Effects of salinity acclimation and eyestalk ablation on Na^+ , K^+ , 2Cl^- cotransporter gene expression in the gill of *Portunus trituberculatus*: a molecular correlate for salt-tolerant trait

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Abstract The Na^+ , K^+ , 2Cl^- cotransporter (NKCC) is an important gene in ion transport. In order to elucidate its function, and regulatory mechanisms, in salinity acclimation, the complete cDNA sequence of NKCC (4218 bp) from *Portunus trituberculatus* (PtNKCC) was first cloned and characterized. It was found to encode 1055 amino acids containing conserved AA-permease and SLC12 motifs. Results show that PtNKCC is expressed to the greatest extent in gills. High salinity stress exposure led to significant increases (9.6-fold) of PtNKCC mRNA expression in the gills 12 h after treatment, declining to less than the levels seen in the control group between 48 and 72 h. During low salinity stress, expression levels of PtNKCC in gills were found to be upregulated at each sampling time, reaching their peak after 6 h (a 12.4-fold increase). Eyestalk ablation also triggered an 11.3-fold increase in PtNKCC mRNA, while re-injection with eyestalk homogenates significantly reduced the expression of PtNKCC mRNA. Four single nucleotide polymorphisms (SNPs) were detected in the PtNKCC open reading frame, and one SNP was associated with salt tolerance. Our results indicate

that PtNKCC plays an important role in the salinity acclimation of *P. trituberculatus*, while there may be a compound present in the XOSG that inhibits the expression of PtNKCC.

Keywords *Portunus trituberculatus* · NKCC · Salinity · Single nucleotide polymorphism

Introduction

Salinity is an important abiotic factor that influences the distribution, abundance, physiology, and well-being of crustaceans (Romano and Zeng 2012), as well as also playing a key role during artificial propagation (Xu and Liu 2011). Although the physiological mechanisms by which euryhaline organisms adapt to salinity change remain largely unexplored at the molecular level (Luquet et al. 2005), one critical adaptation is known. Euryhaline crustaceans keep their hemolymph osmotic and ionic concentrations relatively constant using ion transport to respond to variations in environmental salinity (Henry and Borst 2006).

The Na^+ , K^+ , 2Cl^- cotransporter (NKCC) is an especially important gene that regulates ion transport and belongs to the SLC12A family. NKCC is an integral membrane protein responsible for the simultaneous transport of one Na^+ , one K^+ , and two Cl^- ions from the external to the internal side of epithelial cells (Kang et al. 2010). Although previous studies suggest that NKCC plays a critical role in the osmoregulatory endurance of fish (Kang et al. 2010; Watson et al. 2014; Lorin-Nebel et al. 2006; Ip et al. 2013; Chandrasekar et al. 2014), its role in salinity adaptations has been little studied in crustaceans compared to other ion transport genes (i.e., Na^+ , K^+ -ATPase, V-type H^+ -ATPase, carbonic anhydrase (CA)) (Tsai and Lin 2007; Lucu et al. 2008; Firmino et al. 2011; Han et al. 2015).

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The major endocrine complex of crustaceans, the X-organ/sinus gland complex (XOSG), is located in the eyestalk (Henry and Campoverde 2006) and is known to play a role in salt and water balance (Morris 2001). Corroborating this, the removal of eyestalks (i.e., eyestalk ablation, ESA) in the green crab, *Carcinus maenas*, resulted in 10-fold upregulation of CA mRNA and a doubling of branchial CA activity. Homogenization of the eyestalk and re-injection of ESA into green crabs counteracted the effect of ESA (Henry and Borst 2006; Henry 2006), while in the Blue Crab, *Callinectes sapidus*, CA has also been shown to be under inhibitory neuroendocrine control, possibly by a hormone in the XOSG (Mitchell and Henry 2014). Because eyestalk ablation of shrimps also affects the Na^+ , K^+ -ATPase activity of gills (Nan et al. 2004), results show that a compound is present in the XOSG that causes changes to osmotic and ionic regulation. However, it remains unclear whether, or not, NKCC is regulated by the XOSG.

Portunus trituberculatus (Crustacea: Decapoda: Brachyura) is the most important economic crab species in China (Yu et al. 2003; Lv et al. 2013). This crab lives in a wide range of salinity conditions (i.e., from 5.7 to 47.7) and is a typical euryhaline species (Xu and Liu 2011). Throughout a prolonged cultivation period (8–10 months), the swimming crab often experiences substantial salinity fluctuations, significantly impacting its growth, larval development, molt stage, and productivity (Xu and Liu 2011; Ye et al. 2014). Due to the critical importance and implications of salinity adaptations in these crabs, we have previously examined the gill transcriptome in low and high salinity and found significantly different expression in the NKCC gene in *P. trituberculatus* (PtNKCC) during salinity stress compared to controls (Lv et al. 2013). This result suggests that the PtNKCC gene is important for salinity acclimation in this crab.

In this study, we first clone and characterize the NKCC gene from *P. trituberculatus*. To further understand the function, and regulatory mechanisms of salinity acclimation, the effects of salinity stress ESA on PtNKCC expression were then examined using quantitative real-time PCR (qPCR). Thirdly, the single nucleotide polymorphisms (SNPs) of PtNKCC were detected, and the association between polymorphism and salt tolerance were analyzed. These results will be essential for understanding the role of PtNKCC in the salinity acclimation of *P. trituberculatus*.

Materials and methods

Sample preparation

Healthy crabs at 80 days (5.8 ± 1.1 g in body weight) were collected from a local farm in Weifang, China. All the samples were acclimated in the laboratory environment (33 ppt, 18 °C)

and fed with *Potamocorbula laevis* once a day. The gill, eyestalk, hepatopancreas, hemolymph, heart, and muscle were sampled for tissue-specific mRNA expression analysis on the seventh day. Then, the crabs were randomly divided into three groups (90 crabs per group with three biological replicates): non-challenged group (NC, 33 ppt), low salinity challenged group (LC, 11 ppt), and high salinity challenged group (HC, 45 ppt). For each group, the gills from three crabs were sampled at 3, 6, 12, 24, 48, and 72 h after salinity challenge. Lower salinity challenged group (5 ppt, 300 individuals) was set to screen the crabs with different salt-tolerant trait. The 45 earliest death individuals were considered to be susceptible to low salinity (SG), and the surviving 45 individuals were regarded as resistance to low salinity (RG). Muscles were collected and applied to DNA extraction. For the experiment of eyestalk ablation (ESA), the eyestalks of 30 crabs were cut by the way of Henry, R.P (Henry and Borst 2006), and the gills were sampled at 12, 24, 48, and 72 h after ESA. Then, the eyestalk homogenates were prepared and injected according to corresponding literatures (Mitchell and Henry 2014). The control group was injected with crustacean saline of the same volume ($410 \text{ mmol l}^{-1} \text{ NaCl}$, $11 \text{ mmol l}^{-1} \text{ CaCl}_2$, $21 \text{ mmol l}^{-1} \text{ MgSO}_4$, $8 \text{ mmol l}^{-1} \text{ KCl}$, $10 \text{ mmol l}^{-1} \text{ HEPES}$, $10 \text{ mmol l}^{-1} \text{ NaHCO}_3$ with 10 % DTT), and the gills were sampled at 12, 24, 48, and 72 h after injected. All these samples were stored in liquid nitrogen before being used for the experiments.

DNA, RNA extraction and cDNA synthesis

Genomic DNA was extracted by phenol-chloroform method and assessed by agarose gel. The concentration was measured with a spectrophotometer. Total RNA was extracted by TRIzol Reagent (Invitrogen, USA), according to the manufacturer's protocol. The quality of RNA was detected by 1.0 % agarose gel electrophoresis, and the concentration of RNA was measured with a spectrophotometer. $\text{OD}_{260}/\text{OD}_{280}$ of all RNA samples was between 1.8 and 2.0. The cDNA template for RACE was synthesized using 1-ug pooled RNA (gill, hepatopancreas, and muscle) according to the SMARTTM RACE Amplification Kit (Clontech, USA). The qPCR cDNA template was synthesized using the PrimeScript RT reagent Kit (TaKaRa, Japan).

PtNKCC clone

PCR specific primers NKCC-F and NKCC-R (Table 1) were designed based on the unigene sequence (comp70314_c0) from transcriptome data (Lv et al. 2013). To obtain the 3' unknown region, NKCC-F and universal primer UPM (Table 1) were used for PCR using the Advantage II PCR Kit (Clontech, USA). Similarly, primer NKCC-R and universal primer UPM were used to amplify the 5' unknown region. The PCR reactions were performed as follows: 95 °C for 60 s, followed by 40 cycles

Table 1 Primer used in this experiment

Primer	Sequence (5'-3')
NKCC-F	GTACAATAACAGTGTGCATTGTGTC
NKCC-R	CTTGGATTCGTCCTGTTGG
UPM	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
Q-NKCC-F	CTATGACGGATGAGCCAGTTGTAG
Q-NKCC-R	TCAAGTATCCACCACCACAGTC
RpL8-F	GCGTACCACAAGTATCGCGT
RpL8-R	AGACCGACCTTCCTACCAGC
L-NKCC-F	GGGGGACGCTATTGAGTTG
L-NKCC-R	GCTGCTAATCTTTCATATTCTTCTCC

of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2.5 min. The PCR products were reclaimed by the Agarose Gel Extraction kit (Sangon, China) and ligated to PMD18-T vector (TaKaRa, Japan). Then, the vector was transformed in *Escherichia coli* DH5 α competent cells and plated on LB-agar containing ampicillin (100 μ g/ml). Three positive colonies were sequenced by a commercial biotechnology company (Sunny, China).

Sequence analysis

The cDNA nucleotide and deduced amino acid sequences were analyzed using the ExpASY Proteomic server (<http://web.expasy.org/protparam/>). The amino acid sequences of different species were obtained using the BLAST program (Altschul et al. 1997) from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>), and a multiple sequence alignment was carried out by BioEdit. A phylogenetic tree was constructed by the MEGA 6.0 program (Tamura et al. 2007) using the neighbor-joining (NJ) algorithm, and the reliability was tested using bootstrap re-sampling (1000 pseudo-replicates). The features of protein domains were predicted by a simple modular architecture research tool (SMART) (Schultz et al. 1998) (<http://smart.embl-heidelberg.de/>). The transmembrane domains were predicted using MEMSAT3 and MEMSAT-SVM provided by PSIPRED protein structure prediction server (LJ et al. 2000) (<http://bioinf.cs.ucl.ac.uk/psipred/>).

qPCR analysis

The RpL8 gene was selected as an internal control (Xu and Liu 2011). First strand cDNA was synthesized from 1 μ g of RNA using M PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). SYBR® Premix Ex Taq™ reagent Kit was used to qPCR analysis. The qPCR reaction mixture (25 μ L) consisted of 1 $\times$ Power SYBR Green PCR Master mix, 0.2 μ M each of the forward and reverse primers, and 1 μ L of template cDNA. PCR

amplification was performed under the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 34 s, and a final dissociation curve analysis 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The internal reference gene and PtNKCC both set three biological replicates. The relative expression ratio of PtNKCC in salinity challenge groups (3, 6, 12, 24, 48, and 72 h) versus that in controls (0 h, no salinity challenge) was calculated using the $2^{-\Delta\Delta CT}$ method by the ABI 7500 Fast Real-Time PCR Systems (Version 2.0.6). The results were subjected to one-way analysis of variance (ANOVA) followed by an unpaired, two-tailed *t* test. $P < 0.05$ was considered statistically significant.

Single nucleotide polymorphism loci detection and genotyping

PCR products amplified by L-NKCC-F and L-NKCC-R from one mixed DNA sample (90 DNA samples from SG and RG were pooled in equal amounts) were purified and sequenced directly in both directions with forward and reverse primers using Sanger technology on the ABI 3730 platform (Applied Biosystems). Sequencing chromatograms were visually analyzed using the Vector NTI Suite 11.0 (Invitrogen), and SNPs were detected as overlapping nucleotide peaks. SNPs were genotyped in the 90 crabs from SG and RG with the MassARRAY platform (Sequenom, San Diego, CA, USA) by the way of Lv (Lv et al. 2015). Each SNP site allele frequencies were calculated by SPSS 17.0 and processed the Pearson's chi-square test to confirm their association with salt-tolerant trait.

Results

Sequence analysis of PtNKCC

The complete cDNA of PtNKCC comprises 4218 bp with a 3165 bp open reading frame (ORF) and encodes 1055 amino acids (aa) (Fig. S1) (GenBank accession number: KT428640). In addition, this sequence includes a 5'-untranslated region (UTR) of 94 bp, a 3'-UTR of 959 bp, a putative polyadenylation consensus signal (AATAAA), and a poly A tail.

The amino acid sequences of the NKCC gene from different species were collected using the NCBI database. Protein domain analysis results show that this gene has conserved signatures; an AA-permease domain (including 12 transmembrane regions) and an SLC12 domain (Fig. 1). The deduced amino acid sequence of PtNKCC is 91, 78, 64, 50, and 47 % homologous to NKCC *Callinectes sapidus*, *Halocaridina rubra*, *Carcinus maenas*, *Daphnia pulex*, and *Cherax cainii*, respectively (Fig. S2). An NJ phylogenetic tree of NKCC suggests that all sequences can be classified into two major

Fig. 1 Structural features of PtNKCC gene

groups (i.e., vertebrate and invertebrate) and that the PtNKCC gene falls with the crustacea within invertebrates (Fig. 2).

Expression profiles of PtNKCC

The qPCR results show that the PtNKCC gene is expressed in all tested tissues, but predominantly in gills, and with

relatively high levels of expression in eyestalks, the hepatopancreas, and in hemocytes. Lower levels of expression are seen in the muscles and heart (Fig. 3).

Expression profiles of PtNKCC in the gills were also detected during changes in salinity. Under high salinity, PtNKCC expression took the form of a wave in early stages, rose dramatically to 9.6 times its initial level

Fig. 2 Phylogenetic tree of NKCC amino acid sequences from different species. The sequences were aligned using Clustal W, which was then employed to construct a phylogenetic tree by MEGA 6.0 using neighbor-joining method. The value at the forks indicates the percentage of trees in which this grouping occurred after bootstrapping the data (1000 replicates). The scale bar indicates 0.1 nucleotide substitution per site. The species and GenBank accession numbers of NKCC used for phylogenetic analysis were *Anguilla anguilla* (CAD31112.1), *Apis florea* (XP_012348508.1), *Bactrocera dorsalis* (XP_011201433.1), *Bombus terrestris* (XP_003399490.1), *Callinectes sapidus* (AAF05702.1), *Capra hircus* (XP_005682784.1), *Carcinus maenas* (AAK62044.1), *Cherax cainii* (AJO70198.1), *Ciona intestinalis* (XP_

002131373.1), *Dendroctonus ponderosae* (ENN71798.1), *Dicentrarchus labrax* (ABB84251.1), *Drosophila grimshawi* (XP_001983757.1), *Halocaridina rubra* (AIM43577.1), *Homo sapiens* (ABU69043.2), *Megachile rotundata* (XP_012143079.1), *Microplitis demolitor* (XP_008544302.1), *Nasonia vitripennis* (XP_003423952.1), *Oreochromis mossambicus* (AAR97733.1), *Pediculus humanus corporis* (XP_002428731.1), *Plutella xylostella* (XP_011557859.1), *Rattus norvegicus* (ABU63482.1), *Salmo salar* (NP_001117155.1), *Solenopsis invicta* (EFZ10415.1), *Taeniopygia guttata* (XP_002189174.3), *Wasmannia auropunctata* (XP_011696392.1), *Xenopus laevis* (NP_001116071.1), *Zootermopsis nevadensis* (NP_001116071.1), and *Portunus trituberculatus* (KT428640).

Fig. 3 Expression profile of PtNKCC in various tissues. Different letters indicate significant differences ($P < 0.05$)

after 12 h, and then declined to the same level as the control group after 24 h. Subsequently, expression levels between 48 and 72 h were lower than that of the control group ($P < 0.05$) (Fig. 4). Under low salinity, expression levels of PtNKCC in gills were upregulated by each measurement time, reaching a peak after 6 h, an increase of 12.4 times the initial level. Subsequently, expression levels gradually decreased, but remained significantly higher than those in the control group (Fig. 5).

Expression of this gene as influenced by ESA was also studied. Results show that following ESA, gene expression of PtNKCC in the gills was significantly upregulated between 24 and 72 h, rising to 11.3 times initial levels (compared to the control group) after 12 h (Fig. 6). Subsequently, expression of PtNKCC in the gills significantly decreased between 24 and 72 h after the injection of eyestalk homogenates, reaching a 0.08-fold level after 72 h when compared to the control group (Fig. 7).

Fig. 4 Expression pattern of PtNKCC during high salinity stress in the gill. Different letters indicate significant differences ($P < 0.05$)

Fig. 5 Expression pattern of PtNKCC during low salinity stress in the gill. Different letters indicate significant differences ($P < 0.05$)

SNP detection and association analysis

Four SNP loci (i.e., 250 C/T, 1177 C/T, 991 C/T, 1493 C/T, and 2571 T/C) were found, all located in the ORF (Fig. S1). Of these, three SNPs are synonymous mutations, while one is non-synonymous; results of an association analysis show that SNP 2571 T/C is significantly associated with low salt tolerance ($P < 0.05$). (Table 2).

Discussion

Previous studies have demonstrated that expression profiles of NKCC in *P. trituberculatus* are significantly influenced by salinity (Lv et al. 2013). Thus, to verify the function of this gene in salinity acclimation, we cloned the complete cDNA sequence from the NKCC gene in *P. trituberculatus* for the first time and studied expression patterns under different salinity levels and with, and without, ESA. In addition, SNPs of

Fig. 6 Expression profile of PtNKCC in the gill after ESA. Different letters indicate significant differences ($P < 0.05$)

Fig. 7 Expression profile of PtNKCC in the gill after eyestalk homogenates injected. Different letters indicate significant differences ($P < 0.05$)

the PtNKCC gene were examined and subject to association analysis with respect to salt tolerance.

The PtNKCC gene encodes 1055 aa and includes two highly conservative motifs; an AA-permease domain and SLC12 domains consistent with the characteristics of this gene family. Multiple sequence alignments reveal that the PtNKCC amino acid sequence is highly homologous with the same gene in *Callinectes sapidus* (91 %), another swimming decapod crab in the family Portunidae. Results presented here also indicate that NKCC sequences are highly conserved in crustaceans, while in vertebrates this gene can be divided into two subclasses; NKCC1 and NKCC2 (Kang et al. 2010). Phylogenetic analysis using NJ shows that all species cluster within two major groups, vertebrates and invertebrates, and that the PtNKCC gene is classified to Crustacea within invertebrates, not with a vertebrate-type NKCC. In addition, different NKCC forms from *P. trituberculatus* were not revealed by mass transcriptome data, implying that this gene may not yet be differentiated in Crustacea.

Osmotic and ionic regulation in crustaceans is accomplished mostly by their multifunctional gills (Freire et al. 2008). Indeed, the qPCR data reported here reveals that the PtNKCC gene is mainly expressed in the gills; this implies an important role in osmoregulatory function. Results show that PtNKCC responded rapidly to salinity changes, varying significantly within 3 h subsequent to low or high salinity stress.

Subsequently, NKCC expression remained consistently higher than in the control group under reduced salinity. In contrast to low salinity stress, NKCC expression gradually decreased to below the level in the control group after reaching a peak after 12 h during high salinity stress. This result is partially similar to that reported from another euryhaline crab, *Chasmagnathus granulatus*; during acclimation to dilute seawater, NKCC mRNA levels in this species increased 10–20 times in the gills during the first 24 h. In contrast, during acclimation to concentrated seawater, expression of NKCC was inhibited after 48 h and then increased 60 times in the gills after 96 h (Luquet et al. 2005). These results indicate that the NKCC gene plays different osmotic adjustment roles in low versus high salinity stress and that its operating mechanism is species-specific in crustaceans. Previous studies on fish have shown that mitochondrion-rich cells (MRCs) with basolateral Na^+/K^+ -ATPase and apical NKCC (type II) are freshwater-type ion absorptive cells, while MRCs with basolateral Na^+/K^+ -ATPase, basolateral NKCC, and apical CFTR (type IV) are seawater-type ion secretory cells (Hiroi et al. 2005). These results imply that the location of NKCC on the cell is important in determining its role in osmoregulation. Persistently, high expression levels during low salinity stress suggest that the PtNKCC gene may play an important role in hyper-osmoregulation. However, it remains unclear whether, or not, the location of PtNKCC on the cell in the gills is similar with the type II cell of fish, which must be verified by further work.

The XOSG is the major crustacean endocrine complex located within their eyestalks (Chung et al. 2010). XOSG comprises a family of neuropeptides, some of which play an important role in osmoregulation (Morris 2001; Henry and Campoverde 2006). Subsequent to ESA, activity and gene expression of Na/K ATPase and carbonic anhydrase were significantly induced (Nan et al. 2004), suggesting the presence in the eyestalk of an inhibitory compound that regulates salinity-stimulated ion transport enzyme induction (Henry and Campoverde 2006; Henry and Borst 2006). However, to date the effects of ESA on NKCC expression have not been reported, although in this study, PtNKCC expression significantly increased following ESA. In contrast, results also show that re-injection with eyestalk homogenates significantly

Table 2 SNP distributions of PtNKCC

Locus	Located	Genotype	Synonymous (Y/N) ^a	χ^2 (P) ^b	Association with trait (Y/N) ^c
250	ORF	C-T	Y	0.7 (0.70)	N
1177	ORF	C-T	Y	0.8 (0.67)	N
1493	ORF	C-T	Y	3.6 (0.06)	N
2571	ORF	T-C	N (Val-Ala)	6.6 (0.04)	Y

^a Y synonymous mutation, N non-synonymous mutation

^b P means the chi-square value

^c Y SNP was significantly associated with low salt-tolerant trait ($P < 0.05$)

reduced PtNKCC mRNA expression, a further result that suggests the presence of an inhibitory compound that regulates the expression of PtNKCC in the XOSG. Previous studies have shown that ESA elevates concentrations of methyl farnesoate (MF) in the hemolymph, indicating that MF might be a potential regulatory factor involved in physiological adaptations to changes in salinity (Henry 2006). Whether, or not, NKCC is regulated by MF is also well worth to further study.

This study shows that SNPs are suitable markers for candidate gene association studies because they possess important traits (Garvin et al. 2010; Seeb et al. 2011). Indeed, the SNP variants present in gene coding regions make it possible to identify candidate genotypes for genetic selection programs in economic species (Bester-van der Merwe et al. 2011; Garvin et al. 2010). In this study, four SNPs were found in the PtNKCC gene, all of them located in the open reading frame. Among these, only one, the SNP 2571 T/C, is a non-synonymous mutation associated with salt tolerance. It is clear that this SNP caused changes between valine and alanine, but further studies are needed to understand why this amino acid switch is related to salt tolerance, or if it is just a molecular marker linked to another critical salinity tolerance gene.

In this study, the complete cDNA sequence of the NKCC gene was cloned from the crab *P. trituberculatus*. Tissue-specific mRNA expression and expression profiles influenced by salinity stress and ESA were studied, while SNPs of PtNKCC were detected, and their association with salt tolerance were analyzed. All the results presented here demonstrate that PtNKCC plays an important role in the salinity stress response of *P. trituberculatus*, an expression which is regulated by XOGO. Results also provide useful information for further functional studies on the NKCC gene, while the SNP association with salt tolerance identified here will be essential to accelerate crab breeding programs.

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