A newly identified Hippo homologue from the oriental river prawn *Macrobrachium nipponense* is involved in the antimicrobial immune response

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Abstract

The Hippo signalling pathway plays a vital role in organ size control, cell proliferation, apoptosis, and immune regulation. In this study, a Hippo homologue with three isoforms (named MnHippo-a, MnHippo-b, and MnHippo-c) was isolated and characterized for the first time from the freshwater prawn *Macrobrachium nipponense*. The deduced amino acid sequences of MnHippo-a (698 aa), MnHippo-b (688 aa), and MnHippo-c (656 aa) were highly similar, and they all contained an N-terminal S_TKc (serine/threonine protein kinase catalytic) domain and a C-terminal Mst1_SARAH (Sav/Rassf/Hpo) domain. MnHippo-a and MnHippo-c were derived from alternative splicing. Phylogenetic analysis was performed, and the results revealed that MnHippo was a member of the clade containing STPK4 and Hippo of *Penaeus vannamei*. The expression distribution showed that MnHippo was constitutively expressed in various tissues of uninfected prawns and highly expressed in the hepatopancreas and intestine. In prawns challenged with *Vibrio parahaemolyticus* and *Staphylococcus aureus*, the expression of MnHippo in haemocytes was significantly upregulated. Furthermore, in MnHippo-knockdown prawns injected with *V. parahaemolyticus* or *S. aureus*, the transcription levels of five antimicrobial peptides were downregulated. MnHippo silencing weakened the clearance of *V. parahaemolyticus* and *S. aureus* in prawns. The survival rate of the MnHippo-dsRNA group was obviously decreased from 2 to 6 days post-injection with *V. parahaemolyticus* or *S. aureus*. Hence, MnHippo might be involved in the antibacterial immune defence of *M. nipponense*.

Keywords: *Macrobrachium nipponense*, Hippo, Expression regulation, Antibacterial response, Innate immunity

Introduction

The Hippo signalling pathway is found in a wide variety of animals, from insects to mammals. Since its initial discovery in *Drosophila melanogaster*, the Hippo pathway has attracted much attention because of its strong involvement in controlling organ size, regulating cell proliferation, and promoting apoptosis [1–3]. The Hippo pathway is composed of more than 30 components, including the core kinase module and the transcriptional module [4]. The core members of this pathway were identified through genetic screening of *Drosophila* chimaeras and include four proteins, namely, Hippo (Hpo), Salvador (Sav), Warts (Wts), and monopolar spindle one binder (MOB), as tumour suppressors (Mats) [5]. Under activator stimulation (the Hippo pathway is activated), the four proteins can form a kinase cascade reaction chain. Hpo is phosphorylated first, and activated Hpo phosphorylates a complex composed of Wts and the adaptor protein Mats (Wts-Mats) with the help of Sav. The Hippo
pathway kinase cascade in turn promotes the inhibitory phosphorylation of the transcriptional co-activator Yorkie (Yki) and excludes Yki from the nucleus to promote apoptosis and restrict the overgrowth of organ size [6–8]. When the Hippo pathway is inactivated, unphosphorylated Yki translocates into the nucleus, where it binds to and thus activates the transcriptional partner Scalloped (Sd), which contains a TEA domain [9]. The Yki-Sd complex promotes tissue overgrowth by acting on downstream target genes, such as connective tissue growth factor (CTGF) and cysteine-rich 61 (Cyr61) [10]. The loss of functional mutations in any of the four genes (Hpo, Sav, Wts, and Mats) or overexpression of Yki will lead to excess growth because of increased cell proliferation coupled to decreased cell death [11].

Although the Hippo pathway is an extremely complex signalling network, it is highly conserved throughout the evolutionary process. Most members of the Drosophila Hippo pathway have homologues in mammals. Hpo, Sav, Wts, Mats, and Yki in Drosophila are homologous proteins of mammalian Sterile20-like protein kinase 1/2 (Mstl/2, also known as STK4/3), Salvador family WW domain-containing protein 1 (Savl), large tumour suppressor kinase 1/2 (Lastl/2), MOB kinase activator 1A/B (MoblA/B), and yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ), which are all functionally conserved [10, 12]. Recent research using multiple model systems showed that the Hippo kinase cascade represents a signal transduction module that integrates multiple biological inputs, thereby highlighting its importance in biological growth control [13]. Yki in Drosophila or YAP/TAZ in mammals is the target molecule of the Hippo pathway, and regulation of this pathway is ultimately attributed to the regulation of Yki or YAP/TAZ [7, 14]. Phosphorylation of the YAP/TAZ protein controls the size of the mouse liver, while overexpression of YAP/TAZ leads to overgrowth of the liver and induces tumorigenesis [12, 15]. In addition to its roles in tissue development and tumorigenesis, the Hippo pathway also has extensive functions in immune regulation, including of the innate and adaptive immune systems [16]. For example, Drosophila Hpo and its mammalian homologue Mstl/2 mediate Toll-like receptor (TLR) signalling in flies [17] and mammals [18, 19]. YAP/TAZ binds and inhibits TANK binding kinase 1 (TBK1) or interferon regulatory factor 3 (IRF3) to antagonize antiviral innate immune responses [20, 21]. Although the function of the Hippo pathway has been extensively studied in Drosophila and most mammals, the roles of the homologous members of the pathway in the immune system of crustaceans remain unclear.

Macrobrachium nipponense is an economically important freshwater prawn widely cultivated in China, Japan, and Southeast Asian countries [22]. Whether the Hippo signalling pathway plays a role in the innate immunity of M. nipponense remains unknown. In the present study, we isolated a Hippo homologue with three isoforms from M. nipponense (named MnHippo-a, MnHippo-b, and MnHippo-c) and analysed their sequence features. The tissue distribution profile and temporal mRNA expression of MnHippo in response to bacterial infection were detected. RNA interference (RNAi) was implemented to determine the function of MnHippo in antibacterial responses. The results of our work will provide insights into the immune defence mechanism of the Hippo pathway in crustaceans.

**Materials and methods**

**Experimental animals**

Healthy M. nipponense prawns (approximately 2.5–3.5 g in weight) were obtained from a commercial market in Nanjing city, China. The prawns were cultured at 24 °C–26 °C in a recirculating aquarium system filled with freshwater. After 10 days of acclimatization in our laboratory, the haemocytes, heart, hepatopancreas, gills, intestine, and stomach were dissected from five prawns. The samples were quickly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

**Bacterial challenge**

Ninety prawns were divided into three groups, namely, the Vibrio parahaemolyticus group, Staphylococcus aureus group, and control group. In the two challenged groups, each prawn was injected with 50 μL of V. parahaemolyticus (3 × 10⁷ cells) and S. aureus (3 × 10⁷ cells). Prawns in the control group were injected with 50 μL of phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄; pH 7.4). The prawns were returned to the rearing tanks. At 0, 12, 24, 36, and 48 h post-injection, haemolymph was randomly collected from the ventral sinus of five prawns in the control and experimental groups. The haemolymph was extracted using a 1 mL syringe, mixed with a 1/2 volume of anticoagulant solution (ACD-B) (glucose, 1.47 g; citric acid, 0.48 g; trisodium citrate, 1.32 g; prepared in ddH₂O added up to 100 mL; pH 7.3), and centrifuged at 2000 × g and 4 °C for 10 min to harvest haemocytes. The haemocyte pellets were stored at −80 °C for subsequent experiments.

**RNA extraction and reverse transcription**

Total RNA was isolated from the above tissue samples by using TRIzol Reagent (Invitrogen, USA). The quality and concentration of total RNA were examined by RNA electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). All RNA samples were...
added to RNase-free Dnase I (Takara, Japan) to eliminate genomic DNA. In brief, 5 μg of RNA from the mixed tissues was reverse transcribed to first-strand cDNA by using the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). Total RNA (1 μg) was reverse transcribed using the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Japan) for real-time PCR analysis. The obtained cDNA was kept at −20 °C.

Molecular cloning of MnHippo

The partial fragment of Hippo cDNA was obtained from the transcriptomic cDNA library of gills of M. nipponense from our laboratory. Two gene-specific primers (MnHippo-F and MnHippo-R, Table 1) were designed based on the obtained cDNA sequence to carry out rapid amplification of the cDNA ends (5‘ and 3’ RACE). 5‘ and 3‘ RACE was performed using an Advantage® 2 PCR Kit (Clontech, USA) according to the protocol. PCR amplification was performed in a 50 μL reaction as follows: five cycles at 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 20 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The resulting PCR products were electrophoresed on a 1.2% agarose gel and purified with a gel extraction kit (Takara, Japan). The expected purified fragments were sub-cloned into the pEasy-T3 vector (TransGen Biotech, China), and positive clones were selected and sequenced (Springen, China). The 5‘ and 3‘ sequences from RACE were assembled with the partial cDNA sequence to obtain the complete sequence of MnHippo, as shown in Additional file 1.

Genome sequence amplification

Genomic DNA was extracted from the gills of healthy prawns by using NucleoSpin Tissue (Clontech, USA). Genomic amplification was performed in a 25 μL reaction volume containing 18.3 μL of sterile distilled H2O, 2.5 μL of 10 × PCR buffer, 2.0 μL of dNTPs (2.5 mmol L−1), 0.5 μL of each primer (10 μmol L−1), and 0.2 μL of Taq polymerase (5 U μL−1) (Takara, Japan). PCR amplification was conducted as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 3 min; and 72 °C for 10 min. The detailed methods were described in our previous study [23]. The primer sequences (MnHippo-gFs and MnHippo-gRs) are shown in Table 1.

Nucleotide and amino acid sequence analysis

Sequences for protein sequence similarities were performed using the BLAST algorithm on the NCBI website. Protein prediction was carried out using the ExPASy Translate tool and the open reading frame (ORF) finder. Signal sequence and motif prediction was conducted with the SMART program. The molecular weight (Mw) and theoretical isoelectric point (pl) were calculated with the ExPASy Compute pl/Mw tool. The deduced amino acid sequences of Hippo homologues from M. nipponense and other species were compared by multiple sequence alignment using the ClustalX v2.0 program and GENEDOC software. A bootstrap neighbour-joining (NJ) phylogenetic tree was built using MEGA 7.0 software [24].

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out using the TransStart® Top Green qPCR SuperMix Kit (TransGen Biotech, China)
with the LightCycler® 96 Real-Time PCR Detection System (Roche, USA). Amplifications were conducted with a 10 μL reaction volume containing 5 μL of 2× TransStart Top Green qPCR SuperMix, 1 μL of cDNA template (50 ng), 0.2 μL of forward and reverse primers (10 μM), and 3.6 μL of ddH2O. The PCR temperature profile was as described in a previous study [25]. One pair of primers (MnHippo-qF and MnHippo-qR, Table 1) was designed to analyse the expression pattern of MnHippo. The reference gene used to normalize the expression of MnHippo was the β-actin gene of M. nipponense (β-actin-qF and β-actin-qR, Table 1). Each sample was analysed in triplicate. Differences in gene expression were calculated by the $2^{ΔΔCt}$ method [26].

Semiquantitative reverse transcription-PCR (SqRT-PCR)
MnHippo expression in different tissues was investigated by SqRT-PCR using two primers (MnHippo-RT-F and MnHippo-RT-R, Table 1). The PCR amplification procedure was as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 30 s; and 72 °C for 5 min. β-Actin was amplified as an internal control (β-actin-RT-F and β-actin-RT-R, Table 1). The PCR products were separated on 1.2% agarose gels with 0.5 µg/mL ethidium bromide and photographed under ultraviolet light using Quantity One software (Bio-Rad, Hercules, CA, USA).

Double-stranded RNA (dsRNA) synthesis
DsRNA of MnHippo was synthesized using a T7 in vitro transcription kit (Takara, Japan) according to the manufacturer's instructions. The green fluorescent protein (GFP) gene was used as a control. Primers used in RNA interference (RNAi) were designed according to the cDNA sequences of MnHippo and GFP. Templates were prepared by PCR using gene-specific primers (MnHippo-dsRNA-F and MnHippo-dsRNA-R; GFP-dsRNA-F and GFP-dsRNA-R, Table 1) with the T7 polymerase promoter sequence at their 5’ ends. The mixture consisted of 10 μL of 5× transcription buffer, 2.5 μL of each NTP (100 mM), 80 U of RNase inhibitor, 8 μg of DNA template, and 80 U of T7 RNA polymerase and was incubated at 37 °C for 6 h. The mixture was then treated with RNase-free DNase I (8 U) to eliminate the DNA template. The synthesized dsRNA was purified by phenol/chloroform extraction and ethanol precipitation. The product was dried and dissolved in RNase-free water. The quality of dsRNAs was assessed by 1.5% agarose gel electrophoresis and spectrophotometry. The dsRNA sample was kept at −80 °C until use.

DsRNA interference of MnHippo and detection of antimicrobial peptide (AMP) expression
Twenty healthy prawns were assigned to two groups, namely, the MnHippo-dsRNA and GFP-dsRNA (as control) injection groups, to verify the efficiency of RNAi. Each prawn was initially injected with 15 μg of MnHippo-dsRNA or GFP-dsRNA. After 12 h, 15 μg of MnHippo-dsRNA or GFP-dsRNA was repeatedly injected into the same prawn. At 36 h after the first dsRNA injection, the haemocytes of five prawns from each group were randomly collected. qRT-PCR was used to validate the knockdown of MnHippo.

At 36 h after two injections of MnHippo-dsRNA or GFP-DSRNA (total 30 μg), the prawns were challenged with V. parahaemolyticus ($3\times 10^7$ cells) or S. aureus ($3\times 10^7$ cells). At 24 h post-bacterial challenge, the haemocytes of five prawns from each group were sampled. The expression of MnHippo and five AMP genes, including anti-lipopolysaccharide factor 1 (ALF1), ALF4, crustin 5 (Crus5), Cras7, and lysozyme (Lyso2), was analysed in MnHippo-dsRNA-silenced prawns. The primers for the AMPs were as follows: MnALF1-qF and MnALF1-qR; MnALF4-qF and MnALF4-qR; MnCru5-qF and MnCru5-qR; MnCru7-qF and MnCru7-qR; MnLyso2-qF and MnLyso2-qR. All primers were listed in Table 1. Each experiment was repeated three times. Statistical analysis was performed using SPSS software. Statistical significance was determined by one-way ANOVA ($P<0.05$).

Bacteria clearance assay
We further investigated the role of MnHippo in V. parahaemolyticus or S. aureus clearance in vivo. MnHippo-dsRNA as a treatment or GFP-dsRNA as a control was injected into the prawns twice (total 30 μg) as mentioned above. At 36 h after the first dsRNA injection, 50 μL of V. parahaemolyticus ($3\times 10^7$ cells) or S. aureus ($3\times 10^7$ cells) was injected into the prawns. Haemolymph from five prawns for each sample was collected and mixed with ACD-B buffer at 15 min post-bacterial injection. After serial dilution with PBS, 50 μL of the haemolymph was loaded on Luria–Bertani agar plates and incubated at 37 °C overnight. The number of bacterial clones in the plate was counted. The experiment was repeated three times.

M. nipponense survival rate test
The prawns were randomly divided into four groups (20 prawns per group) to examine whether MnHippo is involved in the anti-bacterial immune defence in vivo. The prawns were injected with 30 μg of MnHippo-dsRNA or GFP-dsRNA twice and then injected with 50 μL of V. parahaemolyticus ($3\times 10^7$ cells) or S. aureus ($3\times 10^7$ cells) at 36 h after the first injection. In a fixed time period of 1–6 days after the last challenge, the number...
of dead prawns in each group was monitored, and the cumulative survival rates of prawns were calculated. The experiments were repeated three times under the same conditions.

Results
Cloning and sequence analysis of MnHippo
Three contigs of MnHippo were identified from the transcriptome data of M. nipponense. These contigs are referred to as MnHippo-a (8022 bp), MnHippo-b (7992 bp), and MnHippo-c (7896 bp) on the basis of their nucleotide sequences (Additional file 1). The full-length cDNA sequence of MnHippo-a consisted of an ORF of 2097 bp encoding a protein of 698 amino acid residues, 136 bp of 5'-untranslated region (UTR), and 5789 bp of 3'-UTR. MnHippo-b possessed a 2067 bp ORF encoding a putative protein with 688 amino acids, a 136-bp 5'-UTR, and a 5789-bp 3'-UTR. MnHippo-c contained a 5'-UTR of 136 bp, a 3'-UTR of 5789 bp, and an ORF of 1971 bp encoding a polypeptide of 656 amino acids. The MWs of mature MnHippo-a, MnHippo-b, and MnHippo-c were estimated to be 79.77, 78.66 and 74.87 kDa, respectively, and their theoretical pIs were 5.35, 5.49, and 5.20, respectively.

The three MnHippo isoforms possessed the characteristic motifs of the serine/threonine protein kinase (STPK) family, including a serine/threonine protein kinase catalytic domain (S_TKc, position 47–298, 252 amino acids) and an Mst1_SARAH (Sav/Rassf/Hpo) domain (48 amino acids) (Figure 1A). When ClustalX was used to align the amino acid sequences of the three transcript isoforms, MnHippo-b and MnHippo-c had partially deletions compared with the complete amino acid sequence of MnHippo-a. MnHippo-b lacked the sequence FSQQQSPQLQLPVKNVQAEQD-QSEGTEVEEQ (10 amino acids), whereas MnHippo-c lacked the sequence HKNNQLNQTHHHHSIPNTQIQ (42 amino acids) (Figure 1B). The function of a protein is defined by its structure. Therefore, STPK3 (SMTL ID: 4lgd.4) from Homo sapiens [27] was used as a template structure for homology modelling of the tertiary structure of S_TKc of MnHippo (Figure 1C). The S_TKc of MnHippo was composed of 11 α-helices and seven β-sheets and was structurally similar to STPK3 and human Mst2 kinase. This finding suggested the possible functional resemblance between MnHippo and other STPKs.

Amplification of the MnHippo genomic sequence
PCR was used to amplify the partial genomic DNA sequence of MnHippo to investigate how the three isoforms of MnHippo were formed. The exon–intron boundaries of the genomic sequence of MnHippo were GT and AG at the 5' and 3' splice sites, respectively. The results of genome amplification suggested that MnHippo-a and MnHippo-c were derived from alternative splicing. MnHippo-c was generated by exon skipping (Figure 2).

Sequence homology and phylogenetic analysis
Multiple protein sequence alignment revealed relatively highly conserved sequences between MnHippo-a and some STPKs in other species. The results showed a similarity of approximately 83.8% with STPK4 of Penaeus vannamei, 75.8% with Hippo of P. vannamei, 63.9% with STPK4 of Armadillidium nasatum, and 61.3% with STPK3 of Hyalella azteca. A phylogenetic tree was constructed based on the deduced amino acid sequence by using MEGA7 (Figure 3). Phylogenetic analysis showed that the three MnHippo isoforms (MnHippo-a, MnHippo-b and MnHippo-c) from M. nipponense shared the closest relationship with STPK4 and Hippo of P. vannamei. The amino acid sequence showed some highly conserved sites.

Tissue distribution of MnHippo
The sequence amplified by the specific primers used for qRT-PCR was in the common region of the three isoforms, so MnHippo was used to represent the sum of three isoforms (MnHippo-a, MnHippo-b and MnHippo-c). The mRNA transcripts of MnHippo were detected in a wide range of tissues in uninfected M. nipponense, including haemocytes, heart, hepatopancreas, gill, intestine, and stomach. MnHippo expression was mostly detected in the intestine and hepatopancreas, and lower expression of this gene was detected in the heart and haemocytes (Figure 4).

Temporal change in MnHippo mRNA after bacterial challenge
The temporal mRNA expression profile of MnHippo in the haemocytes of M. nipponense following V. parahaemolyticus and S. aureus challenge is presented in Figure 5. MnHippo expression was induced and gradually upregulated during the first 12 h after V. parahaemolyticus challenge. The expression peaked at 36 h and then decreased at 48 h (Figure 5A). After S. aureus infection, the level of MnHippo transcripts increased rapidly at 12 h and decreased slightly from 24 to 48 h, but it was still higher than the original expression level (Figure 5B). In the control group, the mRNA expression of MnHippo in haemocytes did not change significantly from 0 to 48 h (after PBS injection).
Figure 1  Sequence analysis of MnHippo from *M. nipponense*. **A** Domain topology of MnHippo-a, MnHippo-b, and MnHippo-c from *M. nipponense*. **B** Multiple alignment of amino acid sequences of the three MnHippo isoforms. The black shaded regions represent the identical residues. Other conserved but not consensus amino acids are shaded in grey. **C** The three-dimensional structure of the S_TKc domain in MnHippo was predicted using the SWISS-MODEL program and PyMOL.
**MnHippo** regulates the expression of antimicrobial peptides (AMPs) in response to stimulation

In the RNAi experiments, *MnHippo*-dsRNA was used to silence *MnHippo* expression in the haemocytes of *M. nipponense* by approximately 72% (Figure 6). Following RNAi treatment and exposure to *V. parahaemolyticus* (Figure 7) and *S. aureus* (Figure 8) for 24 h, the expression levels of five AMPs, namely, *MnALF1*, *MnALF4*, *MnCrus5*, *MnCrus7*, and *MnLyso*, were detected using qRT-PCR. The results revealed that the transcription levels of the five AMPs were significantly downregulated (*P < 0.05*) after the effective knockdown of *MnHippo*. *MnCrus5* was strongly downregulated after *V. parahaemolyticus* or *S. aureus* stimulation. The expression levels of AMP genes in prawns treated with GFP-dsRNA showed no obvious differences compared with those in the control. These results suggested that AMPs might play a role in *MnHippo*-mediated defence against bacterial infection in *M. nipponense* haemocytes.

This finding indicated that silencing *MnHippo* inhibited bacterial clearance in prawns.

**Effects of MnHippo knockdown on the survival of challenged prawns**

Considering that *MnHippo* knockdown could obviously reduce the bacterial clearance rate, we further tested the survival rate after *MnHippo*-silenced prawns were infected with *V. parahaemolyticus* or *S. aureus*. The survival rate of prawns from 2 to 6 days after *MnHippo*-dsRNA plus *V. parahaemolyticus* or *S. aureus* injection was obviously lower than that in the control group (GFP-dsRNA plus *V. parahaemolyticus* or *S. aureus*) (Figure 10). The PBS group was used as a normal reference, and almost no prawns died in this group (data not shown). These data indicated that silencing *MnHippo* significantly reduced the survival rate of prawns infected with *V. parahaemolyticus* or *S. aureus*, thereby promoting their cumulative mortality.

**Discussion**

Various studies have focused on the function and regulation of the Hippo signalling pathway in *Drosophila* and mammals. Many crucial components and new regulators of the Hippo pathway have been cloned and characterized. However, the current understanding of the Hippo pathway in crustaceans is still very limited, especially that of its functions related to defence against pathogen infection.
The Hippo pathway, which was named after Hippo, a Drosophila kinase-encoding gene, was independently shown to restrict tissue growth by several research groups more than ten years ago [5, 28]. In this study, a Hippo homologue with three isoforms (MnHippo-a, MnHippo-b, and MnHippo-c) was identified for the first time from the haemocytes of M. nipponense. The sequences of the three isoforms were highly similar. Compared with the amino acid sequence of MnHippo-a, the sequences of MnHippo-b and MnHippo-c showed partially deletions, with 10 and 42 amino acids deleted, respectively. MnHippo-a and MnHippo-c were derived from alternative splicing. Although the complete genome sequence of MnHippo...
was not yet available, we speculated that MnHippo-b was also formed by alternative splicing. However, the splicing method used to form MnHippo-b remains to be further studied.

Multiple sequence alignments revealed that the two missing parts in MnHippo-b and MnHippo-c were located in unknown regions, and the predicted protein domains of the three isoforms were identical. No signal peptide was found at the N-terminus of the MnHippo protein. The Hippo protein was speculated to be an intracellular protein. The mature MnHippo protein contained an S_TKc domain at the N-terminus and an Mst1_SARAH domain at the C-terminus, consistent with the other Hippo proteins of the Pacific white shrimp *P. vannamei* (ROT79885.1) and insects, such as *D. melanogaster* (NP_611427.1) and *A. nasatum* (KAB7507665.1). Further sequence analysis indicated that MnHippo could be a phosphotransferase and a member of the STPK subfamily. Protein kinases play roles in a variety of cellular processes, such as division, proliferation, apoptosis, and differentiation [29]. Protein phosphorylation is a reversible process mediated by protein kinase and phosphoprotein phosphatase and plays a crucial role in most cellular activities [30]. Phosphorylation usually results in functional changes in the target protein by altering enzyme activity, cell location, or interaction with other proteins [31]. In the Sav and Hpo families, the SARAH domain mediates signal transduction from Hpo via the Sav scaffolding protein to the downstream component Wts [32]. The Mst1_SARAH domain associates with Ras-association domain family 1 (Rassf1) and Rassf5 by forming a heterodimer, which mediates the apoptosis process [33]. MnHippo contains two characteristic domains that can phosphorylate downstream proteins.
to ensure normal signal transmission. Hippo mutants resulted in uncontrolled growth in various tissues owing to excessive cell proliferation and reduced apoptosis [34].

In uninfected adult *M. nipponense*, MnHippo was constitutively expressed in various tissues and highly expressed in the intestine and hepatopancreas. The intestine is a crucial organ for digestion, absorption, and nutrient exchange as well as for immunity [35]. In crustaceans, the intestinal barrier serves as the first line of host defense against environmental stress [36]. The hepatopancreas is an integrated organ of immunity and metabolism and is involved in the synthesis of immune and growth factors as well as in the maintenance of homeostasis in crustaceans [37, 38]. Therefore, the abundance of MnHippo in the two immune-related tissues indicated its potential immune defensive function in *M. nipponense*. Although the expression of the MnHippo gene in the haemocytes of healthy prawns was lower than that in other tissues, haemocytes are one of the most critical cells involved in the innate immunity of crustaceans. In this regard, we selected haemocytes for immune stimulation experiments. In *Drosophila*, Gram-positive bacteria rather than Gram-negative bacteria induced the activation of the Hippo pathway [17]. Here, Gram-negative *V. parahaemolyticus* and Gram-positive *S. aureus* were selected to test whether they can activate the Hippo pathway in *M. nipponense*. After stimulation of *V. parahaemolyticus* or *S. aureus* via the ventral sinus, the expression of MnHippo was significantly upregulated. The expression level of MnMOB1, a central signal adaptor in the Hippo pathway, was also upregulated in the hepatopancreas, gills, and intestine of *M. nipponense* [39]. In the Chinese mitten crab *Eriocheir sinensis*, haemocytes infected with *S. aureus* or *V. parahaemolyticus* obviously increased the phosphorylation of EsHpo at 0.5 and 1 h post-stimulation [40]. This result suggested that MnHippo might be involved in defence mechanisms against *V. parahaemolyticus* and *S. aureus*.

Prawns and other invertebrates rely almost entirely on innate immune systems, namely, humoral and cellular immunity, to defend themselves from pathogen invasion [41, 42]. The Toll signalling pathway is an essential cascade that plays a key role in the production of AMPs and is the main mechanism by which invertebrates eliminate bacteria and fungi [43, 44]. In *Drosophila*, Hippo–Yorkie signalling proved to be acutely activated by only Gram-positive bacteria. Inhibition of the Hippo pathway promoted Yorkie translocation and then regulated the expression of Cactus; the high expression of Cactus prevented the migration of Dorsal/Dif from the cytoplasm into the nucleus, thereby reducing the expression of AMPs [17]. This interesting study demonstrated the important contribution of the Hippo pathway and its crosstalk with the Toll pathway to innate immunity. We suspected that such an immune-related function for the Hippo pathway may also exist in other invertebrate species. In this regard, silencing of MnHippo was performed by specific dsRNA injection to characterize its role in the innate immunity of prawns. MnHippo-silenced haemocytes showed significantly low expression of several AMPs, such as MnALF1, MnALF4, MnCrus5, MnCrus7, and MnLyso2, which are Toll pathway target genes in crustaceans [44]. These results were consistent with a study on MnMOB1; in this work, the expression levels of MnCrus and MnALF were significantly downregulated when MnMOB1 expression was knocked down after *S. aureus* and *V. parahaemolyticus* challenge [39]. In addition, MnHippo silencing weakened the clearance of *V. parahaemolyticus* and *S. aureus* in prawns. In the *V. parahaemolyticus*- or *S. aureus*-challenged group, the survival rate of the MnHippo-dsRNA group was significantly decreased from 2 to 6 days post-injection, corresponding to the MnHippo knockdown periods. However, the survival rates of non-related dsRNAs were 50% and 60% as a
Figure 7 Regulatory effect of MnHippo on the expression of AMPs. A Sequence-specific MnHippo-dsRNA was injected into V. parahaemolyticus-infected prawns to inhibit MnHippo expression. B–F The mRNA expression levels of MnALF1, MnALF4, MnCrus5, MnCrus7, and MnLyso2 after V. parahaemolyticus stimulation in the haemocytes of MnHippo-interfered prawns. Significant differences between the normal group and treated groups were subjected to one-way ANOVA. The different letters indicate significant differences compared with the other groups (P<0.05).
Figure 8 Regulatory effect of MnHippo on the expression of AMPs. A Sequence-specific MnHippo-dsRNA was injected into S. aureus-infected prawns to inhibit MnHippo expression. B–F The mRNA expression levels of MnALF1, MnALF4, MnCrus5, MnCrus7, and MnLyso2 after S. aureus stimulation in the haemocytes of MnHippo-interfered prawns. The significant differences between the normal group and treated groups were subjected to one-way ANOVA. The different letters indicate significant differences compared with the other groups ($P < 0.05$).
result of *V. parahaemolyticus* and *S. aureus* injection, respectively. Hence, *MnHippo* could resist bacterial invasion by regulating the transcription of some AMP genes. The detailed mechanism needs further in-depth research.

In summary, a Hippo homologue with three isoforms was isolated and identified from *M. nipponense*. *MnHippo* expression in haemocytes was significantly induced by *V. parahaemolyticus* and *S. aureus* injection. The RNAi results validated the function of *MnHippo* in accelerating bacterial clearance and increasing the survival rate by regulating the expression of several AMPs. These results showed that *MnHippo* might be involved in the antibacterial immune defence of prawns. Further elucidating the involvement of the Hippo signalling pathway in innate immunity is of great significance (Figure 11).

**Figure 9** Bacteria clearance assay in vivo. *V. parahaemolyticus* (A) or *S. aureus* (B) was injected into normal or *MnHippo*-dsRNA/GFP-dsRNA-injected prawns. The same volume of haemolymph (500 μL) was extracted from prawns at 20 min after injection in different groups, diluted with sterile PBS, and cultured on LB medium plates at 37 °C overnight. The number of bacterial colonies was counted. The data are shown as the mean values ± S.D. (*N* = 10). The asterisks indicate significant differences (** *P* < 0.01).

**Figure 10** Evaluation of prawn survival rate. The prawns were co-injected with *MnHippo*-dsRNA or GFP-dsRNA and *V. parahaemolyticus* (A) or *S. aureus* (B). At various times (1–6 days) after infection, the survival rate of the prawns was examined.
Figure 11  Schematic representation of Hippo-regulated antimicrobial activities in oriental river prawns. In M. nipponense, Hippo–Yorkie signalling was activated by Gram-positive and Gram-negative bacteria. The activation of this signalling axis excluded Yorkie from the nucleus. Inhibition of the Hippo pathway promoted Yorkie translocation, enabling Cactus transcription; the high expression of Cactus prevented the transfer of Dorsal/Dif from the cytoplasm into the nucleus, thereby reducing the expression of AMPs (such as ALF, crustin, and lysozyme).

Supplementary Information
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Additional file 1. Nucleotide and deduced amino acid sequence of MnHippo-a from M. nipponense. The red letters indicate the start codon (ATG) and the stop codon (TAG). The S_TKc domain is underlined, and the italic letters indicate the Mst1_SARAH domain. Compared with MnHippo-a, MnHippo-b lacks the sequence marked in blue, and MnHippo-c lacks the sequence marked in green.

Authors’ contributions
YH: Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Software; Supervision; Validation; Visualization; Writing—original draft; Writing—review & editing. QR: Conceptualization; Project administration; Funding acquisition; Resources; Writing—review & editing. All authors read and approved the final manuscript.

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Declarations
Ethics approval and consent to participate
We declare that appropriate ethical approval and licences were obtained during our research.

Competing interests
The authors declare that they have no competing interests.

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