Crustacean hyperglycemic hormone of *Portunus trituberculatus*: evidence of alternative splicing and potential roles in osmoregulation

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Received: 22 October 2018 / Revised: 1 February 2019 / Accepted: 5 February 2019 / Published online: 14 February 2019
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Abstract

The crustacean hyperglycemic hormone (CHH) gene of *Portunus trituberculatus* (*Pt-CHH*) consists of four exons and three introns spanning 3849 bp in size and generating two mature mRNA, *Pt-CHH1*, and *Pt-CHH2*. The primary gene transcript produces a cDNA encoding for the putative *Pt-CHH2* from exons 1, 2, 3, and 4 and an alternative transcript encodes for a putative *Pt-CHH1* peptide from exons 1, 2, and 4. A promoter fragment of about 3 kb was obtained by genomic walking. The tissue-specific expression pattern is examined by reverse transcriptase chain reaction, and the results show that *Pt-CHH1* is detected in the eyestalk, brain, muscle, and blood. However, *Pt-CHH2* is detected in the ganglia thoracalis and gill. The results indicate that the expression of *Pt-CHH2* in the gill might suggest a potential role in osmoregulation. The *Pt-CHH* transcript level in the gill increases when the crab is exposed to low salinity. The injection of dsRNA for *Pt-CHH* causes a significant reduction in *Pt-CHH2* transcript level and the activity of Na^+/K^+ -ATPase, and carbonic anhydrase (CA) show a serious decrease. In conclusion, this study provides molecular evidence to support the osmoregulatory function of *Pt-CHH2*.

Keywords *Portunus trituberculatus* · Crustacean hyperglycemic hormone · Alternative splicing · Osmoregulation

Introduction

The swimming crab, *Portunus trituberculatus*, is one of the most important commercial species in China (Dai et al. 1977). As a euryhaline crab, *P. trituberculatus* can tolerate a wide range of salinity from 13.7 parts per thousand (ppt) to 47.7 ppt and the half lethal concentration is 11.1 ppt in low salinity (Han et al. 2014). The gill is the main organ of salinity adaptation, which can regulate the osmotic balance pathway in vivo and in vitro through ion transport. The regulation of ion transport is mainly achieved through Na^+/K^+ -ATPase, V-ATPase, and carbonic anhydrase (CA) (Mcnamara and Faria 2012). Previous studies have shown that the regulation of

osmotic and ion transport are controlled by endocrine mechanisms, and the mode of action is that neuroendocrine factors act on ion transport related enzymes to regulate the content of osmotic pressure (Kamemoto 1976; Mantel 1985; Pan and Liu 2005). Neuropeptides are neural signaling molecules that play a vital role in this regulation process (Christie et al. 2010).

The glucagon (CHH) superfamily are unique crustacean neuroendocrine peptides that play a crucial role in crustacean metabolism, osmotic regulation, molting, and reproduction (Christie et al. 2010; Lacombe et al. 1999; Webster et al. 2012). According to the primary structure and the structures of their precursor proteins, the CHH superfamily is divided into two subgroups: the CHH subfamily (type I neuropeptide) and the molt-inhibiting hormone (MIH)/gonad-inhibiting hormone (GIH)/vitellogenesis-inhibiting hormone (VIH)/mandibular organ-inhibiting hormone (MOHH) subfamily (type II neuropeptide) (Lacombe et al. 1999), among which CHH and the ion transport peptide (ITP) belong to type I neuropeptides (Meredith et al. 1996). CHH has a variety of physiological functions including increasing blood glucose concentration, tissue regeneration, lipid metabolism (Sedlmeier 1985), and a novel function of CHH concerns its role in osmo/ionoregulation. The injection of SG extracts or CHH into the posterior gills of the euryhaline crab, *Pachygrapsus*

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marmoratus, can significantly increase the influx of Na^+ in the gill epithelium (Spanings-Pierrot et al. 2000), and the injection of CHH can increase the hemolymph osmolarity and Na^+ levels in eyestalk-ablated crayfish (Laetitia et al. 2003). Low-salt stress experiments and RNAi techniques were used to study the penaeid shrimp *Litopenaeus vannamei*. Salinity stress increased the expression level of LvITP in the pericardial cavity and a high concentration of dsRNA caused gill hemorrhage (Tiu et al. 2007). In the swimming crab, *P. trituberculatus*, two cDNAs (*Pt-CHH1* and *Pt-CHH2*) encoding CHH have been cloned, and semi-quantitative tissue expression analysis shows that *Pt-CHH1* is only expressed in the eyestalks while *Pt-CHH2* transcript is detected in the thoracic ganglia, Y-organ, and mandibular organ. The results of real-time fluorescence quantitative PCR indicate that *Pt-CHH1* inhibits molting and ovary development (Xie et al. 2014). However, research into the function of the CHH gene in the osmotic regulation of *P. trituberculatus* has not been carried out.

In the present study, the promoter sequence is cloned by genome walking, and its transcription factor-binding sites are analyzed. The full-length CHH genes are obtained by PCR amplification, and the direct cloning data reveals an alternative splicing event in CHH gene transcripts. The expression pattern of *Pt-CHH2* in low salinity is examined by RT-quantitative real-time PCR (qPCR), and the relationships among *Pt-CHH2* and Na^+/K^+ -ATPase, V-ATPase, and CA activity are further studied by RNA interference. Our study, as a whole, provides new insights into hormone-regulated crustacean osmotic regulation and may prove instructive to the breeding of new varieties resistant to low salinity.

Materials and methods

Experimental materials

Healthy crabs (*P. trituberculatus*) with a body weight of 20 ± 3 g were collected from a culture farm (Haifeng Aquaculture Co. Ltd., Weifang, China). Crabs were acclimated in a 4 m³ indoor cement pool with an ambient environment salinity (33 ppt) at 20 ± 3 °C and fed with living *Potamocorbula laevis* once a day. The gill, eyestalk, ganglia thoracalis, liver, heart, muscle, and blood were sampled for tissue-specific mRNA expression analysis and genomic DNA extraction.

Cloning of the full-length *Pt-CHH* gene and genome walking

According to the *Pt-CHH* cDNA sequence (*Pt-CHH1* EU395808, *Pt-CHH2* KJ813806) in the NCBI database, Primer Premier 5.0 software was used to design the specific primers to amplify the intron. PCR amplification was

performed using LA Taq DNA polymerase (TaKaRa, Japan), and the 10 µL reaction system included 0.5 µL DNA template (50 ng), 0.1 µL LA Taq (5 U/µL), 1 µL 10 × LA Taq Buffer II (Mg²⁺ Plus), 1.6 µL dNTP Mixture (2.5 mM each), 0.5 µL CHH F1/F2/F3 (10 µM, Table 1), 0.5 µL CHH R1/R2/R3 (10Mm, Table 1), and 5.8 µL ddH₂O. The following program was used for PCR amplification: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 2 min, with a final elongation at 72 °C for 10 min. The PCR product was detected by 1% agarose gel electrophoresis, and the fragment was recovered by using a gel recovery kit (Tsingke, China), ligated with the pClone007 vector (Tsingke, China), and transformed into competent *Escherichia coli* cells. After transformation, six positive clones were picked for sequencing (Tsingke, China).

To obtain the promoter sequence, a PCR gene walking approach was performed by using a universal genomewalk™ 2.0 kit (TaKaRa, Japan). Briefly, the genomic DNA of *P. trituberculatus* was extracted by using an animal genomic DNA extraction kit and assessed by agarose gel. Genome walker libraries were constructed, and the specific reverse primers GSP1 and GSP2 were designed according to the principle of primer design in the kit manual. PCR amplification was performed using 1 µL of DNA library, 0.5 µL of advantage 2 polymerase (50 ×), 0.5 µL of AP1 primer, 0.5 µL of specific primer SP1, 0.5 µL of dNTP, 2.5 µL of 10 × advantage 2 PCR buffer, and 19.5 µL deionized H₂O. The amplification fragments were obtained and cloned into the pClone007 vector (Tsingke, China). Then the vectors were transformed into *E. coli* competent cells and sequenced.

Sequence analysis

To obtain the promoter sequence and full-length DNA sequence, sequence splicing was performed using Vector NTI 11.5 software (Fig. 1). The core promoter and transcription start site (TSS) were predicted by using the online software http://www.fruitfly.org/seq_tools/nnppHelp.html. The potential transcription factor-binding sites of the promoter were detected by using LASAGNA-Search 2.0 http://biogrid-lasagna.engr.uconn.edu/lasagna_search/, and CpG islands were predicted by <http://www.softberry.com/berry.phtml?topic=cpfinder&group=programs&subgroup=promoter> (GC Percent > 50.0, Obs./Exp. > 0.6, length > 100 bp).

Functional study of the *Pt-CHH2*

A salinity exposure experiment was performed to study the changes in the expression pattern of *Pt-CHH2*. The crabs were randomly divided into two groups: control groups (33 ppt) and low salinity group (11.1 ppt). In each group, three crabs were sacrificed at 3, 6, 12, 24, 48, and 72 h after salinity

Table 1 Primers used in this study

Primer	Sequence 5'-3'
dsRNACHH F1	GATCACTAATACGACTCACTATAGGGCACAAGTGCCAAAGACGAGA
dsRNACHH R1	GATCACTAATACGACTCACTATAGGGCTACTCGAGGGGTCACAAGC
dsRNACHH F2	GATCACTAATACGACTCACTATAGGGAAGGCTACGGGACAGAGACA
dsRNACHH R2	GATCACTAATACGACTCACTATAGGGACACGAAGGGGGAAAGAGAT
dsRNAGFP F1	GATCACTAATACGACTCACTATAGGGATGGTGAGCAAGGGGGAGGA
dsRNAGFP R1	GATCACTAATACGACTCACTATAGGGTTACTTGTACAGCTCGTCCA
CHH F	CAGTCTATCAAATCCGTATGCC
CHH R	TGTCCATCAGCAGCAGGTC
qCHH F	ATCATTTTCACACTGCCCTGG
qCHH R	CCTGTCTCTGTCCCGTAGCC
CHH F1	ATTTCGCAGAGGAAGGACA
CHHR1	CTGGAGGTTCTGTAGAGGTTGT
CHHF2	ACAACCTCTACAGAACCTCCAG
CHHR2	ACATTTCGTCAGGCAGCAT
CHHF3	GCTGATGCTGCCTGACGA
CHHR3	CATCAGCAGCAGGTCTTCC
GSP2	GTGTCCAGGGCAGTGTGAAAATGATG
GSP4	ACGGATTGATAGACTGCATGGCTGT
β -actin-F	CGAAACCTTCAACACTCCCG
β -actin-R	GGGACAGTGTGTGAAACGCC

exposure, and total RNAs extracted from the gills were used for RT-qPCR analysis. To further study the function of *Pt-CHH2*, an RNA interference technique was employed. The primers for CHH (experiment group) and green fluorescent protein (GFP, control group) were designed by Online Biology Software http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl and a T7 promoter was added to the 5' end (Table 1). The dsRNA coding the GFP has been widely used as a non-specific control in a variety of RNA interference studies (Ponprateep et al. 2012; Westenberg et al. 2005). We amplified the *Pt-CHH* cDNA with a pair of T7 linked primers, and the *Pt-CHH* DNA fragment with the T7 promoter was used as a template for the synthesis of dsRNA using an in vitro transcription T7 kit (TaKaRa, Japan). About 10 μ g of *Pt-CHH* dsRNA was obtained from the synthesis reaction in a 20 μ L system. For the experiment group, 20 crabs were injected with various doses (1 μ g/g crab weight) of *Pt-CHH*

dsRNA at the arthrodial membrane of the fifth swimmeret. Another 20 crabs, which were injected with the same concentration of GFP dsRNA, were set as the control group. The crabs were put back into normal seawater and sampled after 24, 48, and 72 h of treatment.

Tissue expression of *Pt-CHH* gene transcripts

Tissue distribution of *Pt-CHH* mRNA was examined by using RT-PCR in selected tissues including gill, eyestalk, ganglia thoracalis, liver, heart, muscle, and blood. For total RNA preparation, various tissues were sampled for extraction by TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The quality of total RNA was detected by 1% agarose gel electrophoresis and the concentration was determined by spectrophotometer. The first strand cDNA was generated following the protocol provided in the PrimeScript™ RT

Fig. 1 Schematic diagram for the cloning of the *Pt-CHH* gene. **a** The gene consists of four exons (red boxes) and three introns (gray boxes). The blue boxes show the promoter region. GT-AG represents the

boundary of the intron–exon. *Pt-CHH* gene generates two transcripts *Pt-CHH1* **b** and *Pt-CHH2* **c** by alternative splicing

reagent Kit (TaKaRa, Japan). The CHH-specific primer set CHH F/CHH R (Table 1) was designed to detect the CHH transcripts from various tissues. PCR amplification was performed using LA Taq DNA polymerase (TaKaRa, Japan) described above (“Cloning of the full-length *Pt-CHH* gene and genome walking”) and condition in the detection procedures were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, with a final elongation at 72 °C for 10 min. The amplification of β -actin was performed under the same condition using the primer pair β -actin-F and β -actin-R. The PCR products were detected by 1% agarose gel electrophoresis and then taken picture.

Determination of *Pt-CHH2* transcript levels

Total RNA from crab tissues were isolated by using TRIzol, and the relative mRNA level of *Pt-CHH2* was assessed using qPCR after low salinity exposure and RNAi. Template cDNAs were 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 (TaKaRa, Japan) was used to qPCR analysis. qPCR reaction system (20 μ L) consisted of 1 $\times$ Power SYBR Green PCR Master Mix, 0.2 μ M each of the qCHH F/R (Table 1), and 1 μ L of template cDNA. PCR was performed using an ABI 7500 RT-PCR instrument and the following program: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 34 s, and a final dissociation curve analysis of 1 cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The β -actin gene was selected as an internal control and amplified under the same conditions. Each cDNA sample was assayed three times, and relative expression was determined by using the $2^{-\Delta\Delta CT}$ method. Data expressed as the mean $\pm$ SE were analyzed by using the Student *t* test with SPSS19.0, and data results were standardized with the expression quantity of 0 h as one. Results were plotted by origin Pro9.0, and $P < 0.05$ indicated a significant difference.

Ion-transport enzyme activity assay

The gill samples treated by RNAi were lapped with liquid nitrogen and then placed in nine volumes (W/V) of normal saline. The diced gills were centrifuged at 2000–3000 g for 20 min, and the supernatant was collected to measure the Na^+/K^+ -ATPase activity by using a Na^+/K^+ -ATPase kit (Nanjing Jiancheng, China). At the same time, total protein content was determined by using Coomassie blue reagent (Nanjing Jiancheng, China). The enzyme activity of V-ATPase and CA were determined by using an enzyme-linked immunosorbent assay (ELISA) kit. After grinding, 0.1 g of tissue was obtained, and 1000 μ L PBS (pH = 7) was added according to the specific operation reference manual. Enzyme activity,

expressed as mean $\pm$ SE, was analyzed by using a two-factor repeated measures analysis of variance (ANOVA) with SPSS, and significances were considered as $P < 0.05$.

Results

Analysis of transcription factor binding sites of the promoter sequence

A DNA promoter fragment of about 3 kb was obtained by using primers GSP2, AP1 and GSP4, AP2 for nested PCR. Bioinformatics analysis showed that the transcription initiation site was located at the 86th nucleotide (A) upstream of the translation initiation codon (ATG), a TATA box was present at 31 bp upstream of the TSS, and the core promoter region was located at $-40 \sim +9$ bp. The putative binding sites for octamer binding protein (Oct-1), nuclear transcription factor (NF-kappaB), globin transcription factor (TBP), intracellular transcriptional activator (AP-1), and other binding sites were also found in the upstream region of the *Pt-CHH* gene. Online software did not find CpG islands (Fig. 2).

Construction of the *Pt-CHH* gene

The full-length amplification was performed using primers (CHHF1/R1, CHHF2/R2, CHHF3/R3) designed according to the difference between the *Pt-CHH1* and *Pt-CHH2* sequences. Overlapping genomic clones were used to calculate the full-length sequence. The results show that the *Pt-CHH* gene consists of four exons separating three introns 1, 2, and 3 with sizes 322 bp, 828 bp, and 975 bp, respectively, and *Pt-CHH2* contains all four exons while *Pt-CHH1* contains exons I, II, and IV. The two mature mRNAs have a common start codon but different stop codons, which are located in exon IV of *Pt-CHH1* and in exon III of *Pt-CHH2*, respectively. All the intron–exon boundaries of the *Pt-CHH* gene fitted the GT-AG boundary rules (Fig. 1). Exon I encoded the first six signal peptide amino acids and exon II encoded the rest, including a 35-residue CPRP and the N-terminal end of the mature peptide. Exon 3 and exon 4 encoded the rest of the mature peptides to form *Pt-CHH1* and *Pt-CHH2*.

Expression of *Pt-CHH* gene transcripts

In order to examine the tissue distribution of these transcripts, RT-PCR was carried out using CHH gene-specific primers. A *Pt-CHH1* fragment of the expected size, 370 bp using the CHH F/CHH R primer set, was found in the eyestalk, brain, muscle, and blood. While a *Pt-CHH2* fragment of the expected size, 480 bp using the CHH F/CHH R primer set, was detected in ganglia thoracalis and gill (Fig. 3).

Fig. 2 Nucleotide sequence of the *Pt-CHH* gene and its promoter. Gray represents the core promoter sequence; Potential transcription initiation site is marked by TSS; Potential transcription factor-binding sites are

underlined in the promoter sequence; TAAs represent the termination codon of *Pt-CHH1* and *Pt-CHH2*; Exon sequences are shown in the boxes

Fig. 3 The distribution of the *Pt-CHH* transcripts in different tissues of *P. trituberculatus*. *E* eye, *Br* brain, *Tr* ganglia thoracalis, *G* gill, *B* blood, *H* heart, *L* liver, *M* muscle

Expression profiles of *Pt-CHH2* under low salinity

Tissue-specific expression of *Pt-CHH* transcripts showed that only *Pt-CHH2* expressed in the gills, which were essential for ion transport, so we studied the expression of *Pt-CHH2* in the gill. The results (Fig. 4) revealed that the expression of the *Pt-CHH2* gene in the experimental group changed significantly ($P < 0.05$) and was upregulated in the whole experiment group. After 3 h salt stress, it increased by 4.2 times compared with the control group. After 24 h low salinity, the expression level of the *Pt-CHH2* gene was the lowest, which was 1.7 times that of the control group.

Effect of *Pt-CHH* gene silencing on ion-transporter enzymes by gills

In this study, we mainly studied the role of *Pt-CHH2* in osmotic regulation, so we only measured the expression of *Pt-CHH2* in the gill after interference. The gills, which were sampled from the crabs injected with dsRNA, showed a 52% and 84% drop in the expression of *Pt-CHH2* at 24 h and 48 h after injections, respectively. At 24 h after the injection of dsRNA, the activity of Na^+/K^+ -ATPase in the experimental group did not show any difference compared with the control group, while the activity of Na^+/K^+ -ATPase in the experimental group at 48 h and 72 h was 0.60 and 0.53 times that of the control group. There was no significant difference in V-ATPase activity between the experimental and control groups in the gills (Fig. 5). The activity of CA in the

experimental group and the control group showed a significant difference at 72 h, reducing the enzyme activity by 8.4 units.

Discussion

Analysis of binding factors in the *Pt-CHH* gene

As a key element of gene expression regulation, promoters play an important regulatory role in gene duplication, recombination, and transcription (Bansal et al. 2014; Keller 1992). The results showed that the transcription initiation site was located at the 86th nucleotide (A) upstream of the translation start site (ATG), the TATA box was located 31 bp upstream of the TSS, and the core promoter region was located at $-40 \sim +9$ bp, which is similar to the promoter of *Scylla paramamosain* (Huang et al. 2017). Putative transcription factor-binding sites, such as OCT-1, NF-kappaB, GATA-1, TBP, AP-1, and C/EBP, which are in the flanking region of *Pt-CHH*, were different from that reported in other crustaceans. These data might explain why *Pt-CHH* has a variety of physiological functions. The C/EBP site implied a similar role with CREB, as a cAMP-responsive factor-binding site. The presence of the C/EBP site in *Pt-CHH* might indicate that cAMP mediates expression. This shows that the genes are possibly under the control of other factors or feedback mechanisms (Gu and Chan 1998; Lu et al. 2001).

Gene structure of *Pt-CHH*

CHH has a variety of physiological functions, so understanding the characteristics of the primary structure of the CHH gene can help us better understand the regulatory role of CHH. At present, the CHH gene is reported to produce two or more transcripts by alternative splicing in many crustaceans. A recent article on the structure of the CHH gene also reported that LvCHH-ITP consists of three introns and four exons in *L. vannamei* (Tiu et al. 2007). Similar to reports in other decapods, two CHH gene transcripts *Pt-CHH1* and *Pt-CHH2* were identified in this study. Evidence from direct sequence suggests that these two isoforms come from a CHH gene transcribed in an alternative splicing manner. The CHH gene forms *Pt-CHH2* by the cleavage of all introns, while *Pt-CHH1* removes exon 3 in the original transcript. Analysis of

Fig. 4 Relative expression of the *Pt-CHH2* transcript in gill exposed to low salinity with time

Fig. 5 Osmo-regulation related to the function of the *Pt-CHH2*. **a** qPCR detection of the *Pt-CHH2* transcript level in gills at 24, 48, and 72 h after injection of dsRNA for *Pt-CHH2*. **b, c, d** Enzyme activity of Na^+/K^+ -ATPase, V-ATPase, and CA in gills at 24, 48, and 72 h after the injection of *Pt-CHH* dsRNA

the nucleotide sequences of *Pt-CHH1* and *Pt-CHH2* revealed that there is a stop codon signal at exon 3, which is the signal that makes the number of amino acids in *Pt-CHH2* almost the same as in *Pt-CHH1*. We do not yet know the signaling mechanism of this stop codon, but it may have an effect on the production and tissue specificity of the two CHH proteins.

Tissue distribution of *Pt-CHH* mRNA

A recent study has established that the expression of CHH peptides was not only discovered in neural tissues, such as the XO-SG system and the pericardial organs, but surprisingly, also expressed in other non-neural tissues. The CHH gene was found to express in the heart, gill, and antennal gland in *Macrobrachium rosenbergii* (Chen et al. 2004) and also found in spermatophore epithelial cells of *Fenneropenaeus chinensis* by in situ hybridization and in the intestine of *L. vannamei* (Tiu et al. 2007). Similarly, 10% of CHH can be detected in the blood after the removal of eye stalks in lobster (Chang et al. 1999). In this study, *Pt-CHH* was also found in none eyestalk tissues, such as gill, muscle, and blood, but in the study of Xie et al. (2014), CHH was not found in muscles and gill. There are two main reasons for speculation. One is that the experimental materials are different. We used crabs of about 20 g, however, in the study by Xie et al., they were 40–80 g; the second is the experimentally determined template concentration is different. In the eyestalk and gill, *LvCHH* and *LvITP* transcripts were consistently detected in the same tissues (Tiu et al. 2007), but our study has not found two forms

of CHH in the same tissue. The CHH gene abundantly expressed in the thoracic ganglia, indicating that the thoracic ganglion was an additional source of CHH. At the same time, this may explain why the presence of CHH can be detected after the removal of the eyestalks in some crustaceans (Li et al. 2017).

The expression profile of *Pt-CHH2* in low salinity

It has been reported that the gene *Pt-CHH1* in *P. trituberculatus* has the ability to inhibit molting and gonad development (Xie et al. 2014), while the function of the *Pt-CHH2* gene has not been reported yet. As a blood–water barrier, the gills, which possess a variety of cell types, have respiration, excretion, osmoregulation, disease defense, and other physiological functions (Mcnamara and Faria 2012; Shuanglin et al. 2001). CHH fractions purified by high-performance liquid chromatography significantly increased the transepithelial potential difference and Na^+ influx by about 50% (Chung et al. 1999). Considering the previous study and the expression site of the transcript, the authors believe that *Pt-CHH2* might be involved in the regulation of osmotic pressure in *P. trituberculatus* and verified it by the following experiment. The low salt stress experiment showed that the *Pt-CHH2* gene responds quickly to stress and remains at a high level in gills under low salt stress. This result is similar to the reports of *CHH2* gene expression in *S. paramamosain* (Miaoan et al. 2012) and the *LvITP* gene expression of *L. vannamei* under low salinity stress. From the above

results, it can be inferred that the *Pt-CHH2* gene plays a certain regulatory role in low salt adaptation. Next, RNA interference technology was used to further study the function of *Pt-CHH2*.

The effect of RNA interference on the activities of ion-transport enzymes in gills

Many reports have demonstrated that osmotic pressure and ion transport are regulated by the neuroendocrine system, such as pericardial organs, thoracic ganglion, supraesophageal ganglion, and ventral. Nerve cord removal of the bilateral eyestalk and unilateral eyestalk can change osmotic pressure and ion concentration, as well as the activity of Na^+/K^+ -ATPase in the gill tissue of *Penaeus monodon* (Nan et al. 2004). Similarly, an injection of the extract of sinus gland could increase the activity of Na^+/K^+ -ATPase in *P. marmoratus* by 54% (Eckhardt et al. 1995). The regulation of crustacean gill epithelium osmotic pressure mainly depended on various enzyme activity, in which Na^+/K^+ -ATPase accounts for 70% of total ATPase activity and V-ATPase accounts for 30% (Furriel et al. 2000). In addition, CA also plays a key role in the process (Henry and Campoverde 2006; Henry et al. 2002). To further investigate the relationship between *Pt-CHH2* and enzyme activity, RNAi technique was used with *Pt-CHH*. The expression level of *Pt-CHH2* significantly decreased after injecting dsRNA and then the Na^+/K^+ -ATPase, V-ATPase, and CA activity in gill tissues were measured. The knockdown effect of the *Pt-CHH2* gene was significant at 24 h, and the activities of Na^+/K^+ -ATPase and CA began to decrease significantly at 48 h and 72 h, indicating that *Pt-CHH2* has a certain regulatory effect on Na^+/K^+ -ATPase and CA, and the regulation of the two enzymes has a certain time difference. This also indicates that Na^+/K^+ -ATPase plays a major role in ion regulation in salinity adaptation (Camacho-Jiménez et al. 2017; Han et al. 2014; Lucu and Towle 2003). The change of CA caused by the resection of the eyestalk proves that CA is regulated by the neuroendocrine system (Henry and Campoverde 2006). In addition, rCHH-B1 and rCHH-B2 can regulate the expression of Na^+/K^+ -ATPase and CA genes in gills of *L. vannamei* (Camacho-Jiménez et al. 2018). Removing the endogenous CHH of *L. vannamei* decreased the activity of Na^+/K^+ -ATPase and CA (Li et al. 2017). However, V-ATPase did not change throughout the process of RNAi and further investigations in the present study were unable to show any significant effect of CHH on V-ATPase activity in *Discoplax celeste* (Turner et al. 2013). Therefore, our study suggested that *Pt-CHH2* regulates ion transport by regulating the activity of Na^+/K^+ -ATPase and CA.

In this study, the promoter and DNA sequences of the *Pt-CHH* gene were cloned from the crab *P. trituberculatus*, and the primary structure of the two transcripts of the *Pt-CHH* gene was defined. Tissue-specific mRNA expression and

expression profiles influenced by salinity stress and RNAi were studied, while the activity of ion-transporting enzyme was detected. The above evidence indicates that the *Pt-CHH2* gene regulates osmotic pressure in the crab *P. trituberculatus* by regulating ion transport enzymes.

Funding information This research was supported by the National Natural Science Foundation of China (grant Nos 41776160 and 41576147), Efficient Eco Agriculture Innovation Project of Taishan Leading Talent Project (No. LJNY2015002), Shandong Province Key Development Program for Research (2018GSF121030), Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology (2016LMFS-A12), Qingdao Applied Basic Research Project (17-1-1-95-jch), Special Scientific Research Funds for Central Non-profit Institutes, Yellow Sea Fisheries Research Institute (Project 20603022018027), and the Key Research and Development Plan of Jiangsu Province (BE2017325).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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