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Identification and expression of cysteine sulfinate decarboxylase, possible regulation of taurine biosynthesis in *Crassostrea gigas* in response to low salinity

Received: 10 January 2017
Accepted: 23 June 2017
Published online: 14 July 2017

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Taurine has been reported high amounts in marine animals to maintain osmotic balance between osmoformers and sea water. Approximately 80% of the total amino-acid content is taurine in Pacific oyster *Crassostrea gigas*, an intertidal and euryhaline species. In this study, we cloned the two copies of cysteine sulfinate decarboxylase (CSAD), the key enzyme in taurine biosynthesis pathway, screened in oyster genome data. Sequentially, we compared the expression patterns of CgCSAD1 and CgCSAD2 under low salinity treatment (8‰ and 15‰) using different families from two populations. There was no correlation between the expression of CSAD and the different population. Notably, CgCSAD1 increased significantly in treated groups for 24 h, but CgCSAD2 had no significant differentiation. Moreover, the results of CgCSAD1 interference provided the evidence of the positive correlation between CgCSAD1 expressions and taurine contents. The zinc finger domain showed in multi-alignment results may be the important character of CgCSAD1 as the key enzyme in taurine biosynthesis to regulate taurine pool in response to low salinity. This study provides a new evidence for the important role of taurine in adaptation to low salinity in oyster. In addition, it is a good model to discuss the function and evolution of the duplication in mollusks.

Environmental factors, especially salinity, in intertidal zones uctuated acutely owing to tides, rainfall, surface runo and so on. Osmoconforming marine animals can result in large changes in the osmotic concentration of hemolymph in order to adapt to uctuations in salinity of ambient seawater[1,2]. One of the primary strategies of osmoconforming animals to keep the osmotic balance is the manipulation of intracellular levels of organic osmolytes, especially free amino acids[3]. For marine molluscs, as osmoconforming animals, only a few free amino acids predominantly contribute to the intracellular pool, such as alanine, glycine, proline, taurine and glutamate. e content of taurine is lower in freshwater species due to the minimized osmotic equilibrium between the cells of them and the respective extracellular uids[4]. Unlike freshwater invertebrates, the cells and extracellular uids are very close to sea water in osmotic concentration, and their intracellular inorganic ion composition is only slightly higher than freshwater species. So free amino acids, as organic osmolytes, maintain osmotic balance between extracellular and intracellular uids in marine species[5].

e Paci c oyster is a euryhaline bivalve which can experience rapid and dramatic salinity uctuations from below 10‰ to above 35‰[6]. As was reported, the osmolality of their hemolymph could change from 800 to 380 mOsm/kg within eight hours with the changes of seawater[7]. e decrease in osmolality and increase in water content of the mantle within 8 h a er the exposure to hypo-osmolality were caused by the permeation of water into the oyster. erea er the water content decreased gradually up to 72 h, while the osmolality did not change[7]. ere is no evidence of ionic regulation for Na^{+}, Ca^{2+} and Mg^{2+} in [8]. Hopkins[9] gave 33 to 41% seawater as the lower limit for e ective pumping in the Paci c oyster. Interestingly, taurine, as the primary

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osmolyte accounts for approximately 80% of the total amino-acid content in the Paci c oyster[10] and were signi - cantly in uenced the pattern of the change in total content of FAA in adaptation to hypo-osmolality[7].

seawater at 18 °C for two weeks. For the experiment, oysters were divided randomly into two groups. One group was injected in 100 μl, 1 μg/μl dsRNA, and other group was injected in 100 μl, 150 mM NaCl as control group. A er 48 h post-injection, each group was separated in 3 sub-groups and cultured in 8‰, 15‰, and 30‰ ltered seawater, respectively. e gill tissues of oysters were dissected and divided into two groups. One was saved in RNA store at −80 °C for RNA extraction, the other was frozen immediately using liquid nitrogen and stored at −80 °C for taurine extraction.

Molecular phylogenetic analyses. Alignment of the sequences and construction of neighbor-joining tree with 1,000 bootstrap replications were performed using the MEGA so ware program v5[24]. e tree was rooted by evolution-related enzymes, HDC (histidine decarboxylase) of human.

Reverse-transcription PCR. e cDNA for qRT-PCR was prepared using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). Firstly, 10 μl reaction consisted of 2 μl 5 × gDNA Ereaser Bu er, 1 μl gDNA Eraser, 2 μl total RNA and 5.0 μl RNase-free water under 42 °C for 2 min to eliminate genome DNA. en, 1 μl PrimeScript RT Enzyme Mix I, 1 μl RT Primer Mix, 4.0 μl 5 × PrimerScript Bu er 2 and 4.0 μl RNase-free water were added to the above 10 μl reaction. e reverse-transcription PCR program was set with 37 °C for 15 min and 85 °C for 5 s.

Quantitative real-time PCR analysis. In order to understand the spatio-temporal expression of CgCSAD1 and CgCSAD2 and compare the mRNA levels of the related genes in the taut1(tTc t)-es 6 ti(ut1(tTo)16(5)]T.7(a(
CgCSAD1 geneCgCSAD126339HErTCATTCCCTCCTTGACCT
CgCSAD1 geneCgCSAD126339-3 -628ATGTTCTGCCCTGGCGGTTCTATT3 3[Tw [(R)-4(A)28(CE PCR f)9(o)
CgCSAD2 geneCgCSAD222071HErGAAGAAATTGACATGCTCCCgCSAD2 geneCgCSAD222071-3 -319GCC

analysis. Samples were pre-column derivatized with o-phthaladehyde (OPA)/2-mercaptoethanol and separated with a Zorbax Eclipse C18 column (Agilent, Germany), using a gradient elution[25].

We applied one-way ANOVA to test the changes of taurine levels between RNAi groups and control groups, and di erent salinity treated groups. We considered di erences to be statistically signi cant at $P < 0.05$.

Results

cDNA cloning and phylogenetic analyses. Two copies of CSAD were cloned in and were named as CgCSAD1 and CgCSAD2. e CgCSAD1 cDNA was 2,097 bp in length and contained a 60 bp 5′-untranslated region (UTR), a 369 bp 3′-UTR, and a 1,668 bp open reading frame (ORF). It encodes a predicted polypeptide consisting of 555 amino acid residues and an estimated molecular mass of 63.35 kDa; e CgCSAD2 cDNA was 1,713 bp in length and included a 82 bp 5′-UTR, a 146 bp 3′-UTR, and a 1485 bp ORF. It encodes a predicted polypeptide consisting of 494 amino acid residues and an estimated molecular mass of 56.11 kDa. e two copies have no signal peptide. e molecular structure of the two copies contains pyridoxal phosphate (PLP)-dependent

Tissue specificity of mRNA expression. e transcripts of CgCSAD1 and CgCSAD2 genes were detected in all tissues examined (Fig. 4). e transcript level of CgCSAD1 was higher than that of CgCSAD2 in all tissues. As for CgCSAD1 gene, the transcript level was higher in the gonad, followed by the hemolymph and adductor muscle. Gill and mantle had lower expression of CgCSAD1. e transcript expression of CgCSAD2 was higher in hemolymph and gonad and lower in digestive gland and labial palp.

Effect on the expression of genes in taurine metabolism after CgCSAD1 interference. Based on the result of pre-experiment (Fig. 5), the expression of CgCSAD1 began to decrease at 24 h a er dsRNA injection and it was maintained at a relatively low level from 48 to 72 h. However, oysters injected with 150 mM NaCl did not display signi cant decreases in CgCSAD1 expression a er injection.

We tested the e ect of CgCSAD1 interference on expression of related genes participating in taurine metabolism pathway, including CgCSAD2, CDO, ADO and GTT. Moreover, a er 48 h of dsRNA injection, the oysters injected with dsRNA and 150

showed, compared with control groups, taurine contents decreased in groups of CgCSAD1 interference. In di er-ent salinity treatments, taurine content was signi cantly highest in 15‰. Combined the expressions of CSAD, the signi cantly di erent expression of CgCSAD1 was in accordance with the signi cant change of taurine contents in CgCSAD1 interference groups and control groups injected with 150 mM NaCl under salinity 15‰ (Table 3), but the expression of CgCSAD2 had no signi cant change under di erent salinity treatments.

Discussion

Terrestrial animals or freshwater invertebrates usually seemed to use inorganic ions as osmotic solute. While

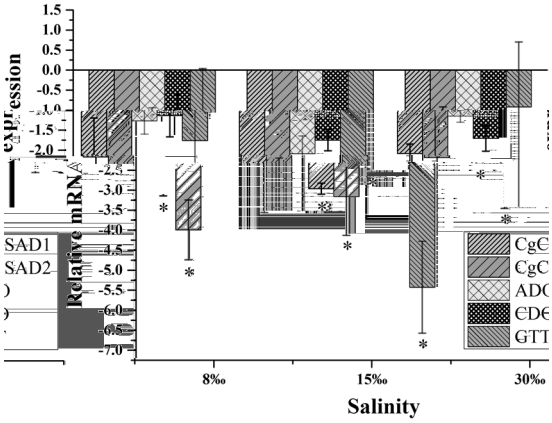

Fig e 6. Relative mRNA expression of genes a er RNAi of CgCSAD1. Elongation factor 1 α (EF) gene expression was used as internal control. e samples injected in 150 mM NaCl solution and acclimated in 8‰, 15‰ and 30‰ are the reference samples, respectively. Vertical bars represent the mean ± SD (N = 6). *Indicated the signi cant di erent (fold change > 2, $p < 0.05$). CSAD: Cysteine sul nate decarboxylase, ADO: cysteamine dioxygenase; CDO: cysteine dioxygenase; GTT: glutathione hydrolase.

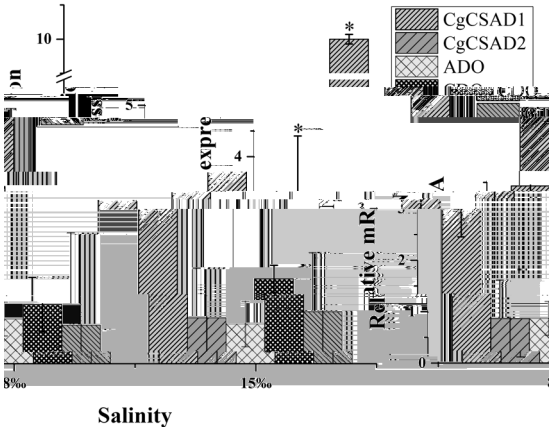

Fig e 7. Relative mRNA expression of genes of samples in di erent salinity a er dsRNA injection. Elongation factor 1 α (EF) gene expression was used as internal control. e samples injected with dsRNA of CgCSAD1 and acclimated in 30‰ are the reference samples. e y-axis represent the fold changes between the expressions of treated samples and reference samples. Vertical bars represent the mean ± SD (N = 6). *Indicated the signi cant di erent (fold change > 2, $p < 0.05$). CSAD: Cysteine sul nate decarboxylase, ADO: cysteamine dioxygenase; CDO: cysteine dioxygenase; GTT: glutathione hydrolase.

G Na e	Ta i e c &e &g (g/g d eigh&) A e age ± SD (= 6)
8‰T	55.37 ± 2.42^{b}
15‰T	60.50 ± 3.11^{a}
30‰T	56.06 ± 3.65^{bc}
8‰C	60.44 ± 2.80^{c}
15‰C	65.57 ± 0.86^{b}
30‰C	61.25 ± 4.44^{c}

Tab e 3. e content of taurine in gill tissues in di erent treatments. T: CgCSAD1 interference group. C: 150 mM NaCl injected group. Di erent superscript letters indicate signi cant di erence among means ($p < 0.05$). Method: one-way ANOVA.

also reported in deep-sea mussel. is result may be attributed to the restriction of amino acid substitutions that is common in functional sequences but also to unique diversi cation in each animal lineage[19]. On the other hand, the natural selection of CSAD gene may rely on habitat, because mollusks and sea urchins live in the sea and insects live on the land.

ere is a zinc nger domain in the C-terminus of the protein of CgCSAD1 by PFAM domain prediction (Figure S1) compared with CgCSAD2 and CSAD in the other molluscs and vertebrate. is result was reported in previous study and speculated that this domain may be a transcriptional regulatory site, or an action site sensing the signal transduction of osmotic pressure in response to salinity uctuation[21].

CgCSAD1 and CgCSAD2 gene expressions were observed in all the tissues examined. Gonad, haemolymph and adductor muscle exhibited higher levels of expression of the two genes, but gill had a lower expression. It is reported that diets supplemented with taurine could improve the muscle growth of rainbow trout[28]. It may have a similar function in oyster, so that CSAD maintains a high expression in gonad and adductor muscle. Haemolymph is an important place to maintain osmotic balance between extracellular region and external environment. To meet the demand, the oysters may express CSAD at high levels in the haemolymph. To test the correlation between the key enzyme of taurine synthesis pathways and the low salinity adaptation of di erent oyster population, we cultured one hybrid family and two parental families using two populations and exposed them to the 8‰, 15‰ and 30‰ ltered seawater. e selection of experimental salinity was based on the reported researches. It deduced that 5‰ may exceed the range of oyster salinity tolerance using transcriptomic data by bioinformatics analysis[20,22]. 8‰ was the lowest known tolerant salinity for [29]. It is con rmed that 15‰ was the low salinity to cause stronger response in taurine[22]. However, based on the results of expression of CSAD under the same treated condition, there was no signi cant di erentiation in CgCSAD1 gene between the oysters from two di erent salinity environments. CgCSAD2 had signi cant di erent expression in the WW families under 8‰ treatment compared with the two other families. It may indicate that the ability of taurine regulation is not the main di erence between the two oyster populations. However, CgCSAD2 is population-speci c in response to low salinity, suggesting it might be correlated with natural selection.

e results of di erent salinity treatments and timepoints showed that 8‰ was an extreme changes of environmental salinity with weaker expression of CgCSAD1. e expression of CgCSAD1 increased to more than one hundred folds under 15‰ for 24 h. In addition, there were no signi cant expression a er 8 h compared with 24 h. It was reported that the water content of the mantle increased in the rst 8 h in response to hypo-osmolality. A er that, the water content decreased gradually without the changes of osmolality[7]. erefore, the taurine regulation might start a er 8 h of exposure to hypo-osmolality. Compared with CgCSAD1, the expression pattern of CgCSAD2 was totally di erent and the expression variation was small in response to low salinity. It was the probable functional di erence between CgCSAD1 and CgCSAD2.

To make the function of CgCSAD1 and CgCSAD2 clear, we conducted RNA interference experiment for the correlation between the expression of CSAD and taurine contents. RNA interference is an e ective way to induce a post-transcriptional homologous gene-silencing by dsRNA in oyster[30,31]. dsRNAs in cells are cleaved into small fragments (about 21–23 bp) by an RNase III, and incorporated with RNase into RNA-induced silencing complex (RSIC), which can degrade target mRNA to inhibit the expression of the target gene[32,33]. In the genome of , we found there are genes annotated as CDO, ADO and glutathione hydrolase (GGT), which were enzymes participating in taurine and hypotaurine metabolism pathway (map 00430). CDO and GGT were at upstream and downstream of CSAD in the pathway, respectively. ADO was the key enzyme in the side pathway of taurine synthesis. Since CgCSAD2 decreased signi cantly with CgCSAD1 in CgCSAD1 interference experiment, it is likely that the conserved domain of the two genes resulted in the lightly interference of CgCSAD2. At 15‰, the expressions of ADO were down-regulated signi cantly, which suggested the side pathway of taurine synthesis did not complement taurine in response to salinity 15‰ when CSAD was silencing. is conclusion was drawn by data , too[22]. GTT took part in the taurine catabolism, and was signi cantly down-regulated with the decreasing of the taurine at 15‰. As showed in Fig. 6, all the gene had the trend of down expression with interference of CgCSAD1, evidencing the key roles of CgCSAD1 in taurine metabolism. Compared even in CgCSAD1 interference groups under di erent salinities, the expression of CgCSAD1 increased signi cantly at 8‰ and 15‰ compared with 30‰. It indicated that CgCSAD1 was closely correlated with hypoosmotic stress.

Based on the results of ANOVA analysis on taurine content, the taurine contents were signi cantly decreasing a er the interference of CgCSAD1 under di erent salinity. It showed there was a positive correlation between the expression of CgCSAD and taurine contents in gill tissues. In addition, taurine contents of samples under 15‰ were signi cantly di erent with those under 8‰ and 30‰ conditions either RNAi groups or control groups. It indicated that the taurine contents were correlated with the expression of CgCSAD1 in response to low salinity stress. In addition to taurine biosynthesis pathways for taurine regulation, there was taurine transporter to uptake taurine into body. Taurine transporter required cotransport of amino acids with Na^+ in integumental tissues[34]. It is reported that acute exposure of isolated gill tissue to 60% arti cial sea water resulted in a greater than 85% inhibition of taurine uptake in mussel, but the apparent accumulation of taurine just reduced by approximately 10%[35]. erefore, we speculated that oysters rapidly swell with the e ux of inorganic ions and organic osmolytes and the in ux of water at the start produced by the hypoosmotic stress. Sequentially, the cell will at least partially recover the original volume with time. With the complement of taurine in cells was from the taurine biosynthesis pathways to help the cell volume recovery. e adaptive range of salinity may be determined by the ability of taurine biosynthesis . However, the taurine contents did not showed hundreds of changes in response to salinity 15 with the up-regulation of CgCSAD1. ere might be no signi cant change at functional levels, such as protein content, enzyme capacities and metabolite concentration, at the same time points. In addition, the oxidation of hypotaurine to taurine remained unknown. is process might be one of the reasons resulting in the di erent changes between gene expression and taurine contents. e further studies need to increase more time points and complement data at functional levels. is study indicated that CgCSAD1 may be an example of gene expanding for salinity adaptation.

However, the expression of CgCSAD2 was not in accordance with taurine. It may have little relationship with osmoregulation. In addition to being an organic osmolyte, taurine play a role in neuroregulation and calcium homeostasis in vertebrates' studies[36,37]. Remy, .[38] found two copies of CSAD in brain and liver of mouse,

respectively, and functions of them was similar. Although CSAD gene is the key enzyme in taurine biosynthesis pathway and the key to maintain cellular taurine balance, the two copies of CSAD in have di erent expression patterns in response to low salinity. In oyster genome research, it is found that oyster stress responsive genes are more likely to retain paralogous duplications[20]. In this study, the results showed that the two copies of CSAD have di erent sub-functions. e speci c function of CgCSAD2 in regulating taurine content needs further study. Comparisons between CgCSAD1 and CgCSAD2 in the molecular mechanism of functional specialization is of interest, and will advance the understanding of life evolution in adaptation to the variable environment.

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Acknowledgements

This study was supported by the grants from National Natural Science Foundation of China (31372524), Shandong Province (2016ZDJS06A06), Shandong Seed Project, and Fundamental Research Funds for the Central Universities.

Author Contributions

X.Z. carried out the molecular genetic studies, participated in the data analysis and dra ed the manuscript. Q.L.