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Crayfish (*Procambarus clarkii*) TRPA1 is required for the defense against *Aeromonas hydrophila* infection under high temperature conditions and contributes to heat sensing

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ABSTRACT

Temperature is an important environmental factor influencing immune responses of crayfish. However, the mechanism underlying how temperature affects immune responses remains unclear. Here, we identified an ortholog of the transient receptor potential ankyrin subtype 1 (TRPA1), a temperature sensor of *Drosophila*, from *Procambarus clarkii* (PcTRPA1-1). Its expression was induced by high temperature and challenge with heat-killed *A. hydrophila* at high temperature, but not at lower temperature. *PcTRPA1-1* silencing led to increased mortality of crayfish challenged with live *A. hydrophila* at high temperature (32 °C), but had no statistically significant effect on crayfish mortality at 24 °C. This suggests that PcTRPA1-1 is involved in the immune responses of crayfish at high temperature as a potential temperature sensor. Further assay exhibited that *PcTRPA1-1* silencing affected immune responses of crayfish, including increase of lipid peroxidation, reduction of total antioxidant capacity, decreased phenoloxidase activity and disruption of circadian rhythm of total hemocyte count entrained by temperature cycles. *PcTRPA1-1* silencing also decreased the expression of *PcHSP70* and *PcHSP90* which are responsive to heat stimuli and bacterial challenge. The results collectively indicate that TRPA1 contributes to heat sensing of crayfish and is required for crayfish defense against bacterial infection.

1. Introduction

Aquatic animals are constantly exposed to environmental temperature changes in different seasons. The temperature changes directly influence biochemical processes of animals. High temperature in the summer is one of the important limiting factors to aquaculture industry in the north of China. Compared to other seasons, high temperature of summer often causes high incidence of diseases and high mortality of aquatic animals, such as crustacean (Guan et al., 2003; Jiravanichpaisal et al., 2004; Moser et al., 2012) and scallop (Wang et al., 2012; Xiao et al., 2005). Effect of temperature on crustacean defense responses against pathogen infection has been reported extensively in crustacean (Guan et al., 2003; Jiravanichpaisal et al., 2004; Moser et al., 2012). However, the underlying mechanism for crustacean to sense the signal of temperature change and further affect the immune responses has remained unknown. No thermal sensor of crustacean has been identified at the molecular level, and little is known about regulation of the temperature on immune responses of crustacean.

In mammals, several ion channels are involved in sensing temperature signals, including TREK-1, DEG/EnaC-family, P2X3 and the proteins of transient receptor potential (TRP) family (Rosenzweig et al., 2005). TREK1 is a background K^+ channel which is activated reversibly by heat. A 10 °C rise enhances TREK1 current amplitude by ~7-fold (Maingret et al., 2000). BNC1, a Na^+ channel of DEG/ENaC family, is modulated by cold temperature. Several other members of DEG/ENaC family exhibit the same characters (Askwith et al., 2001). P2X3 is an ATP-gated cation channel which is required for mice to respond to innocuous thermal stimuli (Souslova et al., 2000). In TRP family, TRPV1-V4 (Patapoutian et al., 2003), TRPM8 (Buijs and McNaughton, 2020), and TRPA1 (Buijs and McNaughton, 2020) are temperature-responsive. They have been shown to be essential for the responses to temperature change. In invertebrates, two temperature sensors have been identified including dANKTM1 and Painless. Painless is required for response to noxious thermal and mechanical stimuli in *Drosophila* larvae (Tracey Jr. et al., 2003), and dANKTM1 can sense warming stimuli and regulates thermotaxis (Rosenzweig et al., 2005; Viswanath

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Table 1
Primers used in the present study.

Primer	Sequence (5′–3′)	Annealing temperature	Amplicon size	Accession number
PcTRPA1-1-FL-F for full length of PcTRPA1-1 cDNA	ATGACCAAACACCCTTACACTACGC	60 °C	3066 bp	GARH01043467 Manfrin et al. (2015)
PcTRPA1-1-FL-R for full length of PcTRPA1-1 cDNA	CACTAACCCACTGACTCTTTCCTTC			
PcTRPA1-1-F for realtime PCR	CAGTCAGGCAGGATTGGC	55 °C	155 bp	MW864052
PcTRPA1-1-R for realtime PCR	GACGGTGAAGAACTTGAGAAAC			
PcHSP70-F for realtime PCR	GTATTGAGATAGACTCCCTG	55 °C	187 bp	KU613184 Baringou et al. (2016)
PcHSP70-R for realtime PCR	ATTCTTGTAGAACCACCC			
PcHSP90-F for realtime PCR	TGACGCTGTCGCTAACTC	55 °C	102 bp	JQ995601 Liang et al. (2013)
PcHSP90-R for realtime PCR	AGCTGCTGGATGCAATAC			
PcACTIN-F for realtime PCR	CAACTTGCCCGCCACTTA	55 °C	228 bp	D14612 Kang and Naya (1993)
PcACTIN-R for realtime PCR	GTGGTGGTGAAGGAATAGCC			
Primers for generation of dsRNA specific for *PcTRPA1-1*, *HSP70* and *HSP90* genes				
PcTRPA1-1-ds-F	TAATACGACTCACTATAGGGCTGATGAAATGAACGGCACG	60 °C	683 bp	MW864052
PcTRPA1-1-ds-R	*TAATACGACTCACTATAGGG*AGACGAGGAAGGCAACGAAC			
PcHSP70-ds-F	*TAATACGACTCACTATAGGG*AGATGAAAGAAACGGCTGAA	60 °C	684 bp	KU613184 Baringou et al. (2016)
PcHSP70-ds-R	*TAATACGACTCACTATAGGG*AGAACCACCCACCATAACAA			
PcHSP90-ds-F	*TAATACGACTCACTATAGGG*GAAGGAAGACGAGGCTGCTA	60 °C	661bp	JQ995601 Liang et al. (2013)
PcHSP90-ds-R	*TAATACGACTCACTATAGGG*GCGTGGATGCCCAGTTTGATA			
Primers for generation of dsRNA specific for *GFP* gene				
GFP-ds-F	*TAATACGACTCACTATAGGG*CGACGTAAACGGCCACAAGT	60 °C	695 bp	JQ064510 Hickey et al. (2012)
GFP-ds-R	*TAATACGACTCACTATAGGG*CTTGTACAGCTCGTCCATGC			

et al., 2003). It has not been established whether crustacean orthologs of these molecules act similar function as temperature sensors.

TRPA1 is a member of TRP family and acts as a nonselective cationic channel. It has been extensively studied as temperature sensors in mammals and *Drosophila*. Mouse TRPA1 can be activated by cold below 17 °C (Buijs and McNaughton, 2020), human TRPA1 is activated by both cold and heat, conferring thermal sensitivity (Moparthi et al., 2016), and *Drosophila* TRPA1 is activated by warming stimuli (Viswanath et al., 2003) and is required for thermotaxis (Rosenzweig et al., 2005). In addition to temperature sensation, TRPA1 is also involved in the regulation of immune responses. TRPA1 expression can be modulated by inflammatory mediators. High concentration of TNFα up-regulates TRPA1 expression and activity (Liu et al., 2020). Both IL-6 and IL-8 also play a direct role in increasing TRPA1 expression (Silverman et al., 2020). Conversely, as regulator of inflammatory signaling, TRPA1 regulated immune responses. TRPA1 can be activated by LPS in a TRL4-independent manner and leads to neuropeptide release and neurogenic inflammation (Meseguer et al., 2014). Recent study found that asthma, an inflammatory disorder of the airways, is accompanied with high TRPA1 expression, and genetic deletion or pharmacological inhibition of TRPA1 alleviated the phenotype of asthma (Li et al., 2020). pharmacological activation of the skin TRPA1 promotes the antigen binding and antibody in the spleen (Kozyreva and Khramova, 2020). Although relationship between TRPA1 and immune responses has been extensively studied in mammals, no invertebrate TRPA1 is reported as immune regulators.

In our previous study, we found that a TRPA1 ortholog in crayfish, PcTRPA1-1, can be induced by high temperature (Li et al., 2019). Here we further investigated whether PcTRPA1-1 plays a role in immune responses of crayfish under different temperatures. We used RNAi-based approach to demonstrate that PcTRPA1-1 contributes to heat sensing and is required for the defense of crayfish against bacterial infection at high temperature.

2. Materials and methods

2.1. Experimental animals

Intermolt crayfish (*Procambarus clarkii*) (13–16 g) were obtained from local market and maintained in aerated water to acclimate for 2 weeks at 24 ± 1 °C before use in experiments. Heated clam mince was used to feed the crayfish three times per week. Given sexual difference affects responses of many animal species to temperature change (Ahnesjo, 1995; Baker et al., 1970; Gomi, 2006) and TRPA1 function (Follansbee et al., 2019; Patil et al., 2013), only male crayfish were used in the experiments to avoid the possible sexual difference.

2.2. Gene cloning and expression analysis

For gene cloning, heptopancreas of crayfish was used for RNA extraction and cDNA synthesis. Total RNA was extracted using TRIzol (Thermo Fisher, USA) according to the manual provided by manufacturer. The first strand cDNA was synthetized using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, China) following the manual. The amino acid sequence of the human (h) TRPA1 was used as query to search against TRA database of crayfish (*P. clarkii*) at the National Center for Biotechnology Information (NCBI) to obtain homologous nucleotide sequence. The primers (Table. 1) were designed according the resultant nucleotide sequence to amplify the coding sequence (CDS) from cDNA. The resultant PCR product was cloned into pMD19-T simple vector and transformed into *Escherichia coli* DH5α strain. PCR-based screen was performed and the positive colonies were sequenced.

Tissue distribution of *PcTRPA1-1* was analyzed using nerve, muscle, hepatopancreas, hemocyte collected from four crayfish. For induced expression analysis of *PcTRPA1-1*, total 80 healthy crayfish were reared at 24 °C for 2 weeks. Subsequently, 20 crayfish were transferred to 32 °C and acclaimated for 1 week before injection with heat-killed *A. hydrophila*. Hepatopancreas was collected at 6 h, 12 h, 24 h and 48 h after injection. Another group including 20 crayfish was treated using the same procedure with CFS replacing heat-killed *A. hydrophila* as control. The third group of 20 crayfish was transferred to 10 °C for low temperature treatment. Samples were collected at 6 h, 12 h, 24 h and 48 h after transfer. The left 20 crayfish were kept in 24 °C as control of low temperature treatment and sampled at the same time points as the third group. For each group, 4 animals was sampled at each time point as four biological replicates. Total RNA isolation and cDNA synthesis were performed as mentioned above. The analysis of gene expression was performed using quantitative real-time PCR on the LighterCycler 480 II system. The reaction was carried out as previous procedure and regents (Li et al., 2019) with β-*actin* gene as inter control. The primers used in the expression analysis were presented in Table 1.

The similar procedure was used for expression analysis of *PcHSP70*

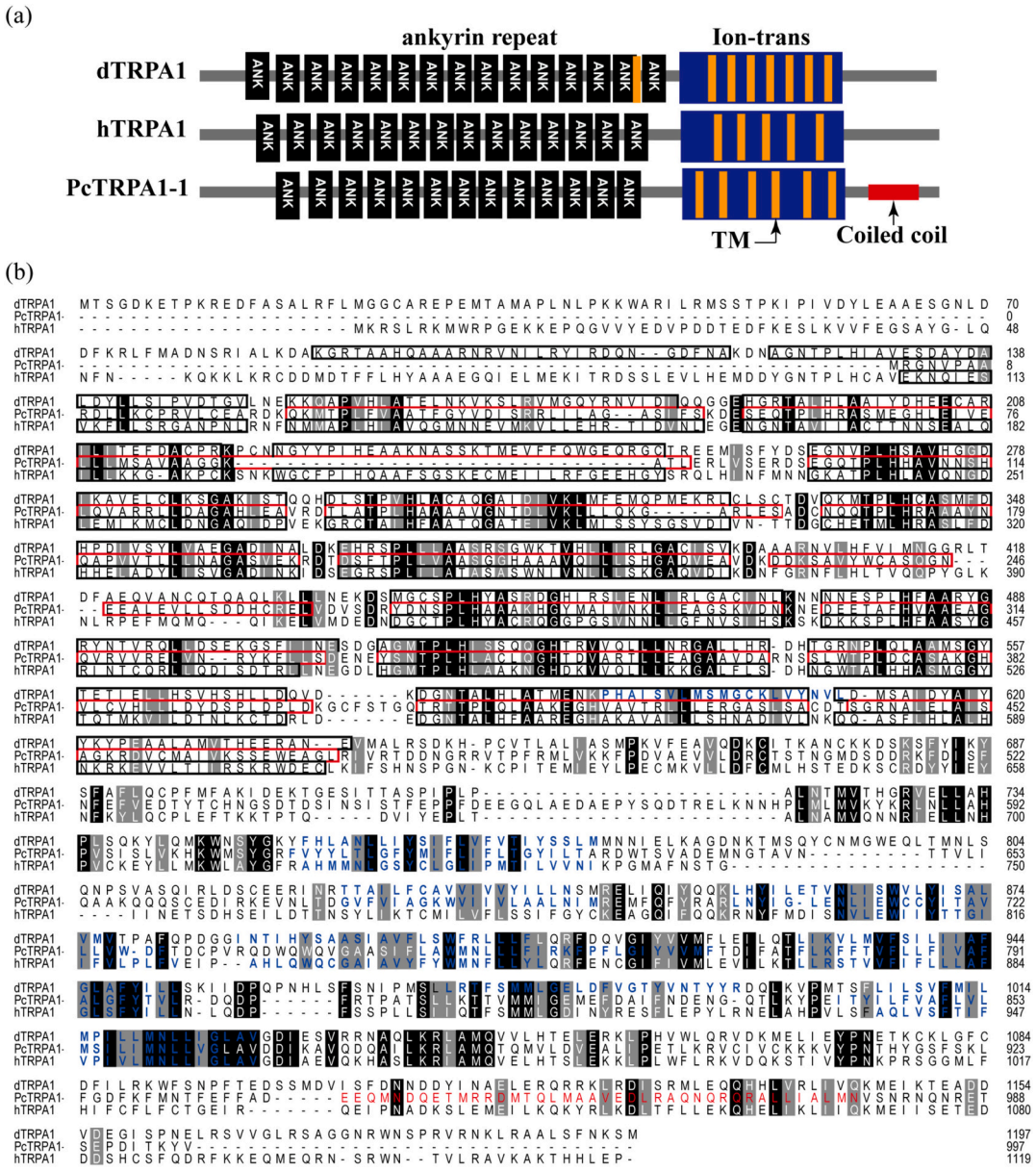

Fig. 1. Structural analysis and sequence alignment of TRPA1 proteins from human, *Drosophila* and red swamp crayfish. a, putative domain composition of the three TRPA1 proteins analyzed by SMART online software (http://smart.embl-heidelberg.de/). TM, transmembrane regions represented with orange bars. b, multiple alignment of TRPA1 proteins. White letters with black background indicate identical amino acids and the similar amino acids are exhibited using white letters with gray background. Black and red frames indicate Ankyrin repeats; Blue bold letters indicate transmembrane regions; Red letters represent coiled coil. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and *PcHSP90* in *PcTRPA1-1* silencing crayfish with some modification in animal treatment. The animals were divided into four groups and each group include five individuals. Two groups were injected with *PcTRPA1-1*-specific dsRNA twice and followed by injection with heat-killed *A. hydrophila* or with CFS, respectively. The other two groups were injected with *GFP*-specific dsRNA twice and followed by injection with heat-killed *A. hydrophila* or with CFS, respectively. The hepatopancreas was collected at 6 h after injection with heat-killed *A. hydrophila* or CFS. The experiments were performed at 24 °C and 32 °C, respectively.

2.3. RNA interference (RNAi) and bacterial challenge

RNAi and bacterial challenge were executed as the procedure reported by Chen et al. (Chen et al., 2016). Briefly, partial *PcTRPA1-1* CDS was amplified with the primers listed in Table 1. The resultant PCR product acted as template to synthetize *PcTRPA1-1*-specific double strand RNA (dsRNA). To silence *PcTRPA1-1* expression, *PcTRPA1-1*-specific dsRNA (10 μg. g^{-1}) was injected into the crayfish. The injection was repeated 12h after the first injection with the same dsRNA concentration (10 μg. g^{-1}). The gene expression was analyzed using RT-PCR and immunobloting at different time points to confirm the gene silencing. The experiments were performed at two different temperatures, 32 °C and 24 °C. For each temperature, the following procedure was conducted. Total 300 animals were divided into three groups. One group was injected with *PcTRPA1-1*-specific dsRNA twice for gene silencing and followed by challenged with 100 μL live *A. hydrophila* (1.0×10^7 CFU.mL^{-1}) 48 h after the first dsRNA injection. The other two groups were subjected to the same procedure but using GFP-specific dsRNA and CFS (Crayfish Saline Buffer, 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2 mM $NaHCO_3$, pH 6.8), respectively, to replace *PcTRPA1-1*-

specific dsRNA. The death of crayfish was recorded per 12 h. The cumulative survival rates of the three groups of crayfish were calculated and survival curves were plotted using software GraphPad Prism 8.0. Statistical significance is analyzed using Log-rank (Mantel-Cox) test. $P < 0.05$ was regarded as significant difference. For *HSP70* and *HSP90* gene silencing, the same procedure were performed. Bacterial growth in crayfish was evaluated with bacterial titer as previously described method (Li et al., 2019).

2.4. Immunobloting

Total protein was exacted from hepatopancreas using One Step Animal Tissue Active Protein Extraction Kit (Sangon, China). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly (vinylidene difluoride) (PVDF) membrane. The immunobloting was performed with polyclonal antibodies which were raised in rabbits immunized with synthesized polypeptides of PcTRPA1-1(EAGAAVDARNSSLWTPLDCA), PcHSP70 (EDEKFKDKVSSTDRSKILDA), PcHSP90 (EKEDEAAKKDDMPNVEDVGA) and PcACTIN (SVGVHETVHSSIMRCDIDIR), respectively. The target proteins were detected by incubating with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG and visualized by enhanced chemiluminescence kit (Beyotime, China).

2.5. Circadian rhythm of total hemocyte count

The animals were divided into three groups and each group contained 300 crayfish. Two groups were injected with *PcTRPA1-1*-specific dsRNA and GFP-specific dsRNA twice, respectively, with 12 h interval. The injected crayfish were treated with temperature rhythm of 24 °C: 12 h/32 °C: 12 h for 7 days and then were maintained at 28 °C. The other group was injected GFP-specific dsRNA twice and kept in constant temperature of 28 °C. Hemolymph was collected at different circadian time points (CT, the stage of constant temperature after temperature rhythm) from ventral sinus cavity using 2.5 mL syringe and mixed with equal volume precooled anticoagulant buffer (0.14 M NaCl, 0.1 M Glucose, 30 mM Na_3Citrate, 26 mM Citric Acid, 10 mM Na_2EDTA, pH 4.6). The hemocyte pellet was isolated centrifugation at 4 °C and 800 ×*g* for 10 min. After being fixed with 10% formalin, the total hemocyte count (THC) were counted using hemocytometer under phase-contrast microscope. For each timepoint, five crayfish were examined. The experiments were performed in three consecutive subjective days (72 h). The data were analyzed based on two-way analysis of variance (ANOVA) followed by the post hoc Tukey's test.

2.6. Phenoloxidase activity, lipid peroxidation and total antioxidant capacity

Phenoloxidase (PO; EC 1.14.18.1) activity was measured according previously reported method (Li et al., 2019). Lipid peroxidation (LPO) and total antioxidant capacity (TAC) were determined using method described by Chen et al. (Li et al., 2019) with slight modification. Briefly, crayfish were acclimated at 24 °C or 32 °C for 1 week followed by injection with dsRNA for gene silencing as mentioned above. For each temperature, six groups of crayfish were prepared and each group contains 15 crayfish. Three groups injected with PcTRPA1-1-dsRNA, GFP-dsRNA and CFS twice, respectively, with 12 h interval were used for measurement of basal PO, LPO and TAC. The other groups used for measurement of induced PO activity, LPO and TAC were injected with PcTRPA1-1-dsRNA, GFP-dsRNA and CFS twice, respectively, with 12 h interval and followed by injection with heat-killed *A. hydrophila* after first injection of dsRNA or CFS. Heamolymph was withdraw at 30 h after first dsRNA injection using the same method as mentioned above. The hemocyte pellet was isolated by centrifugation and homogenized in 100 μL of deionized water. After centrifugation at 7500 ×*g*, the supernatant was used for measurements of LPO and TAC using the method reported by Coggins et al. (Coggins et al., 2017). The data was analyzed with Two-way ANOVA followed by Tukey's test using GraphPad Prism 8.0.

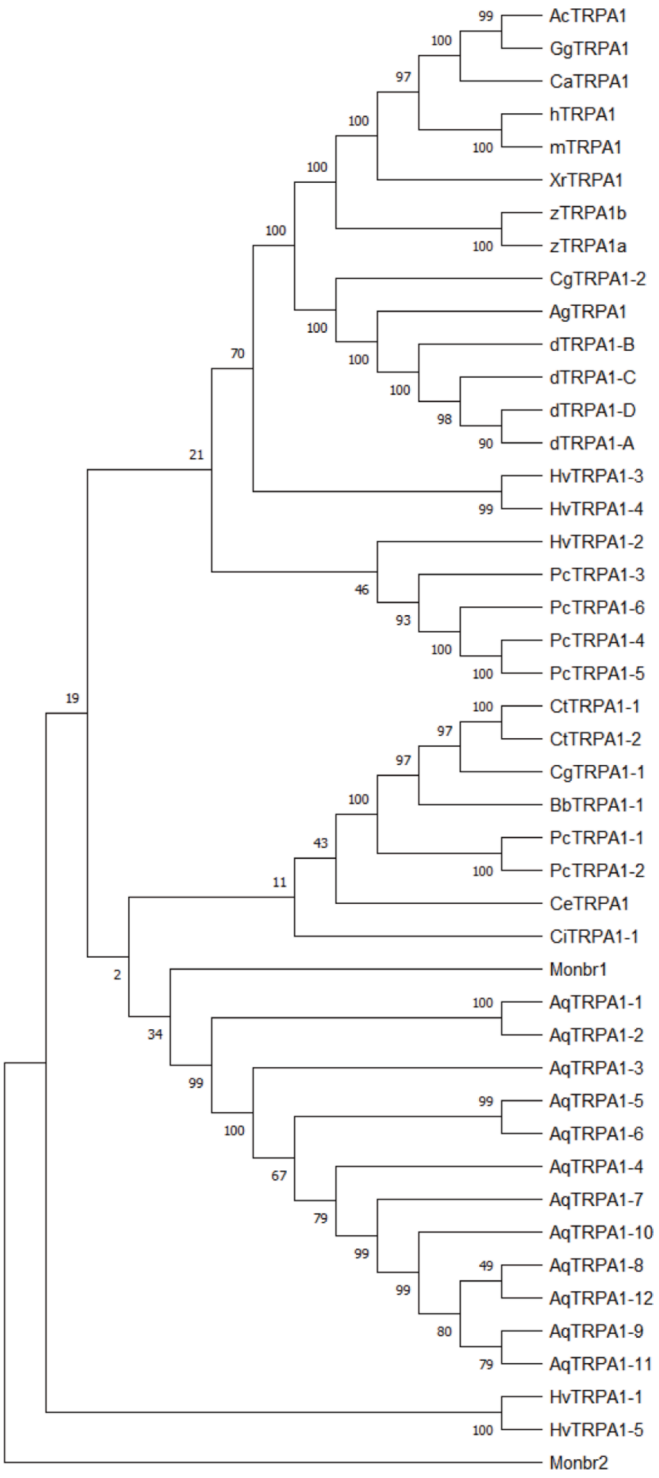

Fig. 2. Phylogenetic tree of TRPA1 protein in species from single cell eukaryotes to mammals. Species are summarized in Table 2. The number at branch node show bootstrap values of 1000 replicates. The tree was constructed using MAGA-X 10.0 with Neighbor-Jointing method.

3. Results

3.1. TRPA1 is induced by bacterial infection at high temperature

To further explore the potential function of crayfish TRPA1, we

Table 2
Proteins used in the present study.

Protein name	Accession numeber	Species	Function	References
Monbr1	JGI22692	*M. brevicollis*	–	Peng et al. (2015)
Monbr2	JGI 28830	*M. brevicollis*	–	Peng et al. (2015)
AqTRPA1-1	XP003386223	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-2	XP003386224	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-3	XP003388066	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-4	XP003390671	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-5	XP003390356	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-6	XP003390078	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-7	XP003389942	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-8	XP003388973	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-9	XP003388069	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-10	XP003390670	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-11	XP003390193	*A. queenslandica*	–	Peng et al. (2015)
AqTRPA1-12	XP003383370	*A. queenslandica*	–	Peng et al. (2015)
HvTRPA1-1	XP002167191.2	*H. vulgaris*	–	Peng et al. (2015)
HvTRPA1-2	XP002169693.2	*H. vulgaris*	–	Peng et al. (2015)
HvTRPA1-3	XP002164531.2	*H. vulgaris*	–	Peng et al. (2015)
HvTRPA1-4	XP002159822.2	*H. vulgaris*	–	Peng et al. (2015)
HvTRPA1-5	XP_012566755.1	*H. vulgaris*	–	Peng et al. (2015)
ceTRPA1	ABQ15208.1	*C. elegans*	Mechanosensation; cold sensor	Thies et al. (2016); Xiao et al. (2013)
CgTRAP1-1	XP_011413964.1	*C. gigas*	–	Gribble et al. (2018)
CgTRAP1-2	EKC39923.1	*C. gigas*	–	Gribble et al. (2018)
CtTRAP1-1	201,917	*C. teleta*	–	Peng et al. (2015)
CtTRAP1-2	192,646	*C. teleta*	–	Peng et al. (2015)
dTRPA1-A	AEU17952.1	*D. melanogaster.*	Heat and chemical sensation	Kang et al. (2011)
dTRPA2-B	AEU17863.1	*D. melanogaster.*	Heat sensation	Kang et al. (2011)
dTRPA3-C	AEU04534.1	*D. melanogaster.*	UV-nociceptor and detector of electrophiles	Zhong et al. (2012)
dTRPA4-D	AET41695.1	*D. melanogaster.*	Noxious thermosensor	Zhong et al. (2012)
AgTRPA1	ACC86138.1	*A. gambiae*	Noxious thermosensor	Hamada et al. (2008); Zhong et al. (2012)
CiTRPA1	XP_018667617.1	*C. intestinalis*	–	Gribble et al. (2018)
BbTRPA1	XP_019617343.1	*B. belcheri*	–	Gribble et al. (2018)
zTRPA1a	ACI26674.1	*D. rerio*	Chemosensation	Oda et al. (2016)
zTRPA1b	ACI26673.1	*D. rerio*	Chemosensation, but not for thermosensation or mechanosensation	Prober et al. (2008)
XrTRPA1	BAM42680.1	*X. ropicalis*	Heat and noxious chemical sensation	Saito et al. (2012)
AcTRPA1	BAM42681.1	*A. carolinensis*	Heat and noxious chemical sensation	Saito et al. (2012)
CaTRPA1	ADD82930.1	*C. atrox*	Infrared radiation sensation	Gracheva et al. (2010)
GgTRPA1	BAO51998.1	*G. gallus*	Heat and noxious chemical sensor	Saito et al. (2014)
mTRPA1	AHB62275.1	*M. musculus*	Mechanical stimuli, cold and heat sensor	Kwan et al. (2006); Sinica et al. (2019)
hTRPA1	NP_015628)).2	*H. sapiens*	Mechanical stimuli, cold and heat sensor	Rhyu et al. (2021); Silverman et al. (2020); Talavera et al. (2020)
PcTRPA1-1	MW864052	*P. clarkii*	Δ	
PcTRPA1-2	GBEV01011871.1	*P. clarkii*	–	
PcTRPA1-3	GBEV01071309.1	*P. clarkii*	–	
PcTRPA1-4	GBEV01009548.1	*P. clarkii*	–	
PcTRPA1-5	GARH01022396.1	*P. clarkii*	–	
PcTRPA1-6	GARH01034338.1	*P. clarkii*	–	

–: There is no reports on the function of TRPA1 of the indicated species; Δ: The function of TRPA1 was investigated in the present study.

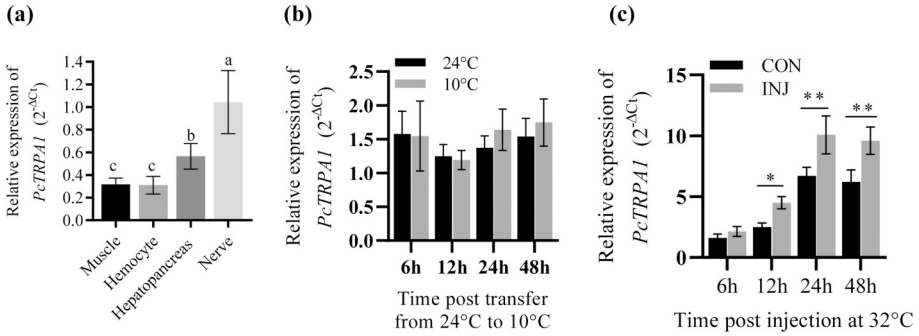

Fig. 3. *PcTRPA1-1* expression in different tissues and its responses to temperature change and bacterial infection. (a) *PcTRPA1-1* expression in different tissues. The tissues were collected from four different crayfish as four biological replicates. Each sample was repeated three times as technological replicates. The relative expression of *PcTRPA1-1* were presented as Mean ± SD ($n = 4$). (b) *PcTRPA1-1* expression in hepatopancreas after transfer from 24 °C to 10 °C. Total (c) *PcTRPA1-1* expression in hepatopancreas after injection with heat-killed *A. hydrophila* at 32 °C. CON represents crayfish injected with CFS. INJ represents crayfish injected with heat-killed *A. hydrophila*. Bars represent Mean ± SD (n = 4). The data in Fig. 2a were analyzed using one-way ANOVA analysis followed by Tukey's test. The data in Fig. 2c and b were subjected to two-way ANOVA analysis followed by Tukey's test.*$P < 0.05$; **$P < 0.01$.

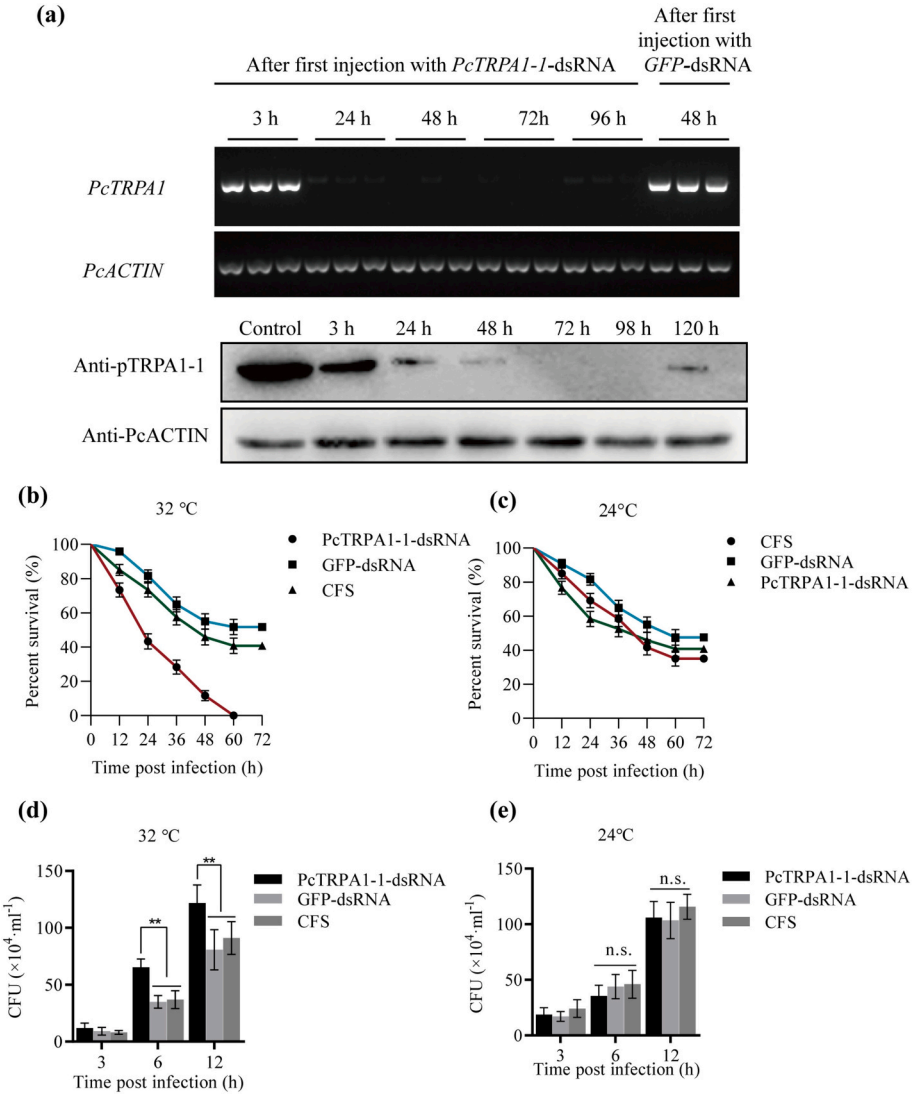

Fig. 4. *PcTRPA1-1* silencing led to high mortality of crayfish challenged by live *A. hydrophila*. (a), PcTRPA1-1 expression was reduced by RNAi as shown by RT-PCR (upper) and Immunobloting (bottom). Samples were collected at indicated time points after first injection of *PcTRPA1-1*-specific dsRNA. Control: the samples were collected from crayfish injected with GFP-specific dsRNA 48 h after first injection with dsRNA. (b) and (c), Accumulative mortality in *PcTRPA1-1* silencing crayfish after challenged with live *A. hydrophila*. GFP-dsRNA- and CFS-injected crayfish were used as control. (d) and (e), Bacterial titers in *PcTRPA1-1* silencing crayfish and control crayfish. Survival curves were plotted using software GraphPad Prism 8.0 and statistical significance is analyzed using log-rank analysis. For bacterial titers, two-way ANOVA was performed and followed by Tukey's test using GraphPad Prism 8.0. Bar represent mean ± SD ($n = 5$). $^{**}P < 0.01$; n.s. indicates no significant difference.

cloned the full-length CDS (2994 bp) from *P. clarkii* and named as *PcTRPA1-1* (Accession Number: MW864052). PcTRPA1-1 protein exhibits similar domain composition, including 13 predicted amino-terminal ankyrin repeats and an ion transport domain composed of five transmembrane domains (Fig. 1a). It shares 21.6% and 22.5% sequence identity with human TRPA1 and *Drosophila* TRPA1 protein, respectively (Fig. 1b). We also constructed phylogenetic tree using TRPA1 from different species (Fig. 2 and Table 2). As reported by Gribble et al., there are two major clades which were referred as A and B, respectively (Gribble et al., 2018). In addition, one TRPA1 from single cell eukaryotes *Monosiga brevicollis* and two from cnidaria *Hydra vulgaris* do not fall in the two major clades. As to six PcTRPA1 isoforms, PcTRPA1-1 and PcTRPA1-2 fall in B group and the other four isoforms fall in A group. Further expression analysis revealed that *PcTRPA1-1* was expressed in nerve, hepatopancreas, hemocyte and muscle with different levels (Fig. 3a). The more abundant expression was observed in hepatopancreas and nerve. Our previously study showed that the expression of *PcTRPA1-1* in hepatopancreas was highly induced by high temperature, but not by heat-killed *A. hydrophila* infection at 24 °C (Li et al., 2019). Here, we further determine whether *PcTRPA1-1* is induced by low temperature and bacterial infection at high temperature. For low temperature treatment, crayfish were transferred from 24 °C to 10 °C, the gene expression was analyzed at 6 h, 12 h, 24 h and 48 h after transfer. No significant difference in *PcTRPA1-1* expression was observed between 24 °C and 10 °C groups (Fig. 3b). To determine whether *PcTRPA1-1* expression is affected by bacterial challenge at high temperature, crayfish were transferred from 24 °C to 32 °C and acclimated for 24 h followed by injection with heat-killed *A. hydrophila*. *PcTRPA1-1* expression demonstrated markedly increase at statistically significance level ($P < 0.05$) after challenged with heat-killed *A. hydrophila* (Figure 3c), suggesting that *PcTRPA1-1* is involved in the defense responses of crayfish against *A. hydrophila* at high temperature.

3.2. *PcTRPA1-1 silencing leads to high mortality crayfish after challenge with A. hydrophila under high temperature*

To analyze the effect of PcTRPA1-1 on immune responses of crayfish, we silenced *PcTRPA1-1* expression using RNAi approach. Injection of *PcTRPA1-1*-specific dsRNA effectively silenced the expression of *PcTRPA1-1* gene in hepatopancreas, but the CFS and GFP dsRNA did not affect *PcTRPA11* gene expression (Fig. 4a). After challenged with live *A. hydrophila*, *PcTRPA1-1*-specific dsRNA injection significantly increased the mortality of crayfish at 32 °C (Fig. 4b). At 24 °C, however, crayfish mortality did not show significant change as compared with control group (Fig. 4c). Further analysis exhibited that bacterial growth was significantly enhanced in *PcTRPA1-1*-silenced crayfish (Fig. 4d) at 32°C as compared to control crayfish. At 24 °C, bacterial growth in *PcTRPA1-1*-silenced crayfish showed no significant change compared to

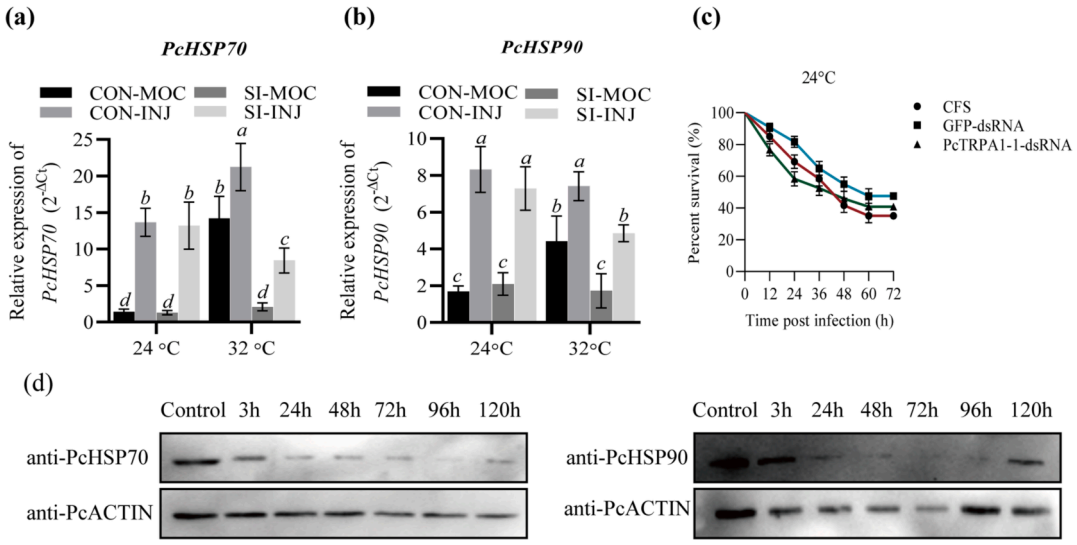

Fig. 5. The expression *PcHSP70* (a) and *PcHSP90* (b) in *PcTRPA1-1* silencing crayfish. CON-MOC represents crayfish injected with GFP-dsRNA followed with injection with CFS as mock infected control; CON-INJ represents crayfish injected with GFP-dsRNA followed by infected with heat-killed *A. hydrophila*; SI-MOC represents (a) *PcHSP70*- or (b) *PcHSP90*-silenced crayfish were injected with CFS; SI-INJ represents (a) *PcHSP70*- or (b) *PcHSP90*-silenced crayfish were challenged with heat-killed *A. hydrophila*. Different letters indicate significant difference. $P < 0.05$. Bars represent mean ± SD (n = 4). (c), the effect of *PcHSP70* and *PcHSP90* silencing on the mortality of crayfish challenged with live *A. hydrophila*. Survival curves were plotted using software GraphPad Prism 8.0 and statistical significance is analyzed using log-rank analysis. $^*P < 0.01$; n.s. indicates no significant difference. (d) PcHSP70 and PcHSP90 proteins in *PcHSP70* and *PcHSP90* silencing crayfish, respectively, as revealed by immunobloting.

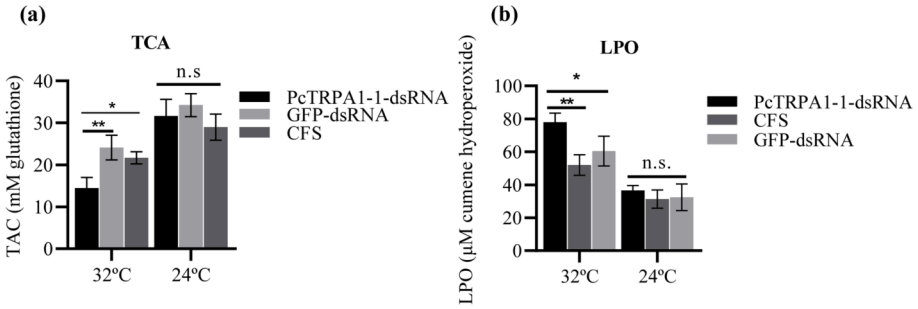

Fig. 6. Changes in TAC (a) and LPO (b) of red swamp crayfish after *PcTRPA1-1* silencing at 32 °C as compared with 24 °C. TAC and LPO at 32 °C were measured 24 h after the crayfish were exposed to 32 °C or 24 °C and expressed as molar units of glutathione and molar units of cumene hydroperoxide, respectively. Bars represents mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control groups (Fig. 4e). The results suggest that *PcTRPA1-1* is required for bacterial defense under high temperature.

3.3. *PcTRPA1-1 silencing reduces the responses of PcHSP70 and PcHSP90 to high temperature*

We examined whether *PcTRPA1-1* silencing influences the expression of *PcHSP70* and *PcHSP90* in crayfish. The experiments were performed at 24 °C and 32 °C, respectively. The induced expression of *PcHSP70* by high temperature was significantly reduced in *PcTRPA1-1*-silenced crayfish, while at 24 °C, *PcHSP70* expression in *PcTRPA1-1*-silenced crayfish was indistinguishable from GFP dsRNA-injected crayfish which were used as control (Fig. 5a). Bacteria-induced *PcHSP70* and *PcHSP90* expressions were not affected by *PcTRPA1-1* silencing at 24 °C, but significantly reduced expressions were observed at 32 °C (Fig. 5b). Further analysis showed that *PcHSP70* silencing increased the mortality of crayfish challenged with live *A. hydrophila* at 32 °C (Fig. 5c, d). However, *PcHSP90* silencing did not affect crayfish mortality at 32 °C (Fig. 5c, d). The results indicate that reduced *PcHSP70* expression has significant influence on the immunity of *PcTRPA1-1*-silenced animals.

3.4. *PcTRPA1-1 silencing reduces LPO and TAC under heat temperature*

To determine whether *PcTRPA1-1* silencing affects immune defense, we examined TAC and LPO in *PcTRPA1-1*-silenced crayfish at different temperatures. We injected crayfish with *PcTRPA1-1*-dsRNA at 24 °C and transferred into 32 °C after injection. The reduced TAC was observed in both *PcTRPA1-1*-silenced crayfish and control crayfish, but the margin of reduction in *PcTRPA1-1*-silenced crayfish was significantly larger than that in control crayfish (Fig. 6a). The similar phenomenon was also observed in LPO measurement. After exposure to 32 °C for 24 h, the *PcTRPA1-1*-silenced crayfish exhibited significantly increased LPO. However, control crayfish including GFP-dsRNA-injected and CFS-injected crayfish did not demonstrate significant changes as compared to the crayfish at 24 °C (Fig. 6b). The results indicate that *PcTRPA1-1* silencing exacerbates the responses of TCA and LPO to high temperature.

3.5. *Circadianrhythm of the total hemocyte count (THC) under high temperature is disrupted by PcTRPA1-1 silencing*

To test the effect of *PcTRPA1-1* on THC and PO activity, we first measured THC changes in *PcTRPA1-1*-silenced crayfish exposed to

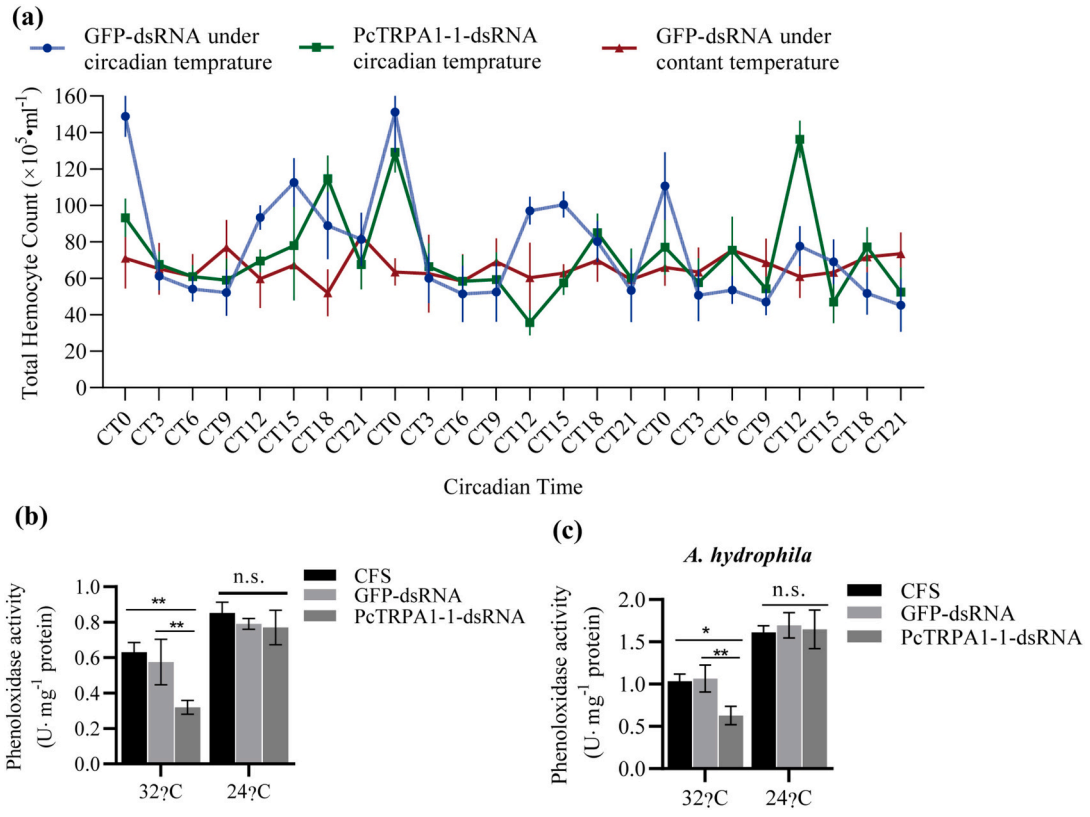

Fig. 7. THC rhythm and PO activity in *PcTRPA1-1*-silenced crayfish. (a) Twenty four hours after injection with dsRNA, crayfish was subjected to treatment with temperature rhythm of 24 °C: 12 h/32 °C: 12 h for 7 days and followed by constant temperature of 28 °C. GFP-dsRNA was used as non-specific control of *PcTRPA1-1*-dsRNA. Constant temperature of 28 °C was used as a control of temperature cycles. The crayfish were kept in dark to avoid the effect of light on circadian rhythm. Total 150 animals were treated in each group and five crayfish were sampled for THC measurement at each time point (n = 5). The data were subjected to two-way analysis of variance (two-way ANOVA) followed by Tukey's test. CT, Circadian Time; (b) Basal PO activity was measured 30 h after first dsRNA injection. (c) Bacteria induced PO activity was measured 6 h after heat-killed *A. hydrophila* challenge which was performed 24 h after first dsRNA injection. For PO measurement, hemolymph from three crayfish was mixed as one sample and three samples were measured. The results were expressed as mean ± SD (n = 3). The data were subjected to two-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$.

temperature cycles. Control crayfish injected with GFP-dsRNA showed obvious THC circadian rhythm after treatment with temperature cycles of 32 °C: 12 h/24 °C: 12 h. In contrast, THC of *PcTRPA1-1*-silenced crayfish did not exhibit circadian fluctuation entrained by the same temperature cycles (Fig. 7a). Although significant THC changes were also observed between different time points, but the changes were irregular. Next, we measured basal and induced PO activity of *PcTRPA1-1*-silenced crayfish. Basal PO activity was obviously decreased in *PcTRPA1-1*-silenced crayfish at high temperature (32 °C) as compared to control animals including GFP-dsRNA injected and CFS-injected crayfish. At 24 °C, however, no significant difference was observed between *PcTRPA1-1*-silenced and control crayfish (Fig. 7b). After injection with heat-killed *A. hydrophila* at 24 °C and 32 °C, the decreased PO activity was observed at 32 °C, but not at 24 °C. The significantly lower PO activity was observed in *PcTRPA1-1*-silenced crayfish under high temperature as compared to GFP-dsRNA injected and CFS-injected crayfish. No significant difference was found between *PcTRPA1-1*-silenced and control crayfish at 24 °C after challenge with heat-killed bacteria (Fig. 7c). The results indicate that PO activity of crayfish was affected by *PcTRPA1-1* silencing.

4. Discussion

Temperature is an important environmental factor regulating the immune defense of aquatic animals. The effect of high temperature on crustacean was extensively reported (Dong et al., 2015; Du et al., 2008; Guan et al., 2003; Jiravanichpaisal et al., 2004; Li et al., 2019). However, the underlying mechanism is unclear. Our previous study indicated that *PcTRPA1-1* gene of crayfish can be induced by high temperature (Li et al., 2019). Here, we show that *PcTRPA1-1* is required for the immune defense of crayfish against bacterial infection at high temperature. This work provides new insight into the mechanism underlying immune regulation of crayfish by temperature.

The expression of TRPA1 in animal tissues varies between different species. It is expressed in the heart (Marguerite et al., 2021), nerve system (Meents et al., 2019), respiratory tract (Caceres et al., 2009), pancreas, the reproductive tissues (Marguerite et al., 2021), inner ear (Takumida et al., 2009), pancreatic islets (Cao et al., 2012), muscle (Marguerite et al., 2021), T cell (Bertin et al., 2017), vascular endothelial cells (Earley et al., 2009) and several different fibroblasts (Jaquemar et al., 1999). Correspondingly, TRPA1 has different functions in these tissues or cells, such as acting as thermal and cold sensors (Rosenzweig et al., 2005; Viswanath et al., 2003), and being involved in the immune responses (Bertin et al., 2017; Kozyreva and Khramova, 2020). Our results show that *PcTRPA1-1* expression was observed in hepatopancreas, hemocyte, nerve and muscle, and the highest expression was found in nerve and hepatopancreas, suggesting that *PcTRPA1-1* plays an important role in hepatopancreas and nerves. Considering the effect of *PcTRPA1-1* silencing on crayfish responses to high temperature, the expression of *PcTRPA1-1* on nerve system may suggest that *PcTRPA1-1* is a potential temperature sensor. Hepatopancreas and hemocyte have been reported to play crucial role in the immune responses of crayfish. The enhanced mortality of crayfish in *PcTRPA1-1* silencing crayfish indicates that the expression of *PcTRPA1-1* in hepatopancreas

and hemocyte is necessary for its function in immune regulation. TRPA1 has been reported to be thermal and cold sensors in different species (Buijs and McNaughton, 2020). *PcTRPA1-1* expression is induced by high temperature of 32°C, but not by low temperature of 10 °C, suggesting that *PcTRPA1-1* may function as a heat sensor, but not as a cold sensor. In mammals, TRPA1 acts as both thermal and cold sensors, such as hTRPA1 and mouse TRPA1 (Moparthi et al., 2016; Moparthi et al., 2014; Patapoutian et al., 2003; Viswanath et al., 2003). However, *Drosophila* TRPA1 has been shown to be a thermal sensors, but not cold sensors (Rosenzweig et al., 2005; Viswanath et al., 2003). These results suggest that TRPA1 function may be different between invertebrates and mammals, but more evidences from different invertebrate species are needed to support this point.

TRPA1 is involved in crayfish immunity by direct and indirect ways. By a direct way, TRPA1 can directly affect the immune responses of animals. Previous studies have demonstrated that TRPA1 is necessary for the inflammatory responses caused by environmental and pharmacal factors (Bautista et al., 2006) and play a role in the progression and exacerbation of asthma (Caceres et al., 2009; Li et al., 2020). TRPA1 also regulates atherosclerosis development (Wang et al., 2020). TRPA1 of Schwann cell is required for neuroinflammation (De Logu et al., 2017). Here, our results demonstrate that *PcTRPA1-1* silencing can directly reduce PO activity, an important immune parameter for crayfish (Liu et al., 2007), which indicates that *PcTRPA1-1* directly affects the immune responses of red swamp crayfish. *PcTRPA1-1*-silencing also influences the expression of *PcHSP70* and *PcHSP90* which have been reported to be involved in both heat stress and immune responses of crustacean (Junprung et al., 2017; Junprung et al., 2019). The decreased *PcHSP70* and *PcHSP90* expressions under high temperature also provide evidences that *PcTRPA1-1* silencing can directly influence the immune responses of crayfish. The decreased expressions of *PcHSP70* and *PcHSP90* contribute to high mortality of *PcTRPA1-1*-silencing crayfish at high temperature.

In contrast, by indirect way, it was speculated that TRPA1 acts as a temperature sensor to sense temperature changes and induce immune system of crayfish to adapt to high temperature. The newly established defense system at high temperature protects animals from pathogen infection. If high temperature can not be sensed by animals, the immune system can not protect animals under high temperature. This point is supported by our results that *PcTRPA1-1* silencing significantly aggravated the TCA reduction and the LPO increase at high temperature. TAC and LPO are routinely used as biomarkers of environmental stress in a variety of organisms. Previous study reported that high temperature led to reduced TAC (Li et al., 2019). These results indicate that *PcTRPA1-1* silencing decreases the defense responses against heat stress, which indirectly reduces the immune defense against bacterial challenge. Consequently, high temperature disturbs the normal physiology and metabolism in *PcTRPA1-1*-silencing crayfish and causes high mortality after challenge with bacterial pathogen.

We also found that *PcTRPA1-1*-silencing prevented temperature-entrained circadian rhythm of THC. Hemocytes play a central role in innate immunity of crayfish and THC is a key immune indicator for crayfish (Lin and Söderhäll, 2011). In reported studies, THC of crayfish has been found showing light-entrained circadian rhythm (Watthanasurorot et al., 2011) and temperature-entrained circadian rhythm (Dong et al., 2015) which are essential for crayfish immunity. In this study, temperature-entrained circadian rhythm of THC was interrupted by *PcTRPA1-1* silencing, indicating that *PcTRPA1-1* silencing prevented crayfish from receiving of temperature signal and further providing evidences that *PcTRPA1-1* acts as a potential temperature sensor. Since the circadian rhythm of animals has closely related to immune responses, the disturbance of THC rhythm also contributes to high mortality of *PcTRPA1-1*-silencing crayfish challenged with bacterial pathogen.

In summary, we identified a TRPA1 ortholog in red swamp crayfish, *PcTRPA1-1*, which is required for crayfish to defend against *A. hydrophila* infection. *PcTRPA1-1* functions as potential thermal sensor, sensing high temperature and playing an important role in immune defense of crayfish by direct and indirect ways.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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