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A novel invertebrate toll-like receptor with broad recognition spectrum from thick shell mussel *Mytilus coruscus*

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ABSTRACT

Toll-like receptors (TLRs) are a category of most well recognized pattern recognition molecules that act on a vital role in both innate and adaptive immunity. In the present study, a novel toll-like receptor (*Mc*TLRw) was identified and characterized in thick shell mussel *Mytilus coruscus*. *Mc*TLRw possesses one intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain, one transmembrane region (TM), one leucine rich repeat N-terminal domain (LRR_NT) and a few of leucine-rich repeats (LRRs), which all are common in typical TLRs. *Mc*TLRw transcripts were constitutively expressed in all examined tissues with higher expression levels in immune related tissues, and were significantly induced in haemocytes with the challenges of live *Vibrio alginolyticus*, lipopolysaccharide (LPS), peptidoglycans (PGN) and β-glucan (GLU), but not induced by polyinosinic-polycytidylic acid (poly I:C). rMcTLRw exhibited affinity to LPS, PGN and GLU while no affinity to poly I:C. Further, the downstream of TLR signaling pathway myeloid differentiation factor 88a (MyD88a), interleukin-1 receptor-associated kinase-4 (IRAK4) and tumor necrosis factor receptor-associated factor 6 (TRAF6) were significantly repressed in *Mc*TLRw silenced mussels while challenged with LPS. These results collectively indicated that *Mc*TLRw is one member of TLR family and involved in immune response to against invaders by taking participate in TLR mediated signaling pathway.

1. Introduction

Thick shell mussel, *Mytilus coruscus*, belongs to Bivalvia, Mollusca. It is mainly distributed in the northwest pacific waters, including Japan's Hokkaido, Jeju Island in the south of Korea, China's Yellow Sea, Bohai Sea, East China Sea and Taiwan Strait. As one of the fastest growing species of marine shellfish, *M. coruscus* was extensively cultured in Zhoushan Islands, Zhejiang, China, in the past decades, bringing the great economic value [1]. *M. coruscus* lives attached to hard substrates and forms subtidal beds playing an important ecological role and affecting the coastal community structure [2]. Due to its economical and ecological importance, *M. coruscus* has developed into model organisms for studying the response mechanism of mussels against biotic and abiotic stresses in recent years [3–7]. However, the relative lack of information seriously hinders the systematic interpretation of immune defence mechanism contrary to environmental stressors, which in turn affects the progress of disease resistance breeding.

Host immunity consists of two arms: adaptive immunity and innate immunity. It is generally recognized that innate immunity is mediated by lymphocytes and is unique to vertebrates, while innate immunity could nonspecifically recognize microbes, an older evolutionary defense strategy common to both vertebrates and invertebrates [8]. In the past decade, rapid progress has been made in understanding innate immune recognition of microbial components and their critical role in host defense against infection [9]. Now, it is well illustrated that the immune recognition is originated from the detection components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs) by host's germline-encoded pattern-recognition receptors (PRRs) [10,11]. Upon recognition PRRs via PAMPs, the downstream cascade is triggered to eliminate invading microorganisms.

Toll-like receptors (TLRs) are the most well studied PRRs, which its history starts with the discovery of phagocytic cells in 1883, followed by the first description of what is now known as IL-1 in 1940 [12]. It was observed that a mammalian ortholog of the *Drosophila melanogaster* (fruitfly) transmembrane protein Toll (now known as TLR4) could induce the activation of certain genes necessary for initiating an adaptive immune response [13,14], and a focus in this direction then onwards started. Several classes of cytosolic PRRs, including RIG-I-like receptors

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(RLRs) and Nod-like receptors (NLRs), were discovered successively [9]. TLRs are structurally conserved and generally characterized with ectodomains containing leucine-rich repeats that mediate the recognition of PAMPs; transmembrane domains; and intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains required for downstream signal transduction [9]. TLRs have broad pattern recognition spectrum as they could recognize diverse PAMPs inclued lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes as bacteria, viruses, parasites and fungi [10]. Recognition and binding of PAMPs by specific TLRs could activate cell signaling cascades through MyD88 (myeloid differentiation primary response 88)-dependent and MyD88-independent pathways resulting in the induction of various cytokines such as TNF-α, IL-1β, IL-12, IL-8, IL-6 and IFN [12].

So far, total of thirteen TLRs have been identified in humans and mice together, with TLR1-TLR9 being conserved in both species [9]. The equivalent forms of many of these TLRs have been detected in other mammalian species [15–17]. However, TLRs in lower vertebrates such as fish and invertebrates have been very much diversified. Twenty TLR types have been identified in more than a dozen of teleost species [12], with the loss of conformability of mammalian TLR6 and TLR10, but TLR22 is present, which is unique in fish. Additionally, recent genome-wide analyses showed that sea urchin, oyster, amphioxus and ascidian possess 222, 83, 72, and 2 TLR candidates [18–22]. The diversification of TLRs in fish and invertebrates may be due to various evolutionary mechanisms, viz. retro-transcription, gene duplication, high gene expansion rate and alternative splicing of the transcripts [23].

Generally speaking, most vertebrates have one gene ortholog for each TLR family, which detect invasion of various pathogens and play key role in innate and adaptive immunity. However, invertebrate like *Drosophila*, TLRs are important for growth (muscle development and heart formation) and antifungal/anti Gram-positive bacterial immunity [24]. In *Caenorhabditis elegans*, MyD88 ortholog and transcription factor NF-κB are not present lacking the central proteins of the canonical TLR signaling cascade, and TLRs are involved only in development [25,26]. In comparison with the intensive studies in vertebrates, the TLR information in invertebrates is still scarce, which severely hinder our understanding on invertebrate defence mechanism against exogenous invasions. Nevertheless, recent studies have identified and functionality characterized several TLR orthologs in specific invertebrates such as in scallop [27–31], oyster [32], mussel [33–35], snail [36], crab [37], shrimp [38,39], blood clam [40] and razor clam [41,42]. These data shed a new light on the regulatory mechanisms of the TLR signaling pathway in invertebrates. Aiming to understand the immune response of diverse TLRs to extrinsic stress in thick shell mussel and enrich the database for functions of TLR, we identified a new molluscan TLR member *Mc*TLRw in the present research. Meanwhile, its response to immune stimuli and affinity capacity towards PAMPs were assessed. Further, its potential role in triggering TLR mediated signal pathway was also explored.

2. Materials and methods

2.1. Experimental animals

M. coruscus adults (shell length, 9.75 ± 0.45 cm; shell width, 4.43 ± 0.48 cm) were obtained from Dongji Island, Zhoushan City, Zhejiang Province, China. Before treated, these mussels were accommodated under a laboratory condition according to our previous description [43,44]. Briefly, *M. coruscus* were temporary cultured in 300 L aquaria with temperature of 24 ± 0.5 °C, salinity of 28 ± 1‰, fed daily with spirulina powder, and filtered seawater was replaced for half aquaria every day.

2.2. Immune challenge and tissue collection

Four immune stimuli containing live *Vibrio alginolyticus* (3×10^6 CFU mL^{-1}), lipopolysaccharide (LPS, 50 μg/mL), peptidoglycans (PGN, 0.5 mg/mL), β-glucan (GLU, 0.5 mg/mL) polyinosinic-polycytidylic acid (poly I:C, 1 mg/mL) and were used for challenge experiments. These stimuli were injected into the adductor muscle of each mussel at the volume of 200 μL, respectively. In the control group, 200 μL PBS (pH 7.4) was injected. The details of immune challenge experiment were described elsewhere [4,45].

Haemolymph was carefully extracted at 0, 3, 6, 12, 24, and 36 h post induction (hpi) from the adductor muscle using 5 mL syringes fitted with 25 gauge needles. Haemocytes were obtained through centrifugation at 700*g* for 10 min at 4 °C. 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. All samples were stored on ice until processed to avoid spontaneous cell clumping [46].

Seven tissues, including the gills, gonads, digestive glands, hepatopancreas, adductor, haemocytes and mantles were chosen for examination of tissue distribution of *Mc*TLRw transcripts. All tissue samples were dissected from eight mussels and immediately frozen at −80 °C with the addition of 1 mL Trizol reagent (Invitrogen, USA).

2.3. Gene cloning and sequence analysis of McTLRw

Total RNAs from all above mentioned samples were extracted using a traditional Trizol extraction method [47]. After extracted, RNA was examined for quality and concentration by a micro-spectrophotometer Nanodrop (Thermo, USA), then diluted RNA was used for the first-strand cDNA synthesis based on M-MLV reverse transcriptase (Promega, USA) with oligo-d(T) primer. The detailed methods were according to the manufacturer's protocol.

The full-length of open reading frame (ORF) sequence of *Mc*TLRw gene was obtained by scanning the *M. coruscus* transcriptome database [4]. After that, three pairs of specific primer TLRw-1, 2 and 3 (Table 1) were designed to amplify the ORF sequence for verifying the correctness of silicon cloning. Rapid-amplification of cDNA ends (RACE) was carried out to amplify the 5′ and 3′ untranslated regions (UTR) with specific and adaptor primers (Table 1) using the RACE cDNA Amplification Kit (Life Technologies, USA) according to the manufacturer's protocol. All 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*TLRw full length cDNA using CAP3 software [48].

The obtained *Mc*TLRw cDNA sequence was analysed using the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/). The ORF of *Mc*TLRw gene was searched using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/), following the amino acid sequence was predicted. The protein molecular weight and theoretical isoelectric point were estimated with an online tool (http://web.expasy.org/compute_pi/). The functional domains of the deduced amino acid sequence were predicted using on line SMART (http://smart.embl-heidelberg.de/) [49]. Phylogenetic relationships were investigated by the neighbour-joining (NJ) method using MEGA 7.0 tested by 5000 bootstrap resampling [50].

2.4. Recombinant expression and purification of McTLRw protein

The LRR fragment of *Mc*TLRw (amino acid 36–653) was amplified with one specific primer pair TLRw-y (Table 1) which incorporated with *Eco*R I and *Sal*I restriction sites at its 5′ end. A recombinant plasmid termed pMAL-c4x-McTLRw-LRR was generated by subcloning McTLRw-LRR cDNA into the pMAL-c4x prokaryotic expression vector. The recombinant plasmid pMAL-c4x-McTLRw-LRR was transformed into *Escherichia coli* BL21 competent cells (DE3) (Takara) for prokaryotic expression of the fusion proteins, and the pMAL-c4x vector without insert fragment was selected as a negative control. The

Table 1
PCR primer pairs used in the present study.

Primer pairs	Sequence (5′to 3′)	Usage
TLRw-1	ATGAATGGTCACCAACAATT GATTGTCCGCCGCAAAAACA	For ORF cloning
TLRw-2	CGAAATGTTTTTGCGGCGGA TGCGCCATCGGTATCTTTCG	For ORF cloning
TLRw-3	TGCCGAAAGATACCGATGGC TTAACCTACAAGGACGTCTTA	For ORF cloning
TLRw-5′	GTTATAGAAAACGTCAT AATGCAGGAATTTCTGTT AGAACAATCCATATTTTGTTTA	For 5′RACE
TLRw-3′	GTCATGTCATGTCACTTTCTAGAATC ACATGGCAAGAATGGAAAGTATTTAT	For 3′ RACE
Real-TLRw	ACTCGTGGAAAGGACCAGTG TCGTAGTATTGCGCCATCGG	For *Mc*TLRw qPCR
Real-MyD88a	AGGATTCGAGGACAGCGAAG GGCCAAACCCATTCTCGTTG	For *Mc*MyD88a qPCR
Real-IRAK4	CCTTTTATGGCAGCAGCGTG AAAATCCAGTGCCCGATGGT	For *Mc*IRAK4 qPCR
Real-TRAF6	TGTGCCAATTCCCTGTCCT GGACACTCTTTATGCAGG	For *Mc*TRAF6 qPCR
β-actin	GCTACGAATTACCTGACGGACAG TTCCCAAGAAAGATGGTTGTAACAT	Internal reference
TLRw-y	CAGAATTCGTGGTTTCTGGCAGATTATGT GAGTCGACAGTATATGATGAACACTCCTT	For LRR fragment amplification
TLRw-ds	TAATACGACTCACTATAGGGAGCAGCACTGATACTCGCGAAT TAATACGACTCACTATAGGGACCTACAAGACGTCTCTTAGC	For *Mc*TLRw gene silencing
GFP-ds	TAATACGACTCACTATAGGGATGGTGAGCAAGGGCGAGGA TAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA	Negative control in RNAi

induction, purification and refolding of the recombinant proteins were performed according previous reports [51,52]. When the bacterial optical density reached absorbance 0.6 at 600 nm through vigorous shaking at 37 °C in LB media, isopropyl-beta-D-thiogalactopy ranoside was added to culture media to a final concentration of 1.0 mM and then incubated at 16 °C at 160 rpm shaking for 12 h. After centrifuged, the whole cell lysate was then added to pMAL Protein Fusion and Purification System (NEB) for recombinant proteins purification. The purified recombinant proteins were analysed using polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R250. The proteins were refolded in 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM oxidized glutathione, and 0.5 mM reduced glutathione overnight at 4 °C. The concentration of purified recombinant proteins was quantified by BCA method [53].

2.5. Pathogen associated molecular patterns binding assay

For the aim to detect the direct binding of rMcTLRw to pathogen associated molecular patterns, an enzyme-linked immunosorbent assay (ELISA) was performed according to previous studies [30,54,55]. A total of 15 μg of LPS, PGN, GLU and poly I:C were used to coat a 96-well microtiter plate, and then the wells were blocked with 3% BSA (w/v) in PBS at 37 °C for 2 h. After washed with PBST, 2-fold serial diluted concentrations of rMcTLRw in TBS were added. The same concentration of MBP-Tag protein was used as negative control. After incubating at 18 °C for 3 h, the plate was washed three times with PBST, and then 100 μL mouse *anti*-MBP tag monoclonal antibody (Life Technologies, USA) diluted to 1:2000 was added and incubated at 37 °C for 1 h. The plate was washed again for 3 times, and 100 μL of goat anti-mouse Ig-alkaline phosphatase conjugate (Life Technologies, USA) secondary antibody (diluted in 1:2000) was added and incubated at 37 °C for 1 h. After the last washing, 100 μL of 0.1% (w/v) *p*-nitrophenyl phosphate (pNPP, Sigma) in 50 mM carbonate bicarbonate buffer (pH 9.8) containing 0.5 mM MgCl2 was added and incubated at room temperature in dark for 30 min. The reaction was stopped by 2 M NaOH and the absorbance was measured at 405 nm. The wells with 100 μL of TBS were used as blank. Each experiment was repeated in triplicates and the results were given in terms of the mean of three individual measurements ± standard deviation (n = 3). Samples with P (sample) - B (blank)/N (negative) - B (blank) > 2.1 were considered as positive [30].

2.6. McTLRw silencing and the expression patterns of downstream genes

Aiming to investigate the role of *McTLRw* in TLR signaling pathway, TLR fragment of *Mc*TLRw was silenced according to previous reports [35,38]. The double-stranded RNA (dsRNA) was generated according to our previous description [45]. Briefly, specific primers of *Mc*TLRw (TLRw-ds, Table 1) and green fluorescent protein (GFP-ds, Table 1) incorporated with the T7 promoter at their 5′-ends were used to amplify the corresponding PCR products as the templates for dsRNA synthesis. The dsRNA was synthesized using the HiScribe™ T7 in vitro transcription kit (NEB, USA) according to the manufacturer's protocol.

*Mc*TLRw dsRNA (100 μg per mussel) was injected into the adductor muscle of each mussel, while the control groups received an injection of GFP dsRNA (100 μg per mussel) or PBS (100 μL per mussel). To enhance the RNAi effect, a second injection was performed 24 h later. Total RNA from haemocytes was sampled at 36 h after the first dsRNA injection. qPCR was used to detect the efficiency of RNAi. 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, then qPCR was carried out to assess the mRNA expression of downstream immune effector genes of myeloid differentiation factor 88a (MyD88a), interleukin-1 receptor-associated kinase-4 (IRAK4) and tumor necrosis factor receptor-associated factor 6 (TRAF6).

2.7. Quantitative real time PCR of McTLRw

The quantitative real time PCR (qPCR) was carried out according to our previous description [45]. Briefly, qPCR assays were performed on a 7500 Real Time PCR System (Applied Biosystems, USA) with one specific primer pair viz. Real-TLRw, SYBR® Green I (Takara) was used as the fluorescent dye and ROX Reference Dye II was added to alleviate the discrepancy between reaction cells. The relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal reference

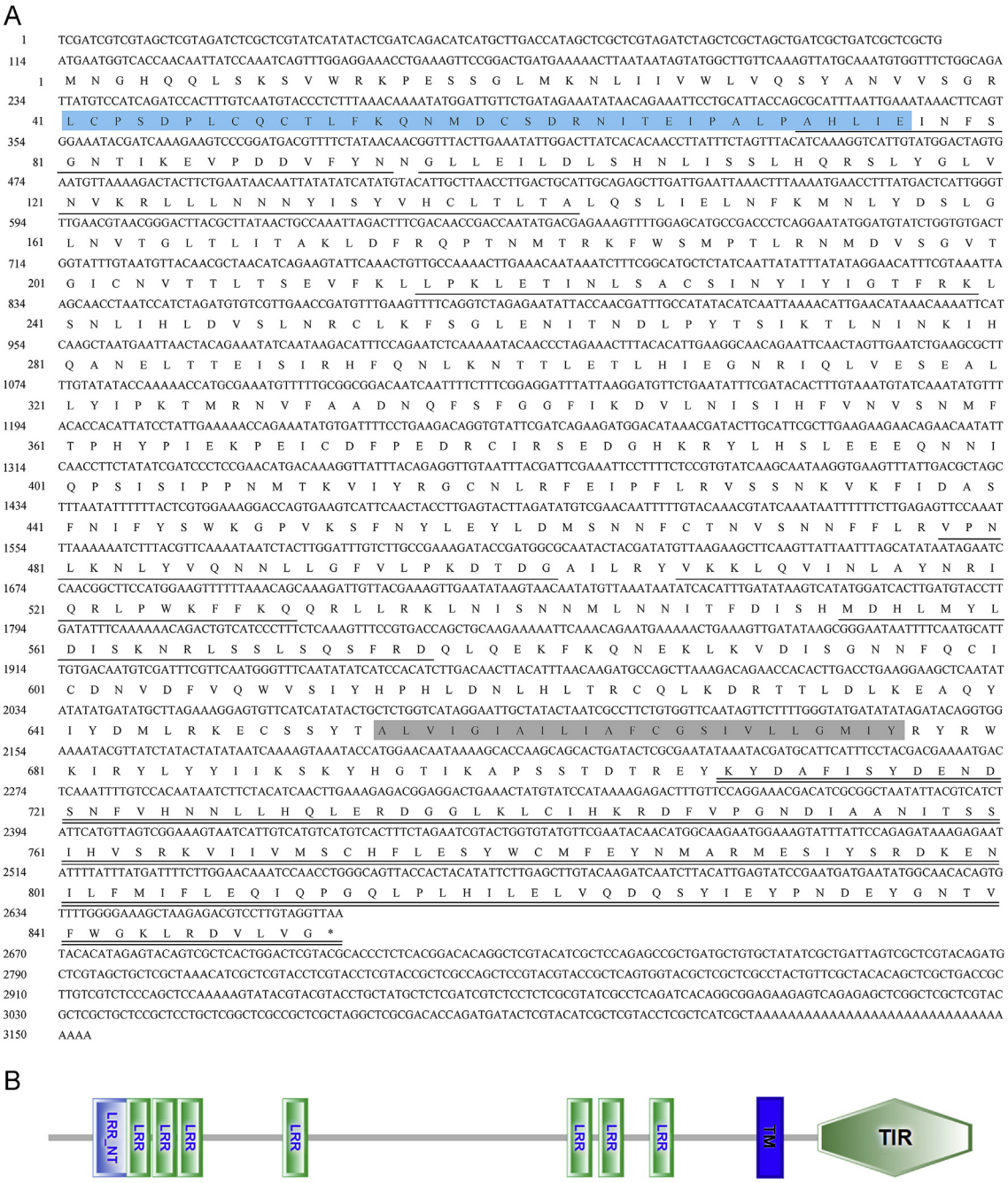

Fig. 1. Molecular characterization of *Mc*TLRw. (A) The nucleotide sequences and the deduced amino acid sequences of *Mc*TLRw. The complete sequence of *Mc*TLRw cDNA is 3153 bp, containing an 113 bp 5′-UTR region and a 484 bp 3′-UTR region, a 2556 bp ORF region coding the protein of 851 amino acid residues. The LRR domains were underlined, the TIR domain was double underlined, the TM domain was marked with gray and the LRR_NT domain was marked with blue. (B) Schematic diagram of *Mc*TLRw functional domains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

[56].

2.8. Statistical analysis

All data were expressed as means ± standard deviation (n = 3) of triplicate experiments. Statistical comparison between two groups was performed with t-tests of independence. Multiple group comparisons were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range tests using SPSS 17.0 software [57]. Differences were deemed significant at $P < 0.05$.

3. Result

3.1. Cloning and sequence analysis of McTLRw

The full length cDNA of a novel TLR was obtained through gene cloning and 5′, 3′ RACE, and the novel TLR gene was termed as *Mc*TLRw. The *Mc*TLRw cDNA of 3153 bp contains 113 bp 5′-UTR, 484 bp 3′-UTR and 2556 bp ORF sequence coding a deduced amino acid sequence of 851 residues (Fig. 1A). The calculated molecular mass of *Mc*TLRw is 98.9 kDa and the protein has a theoretical isoelectric point of 8.54. Blast P revealed that *Mc*TLRw shared the most sequence

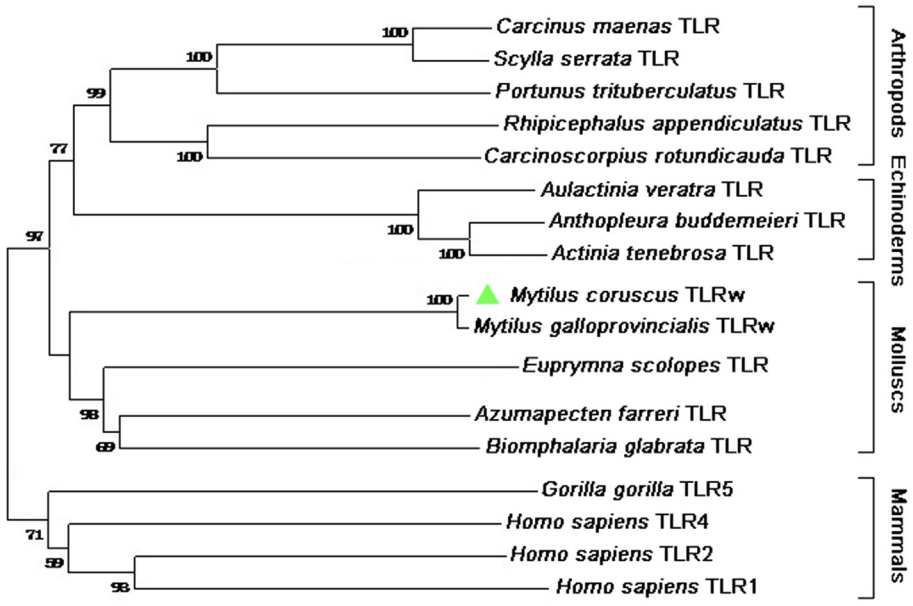

Fig. 2. Phylogenetic analysis of *Mc*TLRw. The phylogenetic tree was constructed using MEGA software 5.0 with 5000 replications of bootstrap in the way of neighbour-joining method. *Mc*TLRw was marked with green triangle. Species included in construction of phylogenetic tree were all retrieved from NCBI database and accession numbers of the TLRs are: *Aulactinia veratra* (ALG40988.1), *Anthopleura buddemeieri* (ALG40987.1), *Actinia tenebrosa* (ALG40986.1), *Sepiella japonica* (AQY56780.1), *Biomphalaria glabrata* (AGB93809.1), *Euprymna scolopes* (AAY27971.1), *Azumapecten farreri* (ABC73693.1), *Mytilus galloprovinciali* (AGI05199.1), *Carcinus maenas* (CDO91661.1), *Portunus trituberculatus* (AUZ62382.1), *Rhipicephalus appendiculatus* (JAP80304.1), *Scylla serrata* (AGG55849.1), *Carcinoscorpius rotundicauda* (ABK88278.1), *Gorilla gorilla* (AGR83259.1), *Homo sapiens* TLR4 (AAY82270.1), *Homo sapiens* TLR2 (AAY85644.1), *Homo sapiens* TLR1 (AAY85639.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identity to TLRw of *M. galloprovincialis* (96.4%), followed by a few other TLR orthologs such as TLRg, e, f and h (42.8%–38.9%) from *M. galloprovincialis* [33]. The functional domain prediction by SMART showed that *Mc*TLRw contained seven Leucine-rich repeats (LRR) domains with an amino acid length of 23–24, a 23 aa transmembrane (TM) domain, a 26 aa Leucine rich repeat C-terminal domain (LRR_NT) and a 142 aa intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain (Fig. 1A and B). In the phylogenetic tree, four conspicuous branches viz. Mammals, Echinoderms, Arthropods and Molluscs branches were presented. *Mc*TLRw firstly clustered with its counterpart of *M. galloprovincialis*, then grouped together with the TLRs of other molluscs, which was consistent with traditional taxonomy and phylogeny (Fig. 2).

3.2. Expression profile analysis of McTLRw transcripts

The tissue distribution of *Mc*TLRw transcripts was examined by qPCR. As shown in Fig. 3A, *Mc*TLRw showed a constitutive expression profile with the highest expression level in hepatopancreas, followed by haemocytes, moderately expressed in gills and digestive glands, and weak expression in mantles, gonads and adductor.

The reaction capabilities of *Mc*TLRw to immune stimuli were assessed in haemocytes. After challenged with live *V. Alginolyticus,* LPS, PGN and GLU, the expression of *Mc*TLRw transcripts was significantly up-regulated despite of the different increase folds. Upon *V. Alginolyticus* challenge, the peak level of *Mc*TLRw transcripts presented at 24 hpi, with a 8.4-fold increase; while at 6 hpi, with a 21.9-fold increase with LPS challenge; at 24 hpi, with a 7.8-fold increase with PGN challenge; at 12 hpi, with a 9.1-fold increase with GLU challenge. Whereas, there was no obvious change to *Mc*TLRw expression level with the challenge of poly I:C.

3.3. PAMPs binding activity of rMcTLRw

The recombinant plasmid pMAL-c4x-McTLRw-LRR was expressed in *E. coli* transetta (DE3). After IPTG induction, the whole cell lysate of the positive clone was analysed by SDS-PAGE, and one distinct band with molecularmass of about 112 k Da (Fig. 4A) were detected, which was consistent with the predicted molecular mass of rMcTLRw.

The binding activity of rMcTLRw to different PAMPs was assessed through ELISA assay and recorded as absorbance at 405 nm. The result revealed that rMcTLRw exhibited higher affinity to LPS (P/N = 5.46), PGN (P/N = 4.68) and GLU (P/N = 4.15), while no affinity to poly I:C (P/N < 2.1). The binding capability of rMcTLRw to LPS, PGN and GLU was of dose-dependent (Fig. 4B), and the P/N values gradually increased when the protein concentration was elevated from 0 to 60 μg/mL, respectively.

3.4. McTLRw was involved in TLR signaling pathway

For the aim to assess the functional role of *Mc*TLRw involved in TLR mediated signaling pathway, *Mc*TLRw was silenced through dsRNA mediated RNAi assay; and hence the transcriptional expressions of some down-stream genes in TLR signal pathway for instance the *Mc*MyD88, *Mc*IRAK4 and *Mc*TRAF6 were assessed by qPCR. The results showed that the expression of *Mc*TLRw transcripts was significantly repressed in McTLRw-dsRNA treated mussels, but had no obvious changes in control group (Fig. 5A). When challenged with LPS, the transcriptional expressions of *Mc*MyD88, *Mc*IRAK4 and *Mc*TRAF6 were increased to higher levels, while in *Mc*TLRw silenced mussels, the expression levels of *Mc*MyD88 (Fig. 5B), *Mc*IRAK4 (Fig. 5C) and *Mc*TRAF6 (Fig. 5D) were down-regulated.

4. Discussion

As a category of widely recognized PRRs, TLRs play a vital immune modulation role in both innate and acquired immunity [58]. To date, a certain number of TLRs have been identified from vertebrates to invertebrates, however, TLRs discriminated in molluscs is still very scarce, especially their roles in immune modulation in response to pathological challenges are still very obscure. In the present study, a novel TLR (designated as *Mc*TLRw), which possessed one TIR, one TM and seven LRRs, was identified from the thick shell mussel, *M. coruscus.* LRRs are found in a large and diverse group of proteins with functions ranging from RNA processing and transcriptional regulation to cell adhesion, bacterial pathogenesis, and signal transduction [59]. TIR domain is a highly-conserved region of 200 residues and may mediate protein-protein interactions between TLRs [9]. Most of TLRs have a TM domain which anchor these proteins on the cell surface or in endosomes [60]. Some identified molluscan TLRs possess these typical functional domains, for instance, in identified *Cf*TLR [29] and *Cg*TLR6 [30], the sole TM and TIR domain, a few of LRR domains were also found. However, in a freshwater pearl mussel *Hyriopsis cumingii*, one TLR homologue *Hc*TLR3 was found to be have tandem TIR domains [35], suggesting the complexity and variety of TLRs in invertebrates. The

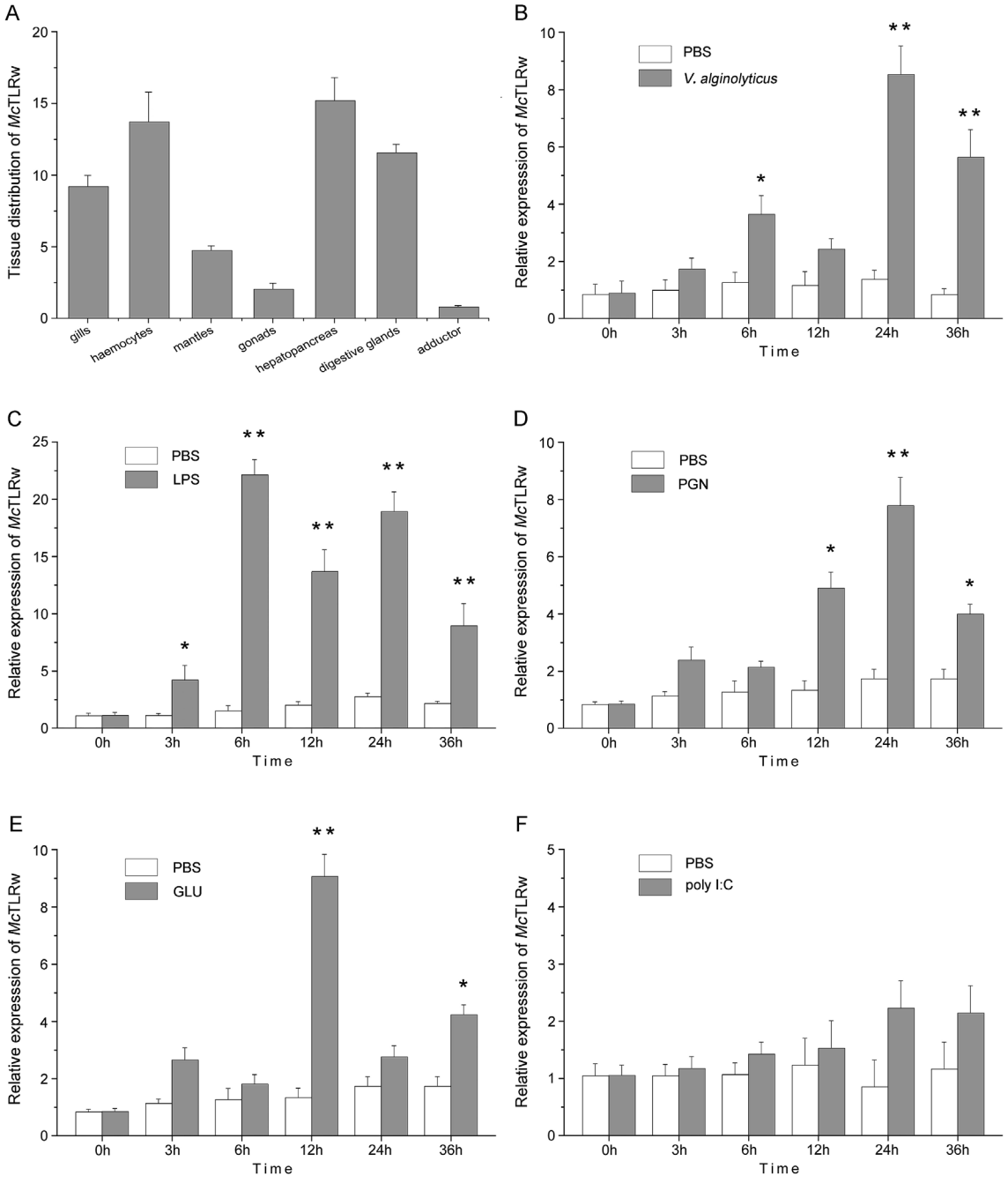

Fig. 3. Expression profile analysis of *Mc*TLRw transcripts in different adult tissues (A), after challenged with live *V. Alginolyticus* (B), LPS (C), PGN (D), GLU (E) and poly I:C (F). The results were expressed as mean ± S.D. (n = 3). Significant difference relative to control was indicated with asterisk symbol (*P < 0.05, **P < 0.01).

amino acid sequence of *Mc*TLRw shared higher similarity with molluscan TLRs like *M. galloprovincialis* and *M. yessoensis*, simultaneously *Mc*TLRw grouped together with TLRs from other molluscs into one apparent cluster, suggesting that these TLRs from the same phylum are close relative. Taken together, it could be speculated that the newly identified TLR congener *Mc*TLRw belongs to the TLR family, and might play the similar pattern recognition receptor role in the innate immune against pathological challenge just as its counterparts play in mammals.

The qPCR results showed that *Mc*TLRw transcripts were constitutively expressed in all examined tissues with the highest expression levels in hepatopancreas and haemocytes. The likewise tissue distribution profile was also presented in other inveterate TLRs, for instance, *Cf*Toll-1 transcripts were constitutively expressed in tissues of haemocyte, muscle, mantle, heart, gonad and gill [27], while *Cf*TLR protein was detected in hemocytes, mantle, gills, hepatopancreas, kidney and gonad of the scallops [29]. *Hc*Toll3 mRNA was broadly expressed in all of the examined tissues of freshwater pearl mussel, *Hyriopsis cumingii* [35]. SpToll2 was widely expressed in all tissues tested, with the highest level found in hemocytes and hepatopancreas while the lowest in heart and muscle in *Scylla paramamosain* [37]. Such constitutive expression in various tissues may be helpful to the interaction between *Mc*TLRw and multiple PAMPs. This notion was alternatively proved by the significant induction of *Mc*TLRw transcripts to different PAMPs. Here, *Mc*TLRw was significantly induced by gram-negative bacteria *V. alginolyticus*, LPS, PGN and GLU. similarly, *Hc*Toll3 is induced by gram-negative bacteria *V. parahemolyticus*, but not gram-

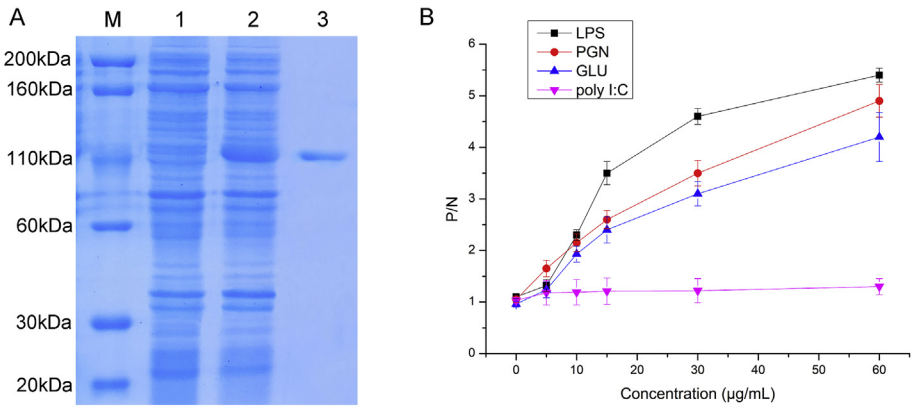

Fig. 4. The affinity activity of rMcTLRw. (A) SDS-PAGE analysis of rMcTLRw. Line M: protein molecular standard; lane 1: negative control for rMcTLRw (without induction); lane 2: induced rMcTLRw; lane 3: purified rMcTLRw. (B) The affinity activity of rMcTLRw to LPS, PGN, GLU and poly I:C was analysis by ELISA. Samples with P/N > 2.1 were considered positive. Values represent mean ± S.D. (n = 3).

positive bacteria *S. aureus* in *Hyriopsis cumingii* [35]. *Cf*Toll transcripts was up-regulated with the treatment of LPS in *C. farreri* [27], two Toll homologue termed *Cg*Toll and *Cg*TLR6 was significantly induced in hemocytes after the pacific oyster was stimulated with LPS, PGN or bacteria *Vibrio splendidus* [30,32]. In the present study, *Mc*TLRw was not significantly induced by poly I:C. However, *Cn*TLR1 transcripts were up-regulated after Poly I:C challenge in *Chlamys nobilis* [61]. This inconsistent results may suggested that the recognition of PAMPs may vary from TLRs. Collectively, we inferred that the identified TLR homologue *Mc*TLRw might belong to the TLR family, and could perform the similar functional role as PRRs in innate immunity as its counterparts act on in molluscs.

To further explore the possible recognition mechanism, four common PAMPs containing LPS, PGN, GLU and poly I:C were selected to observe the binding affinity of rMcTLRw. rMcTLRw showed the higher affinity to LPS, PGN and GLU, but no affinity to poly I:C, which suggested a broad recognition spectrum. The broad recognition was also examined in some molluscan TLRs. In *Crassostrea gigas*, the recombinant protein rCgTLR6 displayed direct affinity to live gram-negative bacteria, gram-positive bacteria, fungi as well as LPS and PGN [30]. In *Chlamys farreri*, rCfTLR could activate NF-κB in response to multiple PAMPs including GLU, PGN, poly I:C and four types of CpG [29]. It has been comprehensively recognized that TLRs in mammals could recognize their ligands specially, such as TLR4 can recognize LPS, TLR3 recognize dsRNA and TLR9 recognize CpG DNA [62]. In short, invertebrate TLRs seem to have a broader recognition spectrum for PAMPs than vertebrate TLRs. The explanation could be, that the invertebrate animals lack of adaptive immunity and need to harbor much more PRRs by gene expansion as a compensation to recognize the diverse pathogens [30].

In mammals, the recognition of PAMPs by TLRs activates NF-κB signaling transduction [63]. This signaling transduction involves the formation of MyD88-Tube-Pelle-TRAF6 complex, followed by the translocation of Dorsal/Dif into nucleus, and the activation of AMPs expression [64]. In previous studies, we have identified some downstream genes such as MyD88s [44], IRAK4 [45] and TRAF6 [65] in *Mytilus coruscus*. To explore whether the *Mc*TLRw was involved in the TLR mediated signaling pathway, *Mc*TLRw was silenced and following the expressions of *Mc*MyD88a, *Mc*IRAK4 and *Mc*TRAF6 transcripts were assessed. RNAi results showed that the expressions of *Mc*Myd88, *Mc*IRAK4 and *Mc*TRAF6 were significantly repressed in *Mc*TLRw silenced mussels when challenged with LPS, suggesting that *Mc*TLRw may be involved in the activation of TLR signaling pathway. Similarly, in *Chlamys farreri*, when *Cf*TLR expression was inhibited by RNAi, the

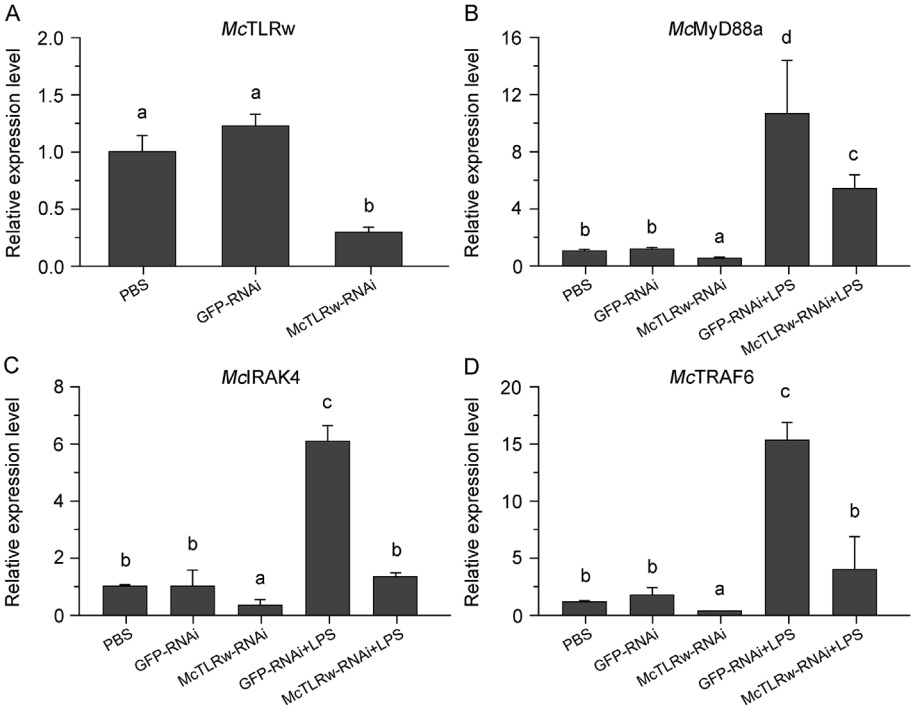

Fig. 5. Temporal expression of some components in TLR signal pathway in *Mc*TLRw knockdown *M. coruscus* with the challenge of LPS. The expressions of *Mc*TLRw (A), *Mc*MyD88a (B), *Mc*IRAK4 (C), *Mc*TRAF6 (D) in *Mc*TLRw knockdown mussels were detected by qPCR. Values represent mean ± S.D. (n = 3), different superscript letters indicate significant differences at $p < 0.05$.

mRNA expression level of *Cf*MyD88, *Cf*TRAF6, *Cf*IκB, *Cf*NFκB and G-type lysozyme were all decreased, while those of superoxide dismutase and catalase were increased [28]. Additionally, in *Scylla paramamosain*, *Sp*Toll1 and *Sp*Toll2 could regulate the transcripts of four immune related mediators as *Sp*MyD88, *Sp*Tube, *Sp*Pelle and *Sp*TRAF6 at different scale [37]. Collectively, these present results suggested that invertebrate TLRs might play a vital role in the TLR signaling pathway as their homologues act in mammals.

In conclusion, a new molluscan TLR member containing traditional functional domains of TLR family was identified from thick shell mussel *M. coruscus*. *Mc*TLRw is constitutively expressed in different tissues and could be induced by bacteria and PAMP stimulation in haemocytes. rMcTLRw could bind to LPS, PGN and GLU, but not to poly I:C. RNAi experiments suggested that *Mc*TLRw might play a vital role in the TLR signaling pathway in *M coruscus*. All the results offered helpful information to characterize the TLRs in molluscs.

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