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Na^+/K^+-ATPase α-subunit in swimming crab *Portunus trituberculatus*: molecular cloning, characterization, and expression under low salinity stress*

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Abstract Na^+/K^+-ATPases are membrane-associated enzymes responsible for the active transport of Na^+ and K^+ ions across cell membranes, generating chemical and electrical gradients. These enzymes' α-subunit provides catalytic function, binding and hydrolyzing ATP, and itself becoming phosphorylated during the transport cycle. In this study, Na^+/K^+-ATPase α-subunit cDNA was cloned from gill tissue of the swimming crab *Portunus trituberculatus* by reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end methods. Analysis of the nucleotide sequence revealed that the cDNA had a full-length of 3 833 base pairs (bp), with an open reading frame of 3 120 bp, 5' untranslated region (UTR) of 317 bp, and 3' UTR of 396 bp. The sequence encoded a 1 039 amino acid protein with a predicted molecular weight of 115.57 kDa and with estimated pI of 5.21. It was predicted here to possess all expected features of Na^+/K^+-ATPase members, including eight transmembrane domains, putative ATP-binding site, and phosphorylation site. Comparison of amino acid sequences showed that the *P. trituberculatus* α-subunit possessed an overall identity of 75%–99% to that of other organisms. Phylogenetic analysis revealed that this α-subunit was in the same category as those of crustaceans. Quantitative real-time RT-PCR analysis indicated that this α-subunit's transcript were most highly expressed in gill and lowest in muscle. RT-PCR analysis also revealed that α-subunit expression in crab gill decreased after 2 and 6 h, but increased after 12, 24, 48, and 72 h. In addition, α-subunit expression in hepatopancreas of crab decreased after 2–72 h. These facts indicated that the crab's Na^+/K^+-ATPase α-subunit was potentially involved in the observed acute response to low salinity stress.

Keyword: cloning; expression; Na^+/K^+-ATPase; α-subunit; *Portunus trituberculatus*; salinity

1 INTRODUCTION

The swimming crab, *Portunus trituberculatus*, is a euryhaline species widely distributed in the coastal waters of Asia-Pacific countries and has become an important commercial species in these countries. In China, farming of *P. trituberculatus* has been under development in recent years, reaching the highest yield to date at 100 000 tons in 2008 (Liu and Liu, 2008). However, because of pollution in coastal waters and endemic crab diseases, *P. trituberculatus* has become an attractive culture species for inland low-salinity farming. Although *P. trituberculatus* has a tolerance to a wide range of salinity, from 15.0 to 35.0 (Lu et al., 2012), ambient salinity exerts great influence on its physiological response and growth performance (Kinne, 1971).

When *P. trituberculatus* is transferred to diluted seawater, its osmoregulatory cells must actively

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absorb Na^+ from its surroundings to counteract passive Na^+ loss. These ions are actively pumped into the hemolymph through gills and other transporting tissues by Na^+/K^+-ATPase. This transport enzyme is considered to play a crucial role in ion regulation and cellular water balance maintenance in many aquatic animals, including crustaceans (Geering, 1990; Horisberger et al., 1991). Na^+/K^+-ATPase belongs to the P-type ATPase family, which are membrane-associated enzymes responsible for establishing and maintaining intracellular ion concentration, using ATP as its energy source (Fagan and Saier, 1994; Axelsen and Palmgren, 1998; Palmgren and Axelsen, 1998; Willms et al., 2004). Normally, osmoregulation capacity of aquatic animals is significantly correlated to Na^+/K^+-ATPase activity (Towle, 1981). Because of Na^+/K^+-ATPase's importance in aquatic animal osmoregulation, many studies have shown that its enzymatic activity and mRNA transcript concentration in gill change when aquatic animals are transferred to water of different salinities (Towle, 1990; Péqueux, 1995; Huong et al., 2010; Sun et al., 2011). Although different species have different adaptation patterns in response to salinity, many studies have reported that gill Na^+/K^+-ATPase activity increases adaptively when salinity sharply decreases (Towle, 1993; Corotto and Holliday, 1996; Cooper and Morris, 1997; Towle, 1997). Moreover, some studies have indicated that expression of gill Na^+/K^+-ATPase α-subunit mRNA increases significantly in the time period (0–12 h) immediately after transfer to lower salinity conditions (Choi and An, 2008; Sun et al., 2011; Feng et al., 2012). However, studies of Na^+/K^+-ATPase in *P. trituberculatus* to date have only focused on measurement of enzymatic activity (Jiang and Xu, 2011).

Therefore, the goal of the present study was to isolate and characterize the *P. trituberculatus* Na^+/K^+-ATPase α-subunit, compare its sequence with other known Na^+/K^+-ATPase α-subunits, examine expression of this α-subunit in tissues of *P. trituberculatus*, and evaluate α-subunit expression concentrations in gill and hepatopancreas of crabs exposed to low salinity conditions. These results yielded insight into osmoregulatory mechanisms that accompany acclimation of these organisms to salinity change.

2 MATERIAL AND METHOD

2.1 Experimental animals

With animal ethics committee approval in our research institute, the experiment was judged acceptable, and all operating procedures strictly complied with relevant laws and regulations. The seawater used here was obtained from Laizhou Bay. Healthy swimming individuals of *P. trituberculatus*, with a total carapace width 105.31±13.80 mm and weighing 74.06±8.62 g, were obtained from Haifeng Aquiculture Co., Ltd. (Weifang, China) and held in tanks containing aerated sand-filtered seawater (35.0 salinity, pH 8.0) at 25 ±0.5°C for at least 1 wk prior to experimentation. During acclimation, one third of the tank seawater volume was renewed weekly and the crabs fed daily with fresh clams at 10% of the crabs' total body weight.

2.2 Total RNA extraction and reverse transcription

After anesthetization with ice, gill tissue from untreated crabs was collected and immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation. Total RNA was extracted from the tissue under RNase-free conditions using Trizol Reagent (Invitrogen, Life Technologies, Grand Island, NY, USA) according to manufacturer's protocol. Quantity, purity, and integrity of RNA were verified by spectrophotometer (A_{260}/A_{280}) and electrophoresis on 1.0% agarose gels. Contaminant DNA was eliminated using RQ1 RNase-Free DNase (Promega, Corp., Madison, WI, USA). First-stand complementary DNA (cDNA) was synthesized from 2 μg of total RNA by M-MLV First-Strand cDNA Synthesis Kit (Promega, Corp.) according to manufacturer's instructions.

2.3 Polymerase chain reaction (PCR) and cloning of Na^+/K^+-ATPase α-subunit cDNA

Full-length Na^+/K^+-ATPase α-subunit cDNA from *P. trituberculatus* was obtained by reverse transcription polymerase chain reaction (RT-PCR) and the 3' and 5' rapid amplification of cDNA ends (RACE) method. The degenerate primers F1 and R1 (Table 1) were designed based on the highly conserved regions of published Na^+/K^+-ATPase α-subunit sequences from other crabs, including *Callinectes sapidus* (AF327439), *Pachygrapsus marmoratus* (DQ173925, DQ173924), and *Carcinus maenas* (AY035550) (http://www.ncbi.nlm.nih.gov). Primers were designed with the assistance of the program AlignX (Vector NTI Suite, Ver. 10.0, Invitrogen), with an engine based on the CLUSTAL W algorithm (Thompson et al., 1994), and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). PCR reactions were carried out as follows: 5 min initial denaturation

Table 1 Oligonucleotide primers used in this study

Prime name	Sequence (5'→3')	Sequence information
F1	CTGCTGAAGTGTGTGGAACT	Degenerate primer
R1	TACCAGCAGAsCGGCACTTG	Degenerate primer
F2	CTATCCACGAGACCCAGGACAAGAAC	3'-RACE PCR
R2	GACATGAGCCCAACAAACCGAAGACC	5'-RACE PCR
F3	GCCTACACCCTCACCTCCAACATCCC	3'-RACE PCR
R3	GCGGAGGTCGTTCTTGTCCTGGGTCT	5'-RACE PCR
18S-F	AGGAGGAGGTTGAGAAGATTGT	Real-time PCR
18S-R	GCAGCTTGGTTTCCAGGTAG	Real-time PCR
F4	CAAGTGCCGTTCTGCTGGT	Real-time PCR
R4	CTTGATGGGAATGTTGAGTCTTTG	Real-time PCR

at 94°C for one cycle, then 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s, followed by a 10 min extension at 72°C and final cooling to 4°C. Partial cDNA sequences encoding the Na^+/K^+-ATPase α-subunit were obtained with the aforementioned degenerate primers (F1, R1).

Gene-specific primers (F2, F3, R2, and R3, Table 1) were designed to complete the sequencing with the aid of 3' RACE and 5' RACE techniques. The gene's 3' and 5' ends were obtained using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Madison, WI, USA). The 3' end-RACE PCR reaction was performed with cDNA templates from gill RNA using the gene specific primers F2 and F3 as well as Universal Primer (UPM). PCR was performed following a schedule of 94°C for 5 min, five cycles of 94°C for 30 s and 72°C for 3 min, five cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min, and then 25 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 3 min, and finally 72°C for 10 min. For 5' end-RACE PCR, the gene specific primers R2 and R3 as well as UPM were employed and PCR conditions the same as the 3' end-RACE PCR above.

PCR products were separated electrophoretically on 2.0% agarose gel and the target band purified using a PCR purification kit (Promega Corp.) and cloned into pMD18-T vector (TaKaRa, Japan) following manufacturer's instructions. After transforming the purified products into competent cells of *Escherichia coli* DH5α, recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega Corp.) and used as a template for DNA sequencing.

2.4 Sequence analysis

The open reading frame (ORF) of the *P. trituberculatus* Na^+/K^+-ATPase α-subunit nucleotide sequence and its protein molecular mass and isoelectric point (pI) were predicted with the aid of DNASTAR software. The nucleotide and deduced amino acid sequences were analyzed for similarity with other known sequences using the BLAST program at the web server of NCBI http://www.ncbi.nlm.nih.gov/BLAST/; (Altschul et al., 1990). A multiple sequence alignment of Na^+/K^+-ATPase α-subunit amino acid sequences was produced with the Vector NTI Advance 10.3 program (Invitrogen). Phylogenetic analysis was performed using a neighbor-joining method, implemented with MEGA 4.0 software (Tamura et al., 2007). The SMART (http://smart.embl-heidelberg.de/) and PROSITE programs (http://kr.expasy.org/prosite) were used to predict the functional sites or domains in the amino acid sequence. A putative signal peptide and cleavage site in the deduced amino acid sequence were analyzed by Signal P 3.0 program (http://www.cbs.dtu.dk/services/SignalP).

2.5 Salinity stress experiment and sampling

Salinity stress was examined using five salinity treatments of 15.0, 13.0, 11.0, and 9.0 as well as a control group at 35.0. Salinity was adjusted by gradually adding fresh water while monitoring with a salinity meter (YSI Inc., Yellow Springs, OH, USA). A total of 450 healthy crabs were randomly assigned to five groups with three replicates. The crabs were transferred directly from the acclimation tanks to the treatment tanks. Gill and hepatopancreas of three crabs from each replicate were randomly sampled at 0, 2, 6, 12, 24, 48, and 72 h after initiation of low salinity conditions. Collected samples were immediately frozen in liquid nitrogen for subsequent RNA extraction and the extracted total RNA product used for reverse transcription and quantification analysis.

2.6 Quantification of Na^+/K^+-ATPase α-subunit mRNA expression in crabs

Hemolymph was collected from the ventral sinus at the base of a crab's last walking leg into a sterile syringe containing an equal volume of precooled (4°C) anti-coagulant (Bachère et al., 1988). The diluted hemolymph was centrifuged at 800×*g* for 10 min at 4°C to collect hemocytes. The mRNA

expression of Na^+/K^+-ATPase α-subunit was assessed in different tissues, including hemocytes, gill, hepatopancreas, and muscle of untreated crabs. In addition, the temporal expression of this subunit in gill and hepatopancreas of crabs exposed to low salinity were evaluated. Both these goals were accomplished by quantitative real-time PCR, as described by Livak and Schmittgen (2001) and Wong and Medrano (2005) (ABI 7500 real-time PCR system, Applied Biosystems, Inc., Foster City, CA, USA).

The gene-specific primers F4 and R4 (Table 1) were used to amplify a PCR product of 148 base pairs (bp). The 18s-rRNA gene-specific primers 18S-F and 18S-R (Table 1) were designed to amplify a 141-bp fragment as the internal control for quantitative real-time RT-PCR (Harasywych et al., 1997). Nuclease-free water was used instead of cDNA templates as a PCR negative control. RT-PCR amplifications were carried out in triplicate in a total volume of 20 μL containing 10.0-μL 2×SYBR Premix Ex Taq™ II (TaKaRa Bio Inc.), 1 μL of the 1/10 diluted cDNA, 0.8 μL each of 10-μmol/L forward and reverse primer, 0.4 μL of 50×ROX Reference Dye II and 7 μL of DEPC-treated water. The real-time PCR temperature profile for the α-subunit was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Fluorescent real-time PCR data were analyzed using 7500 System SDS Software (Applied Biosystems, Inc.).

2.7 Statistical analysis

All data were processed as mean±standard deviation (S.D.) of the relative mRNA expression. Comparisons between different groups were analyzed by a one-way ANOVA using SPSS 17.0, followed by the Duncans multiple comparison test. The level of statistically significant difference was set at $P<0.05$.

3 RESULT

3.1 Molecular characterization of *P. trituberculatus* Na^+/K^+-ATPase α-subunit

A full-length 3 833-bp Na^+/K^+-ATPase α-subunit of *P. trituberculatus* was obtained by the RT-PCR and RACE procedures, and the full-length nucleotide sequence and deduced amino acid sequence are shown in Fig.1. The sequence contained an open reading frame (ORF) of 3 120 bp that encoded 1 039 amino acids, 317 bp of the 5' untranslated region (UTR), and 396 bp of the 3' UTR. The calculated molecular mass was 115.57 kDa and theoretical isoelectric point (pI) of this protein 5.21. No putative signal-peptide amino acids were present in the Na^+/K^+-ATPase α-subunit sequence. Eight transmembrane domains were predicted by hydrophobicity analysis of the amino acid sequence (Fig.1). The *P. trituberculatus* Na^+/K^+-ATPase α-subunit cDNA sequence has been submitted to the GenBank (GenBank accession number: JX173959).

3.2 Homology analysis of Na^+/K^+-ATPase α-subunit

A BLASTP search revealed that the predicted Na^+/K^+-ATPase α-subunit amino acid sequence of *P. trituberculatus* exhibited high identity with those of *C. sapidus*, *P. marmoratus*, *Exopalaemon carinicauda*, *Homarus americanus*, *Litopenaeus stylirostris*, *Penaeus monodon*, and *Fenneropenaeus indicus* (99%, 97%, 95%, 94%, 94%, 92%, and 93%, respectively; Fig.2).

The evolutionary relationships between the species identified with related Na^+/K^+-ATPase α-subunit amino acid sequences were constructed using the neighbor-joining distance method in MEGA 4.0 (Fig.3). The resulting cladogram revealed the existence of three groups, comprising arthropods, mammals, and osteichthyes, according to their α-subunits. The arthropod group was further divided into three subgroups, crustaceans, insects, and arachnids. The Na^+/K^+-ATPase α-subunit amino acid sequence of *P. trituberculatus* fell in the crustacean group and more closely related to the α-subunit of *C. sapidus* than to *F. indicus* and *P. monodon*. This strongly supported a common evolutionary lineage for these species with this crab based on their α-subunits.

3.3 Tissue distribution of *P. trituberculatus* Na^+/K^+-ATPase α-subunit mRNA

Expression of Na^+/K^+-ATPase α-subunit mRNA in different tissues was assessed by real time quantitative PCR (Fig.4). Na^+/K^+-ATPase α-subunit mRNA transcripts were expressed in gill, which was 20-fold that in muscle ($P<0.05$) and significantly higher than the other tissues ($P<0.05$). The mRNA transcript in hepatopancreas was 12-fold that in muscle ($P<0.05$), with only slight gene expression observed in hemocytes and muscle ($P>0.05$).

3.4 Quantitative analysis of *P. trituberculatus* Na^+/K^+-ATPase α-subunit mRNA expression after low salinity stress

Relative expression concentrations of Na^+/K^+-ATPase α-subunit in gill of *P. trituberculatus* after

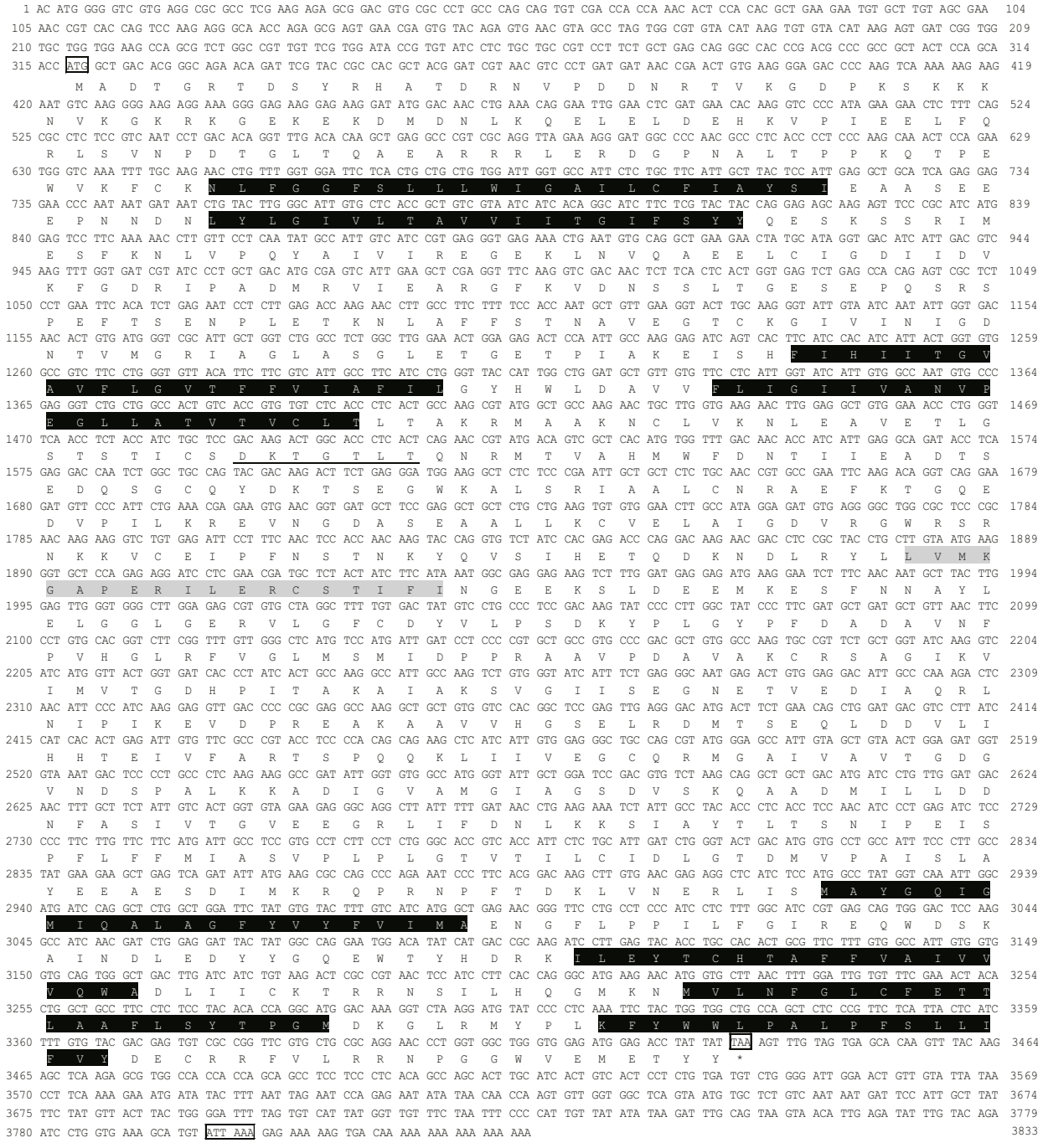

Fig.1 Nucleotide and deduced amino acid sequence of *P. trituberculatus* Na^+/K^+-ATPase α-subunit

Start codon (ATG), stop codon (TAA), and putative polyadenylation-signal sequence (ATTAAA), boxed; transmembrane domains predicted by hydrophobicity analysis, black shadow; likely ATP-binding site, gray shadow; and phosphorylation site, underlined.

low salinity stress are shown in Fig.5. Compared with the control group, concentrations of α-subunit transcripts in the four salinity-challenged groups (15.0, 13.0, 11.0, and 9.0) decreased significantly during the first 6 h after stress initiation (P<0.05). Afterwards, α-subunit expression in the four groups tended to increase significantly and reach their highest concentration at 24 h (P<0.05). Subsequently, transcript expression decreased slightly, again. In the control group, subunit expression exhibited no significant differences over related time periods (P>0.05). Incidentally, a higher crab mortality rate (64.4%) was observed at salinity 9.0.

The relative expression concentration of Na^+/K^+-

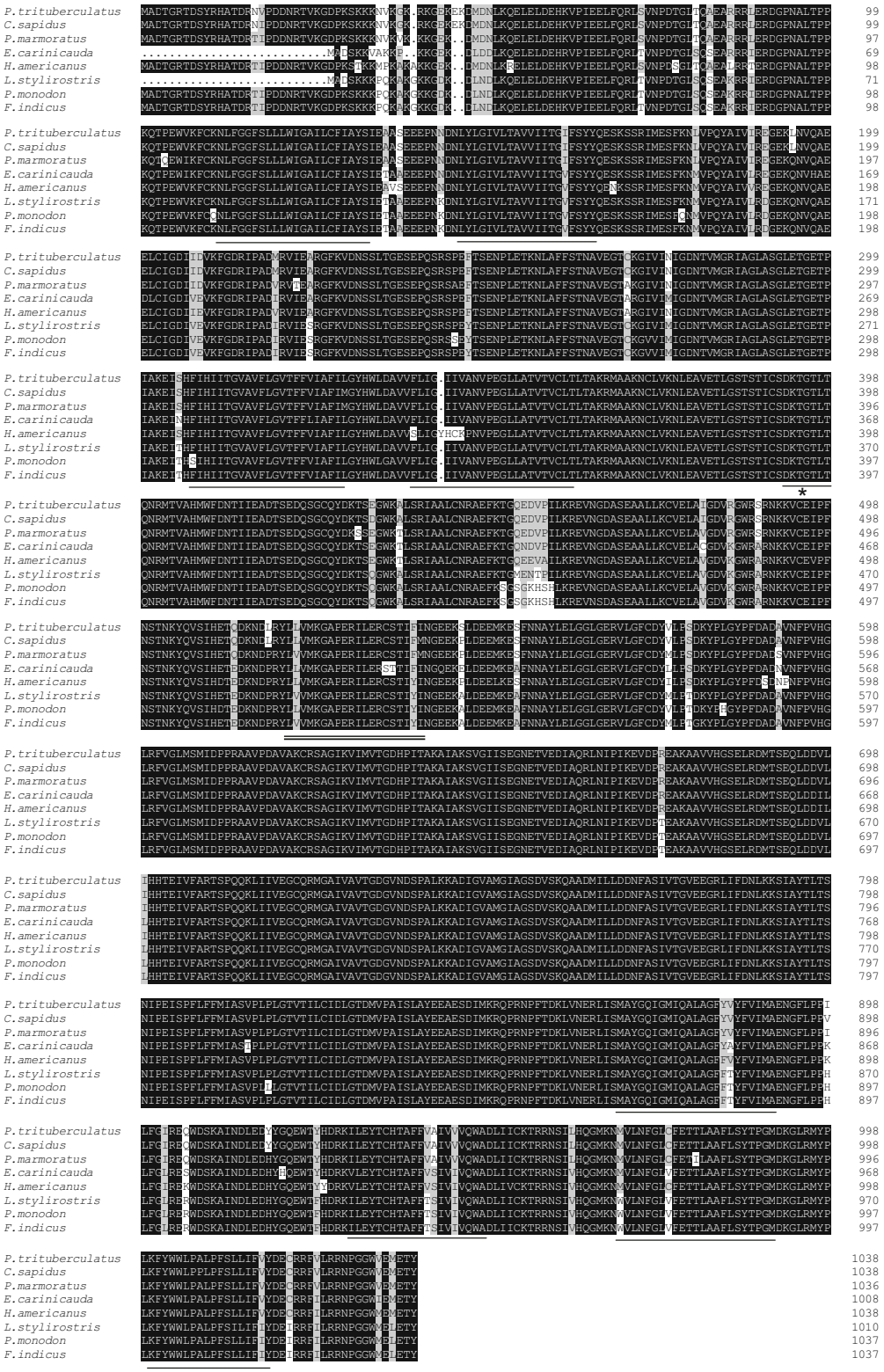

Fig.2 Multiple alignment of the *P. trituberculatus* Na^+/K^+-ATPase α-subunit amino acid sequence with examples from other crustaceans

Other crustaceans: *C. sapidus* (AAG47843), *P. marmoratus* (ABA02166), *E. carinicauda* (AFM54541), *H. americanus* (AAN17736), *L. stylirostris* (AEX07319), *P. monodon* (ABD59803) and *F. indicus* (ADN83843); putative transmembrane domains, solid black lines; likely ATP-binding site, double lines; and phosphorylation site, asterisk (*).

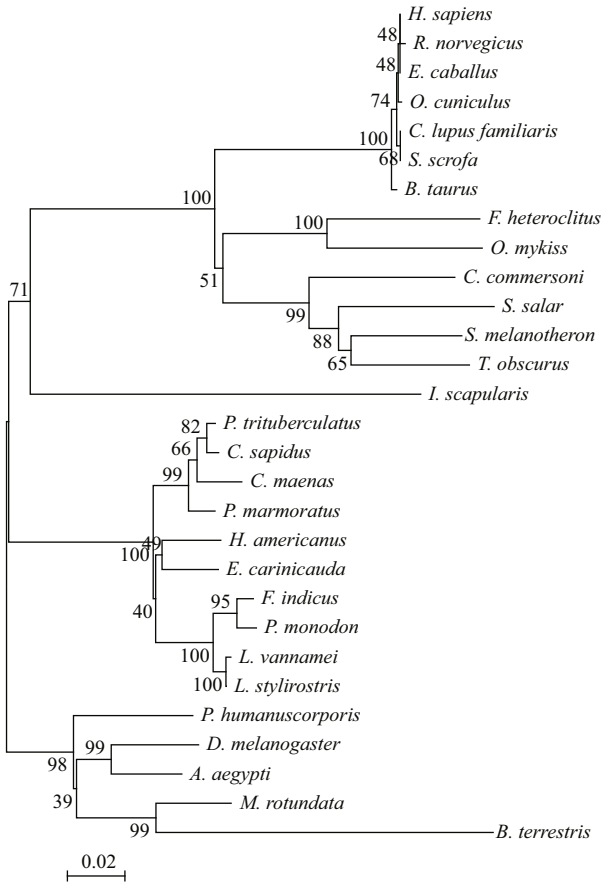

Fig.3 Phylogenetic relationships of Na^+/K^+-ATPase α-subunit on the basis of the amino acid sequence using neighbor-joining distance analysis

Crustacean Na^+/K^+-ATPase α-subunit: *P. trituberculatus*, *C. sapidus* (AAG47843), *P. marmoratus* (ABA02166), *C. maenas* (AAK62046), *H. americanus* (AAN17736), *L. vannamei* (ADM87522), *F. indicus* (ADN83843), *P. monodon* (ABD59803), *E. carinicauda* (AFM54541), and *L. stylirostris* (AEX07319); Arachnida α-subunit: *I. scapularis* (XP_002404061); insect α-subunit: *P. humanuscorporis* (XP_002427714), *D. melanogaster* (NP_996247), *A. aegypti* (XP_001662217), *M. rotundata* (XP_003705422), and *B. terrestris* (XP_003395448); mammal α-subunit: *H. sapiens* (NP_001243143), *R. norvegicus* (P06687), *Equus. Caballus* (XP_001499572), *O. cuniculus* (XP_002724108), *C. lupus familiaris* (XP_855286), *S. scrofa* (NP_001165224), and *B. taurus* (XP_002695120); Osteichthyes α-subunit: *F. heteroclitus* (AAL18003), *O. mykiss* (NP_001117930), *C. commersoni* (P25489), *S. salar* (ACN10460), *S. melanotheron* (ADB03120), and *T. obscurus* (ADD60471).

ATPase α-subunit in hepatopancreas of *P. trituberculatus* after low salinity stress is shown in Fig.6. Expression profiles in hepatopancreas over time after initiation of salinity stress showed distinctly different trends when contrasted with gill. Expression in the four salinity-challenged groups dropped strongly at 2 h post-stress initiation (P<0.05) and were lower than the control group until termination of the experiment. In the control group, no significant change in subunit expression was detected at these times (P>0.05).

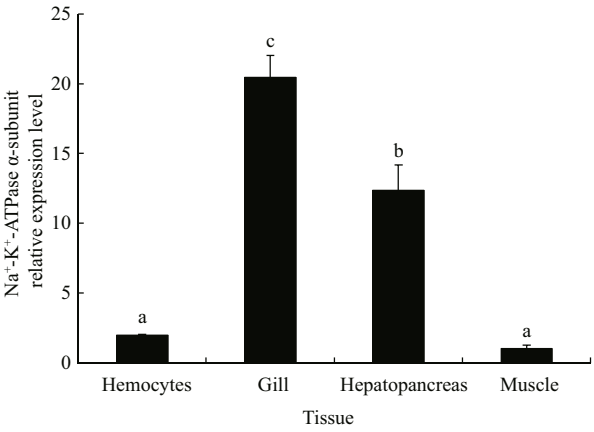

Fig.4 Tissue distribution of Na^+/K^+-ATPase α-subunit mRNA in *P. trituberculatus*

Vertical bars, means±S.D. and bars with different letters significantly different (P<0.05).

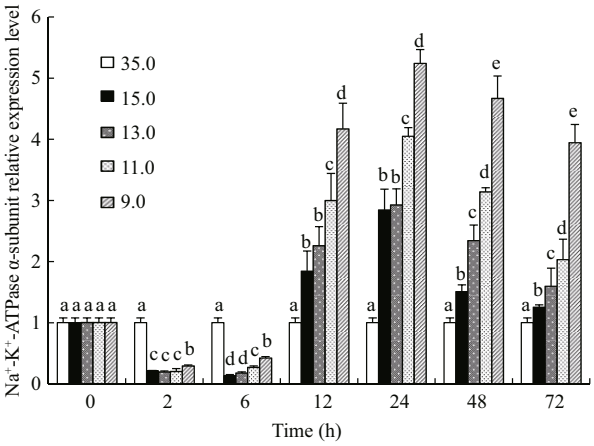

Fig.5 Analysis of Na^+/K^+-ATPase α-subunit expression in gill of *P. trituberculatus* in different low salinity treatments and control group by SYBR green quantitative real-time RT-PCR after 0, 2, 6, 12, 24, 48, and 72 h

Vertical bars, means±S.D. and significant differences (P<0.05) of Na^+/K^+-ATPase α-subunit expression between challenged and control groups at the same time, different letters.

4 DISCUSSION

Na^+/K^+-ATPase is a member of the P-type ATPase family and serves as the sodium pump, as in other membrane-associated enzymes responsible for establishing and maintaining intracellular ion (H^+, Na^+, Mg^{2+}, K^+, Ca^{2+}, Cu^{2+}, and Cd^{2+}) concentrations. These enzymes use ATP as their driving force (Jørgensen and Andersen, 1988; Serrano, 1988). Crustacean Na^+/K^+-ATPase, like that in all animal cells, is composed of three subunits α, β, and γ; (Geering et al., 1996; Béguin et al., 1997). The α and β-subunits were first cloned from mammalian kidney (Shull et al., 1985; Shull et al., 1986). Analysis of

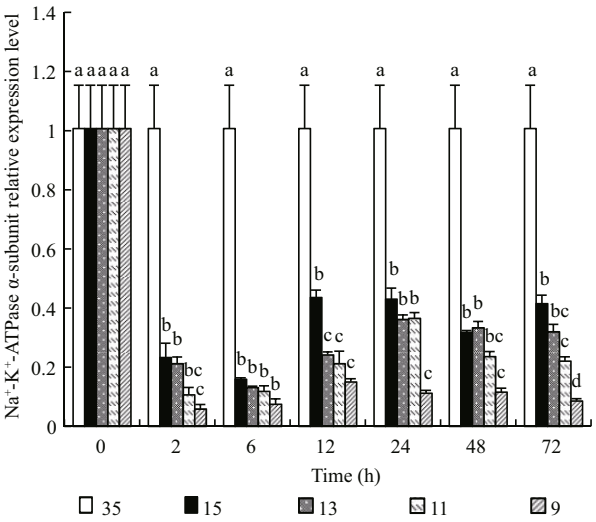

Fig.6 Analysis of Na^+/K^+-ATPase α-subunit expression in hepatopancreas of *P. trituberculatus* in different low salinity treatments and control group by SYBR green quantitative real-time RT-PCR after 0, 2, 6, 12, 24, 48, and 72 h

Vertical bars, means±S.D. and significant differences ($P<0.05$) of Na^+/K^+-ATPase α-subunit expression between challenged and control groups at same time, different letters.

Na^+/K^+-ATPase function has revealed that the α-subunit possesses catalytic function, including binding and hydrolyzing ATP. During the transport cycle, α-subunit becomes phosphorylated (Mercer, 1993; Lingrel and Kuntaweiler, 1994). In the present study, the cDNA sequence of Na^+/K^+-ATPase α-subunit was initially cloned and characterized from *P. trituberculatus*, yielding the full-length cDNA of α-subunit with 3 833 bp and encoding 1 039 amino acids. Conserved sequences and characteristic motifs were found in the deduced amino acid sequence, including eight transmembrane domains (Mitsunaga-Nakatsubo et al., 1996), a putative ATP-binding site (Horisberger et al., 1991), and a phosphorylation site. These were major structural and functional domains typically present in the Na^+/K^+-ATPase α-subunit of other aquatic animals (Baxter-Lowe et al., 1989; Towle et al., 2001; Semple et al., 2002).

A interspecies search for sequence similarities revealed that the deduced amino acid sequence of *P. trituberculatus* Na^+/K^+-ATPase α-subunit shared high identity with other known Na^+/K^+-ATPase α-subunits, especially with those from crustaceans. The crustacean α-subunit amino acid sequence is 71%–74% identical to vertebrate α-subunit sequences (Lucu and Towle, 2003). Phylogenetic analysis revealed that the *P. trituberculatus* α-subunit belonged to the crustacean α-subunit group, which is more similar to insects and arachnids than mammals and osteichthyes. In this study, crustacean, insect, and arachnid α-subunits were concluded to have diverged from a common ancestor after the divergence of invertebrate and vertebrate α-subunits, which was in agreement with an earlier report on *P. trituberculatus* (Pan et al., 2010). Based on sequence alignment, structure comparison, and phylogenetic analysis, the *P. trituberculatus* Na^+/K^+-ATPase α-subunit was suggested here to be a member of the Na^+/K^+-ATPase family.

Analysis of real-time RT-PCR amplification results of the four *P. trituberculatus* tissues examined in this study indicated that the Na^+/K^+-ATPase α-subunit was expressed in all these tissues, but the relative expression concentrations of the α-subunit were the highest in gill, less in hepatopancreas, and lowest in muscle and hemocytes, such that gill appeared to be an important expression site for this α-subunit compared with the other three tissues. Similar results have been previously reported in *C. sapidus* (Towle et al., 2001), *P. marmoratus* (Jayasundara et al., 2007), *A. schlegeli* (Choi and An, 2008), *S. sarba* (Deane and Woo, 2005), *A. anguilla* (Cutler et al., 1995), and *F. heteroclitus* (Semple et al., 2002).

The temporal profile of *P. trituberculatus* Na^+/K^+-ATPase α-subunit expression after salinity stress were important for understanding the protein's role in osmoregulation mechanisms (Deane and Woo, 2005; Choi and An, 2008; Sun et al., 2011). In the present study, α-subunit expression in gill and hepatopancreas dropped strongly at early times after initiation of low salinity stress. However, the behavior of these two tissues differed after 6 h post-stress initiation. Subunit expression increased significantly in gill after 6 h and, by the experiment's end, expression again slightly decreased. It is inferred that Na^+/K^+-ATPase enzyme activity positively correlated to α-subunit transcript abundance, which was in accordance with results reported in *C. sapidus* (Towle et al., 2001), *Acipenser sinensis* (Feng et al., 2012), *Salmo salar* (Nilsen et al., 2007), and *Oreochromis mossambicus* (Tang et al., 2008). While α-subunit expression in hepatopancreas after low salinity stress was always lower than the control group, this difference was likely related to different functions of the two tissues in response to this stress. The observation of higher death rates and lower subunit expression at salinity 9.0 might have indicated that osmoregulatory capacity in these crabs became unstable at this salinity. The fluctuations in α-subunit mRNA expression observed here implied

that the Na^+/K^+-ATPase α-subunit participated gradually in osmoregulation adaptation against salinity stress via a complex mechanism. Together, these results suggested that Na^+/K^+-ATPase performed a very important role of the uptake of Na^+ in *P. trituberculatus* in response to these salinity changes, which might offer an explanation for the species' wide range of salinity tolerance.

In conclusion, a Na^+/K^+-ATPase α-subunit was cloned from the gill of *P. trituberculatus*. Analysis of the nucleotide sequence revealed that the cDNA possessed a full-length of 3 833 bp, an open reading frame (ORF) of 3 120 bp, a 5' untranslated region (UTR) of 317 bp, and a 3' UTR of 396 bp. This sequence encoded a 1 039 amino acid protein with a predicted molecular weight of 115.57 kDa and an estimated pI of 5.21. Also, mRNA encoding the α-subunit could be detected in gill, hepatopancreas, muscle, and hemocytes. Expression of α-subunit mRNA in gill and hepatopancreas altered rapidly at 2 and 6 h after initiation of low salinity stress (9, 11, 13, and 15). The observed variations in α-subunit expression during salinity stress indicated that it might be involved in osmotic regulation and adaptation. Further work will focus on the translational regulation of Na^+/K^+-ATPase α-subunit in response to environmental salinity changes.

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