

Original Research Articles

Identification and functional characterization of Wts from kuruma Shrimp (*Penaeus japonicus*) in response to *Vibrio harveyi* challenge

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Keywords: Wts, bacterial infection, gene expression, RNAi, *Penaeus japonicus*<https://doi.org/10.46989/001c.159349>

Israeli Journal of Aquaculture - Bamidgheh

Vol. 78, Issue 2, 2026

As a key member in Hippo signal pathway, Wts plays an important role in animal growth and immunity. To investigate the role of Wts in innate immunity of kuruma shrimp *Penaeus japonicus*, the cDNA and genome sequence of Wts from *P. japonicus* (designated as PjWts) were isolated and characterized. The PjWts cDNA was 4,123 bp length, including an open reading frame of (ORF) 3,438 bp encoding 1,145 amino acids. No transmembrane structure or signal peptide was found in PjWts. The predicted protein contains a MobB domain and a STKc_LATS domain. The result of multiple alignment showed that these domains were highly conserved across different species. The genomic sequence of PjWts consists 7 exons and 6 introns. PjWts was expressed in all tested tissues, with high expression level in ganglia, gill, ovary and lymph. After *Vibrio harveyi* was injected, the expression of PjWts in the gill and hepatopancreas were significantly upregulated at 3h. After PjWts silencing, the expression of PjWts in hepatopancreas and gill were gradually decreased. The mortality rate of *P. japonicus* co-injected with dsRNA-Wts and *V. harveyi* was significantly higher than that of other groups. All of these results suggest that PjWts may be involved in the innate immunity of *P. japonicus*. This study provides a useful foundation for elucidating the function of Wts in *P. japonicus* innate immunity.

INTRODUCTION

The Hippo signaling pathway plays a crucial role in regulating cell proliferation, apoptosis, and immunity in animals.¹⁻⁴ Warts (Wts), which belongs to the nuclear Dbf2-related (NDR) protein kinase family, was first reported in *Drosophila melanogaster*.¹ Wts, Salvador (Sav), Yki, and Hippo are key component in the Hippo signaling pathway which is highly conserved in *Drosophila* and mammals.⁵ When wts is mutated, cells undergo excessive growth and form tumors.² Wts was phosphorylated and activated by Hippo.² Yki was regulated by Wts through two Wts phosphorylation sites on Yki.^{2,6} After being phosphorylated, Yki was sequestered in the cytoplasm and could not regulate the transcription of downstream genes related cell proliferation in the nucleus.⁷

Currently, few reports about the function of Wts are available in aquatic animals.² The large tumor suppressor (Lats) is a homologous gene of Wts in vertebrates and *Crassostrea hongkongensis*.^{5,8} Lats1/2 play an important role in

the formation of gastrula in *Danio rerio*.⁹ When *lats1/2* were knocked out, the number of cardiomyocyte was increased in atria during early development of *D. rerio*.¹⁰ Lats1/2 could phosphorylate angiotensin, which inhibited endothelial cell migration in vitro and angiogenesis in zebrafish embryos *in vivo*.¹¹ Lats was highly expressed in haemocytes and triggered hemocytic apoptosis in *C. hongkongensis*, which suggested that Hippo signaling pathway was involved in the immune defense process of oyster.⁸ When Hippo and Wts were silenced, the ability of hemocyte phagocytosis become weakened, which proved that the Hippo signaling pathway could positively regulate the cellular immunity in *P. vannamei*.¹² The white spot syndrome virus (WSSV) in shrimp *P. vannamei* increased after Wts and Hippo were silenced *in vivo*, which indicated a positive role of Hippo signaling in antiviral response.¹³

Penaeus japonicus, also named *Marsupenaeus japonicus*, is widely distributed in the Indo-Western Pacific, including China, Japan, Southeast Asia, East Africa, and Australia.¹⁴ It is an economically important shrimp species in China.¹⁵ *P. japonicus* is cultured along southeast coastal areas of

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China due to its fast-growth and strong adaptability.¹⁴ The production of *P. japonicus* was 4.62 ten thousand tons in China in 2022.¹⁵

The shrimp aquaculture industry has been threatened by various pathogens including viruses, bacteria and fungi recently.^{16,17} *Vibrio* species are commonly found in marine and estuarine environments. *Vibrio harveyi* is thought as one of the main pathogens in shrimp, which cause several acute diseases such as hepatopancreatic necrosis, bacterial white-tail disease, black shell disease and light-vibriosis.¹⁷ Understanding the molecular regulatory mechanism of defense against bacteria in shrimp is useful to design strategy to prevent and control bacterial diseases. Nowadays, there are some reports on the molecular mechanism of innate immunity in *P. japonicus*.¹⁸ However, it remains unclear whether Wts is involved in the innate immunity of *P. japonicus*.

In this study, the cDNA and genomic DNA sequences of Wts of *P. japonicus* (named PjWts) were obtained, characterized and analyzed. The role of Wts in response to *V. harveyi* infection was investigated with the real-time quantitative polymerase chain reaction (RT-PCR) and RNA interference (RNAi) techniques. Our study provides a theoretical basis for further study of the role of Wts and the Hippo signaling pathway in the innate immunity of *P. japonicus*.

MATERIALS AND METHODS

2.1. EXPERIMENTAL ANIMALS AND SAMPLE PREPARATION

All of *P. japonicus* individuals were purchased from a shrimp aquaculture company in Fujian Province. Several tissues including the hepatopancreas, muscle, lymph, gills, ganglia, stomach, heart, hemolymph and eye stalks were separately collected from five shrimps (20.7 ± 1.2 g). All of tissues were frozen with liquid nitrogen quickly, and stored in -80°C until used.

A total of 410 healthy and active individuals of *P. japonicus* (31 ± 2.1 g) were chosen randomly and cultured in several concrete ponds (20 m^2) three days for the experiment of bacterial challenge. The salinity and temperature of seawater was 35‰ and 25°C , respectively. Two-third seawater was changed and the residual feed/waste was removed everyday. All shrimps were fed a commercial mixed feed supplied by Haid Group Co., Ltd. (Guangdong, China).

2.2. BACTERIAL CHALLENGE

The shrimps were divided into two groups designated as control group (CG) and experimental group (EG). Each group was set up in triplicate (i.e., three biological replicates). *V. harveyi* was used for the bacterial stimulation experiment. Shrimps in the EG were intramuscularly injected with 50 μL of resuspended bacteria solution (1×10^6 cfu/ml), while the CG were injected with 50 μL phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4).

The hepatopancreas and gills were collected from five shrimps at different time points (0, 3, 6, 12, 24, 48 and 72 h post-injection) for RNA extraction. All samples were frozen immediately with liquid nitrogen quickly, and then stored at -80°C until used.

2.3. RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA from each tissue was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The quality and concentration of RNA were examined by 1.5% agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo Scientific, USA), respectively. A total of RNA 1 μg of total RNA was used as the template for reverse transcription reaction with a Prime-Script™ RT reagent Kit with gDNA Eraser (Takara, Japan), following the manufacturer's instructions.

2.4. VALIDATION OF WTS FROM TRANSCRIPTOME DATA

The full-length of cDNA sequence of Wts was obtained from transcriptomic sequences (SRX2030618) of *P. japonicus*. All primers were designed with Primer Premier 5 (Table 1). A pair of primers (Wts-S1/R1) was used to confirm the accuracy of Wts sequence. Polymerase chain reaction (PCR) was performed in a 20 μL reaction volume containing 12 μL ddH₂O, 2.0 μL 10 × Ex Buffer, 1.6 μL dNTP mixture (2.5 mmol/L), 1.6 μL MgCl_2 (25 mM), 0.8 μL of each of primer (10 μM), 1 μL cDNA (50 ng/ μL), and 0.2 μL Ex Taq DNA polymerase (5 U/ μL) (TaKaRa, Japan). The PCR product was purified by SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China), and then sequenced directly by Sangon Biotech (Shanghai) Co., Ltd. (China).

2.5. BIOINFORMATICS ANALYSIS

The open reading frame (ORF) and predicted protein of Wts were analyzed by ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The physico-chemical property of protein was analyzed using ProtParam (<http://web.expasy.org/protparam/>). The similarity of PjWts with Wts from another species was analyzed with Global Align (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?>). The structure of PjWts protein was analyzed by Conserved domains (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The signaling peptide was predicted by SignalP5.0 (<http://www.cbs.dtu.dk/services/SignalP/>). The transmembrane domain was predicted by TMHMM (<https://services.healthtech.dtu.dk/service.php?TMHMM-2.0>). Multiple alignment of protein was analyzed by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic tree was constructed by MEGA 7.0 software and neighbor joining was used and branching reliability was tested by bootstrap resampling with 1000 pseudo-replicates.

2.6. EXPRESSION OF WTS IN DIFFERENT TISSUES

The relative expression levels of Wts in different tissues from five shrimp were analyzed using quantitative real-time PCR (qRT-PCR) with primers qWts-S and qWts-A

Table 1. Primers used in this study

Primer name	Sequence (5' → 3')	Product Length(bp)	Purpose
Wts-S1	AAAGCCGATGTCTTGAAG	980	cDNA cloning
Wts-R1	GGCTGATTGCTATTGTTGAC		
qWts-s1	TGTTGTAACCCACATCCTG	225	RT-qPCR
qWts-a1	GAAGAGGCATATGAAGGAGG		
RiActin-RT-F	CAGCCTTCCTTCCTGGGTATGG	182	RT-qPCR
RiActin-RT-R	GAGGGAGCGAGGGCAGTGATT		
dsWts-S1	GATCACTAATACGACTCACTATAGGG CAACAAAGACTAGGACGCAAT	219	RNAi
dsWts-A1	GATCACTAATACGACTCACTATAGGG TGATTGCTATTGTTGACC		
dsGFP-F	GATCACTAATACGACTCACTATAGGGCTGGAGTGGTCCCAGTTCTTGTGA	289	RNAi
dsGFP-R	GATCACTAATACGACTCACTATAGGG CATTCTTTGGTTTGTCTCCCAT		

(Table 1). All qRT-PCR assays were carried out in three biological replicates. The qRT-PCR was performed using SYBR Premix Ex Taq (Takara, China) according to the manufacturer's instructions, and carried out on LightCycler® 480 II (Roche, Switzerland). The β -actin (GenBank accession No.: AB055975.1) gene was chosen as the reference gene.¹⁹ PCR programme was as follow: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 15 s, finally a melting curve was obtained at the end of the reaction and a single peak was observed. The acquired data were calculated using a $2^{-\Delta\Delta CT}$ method,²⁰ and SPSS 19.0 software (IBM, USA). One-way analysis of variance (ANOVA) was used to evaluate significant differences ($P < 0.05$).

2.7. RNA INTERFERENCE (RNAI) AND SURVIVAL RATE ANALYSIS

The recombinant plasmids, pD7-Wts and pD7-GFP, were established, respectively. The primers specific to target genes were dsWts-S1/A1 and dsGFP-F/R with T7 promoter sequence (Table 1). Sense and anti-sense DNA templates for transcription *in vitro* were produced by employing pD7-Wts and pD7-GFP recombinant plasmids as templates via PCR. dsRNA-Wts and dsRNA-GFP were synthesized *in vitro* with a Transcription T7 kit (TaKaRa Biotechnology) following manufacturer protocols. dsRNA-GFP was served as a control. The quality of the dsRNA was evaluated using 1.2% agarose gel electrophoresis. dsRNA was stored at -80 °C until used.

dsRNA targeting Wts (dsRNA-Wts) or green fluorescent protein (dsRNA-GFP; control) (50 µg per shrimp) was injected into the abdominal segment of each shrimp.²¹ The hepatopancreas and gills were scissored from four shrimps at 0h, 12h, 24h and 48h post-injection, then immediately frozen in liquid nitrogen. The expression of *PjWts* mRNA at different time points was checked by qRT-PCR as described

in Sections 2.1 and 2.6. All samples were tested in triplicates.

To investigate the role of Wts during *V. harveyi* infection, shrimps were injected with dsRNA-Wts, dsRNA-GFP or PBS firstly, then challenged by *V. harveyi* (10^6 CFU.mL⁻¹) 48h later. The number of dead shrimps in each groups was recorded within 96 h. Statistics were processed by GraphPad Prism 8.0 Software (GraphPad Software, USA).

RESULTS

3.1. CLONING AND SEQUENCE ANALYSIS OF PJWTS

The full-length cDNA sequence of *PjWts* was 4,123 bp in length, including an open reading frame (ORF) of 3,438 bp encoding 1,145 amino acids, a 5' untranslated region (5'UTR) of 225 bp and a 3'UTR 460 bp (Figure 1). The molecular weight and isoelectric point of *PjWts* protein were predicted to be 125.607 kDa and 8.96, respectively. No transmembrane domains or signal peptides were identified. Two conserved domains were detected: a Mob-binding domain (Mob, 677–752 aa) and a serine/threonine kinase catalytic domain of the large tumor suppressor family (STKc_LATS, 753–1115 aa). The *PjWts* cDNA sequence was submitted to GenBank with the accession number PP155416.1.

The genome sequence of *PjWts* was identified from genome sequence of *P. japonicus* (GenBank accession No.:NW_025035089.1). After alignment with the cDNA sequence of *PjWts*, seven exons and six introns were identified in the genomic sequence of *PjWts*. The longest and shortest exon was exon 2 (2,197bp) and exon 1 (81bp), respectively (Figure 2). The longest and shortest intron was intron 1 (1,564bp) and intron 5 (112bp) (Figure 2). The exon-intron junction regions conformed to the GT/AG rule. The genomic sequence of *PjWts* has been submitted to GenBank (Accession number: PP155417.1).

Figure 1. The cDNA and deduced amino acid sequence of *PjWts*. The MobB domain and STKc_LATS domain were represented by yellow-highlighted and single line, respectively.

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1 cgggtgtgaaccaagtggtgattgtgtattgtgtgttataaatctcctgtgtgtgtgtgtgtgtacgaacctg
81 gaaaagtgccctggaagtcgattggaatttaactatgtgcagtgagagaaaaggcgtctgttcaacacatagtactttg
161 ggagctttctcagctgtgacgggttaagttggccatgggtccaccagtggttagctcttcatggcccg
226 ATGGCCCCCTCAGATGCCTCATGCGGCTACCATGAGCACCAACACAATAGGCCGGTCAACTCGAGCAGATTATAATAAG
1 MAP Q M P H A A T M S T N T I G R S T R A D Y N K
304 GAGGCTTTACATCGAATCCGAGCGTCTTTGCTACCCTATGCTGCGGGTGGCATCCCCGGGAGAGTCTTAGCCCCATC
27 E A L H R I R A S L L P Y A A G G I P R E S L S P I
382 AGCCCGGGTGTCTAATGCCAGGGGTCTTTCAATTTGTGCCCCACCGATGATGAACTACAAAGATCTACAATGGGTAC
53 S P G A Q C Q G S F N L C P T D D E L Q R S Y N G Y
460 CCGGCCCAGGTGGCCAGGTGGAGGGCAGCAAGATAATCCGCAAGTCCAGCTTTGATCGCGAGCTGAATTTGCAACGG
79 P A Q V A Q V E G S K I I R K S S F D R E L N L Q R
538 GGAAGTCCTGCCCTTAGACAGCAGCAATCGACGTTTCAGACAGTCTGGCATCGGGGGGCAGATGCTGTACAAC
105 G S P A L D S S N A S T R S D S P G I G G Q M L Y N
616 AACAAATATGCCTGTAGGCAATATGCTGAGGCTCCACCTCCAAGAATGGGAAGTGGGGAGCAAATTGTTGATGTCAAC
131 N N M P V R Q Y A E A P P P R M G S G E Q I V D V N
694 CACCCAGCTGTGTCCAGACAATACAGCAGCTCGTACCCTGTAGCACCAGATGCACCCCCACCTCCCCACCCCGCAGT
157 H P A V S R Q Y S S S Y P V A P D A P P P P P R S
772 CAAGGGAGCAGTGCTCCTCCCCAACACCACCTCGGGGCACCACCCACCTCCACCTCCTTACCCTTGTGCCACCAGC
183 Q G S S A P P T P P R G T T P P P P Y P C A T S
850 TGTGCCCCCACCATGGTAACTACTATTGGCCAGAGACCAATATTACTATCAAGACTCTCTCCAGGTCCAAGCAAGAAT
209 C A P T M V T T I G Q R P I L L S R L S P G P S K N
928 AAATCGCAAGCTAATTATGTAATATTAATGTGTACAAAGCTCAACTCCTCAAAGGGGTACTAGTCTGTCTCA
235 K S Q A N Y V N I N N V S Q S S T P Q R G T S P V S
1006 TTTGGTGAGCAGCAGCAGCAGTGTGTGTGTCAGAAATAGTGCTCAGGTGCAACAGCAGCTTACTAACCAGATACAGGCT
261 F G G R Q Q P V V V Q N S A Q V Q Q Q L T N Q I Q A
1084 TTCTCTTTAAATAATGGTAATAACTATGAAACCAAGTGAACGCCCGCTCCATATCCATTAGGATCTGCATTAAAG
287 F S L N G N N Y G N Q L E R P P P Y P L G S A L K
1162 CATGTAGGAGGTATGAACTAACAGCACCCCCACCGTCATATAATGCAACAATGCAGAATAGGCAAAGTCCCACCCAA
313 H V G G M K L T A P P S Y N A T M Q N R Q S P T Q
1240 GATGCAAGAAAAAGCCCTGCTCAGCTAAATTTCCAGGGCCCAAGTCTGTTGTAATCCACATCCTGTATCACCATCA
339 D A R K S P A Q L N F Q G P S P V V T P H P V S P S
1318 TCATCAGTACCTCCATCGTCTCCTGCTCGCTTCCACGTGGTGCCAAACTCCCGACAAGCAAAGACTCCC
365 S L S P P S S M S P V S L P R G G Q T S R Q A K T P
1396 ACACCAATTATCATGCAGTCTGTCAAAGTACCCAGGTTCAGAAACAGTGTGCAGACAGCTGTTGCTCCAGCTTCA
391 T P I I M Q S V K S T Q V Q K P V L Q T A V A P A S
1474 CCTCCAGTCTGGCTCCACCTCCTTCATATGCCTCTTCCATCCAGCAGAAGCAAGCGCAGGCTGCTGCTGCTGCTCAG
417 P P V S A P P S Y A S S I Q Q K Q A Q A A A A A Q
1552 GCTGTACCTGGGATGGGATTAGCACAAATGATGCCACAAGAGAGTGGAGGATCATCATCCTGGACTTTCATCTGT
443 A V P G M G L A Q M M P Q E S G G S S S P L S S C
1630 AACTATCAGTTAGCAGTAACGTGCAATAGTGAAGTAGTGCCAACCACAGATCCCCCAGCTATCATTCAACTATACAA
469 N Y Q L A V T C N S E V V P T T D P P S Y H S T I Q
1708 GCAATGGCAGCTGCTAGAGCTTATAATCCAGGATATATGGCTCAGCCTGTAGCCAGTATACCAGTTCAAGATACTCCA
495 A M A A A R A Y N P G Y M A Q P V A S I P V Q D T P
1786 GTGCAGGAATTAGATTGTCTCCACACCCTTGGCTCACATACCAATAACTCAAGTTAGTACGGTTGATTCCATCATA
521 V Q E L D L S S T P L A H I P I T Q V S T V D S I I
1864 ACAACACAGCCAACAAGTCAGTAGAAACAGTAAGCAGTTGTTATGATCCCAACCTAACACCAGGTCTGAGTCATCCC
547 T T Q P T S A V E T V S S C Y D P N L T P G L S H P
1942 CCTCTCAAAGAAAATTCTCTCTGTAATGGCTGATACAGCTCCAGTGCCTCTCGGTCTGAGAGTCTGTTTCTTCT
573 P L Q R K F S P V M A D T A S S A S R S E S P V S S
2020 GTAAATTCCTGACACTCATGCGTTATCTCAATCACCTGTATCGTGCTTCTACTGCCACGTGCTCTCCCTCCACT
599 V N S H D T A L S Q S P V S C L S T A T S S P S T
2098 CAGTCAGATTTAACCTTGGAATCTGGATATCTCTCTGGGAGTCTGTGAGGAGTGCCCCACCACCACCTCCAACA
625 Q S D L T L E S G Y S S G Q S C R G V P P P P P T
2176 GAACAGAAAACTACTCATCAATCACCTATACCTGAACGAAAGAAATTAAGCTCTGAAATGGAATTCAGAGATGCGAG
651 E Q K T T H Q S P I P E R K K L S S E M E L Q R C E
2254 TCAAAAGTTCGAAATTTATCTCCTCAGGCCTTTAAATTTACATGGAACAACACATTGAAAATGTGATAAAATCTTGT
677 S K V R N L S P Q A F K F Y M E Q H I E N V I K S C
2332 GAAGAACGAGAAAAACAGAAGAATGCAGCTTGAGAAAGAAATGAAAAAATAGGATTATCAGACAAGGATCAATCCCAA
703 E E R E N R R M Q L E K E M K K I G L S D K D Q S Q
2410 ATGCGTAAAATGCTATCCCAAAGGAATCCAACCTTCAAGATTAAAGAGAGCAAAAATGGACAAACAGCTCTTCCGC
729 M R K M L S Q K E S N F I R L K R A K M D K Q L E R
2488 AAAATAAAACCATAGGTGTTGGCGCATTTGGTGAGGTTGCCCTCGTCTGCAAGCTAGATACACACCGTCTCTATGCA
755 K I K T I G V G A F G E V A L V C K L D T H R L Y A
2566 ATGAAGACCTTACGCAAAGCCGATGCTTGAAAAGGAATCAAGTGCTCACGTGAAAGCTGAGAGAGACATTCTAGCA
781 M K T L R K A D V L K R N Q V A H V K A E R D I L A
2644 GAAGCTGACAACGAGTGGGTGTCAAACCTATTATTATTCAAGATAAAGACAACCTTTACTTTGTTATGGACTAC
807 E A D N E W V V K L Y S F Q D K D N L Y F V M D Y
2722 ATCCCTGGTGGTGACCTTATGCTCTGCTCATCAAATTCGGAATCTTTAAGGAGCAGTTGGCAAGATTCTATATAGGA
833 I P G G D L M S L L I K F G I F K E Q L A R F Y I G

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genome

5' 0kb 1kb 2kb 3kb 4kb 5kb 6kb 7kb

Legend: CDS upstream/ downstream Intron

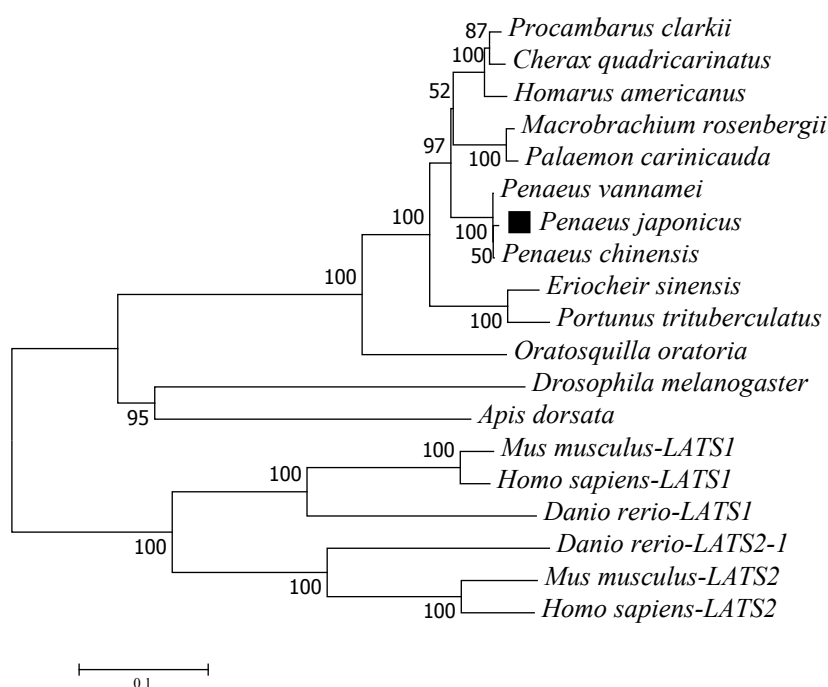


The black straight line represents introns. Yellow boxes represent exon, blue arrow represent upstream/downstream sequence.

The results of the *PjWts* mRNA expression in gills and hepatopancreas after *V. harveyi* infection are shown in [Figure 6](#). The mRNA expression level of *PjWts* was significantly upregulated at 3 h post-infection. It reached peak levels at 6 h in the gills and 12 h in the hepatopancreas, respectively. At 72 h post-infection, the mRNA expression of *PjWts* was significantly higher than that in the control group ($P < 0.05$).

Table 2. The information of Wts or LATS protein from another species and its identity with PjWts

Species	Protein	Accession No.	Identity(%)
<i>Danio rerio</i>	DrLATS2-1	ABS88161.1	38
<i>Homo sapiens</i>	HsLATS2	NP_055387.2	39
<i>Mus musculus</i>	MmLATS2	AAH53028.1	39
<i>Danio rerio</i>	DrLATS1	AAX56091.1	40
<i>Homo sapiens</i>	HsLATS1	AAD16882.1	40
<i>Mus musculus</i>	MmLATS1	NP_034820.1	41
<i>Drosophila melanogaster</i>	DmWts	NP_733403.1	45
<i>Apis dorsata</i>	AdWts	XP_006611780.1	51
<i>Eriocheir sinensis</i>	EsWts	XP_050697485.1	80
<i>Portunus trituberculatus</i>	PtWts	XP_045130442.1	80
<i>Cherax quadricarinatus</i>	CqWts	XP_053638150.1	90
<i>Homarus americanus</i>	HaWts	XP_042211293.1	91
<i>Procambarus clarkii</i>	PclWts	XP_045614888.1	91
<i>Penaeus chinensis</i>	PchWts	XP_047497193.1	99
<i>Penaeus vannamei</i>	PvWts	UZX50346.1	99
<i>Penaeus japonicus</i>	PjWts	PP155416.1	-


Figure 4. NJ phylogenetic tree based on amino acid sequence of Wts and LATS.

3.4. RNA INTERFERENCE EFFICIENCY AND SURVIVAL RATE

The effect of *PjWts* knockdown in hepatopancreas and gills was evaluated using RNAi technology (Figure 7). The expression levels of *PjWts* in the hepatopancreas and gills were significantly decreased at 12h after dsRNA-Wts injection (Figure 7). Compared with the control group, the interference effect was significant at 12 h, 24 h, and 48 h after dsRNA-Wts injection in the hepatopancreas, with

knockdown percentages of 60.54%, 75.64%, and 97.30%, respectively. In the gills, the knockdown percentages were 54.87%, 75.55%, and 87.12%, respectively. These results indicate *PjWts* were significantly knocked down in hepatopancreas and gills.

The survival rates of the shrimps in three groups were recorded after *V. harveyi* infection (Table 3). The survival rates of dsRNA-*PjWts* + *V. harveyi*, dsRNA-GFP + *V. harveyi* and dsRNA-GFP + PBS group were 20%, 47.5% and 92.5%, respectively (Figure 8, Table 3).

[illegible]

IAPEVL\$RTGYTQLCDWW 949
IAPEVL\$RTGYTQLCDWW 947

MDTSNFDPVVEEGGPGAWSD 1056
MDTSNFDPVDEES---PWHE 971
MDTSNFDPVDEES---PWND 1013
MDTSNFDPVDPDK---LWSE 991
MDTSNFDPVDPDK---LWSD 1049
MDTSNFDPVDPDK---LWSD 1050
MDSNFDPVDPDK---LRNS 1059
MDSNFDPVDPDK---LRNS 1082
MDSNFDPIDPDK---LRPS 1096
MDSNFDPIDPDK---LRPS 1091
MDSNFDPIDPDK---LRPS 1088
MDSNFDPIDPDK---LRPS 1098
MDSNFDPIDPDK---LRPS 1095
MDSNFDPIDPDK---LRPS 1094
MDSNFDPIDPDK---LRPS 1065
MDSNFDPIDPDK---LRPS 1063

28h	32h	36h	40h	44h	Number of survivors
26	22	22	20	20	19
16	14	10	10	8	8
38	38	38	37	37	37

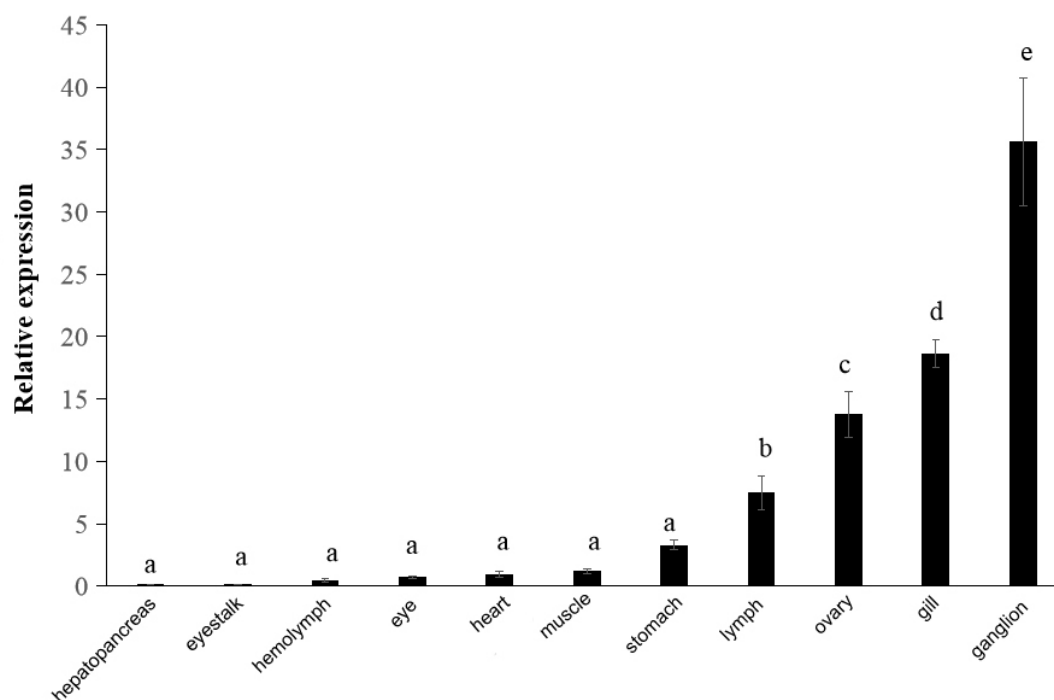


Figure 5. Distribution of PjWts of mRNA expression in different tissues.

Different letters indicate statistically significant differences ($P < 0.05$)

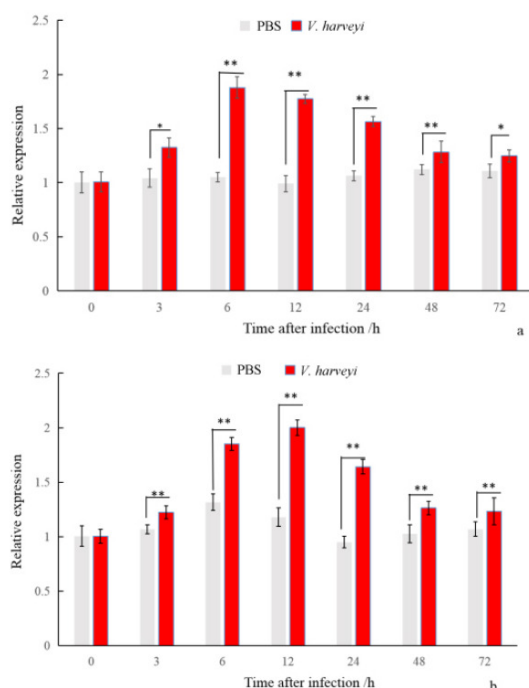


Figure 6. Relative expression levels of PjWts in gill(a) and hepatopancreas(b) after *V. harveyi* infection.

Asterisks indicate significant differences of mean values (*, $P < 0.05$; **, $P < 0.01$).

DISCUSSION

The Hippo signaling pathway plays an important role in innate immunity of many animals such as *D. rerio*, *C. hongkongensis* and *P. vannamei*.^{2,8,12,13,22} The Hippo signal-

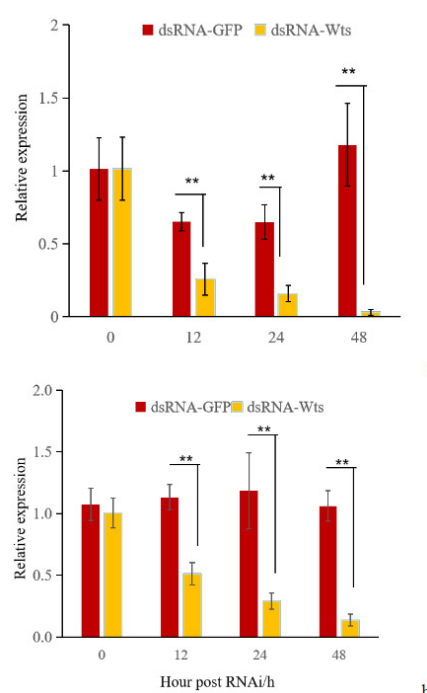


Figure 7. After dsRNA-Wts or dsRNA-GFP infected, the expression of PjWts in hepatopancreas (a) and gills (b).

ing pathway was involved in immune responses to bacteria and viruses in *P. vannamei*.^{12,13} However, no studies have reported on the Hippo signaling pathway in *P. japonicus*.

As a core component of the Hippo signaling pathway, Wts is essential for its function.^{4,22} The cDNA and genomic sequences of PjWts were isolated and characterized in this

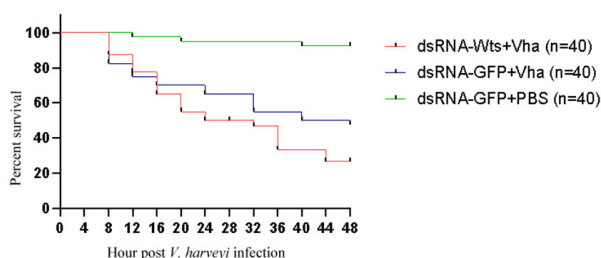


Figure 8. The survival rate of *V. harveyi*-infected shrimp after *PjWts* silencing.

study. The ORF of *PjWts* was 3,438bp in length, encoded 1,145 putative amino acids (Figure 1), which was similar with the length of *PcWts* (1,137 amino acids) and *LvWts* (1,140 amino acids).

The identity of *PjWts* protein sequence with LATS from vertebrate was ranged from 38% to 41% (Table 2). In contrast, *PjWts* shares a high sequence identity (99%) with Wts proteins from other *Penaeus* species (Table 2). Both of MobB and STKc_LATS domain of Wts were detected, which maintain high conservation among various species in this study (Figure 2). These results suggest that the Wts protein, particularly its MobB and STKc_LATS domains, is evolutionarily conserved. This conservation implies that Wts may exert similar physiological functions across different species. Notably, STKc_LATS domain could phosphorylate protein and maintain cell activity²³—a function that likely contributes to *PjWts*' role in immune regulation.

The genomic sequence of *PjWts* consists of 7 exons and 6 introns, which is consisted with the structure of Lats1/2 genome in human and mice but differs from that of Lats1/2 in zebrafish (which contains 8 exons).⁹ This observation indicates that the genomic structure of Wts is highly conserved with minimal evolutionary variation.

Phylogenetic tree analysis revealed that *P. japonicus* clusters with other crustaceans—a result consistent with traditional taxonomic classifications (Figure 4). Meanwhile, LATS1 and LATS2 from vertebrates each form distinct sub-clusters within the vertebrate branch (Figure 4). To date, no Wts homologs have been reported in shrimp (beyond the present study). Evolutionarily, vertebrate LATS1 is thought to originate from invertebrate Wts, whereas LATS2 may have emerged via whole-genome duplication during evolution⁹—a process that could explain the functional divergence of LATS1/2 in vertebrates compared to invertebrate Wts.

PjWts mRNA is expressed in various tissues of *P. japonicus* (Figure 5), which is consistent with the tissue expression pattern of Wts in *P. vannamei*.¹³ These results suggest that Wts may perform multiple physiological functions in shrimp. Additional experiments are required to verify this hypothesis.

In mammals, the Hippo signaling pathway plays an important role in the development of the nervous system, including regulating the proliferation and differentiation of neural cells, and promoting the development of synapses, the corpus callosum, and the cerebral cortex (Li X, et al.,

2021). Notably, the expression of *PvWts* in *P. vannamei* is highly abundant in ganglia¹³—a finding consistent with our observation of high *PjWts* expression in *P. japonicus* ganglia (Figure 5). This conservation in ganglionic expression implies that Wts may be involved in neurodevelopment in shrimp.

In mice, only a few follicles and no corpus luteum were observed in the ovaries of Lats1-knockout individuals, suggesting that LATS1 supports fertility in mammals.²⁴ Tsoi et al.²⁵ were the first to demonstrate that LATS1/2 play a critical role in maintaining the genetic program of ovarian granulosa cells. In the present study, we also detected high *PjWts* expression in the ovary of *P. japonicus*—a result consistent with the aforementioned mammalian studies (Tsoi et al., 1999; St John et al.²⁴). However, the specific function of Wts in shrimp ovaries remains unclear, and further research is needed to elucidate its role in shrimp reproduction.

In shrimp, hemolymph, gills, and lymphoid organs play critical roles in innate immunity, including both cellular and humoral immune responses.²⁶ For instance, *Lats* mRNA is highly expressed in the hemocytes and gills of *C. hongkongensis*.⁸ In the present study, we detected high *PjWts* expression in the gills and low expression in the hemolymph of *P. japonicus* (Figure 5)—a pattern consistent with the tissue expression of *PvWts*.¹³

Following *V. harveyi* challenge, the expression of *PjWts* was significantly upregulated, reaching peak levels at 6 h in the gills and 12 h in the hepatopancreas, respectively (Figure 6). This result suggests that *PjWts* may be involved in the defense response against *Vibrio* species, which aligns with findings from previous studies. For example, in *C. hongkongensis*, *Lats* expression in hemocytes was significantly increased at 3 h post-injection with *V. coralliilyticus* and reached a maximum at 6 h.⁸ In *P. vannamei*, *PvWts* expression was significantly upregulated at 12 h post-*Vibrio* infection and increased rapidly from 48 h to 72 h.¹² Additionally, *PvWts* expression was elevated in *P. vannamei* following WSSV infection, accompanied by increased hemocyte activity.¹³

Previous studies have shown that animals infected with bacteria exhibit injury or mortality when Wts or Lats is knocked out or silenced. For example, the mortality rate of *D. melanogaster* infected with gram-positive bacteria increases following Wts knockout.² In *P. vannamei*, silencing of *PvWts* enhances the infectivity of WSSV.¹³ Additionally, silencing Wts in shrimp reduces the phagocytic activity of hemocytes. Consistent with these findings, the mortality rate of shrimp infected with *Vibrio* exceeds 80%—a rate higher than that of uninfected shrimp.¹²

In mammals, knockout of Lats1 in mice leads to the development of soft tissue sarcomas and ovarian stromal cell tumors, indicating that Lats1 functions to inhibit tumorigenesis.²⁴ In the present study, we also observed that the expression of *PjWts* in the gills and hepatopancreas gradually decreased within 48 hours after *PjWts* silencing, which was significantly different from the results in the control group (Figure 7). This finding confirms that *PjWts* was successfully inhibited.

Notably, the survival rate of *P. japonicus* infected with *V. harveyi* was only 16% following *PjWts* silencing. In contrast, the survival rate of *P. japonicus* infected with *V. harveyi* was 40% when *PjWts* was not silenced (Figure 8). These results suggest that *PjWts* may inhibit the virulence of *V. harveyi* in shrimp.

In summary, the cDNA and genomic DNA sequences of the *PjWts* gene were isolated and characterized in this study. The Mob and STKc_LATS domains of *PjWts* are highly conserved across various species. *PjWts* mRNA is highly expressed in the ganglia and gills of *P. japonicus*. Following *V. harveyi* injection, the mRNA expression of *PjWts* was significantly upregulated in the gills and hepatopancreas. After *PjWts* silencing, the survival rate of *P. japonicus* infected with *V. harveyi* was significantly lower than that of shrimp in the other groups. These results suggest that *PjWts* may be involved in the innate immunity of *P. japonicus*, laying a foundation for further investigations into the function of the Hippo signaling pathway in this species.

ACKNOWLEDGMENTS

This work was supported by Open project of Key Lab of Freshwater Biodiversity Conservation, Ministry of Agriculture and Rural Affairs of China (LFBC1114); Open Program of Key Laboratory of Cultivation and High-value Utilization of Marine Organisms in Fujian Province(2025fjscq04); Hunan Natural Science Foundation Youth Project (2024JJ6331).

AUTHORS' CONTRIBUTION

Chao Peng: Methodology, formal analysis and writing - original draft. Shuiqing Wu: Methodology, data curation. Haihong Huang: Methodology, data curation. Liye Shao: collected the samples and performed the bioinformatic. Yibin Yang: designed, conceived of the study, supervision. All authors gave final approval for publication.

COMPETING OF INTEREST – COPE

No competing interests were disclosed.

ETHICAL CONDUCT APPROVAL – IACUC

P. japonicus used in this experiment do not belong to vertebrates or regulated invertebrates, and are not endangered protected animals.

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

Submitted: September 03, 2025 CDT. Accepted: November 20, 2025 CDT. Published: April 01, 2026 CDT.



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