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A crayfish *Ras* gene is involved in the defense against bacterial infection under high temperature

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ABSTRACT

Temperature is an important environmental factor influencing crustacean resistance to pathogen infection. However, the mechanism underlying immune regulation by temperature remains unclear in crustacean. Here, we report a *Ras* gene of crayfish (designated as *PcRAS1*) which is involved in immune regulation of crayfish under high temperature. *PcRAS1* is induced by both high temperature and bacterial infection and the induction by bacterial infection is associated with temperature. Significant changes of *PcRAS1* expression was observed at 32 °C and 24 °C after infection with *Aeromonas hydrophila*, but relative moderate alternation was found at 16 °C after challenged with *A. hydrophila*. *PcRAS1* silencing significantly reduced crayfish survival from high temperature (32 °C and 24 °C) or bacterial infection at 32 °C, but there was no significant effect on survival from bacterial infection at 24 °C or 16 °C. Further analysis reveals that PO activity is reduced by high temperature or enhanced by bacterial infection. Moreover, both the decreased PO activity and the enhanced PO activity are affected by *PcRAS1* expression. *PcRAS1* silencing further reduces PO activity under high temperature and compromises the enhanced PO activity by bacterial infection. Lipid peroxidation (LPO) and total antioxidant capacity (TAC) are also involved in the responses to high temperature. LPO is enhanced by lower temperature. TAC is reduced by high temperature and TAC change resulting from high temperature is amplified by *PcRAS1* silencing. These results collectively indicate that *PcRAS1* is involved in immune regulation against bacterial infection mediated by temperature.

1. Introduction

As aquatic poikilotherm, body temperature of crustaceans varies with water temperature. During the summer months, high temperature often results in high incidence of diseases and causes high mortality in crustacean. For example, WSSV infection to shrimp is affected by water temperature [1–3]. Viral load and host mortality were markedly increased at a temperature higher than 22 °C, but significantly decreased at a temperature lower than 15 °C or higher than 32 °C [1,2]. The temperature effect on WSSV infection was also observed in crayfish. In spite of being injected or orally inoculated with WSSV, no death of crayfish was observed at 4 °C and 12 °C. However, 100% mortality occurred after being infected with WSSV under a temperature of 22 °C. If moribund crayfish at 22 °C were transferred to 16 °C, death was significantly delayed [4,5]. Not only viral infection but also bacterial infection is affected by temperature [6,7]. Survival rate of bacterial infected shrimps kept at 34 °C or 32 °C was significantly lower than that of shrimps maintained at lower temperatures [7]. Higher temperature also enhances the susceptibility in giant freshwater prawn *Macrobrachium rosenbergii* to *Lactococcus garvieae* [8]. In comparison, the increased susceptibility of tiger shrimp *Penaeus monodon* to *photobacterium damselae* subsp. damselae appeared at both higher (34 °C) and lower (22 °C) temperature compared with that at optimal temperature (26 °C) [6]. The same phenomenon has also occurred in other aquatic animals, such as scallop, in which mortality was significantly affected by high temperature [9,10]. These phenomena indicate that temperature, as an important environmental factor in aquaculture, exerts significant influence on the resistance of animals to pathogenic infection.

Although temperature effects on pathogen infection are widely reported in crustacean, it has not yet been determined whether these effects are the results of immune regulation by temperature. They may also be the results from direct effect of temperature on gene expression and pathogenicity of pathogens. For example, optimum temperature for *Litopenaeus vannamei* benefits the expression of some WSSV genes [11]. High temperature triggers molecular and physiological responses of crustacean. It has been reported that high temperature increases lipid peroxidation (LPO) and alters lipid composition [12,13]. The antioxidant capacity of animals can also be reduced by heat stress and

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Table 1
Primers used in the present study.

Primer	Sequence (5'-3')
PcRAS1-F	ATGTCGAAACCACCATCAGA
PcRAS1-R	TTACATAATCACACACTTTG
PcRAS2-F	ATGGGGAGGAGAAGTCCCAG
PcRAS2-R	TCAAACAATAACACAATTCT
PcRAS3-F	ATGACGGAGTACAAGCTGGT
PcRAS3-R	CTAAAATACAATGCACTTCC
PcRAS4-F	ATGGCACTCACAGTGGGACA
PcRAS4-R	TTACTTGTATCGTGGCCGAC
PcRAS5-F	ATGTTGGGGCTGCTGCTGCT
PcRAS5-R	CTAAAGAATGCAGCACTTGG
PcTRPA1-F for realtime PCR	CAGTCAGGCAGGATTGGC
PcTRPA1-R for realtime PCR	GACGGTGAAGAACTTGAGAAAC
PcTRPV1-F for realtime PCR	CATCATCTTCAGCTCGTTT
PcTRPV1-R for realtime PCR	ACCTTGCCAGTAATCTCG
PcTRPM8-F for realtime PCR	ATCACCGTCTTCCGTTTG
PcTRPM8-R for realtime PCR	TCTGGCTATGTCCACTCG
PcHSP70-F for realtime PCR	GTATTGAGATAGACTCCCTG
PcHSP70-R for realtime PCR	ATTCTTGTAGAACCACCC
PcHSP90-F for realtime PCR	TGACGCTGTCGCTAACTC
PcHSP90-R for realtime PCR	AGCTGCTGGATGCAATAC
PcRAS1-F for realtime PCR	CCAACGGCTGTTTGTGAC
PcRAS1-R for realtime PCR	CTCCCGCATAGCACTGAA
PcRAS2-F for realtime PCR	CTGGTGAAGGTTTCCTGCTA
PcRAS2-R for realtime PCR	TCTGATGCTCCAAATCTGCT
PcRAS3-F for realtime PCR	GATCTACAAGTGCGGGCTAT
PcRAS3-R for realtime PCR	CGATGTTCCCTATCTTTCCT
PcRAS4-F for realtime PCR	GTCGTAGCTTTGAGGAGGTT
PcRAS4-R for realtime PCR	GTCGTAAGGCAGCAGATGTT
PcRAS5-F for realtime PCR	TGCTAGTAGGCAACAAAGCT
PcRAS5-R for realtime PCR	GAATCTCACGCATCAAATCA
PcACTIN-F for realtime PCR	CAACTTGCCCGCCACTTA
PcACTIN-R for realtime PCR	GTGGTGGTGAAGGAATAGCC
Primers for generation of dsRNA specific for PcRAS1 gene	
PcRAS1-ds-F	*TAATACGACTCACTATAGGG*ATGTCGAAACCACCATCAGA
PcRAS1-ds-R	*TAATACGACTCACTATAGGG*TTACATAATCACACACTTTG
Primers for generation of dsRNA specific for GFP gene	
GFP-ds-F	*TAATACGACTCACTATAGGG*CGACGTAAACGGCCACAAGT
GFP-ds-R	*TAATACGACTCACTATAGGG*CTTGTACAGCTCGTCCATGC

acclimation at high temperature improves antioxidant capacity of animals [12]. High temperature changes the capacity of hemocyanin to carry oxygen. With the increase of temperature, the affinity of hemocyanin to oxygen increases and isoelectric point of hemocyanin is changed with temperature change, suggesting a change of protein structure [14,15]. Heat stress also causes a loss of ion homeostasis in crustacean which further impairs neuromuscular function [16]. In immune aspects, hemocyte count, phenoloxidase activity, respiratory burst, superoxide dismutase (SOD) activity and phagocytic activity in shrimp significantly decrease at lower and higher temperature compared with those at optimum temperature [6,7]. At the molecular level, the expressions of heat shock proteins are significantly changed in response to temperature change. Heat shock proteins HSP70, HSP90 and GRP78 were significantly induced responding to high temperature stress in *Acartia tonsa* and *Eurytemora affinis* [17]. In addition, the expressions of some immune genes are regulated by temperature change only in infected animals, but not in non-infected animals. For example, the expressions of both *Cas-ecCuZnSOD-2* and *CasTPS* genes are enhanced in the hemocytes of reo-like virus-infected blue crab, *Callinectes sapidus,* compared with those of the uninfected ones after exposed to 23 °C for 10 days [18]. Although physiological or immune responses at different temperatures are widely reported, the mechanism underlying immune regulation by temperature is seldom reported in crustacean.

Ras proteins are a conservative GTPases family which plays a role in initiating signaling transduction with their active GTP-bound forms interacting with downstream protein factors. M-Ras is a member of Ras family and widely expressed in various tissues [19–21]. It promotes cell survival, neurite outgrowth and differentiation [22,23], is involved in osteogenesis and transdifferentiation [24], induces lymphocyte adhesion [25] and regulates phagosome formation in macrophages [26]. In addition, R-Ras3/(M-Ras) identified from chick was reported to be involved in thermal adaption [27]. It is induced by both high (37.5 °C) and low (16 °C) temperature in chick, playing crucial roles in thermal adaption of chick [27]. In the present study, we also identified crayfish *Ras* gene, a homologue of chick R-Ras3/(M-Ras), from several candidate genes which are homologous to reported heat-responsive genes in other species and found that it is involved in bacterial immunity of crayfish at high temperature. Our results suggest that this crayfish *Ras* gene is a component of the mechanism underlying immune regulation mediated by temperature in crayfish.

2. Materials and methods

2.1. Experimental animals

Red swamp crayfish (*Procambarus clarkii*) (12–14 g body weight) were purchased from local market in Qingdao of China and kept in aquatic tanks with tap water of 24 ± 1 °C to acclimate for a week under constant aeration. The crayfish were fed three times a week with heat-killed clam. To exclude the difference resulted from sexes, only male animals were used.

2.2. Temperature treatment and bacterial infection

Crayfish were acclimated at 24 °C for a week and then were divided into four groups for treatment with four different temperatures: 16 °C, 24 °C, 32 °C and 38 °C, respectively. The survival rate was recorded every 12 h for 96 h.

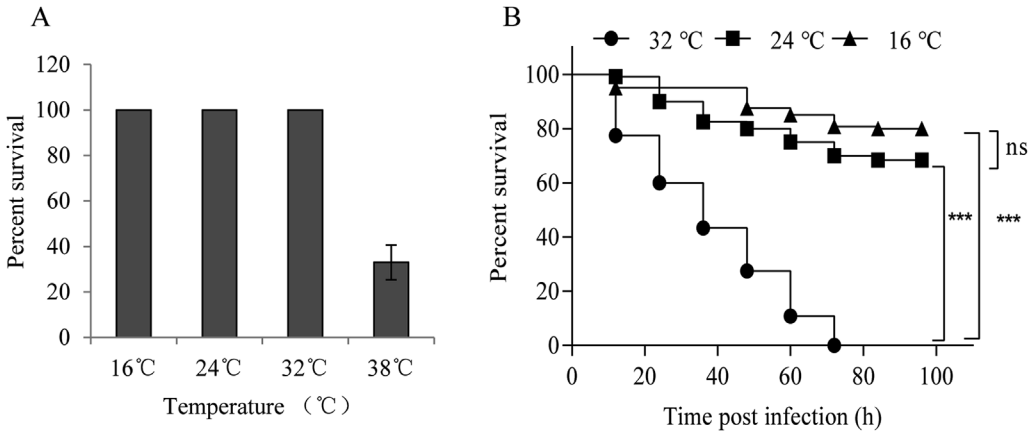

Fig. 1. Survival rate of crayfish at different temperataures. (A) Percent survival of crayfish after treatment with different temperatures for 96 h. The survival was calculated at 96 h. (B) Kaplan-Meier curves of crayfish challenged with *A. hydrophila* at different temperatures. Three animal groups were exposed to 16 °C, 24 °C and 32 °C, respectively, for a week and then challenged by injection with *A. hydrophila* and the survival was recorded every 12 h for 96 h. Statatical significance was determined by log-rank (Mantel-Cox) using GraphPad Prism 7 (***$p < 0.0001$; ns, not significant).

For bacterial infection, animals in each group were exposed to three different temperatures: 16 °C, 24 °C and 32 °C for a week and then challenged by injection with 1.0×10^5 CFU mL^{-1} of *Aeromonas hydrophila*. In each group, forty individuals were challenged and dead animals were counted every 12 h for 96 h. The experiment was repeated three times. Kaplan-Meier curves are shown and significance was determined by log-rank (Mantel-Cox) using GraphPad Prism.

2.3. Gene cloning and sequence analysis

Total RNA was isolated from hemocytes of red swamp crayfish using RNAiso plus regent (TaKaRa, China), and reversely transcripted into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, China) according to user's manual. With R-RAS3 as query to search against NCBI transcriptome shotgun assembly (TSA) database using tBLASTn algorithm, four sequences with high identity to R-RAS3 were chosen for further analysis. The four sequences were subjected to FGENESH analysis to predict CDS. Primers for cloning of the genes were designed according to obtained CDS (Table 1). These genes were cloned and confirmed by sequencing. Structural analysis of proteins was performed with the Expert Protein Analysis System (http://www.expasy.org/) and Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de). The same procedure was carried out to obtain homologous sequences of the thermal-responsive genes.

2.4. Quantitation of gene expression

The gene expressions were quantified using quantitative real time PCR (qRT-PCR). The qRT-PCR was performed according to previously reported method with slight modification [28]. Briefly, the haemolymph was collected at different time points after being exposed to different temperatures or bacterial infection and the hemocytes were harvested by centrifuge at 3000 × *g* for RNA extraction and cDNA synthesis. Total RNA extraction and cDNA synthesis was executed as described above, and 5 × diluted cDNA (1 μL for each PCR reaction) was used as template for PCR. The primers used for qRT-PCR are listed in Table 1. Quantitative real-time PCR were performed using TB Green™ Fast qPCR Mix (Takara, China) on LightCycler®480 system (Roche, Switzerland) following user manual. Thermal cycles were set up as follows: predenature 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. To determine the specificity of PCR amplification, an additional procedure was set up to obtain melting curve: 95 °C for 5 s, 65 °C for 1 min, and 97 °C continuous. Relative expression of genes was calculated using modified $2^{-\Delta\Delta CT}$ method and expressed as mean ± S.D. Amplification efficiency of all genes was measured using the same procedure with 10-fold serial diluted cDNA as template. The obtained data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey's test. Statistical analysis was performed with *SPSS11.0 software*.

2.5. In vivo PcRAS1 silencing

PcRAS1 silencing was carried out using RNA interference according to previously reported methods with slight modification [29]. Double strand RNAs (dsRNA) were generated by *in vitro* transcription according to previously reported method. To knockdown *PcRAS1* gene, healthy crayfish were injected with *PcRAS1*-specific dsRNA (10 μg g^{-1}) twice at 12 h interval. The animals were kept at 24 °C for recovery of 12 h after second injection and then divided into three groups. Two groups were transferred to 32 °C and 16 °C, respectively, the other one was maintained at 24 °C. Gene silencing was confirmed by RT-PCR 24–120 h after first injection of dsRNA. The same procedure was performed in control animals which were injected with GFP-specific dsRNA or CFS. The primers used in RNA interference were listed in Table 1.

2.6. Assay of phenoloxidase (PO; EC 1.14.18.1) activity

PO activity was analyzed according previously reported method. The haemolymph harvested from five crayfish was mixed together and used as a sample for PO assay, and the assay was repeated three times. PO activity was determined by measuring the generation of dopachrome conversed from L-3, 4-dihydroxyphenylalanine (L-dopa). Briefly, haemolymph (50 μL) and L-dopa (50 μL, 3 g L^{-1}) in PBS (0.1 M, pH7.0) were mixed in 24-well plates and incubated for 20 min at room temperature. Change of absorbance at 490 nm for initial 10 min was recorded using Multiskan MK3. Excessive substrate was used to saturate enzyme activity. The Linearity of dopachrome formation from L-dopa in 50 μL of haemolymph was demonstrated up to at least 13.5 min of incubation time. PO activity was recorded as the maximum change in absorbance over any 1 min interval (OD490 nm min^{-1}). PO activity was expressed as relative value to total protein content (ΔA490/mg protein/min). Total protein content was determined based on Bradford method using bovine serum albumin as standard. The obtained data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey's test. Statistical analysis was performed with SPSS11.0 software.

2.7. Measurements of lipid peroxidation and total antioxidant capacity

Crayfish were kept at 24 °C for at least one week, and then some of them were transferred to 32 °C or 16 °C. The other animals were kept at 24 °C and used as control. For *PcRAS1*-silencing, crayfish were injected with dsRNA twice with an interval of 12 h at 24 °C and transferred to 32 °C or 16 °C 24 h after first injection. The hemocytes were collected at 0 h, 12 h, 24 h and 48 h after transfer. Collected hemocytes were homogenized in 100 μL of deionized water and centrifuged for 15 min at

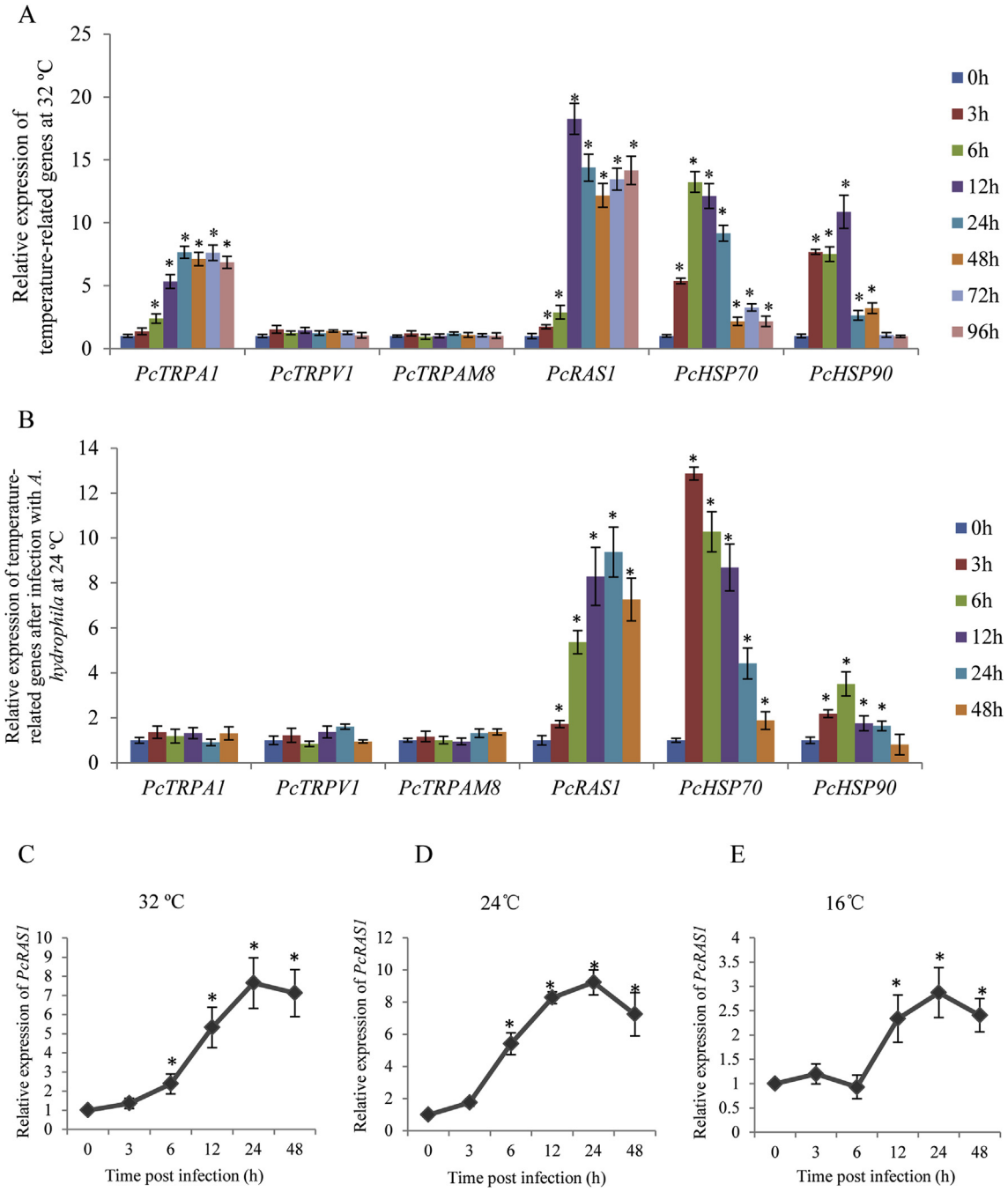

Fig. 2. Expression of temperature-related genes at different temperatures or induced by bacterial infection as revealed by quantitative real time PCR. (A) Expression of temperature-related genes at high temperature of 32 °C; (B) Expression of temperature-related genes induced by bacterial infection at 24 °C; (C–E) *PcRAS1* expression induced by *A. hydrophila* infection at different temperatures. The experiments were performed three times and at least 5 animals were examined in each group at each time points. The data were subjected to one-way ANVOA analysis followed by Tukey's test. Asterisks indicate significantly difference at $P < 0.05$ compared to the expression value at 0 h.

7200 × *g*. The supernatant was placed on ice until being used. Measurements of lipid peroxidation (LPO) and total antioxidant capacity (TAC) were performed using the method described by Coggins et al. [12]. The obtained data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey's test. Statistical analysis was performed with SPSS11.0 software.

3. Results

3.1. High temperature reduces the survival of crayfish infected with *A. hydrophila*

Crayfish maintained at 16 °C, 24 °C and 32 °C survived 100% and crayfish activity did not show visible difference at the three temperatures. Death of crayfish occurred only in the 38 °C group with 67.67% mortality (Fig. 1A). After being infected with *A. hydrophila*, the crayfish exposed to 32 °C demonstrated significantly lower survival than those exposed to 24 °C or 16 °C. All animals in 32 °C group were dead 72 h

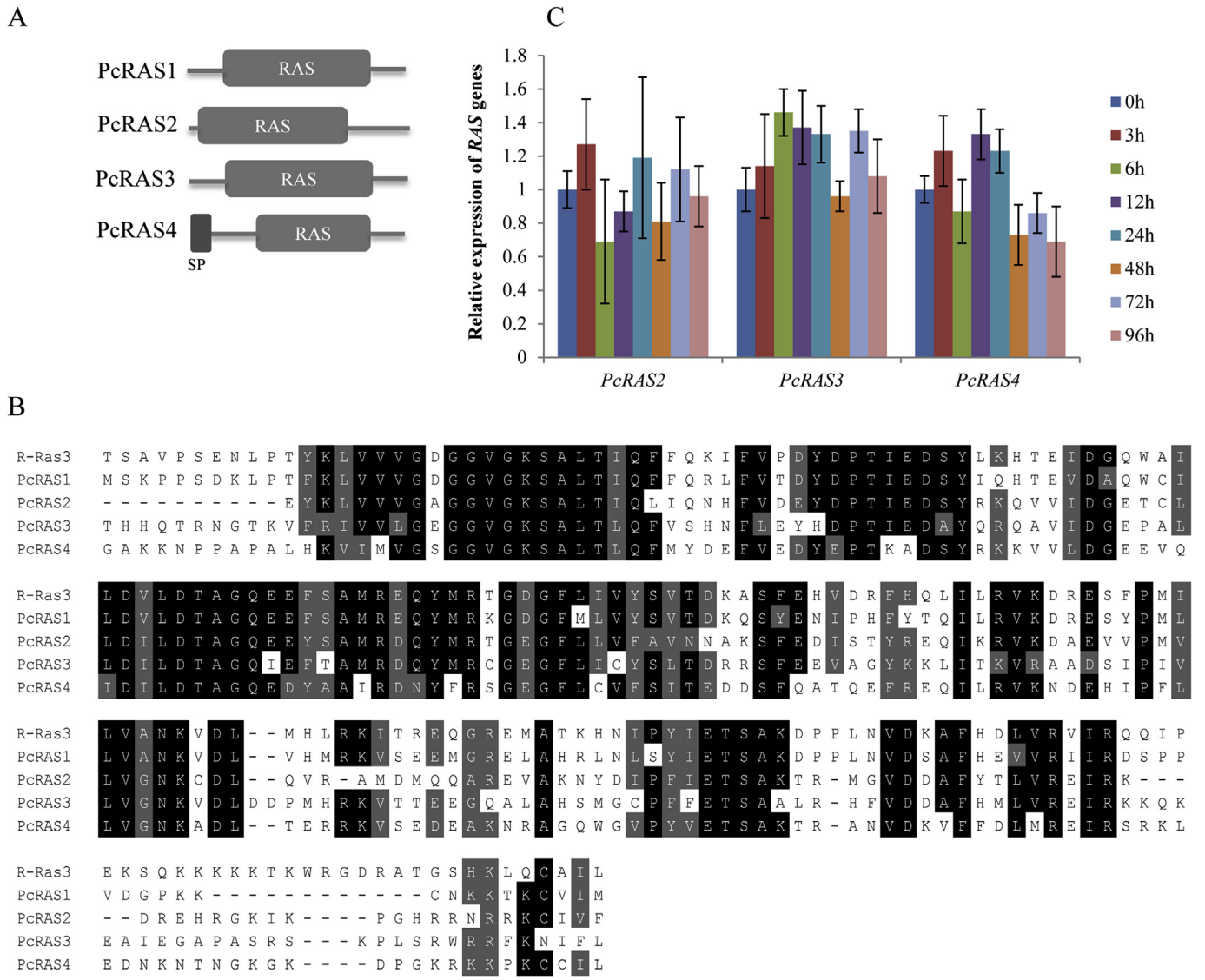

Fig. 3. Structure and homology of crayfish RAS proteins. A. Domain components of RAS proteins; B. Multiple alignment of RAS proteins. White letters with black background indicate identical amino acids and letters with gray background indicate similar amino acids; C. Expression of *Ras* genes in response to high temperature of 32 °C. The experiments were performed three times and at least 5 animals were examined in each group at each time points.

after infection with *A. hydrophila,* whereas 24 °C and 16 °C groups exhibited 68.33% and 80% survival rates, respectively, 96 h after infection. Survival difference between 24 °C and 16 °C groups was much smaller than that between 24 °C and 32 °C groups and the difference was not statistically significant (Fig. 1B). The results indicate that high temperature reduces the resistance of crayfish to *A. hydrophila*.

3.2. *PcRAS1 is induced by both high temperature and bacterial infection*

To explore genes related to immune defense under heat stress, we investigated the expression changes of several temperature-related genes that were reported in other species, including *HSP70* [30], *HSP90* [30] and the homologues of *TRPA1* [31,32], *TRPV1* [33], *TRPAM8* [34,35] and *R-Ras3/(M-Ras)* [27] (designated as *PcTRPA1*, *PcTRPV1, PcTRPAM8* and *PcRAS1,* respectively). We obtained the partial CDS sequences of these genes from TSA database of *P. clarkii* by homology search using TBLASTN algorithm. Primers were designed based on obtained sequences and used for quantitative real time PCR (Table 1). We first examined the effect of temperature on the expression of these genes. Among six genes examined here, four of them (*PcRAS1*, *PcHSP70*, *PcHSP90 and PcTRPA1*) were significantly induced by enhanced temperature from 24 °C to 32 °C (Fig. 2A). Gene expressions in response to bacterial infection were also examined at 24 °C and three genes (including *PcRAS1*, *PcHSP70* and *PcHSP90*) were found to exhibit significant alternation in response to bacterial infection (Fig. 2B). These results suggest that *PcRAS1*, *PcHSP70* and *PcHSP90* are involved in crayfish responses to both high temperature and bacterial infection. Among the three genes, *PcRAS1* was seldom reported in crustacean. We next focused on *PcRAS1* to investigate its temporal expression induced by bacterial infection at different temperatures. The peak values of *PcRAS1* expression were observed 24 h after inoculation with *A. hydrophila* at all three temperatures (Fig. 2C–E). The larger changes were observed at 32 °C and 24 °C. *PcRAS1* expressions were enhanced to 7.65 folds and 9.23 folds, respectively. However, relative moderate alternation (2.87 folds) was observed at 16 °C after challenged with *A. hydrophila* (Fig. 2C–E). These results suggest that *PcRAS1* induction by *A. hydrophila* is associated with temperature.

3.3. *Other RAS genes of crayfish tested here are not induced by high temperature*

To test whether other *RAS* genes of *P. clarkii* are also induced by

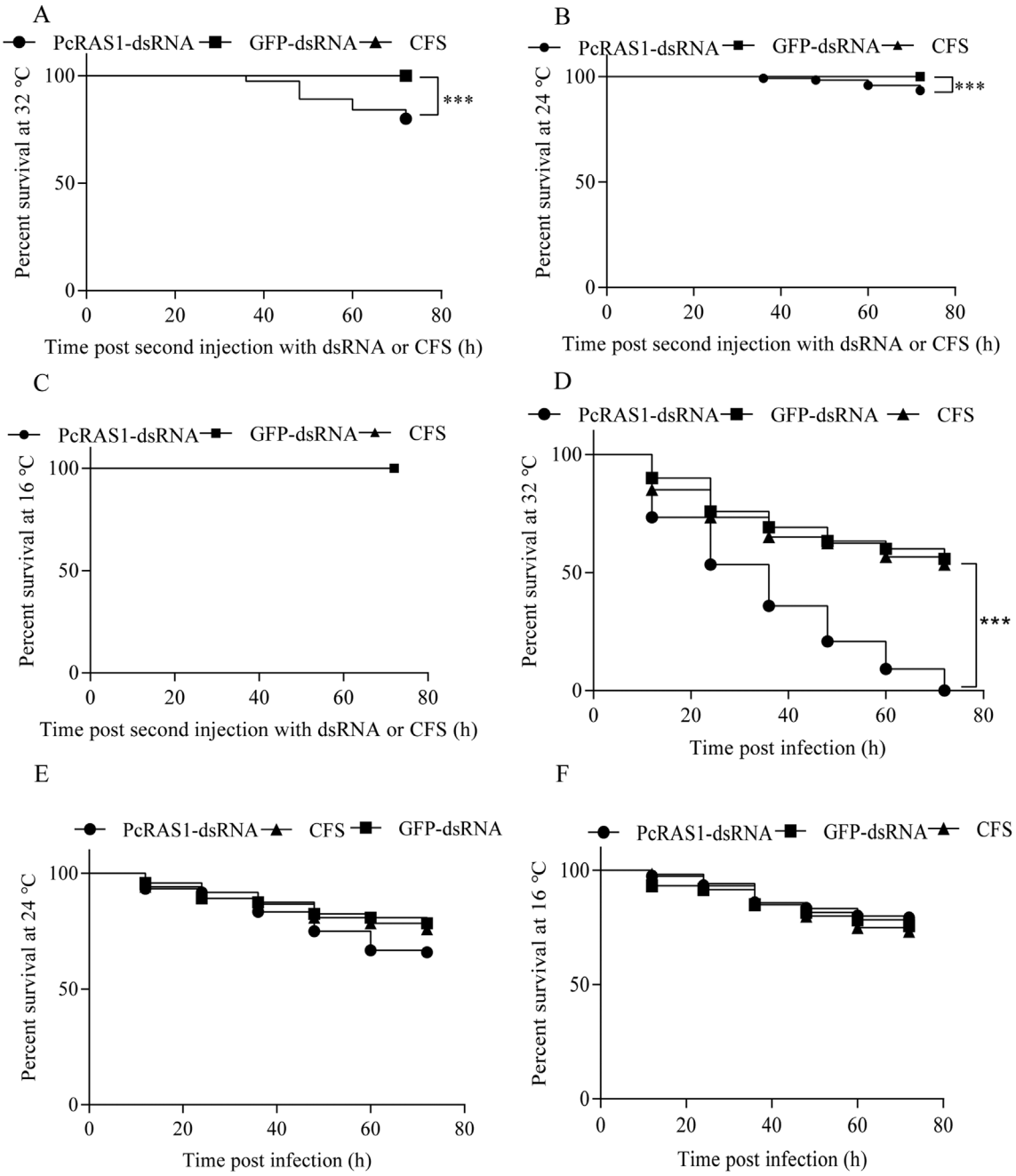

Fig. 4. The effect of *PcRAS1* gene on crayfish responses to temperature change and bacterial infection. (A–C) Kaplan-Meier survival curves exhibiting *PcRAS1* effect on crayfish survival at different temperatures. (D–F) Effects of *PcRAS1* silencing on crayfish survival after being infected with *A. hydrophila* at different temperatures. Three animal groups were exposed to 16 °C, 24 °C and 32 °C, respectively, and injected *PcRAS1*-dsRNA twice with 12 h interval and then challenged with *A. hydrophila* 24 h after first dsRNA injection. The survival was counted every 12 h for 72 h. Statatical significance was determined by log-rank (Mantel-Cox) using GraphPad Prism 7 (***$p < 0.0001$).

heat, we cloned the full length of *PcRAS1* and other three homologous genes of *R-RAS3* from hemocytes. The full-length cDNAs of the four genes contain open reading frame (ORF) ranging from 564 bp to 984 bp encoding proteins of 187–327 amino acids. All of the four proteins contain a RAS domain as revealed by SMART analysis (Fig. 3A) and they were therefore referred as *PcRAS1-4*, respectively. Multiple alignment shows that the proteins encoded by *PcRAS1-4* share identity with chick R-RAS3 ranging from 28.1% to 54.7% (Fig. 3B). Among the four proteins, PcRAS1 shares the highest identity (54.7%) with chick R-RAS3. The expressions of *PcRAS2-4* genes were also examined at different temperatures. The three *PcRASs* did not demonstrate significant expression changes in crayfish transferred from 24 °C to 32 °C (Fig. 3C), suggesting that *PcRAS1*, different from other members of Ras family, plays an important role in response to heat stress.

3.4. Knock-down of PcRAS1 reduces the survival of crayfish under high temperature

To further understand the role of *PcRAS1* in the responses of crayfish to heat stress, *PcRAS1* gene was knocked down using dsRNA mediated RNAi technology and then the survival of crayfish was calculated under various conditions. Firstly, 24 °C group and 32 °C group showed 8.32% and 22.5% cumulative mortality, respectively, until 72 h after the animals were exposed to their respective temperatures (Fig. 4

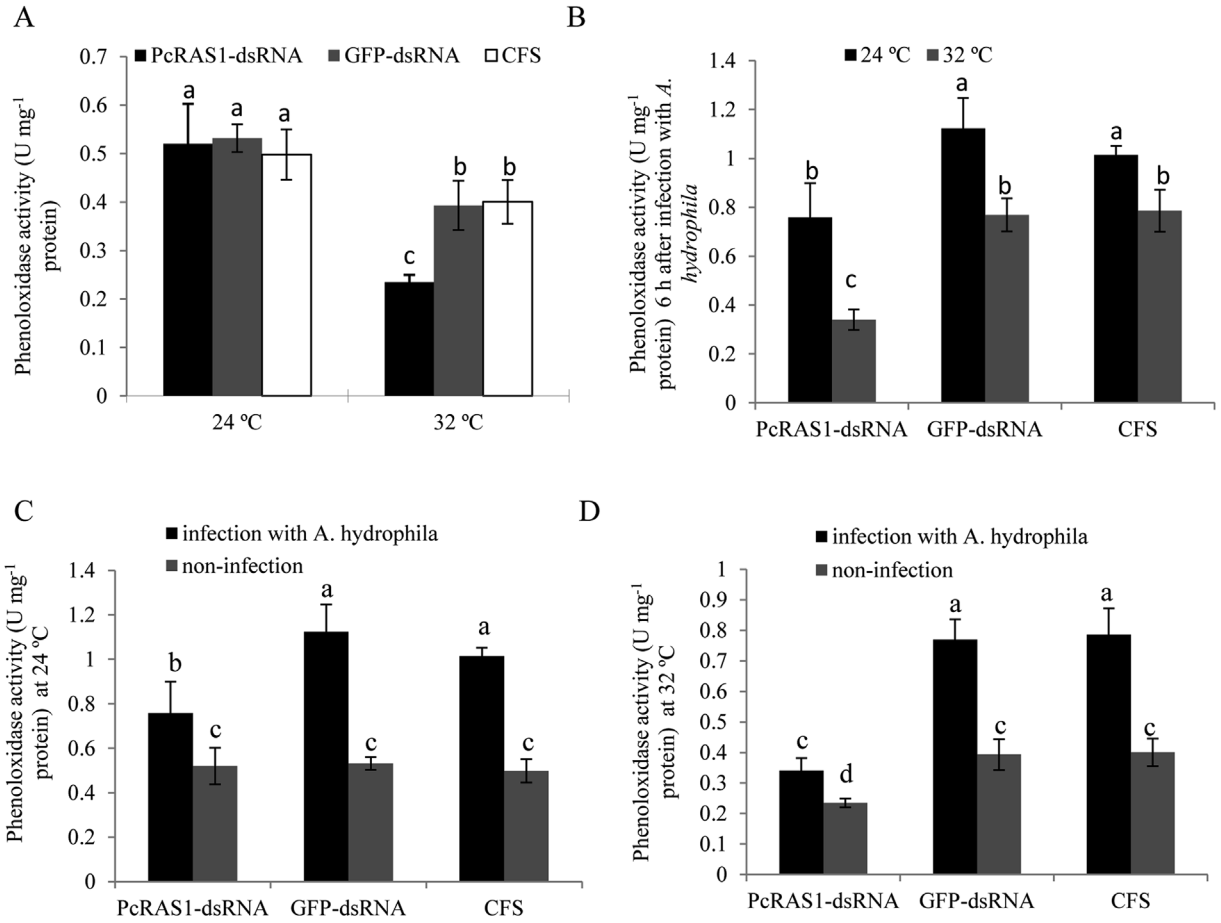

Fig. 5. Effect of *PcRAS1* gene on PO activity. A. PO activity at different temperatures. B. PO activity after bacterial infection. C-D. The effect of *PcRAS1* silencing on PO activity of crayfish infected by *A. hydrophila* at different temperatures. PO activities were measured at 6 h after the animals were transferred to 32 °C or infected with *A. hydrophila.* Bacterial infection at 32 °C was performed 12 h after transfer from 24 °C. Five biological replicates were set up for each group and each replicate is the mixture of the sample from 5 individuals. Different letters indicate significant difference ($P < 0.05$).

A, B). However, *PcRAS1* knock-down did not result in any death of crayfish at 16 °C (Fig. 4C). This result indicates that *PcRAS1* is required for crayfish to tolerate high temperature. After being infected with *A. hydrophila*, *PcRAS1*-silencing crayfish exhibited significantly increased mortality at 32 °C compared with control animals. At 32 °C, animals injected with *PcRAS1*-dsRNA demonstrated 100% mortality at 72 h after bacterial infection which is significantly higher than that observed in animals injected with GFP-dsRNA (55.83%) and CFS (53.33%). By contrast, at 16 °C or 24 °C, *PcRAS1*-silencing crayfish did not demonstrate significantly different mortality from that of control animals after infection with *A. hydrophila*. These results indicate that *PcRAS1* is not only related with heat tolerance but also associated with immune responses of crayfish under heat stress.

3.5. Knock-down of PcRAS1 influences PO activity in response to high temperature and infection of A. hydrophila

No significant difference of PO activity was observed among animals injected with *PcRAS1*-dsRNA, GFP-dsRNA and CFS under non-infected condition at 24 °C (Fig. 5A). PO activity of crayfish at 32 °C was significantly lower than that at 24 °C under the condition of both infection and non-infection (Fig. 5A and B). *PcRAS1*-silencing animals exhibited further reduced PO activity compared with GFP-dsRNA or CFS-injected animals at 32 °C (Fig. 5A), indicating that *PcRAS1*-silencing regulates PO activity at high temperature. Obviously, bacterial infection resulted in significantly enhanced PO activity at both 24 °C and 32 °C (Fig. 5C and D). However, the enhanced PO activity by bacterial infection was compromised by *PcRAS1* silencing. After being infected with *A. hydrophila*, *PcRAS1*-silencing crayfish demonstrated significantly lower PO activity than control animals which were injected with CFS or GFP-dsRNA (Fig. 5B–D) at 24 °C or 32 °C. Although *PcRAS1* silencing compromised PO activity responding to infection at 24 °C, the alteration was relative moderate compared with that at 32 °C (Fig. 5B–D).

3.6. PcRAS1 silencing reduces TAC under heat stress

TAC and LPO were measured to examine the effect of *PcRAS1* on crayfish immunity and heat acclimation. TAC of crayfish was significantly reduced when the animals were transferred from 24 °C to 32 °C. The same effect was not found at 16 °C (Fig. 6A). On the contrary, LPO of hemocyte is significantly enhanced by both high temperature (32 °C) and low temperature (16 °C). LPO of hemocytes at 12, 24, 48 h were significantly higher than that measured at 0 h in crayfish transferred from 24 °C to 32 °C or 16 °C (Fig. 6B).

Next, we tested if *PcRAS1* silencing could alter TAC or LPO level. The crayfish exposed to 32 °C or 16 °C for 24 h were used for measurement of TCA and LPO. TCA was further decreased in *PcRAS1*-silencing animals at 32 °C compared with control animals which were injected with CFS and GFP-dsRNA, but no further TAC change was found in *PcRAS1*-silencing animals at 16 °C (Fig. 6C). By contrast, *PcRAS1*-silencing did not influence LPO at 32 °C or 16 °C. These results indicate that TAC is regulated by *PcRAS1* expression, whereas LPO is not. (Fig. 6D).

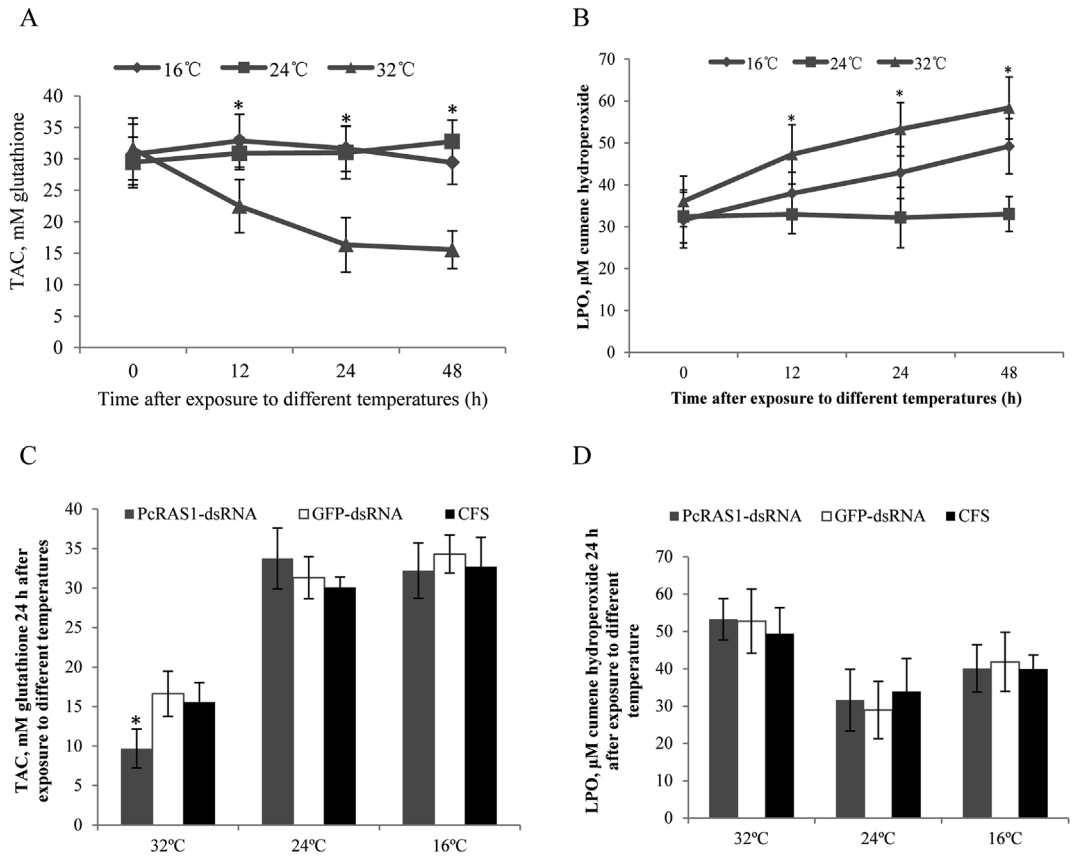

Fig. 6. Total antioxidant capacity (A, C) and Lipid peroxidation (B, D) in *P. clarkii* 24 h after exposed to different temperatures. LPO values were calculated as cumene hydroperoxide molar units and TAC were calculated as glutathione molar units according to the method described by Coggins et al. (2017). Five biological replicates were set up for each group and each replicate was the mixture of the sample from 5 individuals. Asterisks indicate significantly difference at $P < 0.05$.

4. Discussion

It is generally assumed that a temperature significantly higher or lower than optimum temperature may damage the immune defense of crustacean against bacterial or viral pathogens. This point was supported by some earlier studies that temperature changes resulted in increased susceptibility to bacterial or viral pathogens [4,6–8]. Here, red swamp crayfish *P. clarkii* exposed to higher temperature (32 °C) but not lower temperature (16 °C) showed higher susceptibility to *A. hydrophila*. These results further present evidences that temperature is an important factor in pathogen infection to crustacean.

The involvement of R-Ras3/(M-Ras) in temperature acclimation were reported in previous study [27], but no crustacean R-Ras3/(M-Ras) or crustacean other Ras genes were reported on their roles in temperature acclimation. Here, we show *PcRAS1* is also induced by heat stress like chick R-Ras3, suggesting that *PcRAS1* gene is involved in thermoregulation of crayfish. Not all Ras gene of crayfish are induced by high temperature (Fig. 3C). Among four Ras genes of crayfish examined here, only *PcRAS1* exhibited high expression at high temperature, suggesting that *PcRAS1* is specific for responding to heat stress. Different from chick *R-Ras3*, *PcRAS1* was not induced by cold stress, suggesting that *PcRAS1* exhibited different function from that of chick *R-Ras3*. *PcRAS1* silencing caused death of some crayfish under the condition of heat stress (Fig. 4), which further confirmed that *PcRAS1* is required for crayfish to tolerate high temperature. Further decreased survival from bacterial infection under heat stress in *PcRAS1*-silencing animals indicates that *PcRAS1* silencing reduces the defense responses of crayfish.

The mechanism underlying various biological roles of R-Ras3/M-ras was reported in several researches. It promotes cell survival by activating Akt in a PI3 kinase-dependent manner in PC12 cells [23] and promotes PC12 differentiation through the MAPK pathway, but it cannot efficiently activate MAPK pathway in fibroblasts [22]. In human breast cancer cells, R-Ras3/M-RAS induces MEK/ERK-dependent and -independent Elk1 activation as well as PI3K/Akt and JNK/cJun activation [36]. In chick, R-Ras3/(M-Ras) is involved in thermal adaptation by activating Jun and further inducing expression of thermal adaptation-related genes [27]. M-Ras also promotes phagocytosis by regulating phagosome formation through FcγR-mediated phagocytic pathway [26] and induces lymphocyte function-associated antigen 1 (LFA-1)-mediated cell aggregation [25]. In the present study, we found that *PcRAS1* expression regulates PO activity and TAC, suggesting PO and TAC are involved in *PcRAS1*-mediated immune regulation under high temperature. However, the signaling pathway involved remains unclear and needs to be further investigated.

PO is important for crayfish immunity. We found that enhanced temperature resulted in decreased PO activity, which is consistent to previous observation in other species [7,37]. This suggests that the effect of temperature changes on PO activity is conservative across species. PO plays important role in melanin synthesis. In some insect species, PO activity is decreased at high temperature so that animals can reduce melanin accumulation to decrease absorption of heat [37]. Low temperature results in increased melanin level which needs enhanced PO activity, whereas PO activity was also important to invertebrate immunity. Enhanced PO activity contributes to increased pathogen resistance [38–40]. Thus PO may play crucial role in connection between heat acclimation and immunity. Our results show that PO activity is changed by *PcRAS1* silencing. Since PO activity is required for survival of freshwater crayfish from bacterial infection [41], we therefore reasonably infer that the decreased survival of infected crayfish under heat stress is related to reduction of PO activity resulting from *PcRAS1* silencing. However, *PcRAS1* silencing also compromised

the increased PO activity resulting from bacterial infection at 24 °C, which cannot explain why *PcRAS1* silencing did not result in significantly different mortality. This result suggests that some other factors are involved in *PcRAS1*-medicated responses to high temperature and bacterial infection.

Antioxidant capacity and lipid peroxidation are routinely used as biomarkers of environmental stress in a variety of organisms. As predicted, TAC and LPO in crayfish were significantly altered at 32 °C compared with that at 24 °C, indicating that 32 °C resulted in heat stress to red swamp crayfish. Different from high temperature, low temperature of 16 °C did not result in TAC change. However, LPO was significantly enhanced by low temperature of 16 °C compared with that at 24 °C, although its level was far lower than that at 32 °C. These results suggest that damage resulted from low temperature of 16 °C is less than that resulted from high temperature of 32 °C. TAC but not LPO at high temperature was affected by *PcRAS1* silencing, further confirming that *PcRAS1* is involved in adaption of crayfish to high temperature but not low temperature.

Taken together, we present new evidences to confirm that temperature is an important environmental factor affecting disease resistance of crayfish. PO activity, TAC and LPO are influenced by temperature change. *PcRAS1* was involved in temperature-mediated immune regulation by exerting influence on PO activity and TAC.

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