Cloning and characterization of calreticulin and its association with salinity stress in *P. trituberculatus*

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Abstract Calreticulin (CRT) is a highly conserved and multi-functional endoplasmic reticulum (ER) chaperone protein and plays important roles in salinity stress response. *Portunus trituberculatus* is a commercially important fishery species, and water salinity conditions influence its commercial farming significantly. In order to research the function of calreticulin under salinity stress, the full-length cDNA sequence of calreticulin from *P. trituberculatus* (PtCRT) was firstly cloned and characterized. The complete cDNA sequence of PtCRT is 1676 bp with 1218 bp open reading frame (ORF), encoding a polypeptide of 405 amino acids. Multiple sequence alignments showed that the deduced acid amino sequences of PtCRT shared the highest homology to CRT of *Fenneropenaeus chinensis* (89 %). Fluorescent quantitative real-time PCR analysis indicated that PtCRT was expressed in all detected tissues and showed the highest expression level in hepatopancreas. In addition, salinity challenge significantly influenced the expression level of PtCRT in gill. Six single nucleotide polymorphisms (SNPs) were detected in cDNA sequence of PtCRT, and one SNP was associated with the salt tolerant trait. All results indicated that PtCRT plays an important role in mediating the salinity adaption of *P. trituberculatus*.

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

Introduction

Calreticulin (CRT) is an endoplasmic reticulum (ER) chaperone protein (Michalak et al. 1999) comprised of three highly conserved domains: a globular N-domain, a high-affinity, low-capacity calcium-binding P-domain, and a low-affinity, high-capacity calcium-binding C-domain (Nakamura et al. 2001), which is ubiquitously expressed in all of the multicellular eukaryotes (Coppolino and Dedhar 1998). CRT have diverse functions in a wide range of organisms, including lectin-like chaperoning (Liu et al. 2011), regulation of cellular Ca^{2+} homeostasis (Michalak et al. 1998) and Ca^{2+} -dependent signal pathways (Michalak et al. 2002), contribution to the immune system (Guo et al. 2002) and apoptosis, control of cell adhesion (Opas et al. 1996), modulation of nuclear-hormone receptor-mediated gene expression (Burns et al. 1994), and regulation of the process of folding proteins (Williams 2006). In crustaceans, CRT plays important roles in immune function, stress responses, and molting (Visudtiphole et al. 2010; Luana et al. 2007).

The swimming crab, *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), is a commercially important fishery species and is widely distributed in the coastal waters of China, Korea, Japan, and Taiwan (Dai et al. 1986). *P. trituberculatus* often experience substantial salinity fluctuations throughout their cultivation, which could have a significant impact on productivity (Lv et al. 2013). Due to the critical importance and implications of salinity adaption to the commercial farming of crabs, we examined the whole transcriptome challenged by different salinity stresses of the *P. trituberculatus* and found a significant difference in

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CRT expression during salinity stress compared to the controls (Lv et al. 2013). Several recent studies have showed that CRT is closely related to a number of stress responses including Ca^{2+} level (Williams 2006), virus infection (Luana et al. 2007; Watthanasurorot et al. 2013), heat shock (Visudtiphole et al. 2010; Conway et al. 1995), starvation (Heal and McGivan 1998), oxidation (Ihara et al. 2005), and heavy metal contamination (Park et al. 2001); however, few studies have focused on the function of salinity adaption in CRT.

In this study, we first report the molecular cloning, sequence analysis, and characterization of CRT from *P. trituberculatus*. To further understand the function of salinity adaption, the effects of salinity stress on PtCRT expression were examined by quantitative real-time PCR. In addition, the single nucleotide polymorphisms (SNPs) of PtCRT were detected and association analysis between polymorphism and salt tolerant trait was carried out. These results will be essential to understanding the role of PtCRT in salinity stress response in *P. trituberculatus*.

Materials and methods

Salinity challenge experiments and sample preparation

Healthy *P. trituberculatus* at 80 days (5.78 ± 1.11 g in body weight) were collected from Chang-yi Aqua-farming Company in Weifang, China. During the experimental period, all the samples were acclimated in aerated seawater and fed with *Potamocorbula laevis* twice a day in the laboratory (33 ppt, 26 °C). Gill, hepatopancreas, muscle, heart, eyestalk, and hemolymph were sampled on the 7th day for tissue-specific mRNA expression analysis. Then the crabs were randomly divided into three groups: a low salinity challenged group (LC, 11 ppt), a high salinity challenged group (HC, 45 ppt) and a non-challenged group (NC, 33 ppt). Every group (LC, HC, and NC) had 270 crabs with 3 biological replicates (each replicate having 90 individuals). For each group, gills from 3 crabs were sampled at 0, 3, 6, 12, 24, 48, and 72 h after challenge, respectively. In addition, the lower salinity challenged group (5 ppt, 300 individuals) was set to screen the individuals with different tolerances to salt. The 60 earliest death individuals were considered to be susceptible to low salinity, and the surviving individuals were regarded as low salinity tolerant. Muscles were collected and applied to DNA extraction. All these samples were stored in liquid nitrogen before being used for the experiments.

DNA, RNA extraction, and cDNA synthesis

Genomic DNA was extracted from the *P. trituberculatus* muscles by the classical phenol-chlorophenol method and was stored at -20 °C. The genomic DNA quality was assessed

by agarose gel and the concentration was measured with a spectrophotometer. All $\text{OD}_{260}/\text{OD}_{280}$ were between 1.8 and 2.0. Total RNA was extracted from collected samples using 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 determined spectrophotometrically. All $\text{OD}_{260}/\text{OD}_{280}$ were between 1.8 and 2.0. The RACE cDNA template was synthesized using 1-ug pooled RNA (gill, hepatopancreas, and muscle) and SMARTTM RACE Amplification Kit (Clontech, USA). The fluorescent quantitative real-time RT-PCR cDNA template was synthesized using a PrimeScript RT reagent kit (TaKaRa, USA). The reaction followed the conditions recommended by the manufacturer.

Cloning the full-length cDNA of PtCRT

PCR specific primers CRT-F, CRT-R, and CRT-F' (Table 1) were designed based on the sequence from our transcriptome data. To obtain the 3' unknown region, two primer-pairs, specific primer CRT-F, and universal primer UPM (Table 1), specific primer CRT-F', and nested universal primer (NUP) (Table 1), were used for the primary PCR and the nested PCR respectively using Advantage 2 PCR Kit (Clontech, USA). Similarly, the primer-pairs, specific primer CRT-R, and universal primer UPM were used to amplify the 5' unknown region. The PCR reactions were performed as follows: 30 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min.. The amplified PCR products were reclaimed by Agarose Gel Extraction kit (sangen, China). The recycled products were ligated into a PMD18-T easy vector (TaKaRa, USA). Competent *Escherichia coli* DH5 α cells were transformed and plated on LB-agar containing ampicillin (100 $\mu\text{g}/\text{ml}$) for selection. Positive colonies containing an insert of the expected sequence were screened by colony PCR. Three colonies were sequenced by a commercial biotechnology company (Sunny, China).

Table 1 Primer used in this experiment

Primer	Sequence (5'-3')
CRT-F	CTTCCAGGAGACTTTTCAGTAGCG
CRT-F'	ATCGTGAAGCCCGACAACACGTATG
CRT-R	CCTCATAGGTGTTGTCTGGGC
UPM	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
Q-CRT-F	GCCCTTCACAAACAAAGACAA
Q-CRT-R	TCAAGTATCCACCACCACAGTC
RpL8-F	GCGTACCACAAGTATCGCGT
RpL8-R	AGACCGACCTTCTACCAGC
C-CRT-F	GTGTACTTCCAGGAGACTTTCAG
C-CRT-R	GGTCTATTGGCATTACACTCTTA

Sequence analysis

The cDNA nucleotide and deduced amino acid sequences of PtCRT were analyzed using DNASTar (<http://web.expasy.org/protparam/>). The signal peptide was predicted by SignalP 4.1 Server software (<http://www.cbs.dtu.dk/services/>). The amino acid sequences of different species were obtained using 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 ClustalW (Thompson et al. 1997) multiple alignment program. A phylogenetic tree was constructed by the MEGA 4.0 program (Tamura et al. 2007) using the neighbor-joining (NJ) algorithm, and the reliability was tested using bootstrap resampling (1000 pseudo-replicates).

Quantitative real-time RT-PCR analysis

The RpL8 gene was selected as an internal control for qPCR analysis and the primers referenced the study by Xu and Liu (Xu and Liu 2011). The standard curve testing was performed using a series of diluted samples (tenfold serial dilutions: 1 ×, 10 ×, 100 ×, 1000 ×, and 10,000 ×), respectively, for each primer. The slopes of the standard curves were calculated. According to the formula $E=10^{(-1/\text{slope})}$, the values of PCR efficiency for all the genes were calculated. In all cases, PCR efficiencies were >90 %. First strand cDNA was synthesized from 1 mg of RNA using M-MuLV reverse transcriptase (Qiagen). The qPCR reaction mixture (20 uL) consisted of 2× Power SYBR Green PCR Master mix, 0.9 uM each of the forward and reverse primers, and 1 uL of template cDNA. PCR amplification was performed under the following

2	TGG	GGA	GTC	GTG	GGC	TAG	AAG	AGA	GGG	AGA	GGA	GAC	GCG	CTC	ACC	CGC	CAC	TCC	AGT	ACA	TCA	GTC	GAC	GCA	ATG	AAG	ATC	TAC	ATT	CTC	91
																									M	K	I	Y	I	L	
92	CTC	GCC	CTC	CTG	GGT	GTG	GCC	CTG	GTG	GAA	GCC	AAG	GTG	TAC	TTC	CAG	GAG	ACT	TTC	AGT	AGC	GAT	GAC	TGG	GCG	TCT	CGA	TGG	GTT	CAA	181
	L	A	L	L	G	V	A	L	V	E	A	K	V	Y	F	Q	E	T	F	S	S	D	D	W	A	S	R	W	U	Q	
182	TCA	GAA	CAC	AAA	GGG	AAA	GAG	TTT	GGC	AAG	TTC	AAG	TTG	ACA	GCT	GGC	AAG	TTC	TAT	GGT	GAT	GCT	GAG	AAG	GAC	AAG	GGC	ATC	CAG	ACC	271
	S	E	H	K	G	K	E	F	G	K	F	K	L	T	A	G	K	F	Y	G	D	A	E	K	D	K	G	I	Q	T	
272	TCC	CAG	GAT	GCT	CGC	TTC	TAT	GGT	CTG	TCC	AGC	AAG	TTC	GAG	CCC	TTT	ACA	AAC	AAA	GAC	AAG	CCT	CTA	GTC	ATC	CAG	TTC	ACA	GTC	AAG	361
	S	Q	D	A	R	F	Y	G	L	S	S	K	F	E	P	F	T	N	K	D	K	P	L	V	I	Q	F	T	V	K	
362	CAC	GAA	CAG	AAC	ATC	GAC	TGT	GGT	GGT	GGA	TAC	TTG	AAG	GTG	TTT	GAC	TGC	TCT	CTA	GAC	CAG	AAG	GAC	ATG	CAT	GGA	GAG	TCT	CCC	TAT	451
	H	E	Q	N	I	D	C	G	G	G	Y	L	K	V	F	D	C	S	L	D	Q	K	D	H	H	G	E	S	P	Y	
452	CTC	CTC	ATG	TTT	GGG	CCT	GAC	ATC	TGT	GGT	CCG	GGA	ACA	AAG	AAG	GTG	CAT	GTG	ATC	TTC	AAC	TAC	AAG	AGC	CAG	AAC	CAC	CTC	ATC	AAG	541
	L	L	H	F	G	P	D	I	C	G	P	G	T	K	K	V	H	V	I	F	N	Y	K	S	Q	N	H	L	I	K	
542	AAG	GAG	ATC	CGC	TGC	AAG	GAT	GAT	GTG	TTC	TCT	CAC	CTG	TAC	ACC	CTC	ATC	GTG	AAG	CCC	GAC	AAC	ACC	TAT	GAG	GTG	CTC	ATT	GAC	AAC	631
	K	E	I	R	C	K	D	D	V	F	S	H	L	Y	T	L	I	V	K	P	D	N	T	Y	E	V	L	I	D	N	
632	GAG	AAG	GCA	CAG	TCT	GGT	GAG	CTG	GAG	GAG	GAC	TGG	GAC	ATG	CTG	CCA	CCC	AAG	AAG	ATT	AAG	GAT	CCC	GAT	GCA	AGC	AAA	CCC	GAC	GAC	721
	E	K	A	Q	S	G	E	L	E	E	D	W	D	H	L	P	P	K	K	I	K	D	P	D	A	S	K	P	D	D	
722	TGG	GAT	GAC	CGT	GCC	ACC	ATC	CCT	GAC	CCT	GAT	GAC	ACC	AAG	CCT	GAG	GAC	TGG	GAC	CAG	CCT	GAG	CAC	ATC	CCC	GAC	CCT	GAT	GCC	ACC	811
	W	D	D	R	A	T	I	P	D	P	D	D	T	K	P	E	D	W	D	Q	P	E	H	I	P	D	P	D	A	T	
812	AAA	CCC	GAG	GAT	TGG	GAT	GAT	GAG	ATG	GAT	GGA	GAG	TGG	GAA	CCC	CCC	ATG	ATT	GAC	AAC	CCT	GAC	TAC	AAG	GGC	GAG	TGG	AAA	CCA	AAG	901
	K	P	E	D	W	D	D	E	H	D	G	E	W	E	P	P	H	I	D	N	P	D	Y	K	G	E	W	K	P	K	
902	CAG	ATT	GAT	AAC	CCT	GAC	TAC	AAG	GGA	CCC	TGG	CAC	CAC	CCT	GAG	ATC	GAC	AAC	CCA	GAG	TAC	ACT	GCC	CAC	CCT	GAG	CTG	TAC	AAG	TAC	991
	Q	I	D	N	P	D	Y	K	G	P	W	H	H	P	E	I	D	N	P	E	Y	T	A	D	P	E	L	Y	K	Y	
992	GAT	GAG	ATC	TGC	TCT	GTG	GGT	CTG	GAT	CTG	TGG	CAG	GTA	AAG	AGC	GGC	ACC	ATC	TTT	GAC	AAC	TTT	CTC	GTT	GGC	GAC	GAC	ATC	GAG	GAG	1081
	D	E	I	C	S	V	G	L	D	L	W	Q	V	K	S	G	T	I	F	D	N	F	L	V	G	D	D	I	E	E	
1082	GCG	AAG	CGC	ATT	GGA	GAA	GAG	ACC	TGG	GGT	GCC	ACC	AAG	GAC	GAC	GCC	AAG	ATG	AAG	GAC	GCT	CAG	GAC	GAA	GAG	GAG	AGG	AAG	AAG		1171
	A	K	R	I	G	E	E	T	W	G	A	T	K	D	E	A	K	K	M	K	D	A	Q	D	E	E	E	R	K	K	
1172	GCT	GAA	GAG	GAA	GCC	AAG	GCA	AAC	GCA	GCT	GAG	GAC	AGT	AAG	GAA	GAT	GAT	GAG	GAG	GAC	GAC	GAT	GAG	CTG	GAT	GAG	GAT	GAC	GAT	GAC	1261
	A	E	E	A	K	A	A	E	D	S	K	E	D	D	E	E	E	D	D	D	E	L	D	E	E	E	D	D	D	D	
1262	ATT	GAT	GAG	CTG	GGT	CAT	GAC	GAG	CTC	TAA	TTT	TTC	TCT	ATC	CCT	TTC	CCC	TCA	ACC	CTC	CCA	CCT	GAT	TAC	AAC	CAT	CAC	CAC	CTG	TAC	1351
	I	D	E	L	G	H	D	E	L	*																					
1352	AGA	TTG	AAG	CCT	CTG	GTG	ATG	CGT	TAA	TGT	TAG	GAT	TAG	AAG	CTT	CAA	TCT	TAT	AGG	CCT	GAG	ATC	TGG	GCA	GGC	ATG	GCA	GTG	TGC	TGT	1441
1442	GTC	CAG	TGG	ATT	TCC	AGG	CTT	TTG	TGA	CTA	TGC	CCT	GTG	TGG	GTG	GGT	TAT	GCA	TCA	CCA	TAT	TCA	TCT	TTA	ATC	ATC	CTC	ATC	GCC	ATA	1531
1532	GTG	TAT	GCA	GCA	CAA	CCC	TGT	ATG	TTT	GTA	CGA	GCC	ATA	GGA	GTG	TAA	ATT	CCA	ATA	GAC	CTA	GAA	TGG	TTT	GCA	TCA	TTG	AGG	TGT	ATG	1621
1622	ATT	GAC	TGA	TTT	CCA	ATA	AAC	AGA	ATG	TGG	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	1676

Fig. 1 Nucleotide and deduced amino acid sequence of PtCRT cDNA. The *italic letters* indicate the start codon and an *asterisk* indicates the stop codon. The predicted signal peptide is marked with *dashed lines*. The two CRT family signatures are *boxed*. The six SNPs are *boxed in red color*. The *underlined letters* show the three

repeats (position 206–218, 223–235, and 240–252) motifs. The *double-underlined letters* represent the endoplasmic reticulum targeting tetrapeptide HDEL. The *shaded letters* (AATAAA) indicate the polyadenylation consensus signal

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, 95 °C for 15 s. The internal reference gene and target gene PtCRT both set three biological replicates. The relative expression ratio of the target gene in the treated groups (3, 6, 12, 24, 48, and 72 h after salinity challenge) versus that in controls (0 h, no salinity challenge) was calculated using the $2^{-\Delta\Delta CT}$ method by 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

The PCR products amplified by primers C-CRT-F and C-CRT-R from 60 low salinity-susceptible and 60 tolerant individuals were purified and sequenced with the automated sequencer ABI 3730 (Applied Bio-system). The SNPs were detected by sequence alignments of different individuals with Vector NTI Suite 11.0 (Invitrogen). The distributions of each SNP site allele frequencies were calculated by SPSS 17.0 and compared by the Pearson chi-square test or Fisher exact test for the significance tests to confirm their association with the

Fig. 2 The multiple amino acid sequences alignment of PtCRT with other species CRT. The two CRT family signatures are indicated with underlining. The three repeats (position 206–218, 223–235, and 240–252) motifs are boxed. The endoplasmic reticulum targeting tetrapeptide H(K)DEL is indicated with double-underlining. The amino acid sequence

data were acquired from the GenBank database with the following accession numbers: *F. chinensis* (ABC50166), *P. monodon* (ADO00927), *P. fucata* (ABR68546), *H. sapiens* (BAD96780), *C. porcellus* (XP_003468401), and *O. cuniculus* (NP_001075704)

susceptibility/tolerance to salinity challenge. Results were considered statistically significant when $P < 0.05$.

Results

PtCRT cDNA sequence

The full-length PtCRT cDNA was 1676 bp with a 1218 bp open reading frame (ORF), encoding 405 amino acids (aa) (Fig. 1) (GenBank accession number: KF548042). The cDNA contained a 5' un-translated region (UTR) of 73 bp and a 3'-UTR of 385 bp, including a putative polyadenylation consensus signal (AATAAA) and a poly A tail. The deduced protein had a calculated molecular weight of 46.74 kDa and

isoelectric point of 4.33. The total number of negatively charged residues (Asp+Glu) and positively charged residues (Arg+Lys) was 107 and 50, respectively. The instability index and grand average of hydropathicity (GRAVY) were computed to be 35.95 and -1.055 , which indicated the PtCRT protein was a hydrophilic stable protein.

Sequence analysis showed that PtCRT contains a signal peptide, two conserved CRT family signature motifs: KHEQNIDCGGGYLKVF (96–111 aa) and LMFGPDICG (128–136 aa), and three CRT family repeat motifs: IKDPDASKPDDWD (206–218 aa), IPDPDDTKPEDWD (223–235 aa) and IPDPDATKPEDWD (240–252 aa). In addition, the C-terminal has the putative endoplasmic reticulum targeting tetrapeptide HDEL (His-Asp-Glu-Leu, 402–405 aa).

Fig. 3 Phylogenetic tree of CRT amino acid sequences from different species. The sequences were aligned using Clustal W, which was then employed to construct a phylogenetic tree by MEGA 4.0 using neighbor-joining method. The value at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping the data (1000 replicates). The scale bar indicates 0.05 nucleotide substitution per site. The species and GenBank accession numbers of CRT used for phylogenetic analysis were: *F. chinensis* (ABC50166), *Pacifastacus leniusculus* (AEC50079), *P. monodon* (ADO00927), *L. vannamei* (AFC34501), *Culex quinquefasciatus* (XP_001848824), *Plutella*

xylostella (ADN06079), *Galleria mellonella* (BAB79277), *Danaus plexippus* (EHJ72848), *Megachile rotundata* (XP_003701110), *Drosophila sechellia* (XP_002031864), *Amblyomma americanum* (AAR29932), *Gallus gallus* (AAS49610), *O. cuniculus* (NP_001075704), *Anolis carolinensis* (XP_003216823), *Paralichthys olivaceus* (ABG00263), *H. sapiens* (BAD96780), *Maylandia zebra* (XP_004555132), *Oreochromis niloticus* (XP_003448535), *P. fucata* (ABR68546), *Lates calcarifer* (ADQ92842), *Ictalurus punctatus* (NP_001187111), and *C. porcellus* (XP_003468401).

Fig. 4 Expression pattern of PtCRT in various tissues. Different letters indicate significant differences ($P < 0.05$)

Multiple sequences and phylogenetic analysis

Amino acid sequences of CRT from different species were collected through the NCBI database. Multiple sequence alignments results showed that CRT had conserved CRT family signatures containing three repeats motifs and HDEL sequence. The deduced amino acid sequence of PtCRT showed 89, 88, 75, 72, 72, and 72 % homologous to CRT of *Fenneropenaeus chinensis*, *Penaeus monodon*, *Pinctada fucata*, *Homo sapiens*, *Cavia porcellus*, and *Oryctolagus cuniculus*, respectively (Fig. 2). The neighbor-joining phylogenetic tree of CRT showed that all cluster species are classified to two major groups including the vertebrate group and the invertebrate group, and PtCRT was classified into invertebrate group (Fig. 3). The results here were in accordance with biological classification.

Expression profiles of PtCRT

The fluorescent quantitative real-time RT-PCR result showed that PtCRT gene was expressed in all the tested tissue and was mainly expressed in the hepatopancreas, followed by relatively high expression levels in gill and eyestalk. Lower expression levels were observed in muscle and heart, and a weak expression level in hemocytes (Fig. 4).

Expression profiles of PtCRT in gill and hepatopancreas were detected during salinity challenges. Under high salinity challenge, the expression level of PtCRT in gill rose dramatically to 1.94-fold at 3 h and then remained almost the same at 6 h. Subsequently, the expression level from 12 to 72 h declined but was still significantly higher than that of the control group ($P < 0.05$) (Fig. 5). Under low salinity challenge, the

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

Fig. 6 Expression pattern of PtCRT during low salinity stress in gill. Different letters indicate significant differences ($P<0.05$)

expression level of PtCRT in gill showed no significant difference ($P<0.05$) from 0 to 12 h and then gradually increased to 2.2-fold at 72 h (Fig. 6). In hepatopancreas, the expression of PtCRT was suppressed significantly at all time points after salinity challenges, the lowest down-regulations were 0.057 and 0.074 fold at 6 h and 72 h after low and high salinity challenges, respectively (Figs. 7 and 8).

SNP detection and association analysis

Six SNP loci (310 C/T, 355 A/T, 991 C/T, 1009 G/A, 1061 G/A, 1186 C/T) were discovered, and all of them were located in the open reading frame (Fig. 1). Among these, five SNPs belonged to a synonymous mutation and one SNP belonged to

a non-synonymous mutation. The association analysis result showed that SNP 1269 G/A was significantly associated with a low salinity tolerance trait ($P<0.05$) (Table 2).

Discussion

Calreticulin (CRT) is a highly conserved protein with multiple functions and is implicated in a variety of physiological/pathological processes. In crustaceans, CRT has been cloned from *F. chinensis* (Luana et al. 2007), *P. monodon* (Visudtiphole et al. 2010), *Litopenaeus vannamei* (GenBank accession number: AFC34501), and *Exopalaemon carinicauda* (Duan et al. 2014). In most reported cases, CRT was related to

Fig. 7 Expression pattern of PtCRT during high salinity stress in hepatopancreas. Different letters indicate significant differences ($P<0.05$)

Fig. 8 Expression pattern of PtCRT during low salinity stress in hepatopancreas. Different letters indicate significant differences ($P < 0.05$)

the functions of stress responses (temperature, air exposure) and immune defenses (virus and bacteria); however, no research focused on its function in salinity adaptation until now. Our previous study results found that the expression pattern of calreticulin changed significantly in salinity stress. To verify its function in salinity adaptation, we cloned the whole length cDNA of calreticulin from *P. trituberculatus* for the first time and studied the expression pattern in different salinity stresses, while also exploring SNP and carrying out association analysis with salt tolerant trait.

In the present study, the full-length cDNA of CRT encoding 405 amino acids was cloned, including the typical signature motifs of the CRT family and three typical conserved N-, P-, and C-domains. Multiple sequence alignments revealed that the deduced amino acid sequence of PtCRT shared high homology with *F. chinensis* and *P. monodon* (89 and 88 %). The results indicated that CRT has highly conserved sequences in crustaceans.

Quantitative real-time RT-PCR data revealed that PtCRT was expressed in all the tested tissues, including gill,

Table 2 SNP distributions of PtCRT

Locus	Located	Genotype	Synonymous (Y/N)	Susceptible <i>N</i> (%)	Tolerant <i>N</i> (%)	χ^2 (<i>P</i>)
310 C/T	ORF	CT	Y	17(0.20)	15(0.17)	0.714(0.700)
		CC		22(0.25)	25(0.29)	
		TT		3(0.03)	5(0.06)	
355 A/T	ORF	AT	Y	32(0.40)	27(0.34)	2.696(0.260)
		AA		5(0.06)	5(0.06)	
		TT		3(0.04)	8(0.10)	
991 C/T	ORF	CT	Y	3(0.04)	0(0.00)	2.759(0.097)
		CC		41(0.49)	39(0.47)	
1009 G/A	ORF	AG	Y	19(0.22)	16(0.18)	2.326(0.313)
		GG		24(0.28)	26(0.30)	
		AA		0(0)	2(0.23)	
1061 G/A	ORF	GG	N	23(0.28)	14(0.17)	3.632(0.020)
		AG		18(0.22)	26(0.32)	
1186 C/T	ORF	TC	Y	18(0.21)	15(0.17)	2.404(0.301)
		CC		24(0.28)	27(0.31)	
		TT		0(0)	2(0.02)	

The SNP distributions between low salinity-susceptible and tolerant groups were significantly different ($P < 0.05$)

Y synonymous mutation, N non-synonymous mutation, *N*(%) percentage of each genotype of susceptible or tolerant individual share in the total number of individuals, χ^2 (*P*) chi-square value

hepatopancreas, muscle, heart, eyestalk, and hemocytes, suggesting its multifunction in several biological processes based on different tissues. Hepatopancreas was regarded as the metabolic center for reactive oxygen species (ROS) production and the major site for the synthesis of immune defense molecules involved in eliminating pathogens or other particulate matter in crustaceans (Bianchini and Monserrat 2007; Duan et al. 2014), and PtCRT was mainly expressed in hepatopancreas suggesting that one of its main functions involves stress and immune response mechanisms. Many studies in crustaceans have proven the inference (Luana et al. 2007; Visudtiphole et al. 2010; Duan et al. 2014), that the expression of CRTs was up-regulated in hepatopancreas after temperature and pathogen challenges. Interestingly, the expression of CRT was suppressed after salinity challenges in this study, which suggests that the stress and immune response functions of CRT were disturbed in salinity stress (Joseph and Philip 2007). This inference is consistent with the phenomenon of increased mortality and decreased immunity caused by salinity changes in the crustaceans (Joseph and Philip 2007; Romano and Zeng 2006; Prangnell and Fotadar 2006); however, the specific mechanism needs further verification.

Osmotic and ionic regulation in the crustacea is mostly accomplished by the multifunctional gills. In addition to their role in gas exchange, the gills are vital in many essential physiological functions such as osmoregulation, calcium homeostasis, ammonium excretion, and extracellular pH regulation (Freire et al. 2008), which are important to salinity adaption. The relatively high levels of PtCRT expression in gill and eyestalk tissues revealed that it may play an important role in osmoregulation. Previous studies showed that salinity stress could induce the expression of CRT in animals and plants (Barman et al. 2012; Shaterian et al. 2005; Shekhar et al. 2013). In this study, the expression of PtCRT up-regulated in gill during salinity challenges further verified that PtCRT plays an important role in salinity adaptation. Further analysis found that the time of PtCRT up-regulated in low salinity challenged was significantly later than the high salinity challenged (48 vs. 3 h), and the different expression profiles indicated that PtCRT may have different mechanisms in low and high salinity adaption.

Single nucleotide polymorphisms (SNPs) are highly abundant markers, which are evenly distributed throughout the genome, and due to their potential for high genotyping efficiency, automation, data quality, genome-wide coverage, and analytical simplicity, they have rapidly become the marker of choice for many applications in genetics and genomics (Garvin et al. 2010; Seeb et al. 2011). SNPs are suitable markers for fine mapping of genes and candidate gene association studies aimed at identifying alleles, which potentially affect important traits. Additionally, the SNP variants present in coding regions allow for associations with phenotypic variations specific to SNP, which makes it possible to identify

particular genotypes, for genetic selection programs or perform molecular studies of adaptation (Bester-van der Merwe et al. 2011; Garvin et al. 2010). In this study, six SNP loci were identified from PtCRT and all of them were located in the open reading frame. The SNP 1269 G/A was found to be associated with a salinity tolerance/susceptibility trait by association analysis, which belonged to the non-synonymous mutation and caused the changes between glutamate and lysine. Further analysis found that this SNP locus lacked the AA genotype, and the GA genotype had higher salinity tolerance than the GG genotype. However, further studies are needed to understand why the amino acid change caused by SNP 1269 G/A were related to the trait of salinity tolerance/susceptibility, or it was only a molecular marker linked with another critical salinity adaptation related genes.

In summary, a full-length cDNA sequence of CRT was cloned from *P. trituberculatus*. We studied the tissue-specific mRNA expression and the expression patterns during high and low salinity challenge in gill and hepatopancreas. In addition, we identified the single nucleotide polymorphism and analyzed its association with low salinity tolerance and susceptibility. All results demonstrated that PtCRT plays an important role in *P. trituberculatus* salinity stress response, which provided useful information for functional research on CRT.

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