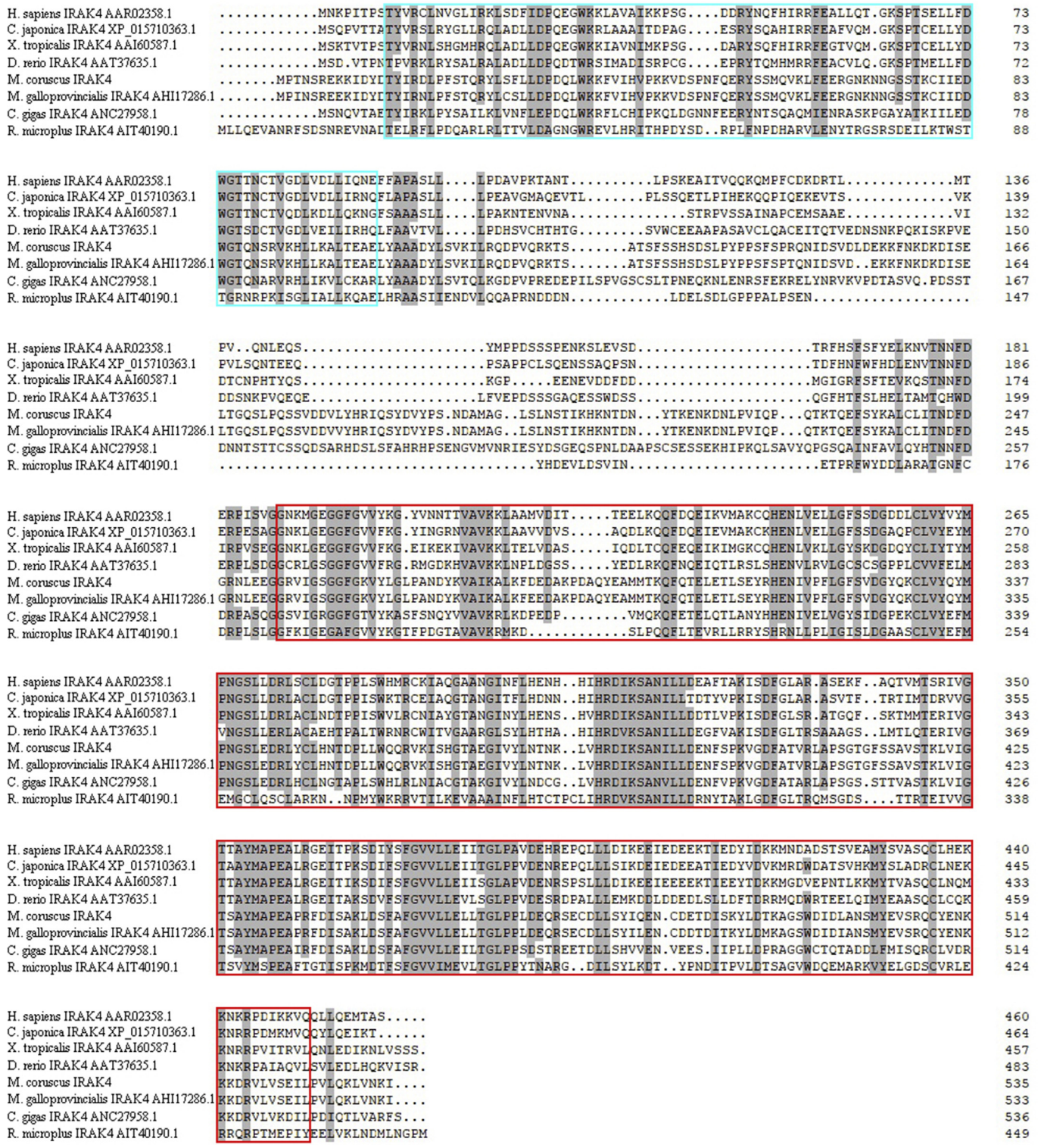

Fig. 3. Multiple alignments of deduced amino acid sequence of *Mc*IRAK4 with its counterparts in other eukaryotes. Surpass 75% conserved residues were marked with 60% gray. DD and STKc domains were marked with blue and red boxes, respectively. The species names and accession numbers were labeled on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Molecular cloning and characterization of McIRAK4

A full length cDNA encoding IRAK4 homologue was firstly cloned from *M. coruscus* (the new gene named '*Mc*IRAK4', Accession No. MH603332). The *M. coruscus* cDNA of 1833 bp contains 82 bp 5′-UTR, 143 bp 3′-UTR and 1608 bp ORF sequence coding a deduced amino sequence of 535 residues (Fig. 1A). The calculated molecular mass of *Mc*IRAK4 is 60.7 kDa and the protein has a theoretical isoelectric point of 5.56. A comparison of homology revealed that the deduced *Mc*IRAK4 shares 48.2%–98.5% sequence similarity and 30.8%–97.0% sequence identity with IRAK4 of other metazoan, with it being the most similar to that of the *M. galloprovincialis* (98.5% sequence similarity and 97.0%

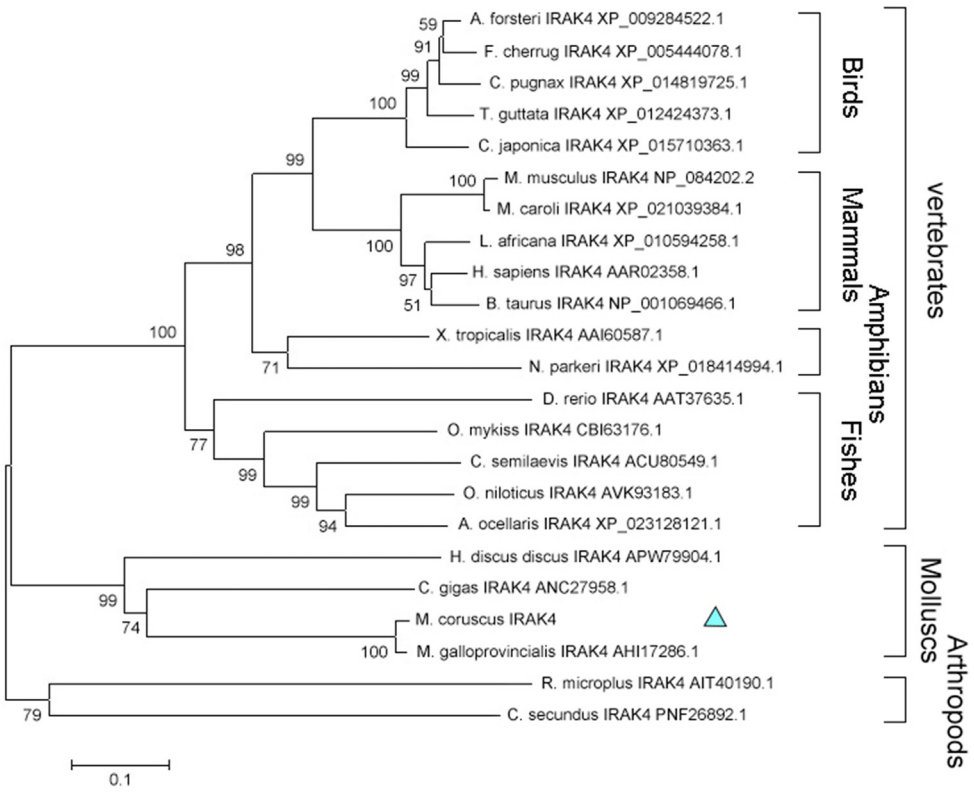

Fig. 4. Phylogenetic analysis of *Mc*IRAK4. The phylogenetic tree was constructed using MEGA software 5.0 with 5000 replications of bootstrap in the way of neighbour-joining method. *Mc*IRAK4 was marked with blue triangle. Species included in construction of phylogenetic tree were all retrieved from NCBI database and accession numbers were also listed in the tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sequence identity), followed by the *C. gigas* (68.3% sequence similarity and 48.5% sequence identity) (Fig. 2). Using on line SMART program, it was found that *Mc*IRAK4 contained a N-terminal death domain (DD) and a C-terminal catalytic domain of serine/threonine kinases (STKc) (Fig. 1A and B). These two functional domains were also highly conserved in other examined metazoa through the multiple sequence alignment (Fig. 3). Phylogenetic analysis showed that *Mc*IRAK4 firstly clustered with its counterpart of *M. galloprovincialis*, then grouped together with the IRAK4s of *C. gigas* and *H. discus discus* to construct the mollusc branch (Fig. 4). In the phylogenetic tree, three conspicuous branches containing molluscs, arthropods and vertebrates (inclusive of four branchlets: mammals, birds, amphibians and fishes) were presented, and these IRAK4s affiliate to the same animal phyla grouped together to form the respective cluster, which was consistent with traditional taxonomy and phylogeny (Fig. 4).

3.2. Expression profile analysis of McIRAK4 transcripts

For the aim to further understand the functional role of IRAK4 acted in *M. coruscus* physiological activities, the expression profiles of *Mc*IRAK4 transcripts were analysed. The tissue distribution of *Mc*IRAK4 transcripts was examined in gills, gonads, digestive glands, hepatopancreas, adductor, haemocytes and mantles. As shown in Fig. 5A, *Mc*IRAK4 showed a constitutively expressional profile with the highest expression level in gills, followed by haemocytes and mantles, moderately expressed in gonads and hepatopancreas, and weak expression in digestive glands and adductor. The expression level changes of *Mc*IRAK4 transcripts under stimulation by LPS and poly I:C were investigated. After poly I:C challenge, the expression level of *Mc*IRAK4 mRNA was gradually up-regulated and reached a significant level at 3 hpi with a 3.8-fold increase to control, following it being a highest expression level at 12 hpi with a 23.1-fold increase. After that, the transcriptional level of *Mc*IRAK4 showed a downward trend, however it was still significantly higher than that of the control group (Fig. 5B). Upon LPS stimulation, *Mc*IRAK4 mRNA was significantly induced at 3 hpi, followed by down-regulated at 6 hpi and abruptly up-regulated to the peak value at 12 hpi with a 26.8-fold increase. Hereafter, the transcriptional level of *Mc*IRAK4 shortly decreased from 24 to 36 hpi, even so, it was still higher than that of the control group.

3.3. McIRAK4 knockdown

For the aim to explore the function of IRAK4 in inflammatory response, *Mc*IRAK4 was knocked down in *M. coruscus* by sequence-specific dsRNA-mediated RNAi. The silencing efficiency of *Mc*IRAK4 was checked with qPCR in the mussel haemocytes at 24 h, and the results showed that the expression of *Mc*IRAK4 was significantly repressed in *Mc*IRAK4-dsRNAs treated mussels, but had no obvious changes in control group (Fig. 6A). Meanwhile, the mRNA levels of inflammatory cytokines *Mc*TNF-α, *Mc*IL-17 and *Mc*TGF-β were also measured at 24 h after *Mc*IRAK4 silencing, and as shown in Fig. 6, the mRNA expressions in haemocytes were remarkably down-regulated in *Mc*TNF-α and *Mc*IL-17 (Fig. 6B and C) while significantly up-regulated in *Mc*TGF-β (Fig. 6D).

The*Mc*IRAK4 knocked down mussels were challenged with LPS, and the mRNA expressions of *Mc*IRAK4, *Mc*TNF-α, *Mc*IL-17 and *Mc*TGF-β were assessed at 12 hpi of LPS. As shown in Fig. 7, the expressions of *Mc*IRAK4, *Mc*TNF-α, *Mc*IL-17 and *Mc*TGF-β transcripts were all significantly elevated after LPS challenge, nevertheless, the mRNA levels of *Mc*IRAK4, *Mc*TNF-α and *Mc*IL-17 in *Mc*IRAK4 silenced mussels were remarkably lower than that of control mussels (Fig. 7A, B, 7C) while *Mc*TGF-β mRNA level was significantly higher than that of control mussles (Fig. 7D).

3.4. Subcellular localization of McIRAK4

To examine the subcellular localization of *Mc*IRAK4, the recombinant plasmid expressed pEGFP-N1-*Mc*IRAK4 was transfected into HEK293T cells and visualized using a fluorescence microscope. The green fluorescence of the GFP-tagged *Mc*IRAK4 was mainly distributed

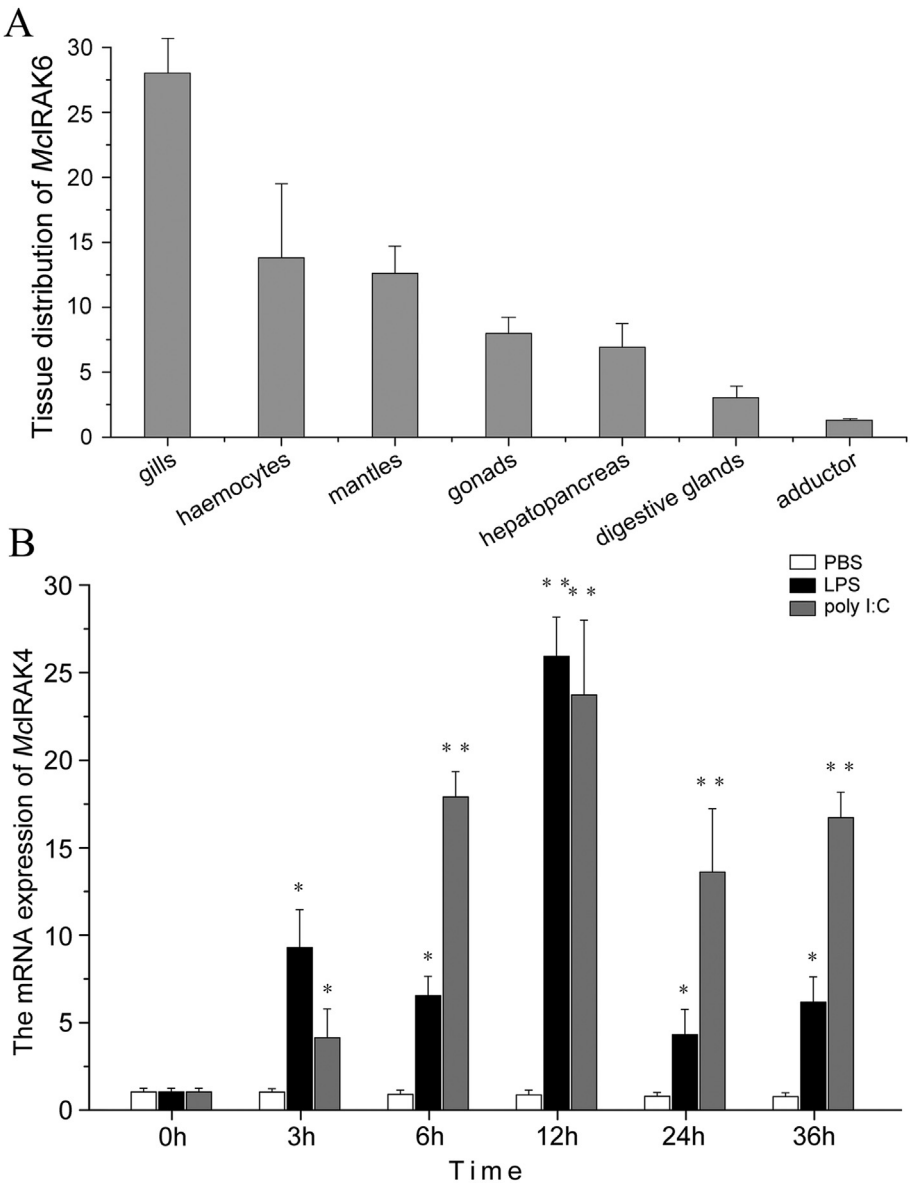

Fig. 5. Expression profile analysis of *Mc*IRAK4 transcripts (A) The mRNA expression of IRAK4 in seven adult tissues. Vertical bars represent the mean ± S.D. from eight mussels (n = 8). (B) Expression analysis of *Mc*IRAK4 gene in haemocytes after injection with LPS and poly I:C. The results were expressed as mean ± S.D. (n = 3). Significant difference relative to control was indicated with asterisk symbol (*P < 0.05, **P < 0.01).

in the cytoplasm, whereas the control protein was dispersed throughout the cytoplasm and the nuclear areas (Fig. 8).

4. Discussion

IRAK4 is considered as the most upstream kinase of IRAKs and plays a vital role in TLR/IL-1R signaling pathway [24,25], additionally, IRAK4 may be also involved in signal crosstalk between the innate and adaptive immune responses [29]. Despite of its remarkable function role in immune activities in mammals, the data about IRAK4 in immune modulation in invertebrates is still scarce. Here, a novel invertebrate member of IRAK4 subfamily was determined from a marine mussel, thick shell mussel, the new gene was denominated as *Mc*IRAK4. A comparison of homology showed that *Mc*IRAK4 shared higher sequence similarity and identity with IRAK4s of other metazoan and showed the most similar to that of the bivalves *M. galloprovincialis* and *C. gigas*. Online SMART predicted a DD domain and a STKc domain in *Mc*IRAK4, meanwhile these two domains were also found to be highly conserved from mollusc to human as shown by multiple alignments. It has been demonstrated that IRAK4 activation relyed on the interaction of the DD domain with MyD88 [50], once activated, IRAK4 transduces the signal to the other downstream components [51]. Phylogenetic analysis showed that *Mc*IRAK4 had a close relative with its counterparts in bivalves *M. galloprovincialis*, *C. gigas* and *H. discus discus* which were consistent with traditional taxonomy and phylogeny. Taken together, the presence of conserved DD, STKc domains and the close relationship with its counterparts in bivalves suggested that *Mc*IRAK4 should belong to IRAK4 subfamily and might play the similar signaling mediator role in TLR/IL-1R signal transduction just as their counterparts play in mammals.

For the aim to explore the possible function of *Mc*IRAK4, the tissue distribution of *Mc*IRAK4 transcripts was examined using qPCR. A constitutive expression profile was found in *Mc*IRAK4 transcripts with the higher expression levels in gills, haemocytes and mantles which are generally considered as the immune involvement tissues in molluscs [52,53]. In our previous studies, one of the key adapter molecules in TLR signal transduction termed as *Mc*TRAF6 was also found to be highly expressed in gills and haemocytes [47]. Collectively, these

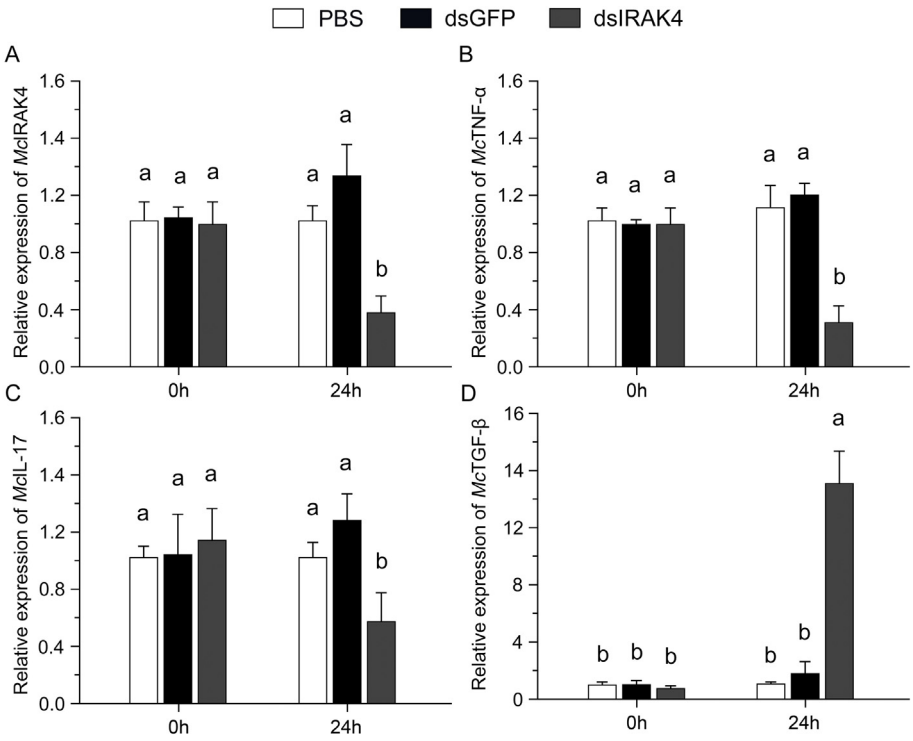

Fig. 6. Temporal expression of the *Mc*IRAK4 (A), *Mc*TNF-α (B),*Mc*IL-17 (C),*Mc*TGF-β (D) in haemocytes of *Mc*IRAK4 knockdown mussels. Values represent mean ± S.D. (n = 3), different superscript letters indicate significant differences at $p < 0.05$.

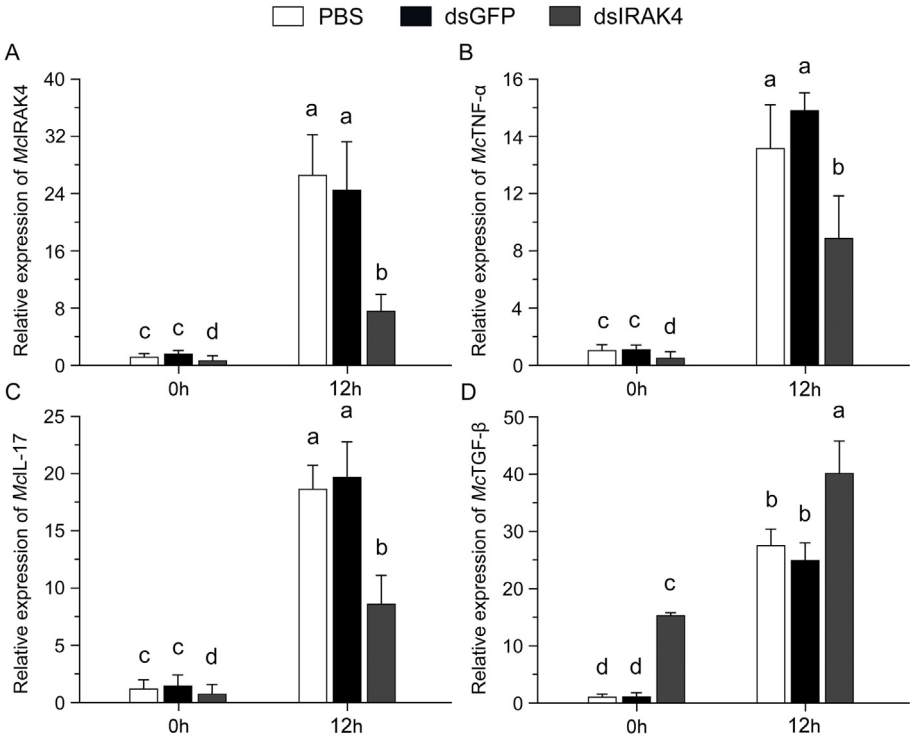

Fig. 7. Temporal expression of the *Mc*IRAK4 (A), *Mc*TNF-α (B),*Mc*IL-17 (C),*Mc*TGF-β (D) in haemocytes of *Mc*IRAK4 knockdown mussels with the challenge of LPS. Values represent mean ± S.D. (n = 3), different superscript letters indicate significant differences at $p < 0.05$.

results suggested that these genes might play roles in the regulation of mussel innate immunity through TLR signaling pathways. This viewpoint was further determined by the obvious induction of *Mc*IRAK4 response to LPS and poly I:C challenge. LPS is the major outer surface membrane components present in almost allgram-negative bacteriaand act as extremely strong stimulators of innate or natural immunity [54], while poly I:C is a structural analogue of dsRNA which considered to be the ligand of mammalian TLR3 [14]. The expression trends of *Mc*IRAK4 in responses to these two PAMPs were different, which could reflect different levels of *Mc*IRAK4 activation during different pathogen infections. Nevertheless, the significant up-regulation of *Mc*IRAK4 response to LPS and poly I:C challenge, together with its higher

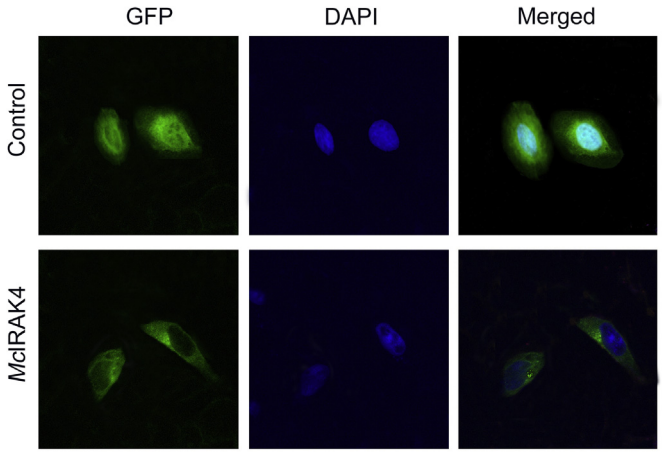

Fig. 8. Subcellular localization of *Mc*IRAK4 in HEK293T cells. Recombinant pEGFP-N1-*Mc*IRAK4 plasmid was transfected into HEK293T cell using lipofectamine 3000, the location of proteins was shown by the green fluorescence and cell nucleus location was indicated by blue DAPI staining, empty EGFP-N1 plasmid was used as control. The*Mc*IRAK4 was mainly localized in the cytoplasm of HEK293T cells while the green fluorescence signal of the control was distributed in both cytoplasm and nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expression levels in immune related tissues suggest that *Mc*IRAK4 is involved in the mussel defense against exogenous invasions and *Mc*IRAK4 mediated TLRs signaling pathway exists.

In previous study, it has been demonstrated that the DD-containing proteins are involved in the signaling leading to apoptosis and inflammatory responses [55]. Studies on mammals showed that IRAK4-deficient mice are completely resistant to LPS- or CpG-induced shock, and severely impaired in responses to viral and bacterial challenges, which might resulted from the reduced production of proinflammatory cytokines and chemokines in impaired TLR/IL-1R-mediated induction [26,27]. The functioning of IRAK4 in inflammatory response has attracted wide concern in mammals, to our knowledge, researches on this topic have not been reported in invertebrates to date. Although there are significant differences in certain molecular characteristics, immune cells involved, and their arrangement in injured tissues, there is no significant change in its functional basis during evolution, and thus, similar functional events seem to occur in the inflamed tissues of mollusks as in vertebrates [21,22]. In this study, *Mc*IRAK4 was silenced by dsRNA mediated RNAi and the result showed that repressed expression of *Mc*IRAK4 could inhibit the expression of proinflammatory cytokines *Mc*TNF-α, *Mc*IL-17 and elevate the expression of antiinflammatory cytokines *Mc*TGF-β upon or no LPS challenge. The result was consistent with the previous report, it was found that TNF-α and IL-6 concentrations was significantly down-regulated in LPS challenged IRAK4 kinase-inactive knock-in mice [27]. On the other hand, these results suggested that *Mc*IRAK4 could induce inflammatory response through promoting the release of proinflammatory cytokines meanwhile restraining the production of antiinflammatory cytokines when exposed to pathogenic stimuli. Given the complex role of *Mc*IRAK4 in pathogens infection, the interaction between *Mc*IRAK4 and pathogen induced inflammatory response needs further investigations.

It is very important for us to learn the localization of the components in TLR signaling pathways [56,57], so that their actual functions could be predicted. Here, a global cytosolic localization of *Mc*IRAK4 was examined in HEK293T cell system, while the control protein was detected in both the cytoplasm and the nucleus. In consistent with previous study, *Lc*IRAK4 and *Ec*IRAK4 were subcellular located in the cytoplasm of HEK293T and HeLa cells, respectively [34,36]. The result suggested the putative function role of *Mc*IRAK4 as a signaling mediator in TLR/IL-1 signaling transduction.

In conclusion, one bivalve ortholog of IRAK4, *Mc*IRAK4 was identified and characterized from thick shell mussel *Mytilus coruscus*. *Mc*IRAK4 is highly conserved in all examined animals, especially to those IRAK4 identified in bivalve species, and is constitutively expressed in different tissues, which can also be up-regulated after poly I:C and LPS stimulation. Further, *Mc*IRAK4 function as a cytosolic protein and is widely involved in inflammatory response. These results collectively indicate that *Mc*IRAK4 is member of IRAK4 subfamily and play potential roles in the signaling transduction leading to inflammatory response. The present research contributes to our understanding of innate immune defense against pathogenic invasions in thick shell mussel and could shed new light on the elucidation of mussel TLR-mediated signaling pathway.

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