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A regulatory variant of tryptophan hydroxylase gene modulates transcription activity and biases growth rate in the Pacific oyster, *Crassostrea gigas*

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ABSTRACT

Growth is an important trait with significant impact on economic value of cultured organisms. Identification of critical genes and nucleotide variations associated with growth is an important step toward molecular breeding of growth-related traits. In this study, we showed that tryptophan hydroxylase (TPH), the rate-limiting enzyme of serotonin (5-HT) biosynthesis, played an indispensable role in growth regulation in the Pacific oyster, *Crassostrea gigas*. We identified the *C. gigas* TPH (*CgTPH*) and performed comparative analyses of its expression and nucleotide variations between selectively bred fast-growing Pacific oysters ("Haida No. 1") and wild oysters. Expression profiling and RNA localization showed that *CgTPH* was specifically expressed in the nerve-rich tissues. Notably, expression level of *CgTPH* was significantly higher in fast-growing "Haida No.1" than wild oysters. Association and haplotype analysis of single nucleotide polymorphisms (SNPs) in upstream regulatory region of *CgTPH* revealed four SNPs (located at −904, −522, −272, −262 bp) and two haplotypes (CCCC and TCTC) that were significantly associated with growth rate of *C. gigas*. Luciferase reporter assay identified the core transcription regulatory element located at −403 ~ −179 bp, in which the SNP -272C/T was critical for transcription activity of *CgTPH*. Further analysis revealed that the different transcription activity was due to the distinct binding efficiency with two transcription factors, FoxO and CUX. Together, we showed that a critical SNP in the regulatory region of *CgTPH* leading to differential transcription regulation by FoxO and CUX, which would affect 5-HT synthesis and growth in *C. gigas*. This work is of great significance for elucidating the role of 5-HT system in regulation of growth in mollusks and provides a marker for molecular breeding of growth with other production and performance traits in *C. gigas*.

1. Introduction

Traits associated with fast growth have long been one of the major target traits for genetic improvement in all food animals. Identification of genetic variations associated with growth traits is critical for molecular breeding which can greatly enhance selection accuracy and shorten breeding process (Beauchemin et al., 2006; Lin et al., 2021; Wang, 2017). Numerous SNPs associated with growth traits can be identified through genome-wide association analysis, while only a limited portion had practical application (Ahsan et al., 2013; Wang et al., 2019; Wang, 2017). Therefore, identification of genetic variations within functional genes contributing to phenotypic changes is of great interest.

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme for biosynthesis of serotonin (5-HT), an important monoamine neurotransmitter and neuromodulator effect on mood, sleep, appetite, reproduction, development and growth (Alenina et al., 2009; Pennati et al., 2001; Yildirim and Derksen, 2013). Mature studies have proved that TPH function on the regulation of growth and cell proliferation in some model species. *TPH2*-knockout mice resulted in the depletion of 5-HT in the central nervous system, leading to growth retardation and 50% mortality in the first 4 weeks of life (Alenina et al., 2009). *TPH* loss of function mutations in *Daphnia magna* also down-regulated growth and

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fecundity (Campos et al., 2019), indicting their conservative and incontestable roles in growth regulation function. The genetic variations of *TPH* genes have been reported in some complex disorders and syndromes, which induce the depressive disorders (Wigner et al., 2018), suicidal behavior (Nielsen et al., 2020), mood (Gutknecht et al., 2007), aggressiveness (Kulikov et al., 2005) and irritable bowel syndrome (Katsumata et al., 2018). However, the genotype-phenotype correlation contributing to the variable phenotypes of growth traits associated with *TPH* gene polymorphisms have not been reported, especially in invertebrates. Therefore, it's of great significance to investigate the nucleotide variations within *TPH* gene, which will be valuable for unravelling the roles of 5-HT system in biological functions.

Nucleotide variations in regulatory DNA elements such as promoters can affect the binding efficiency with transcription factors, thus controlling gene expression and phenotypes. Previous studies have found that *TPH* gene was under control of the transcription factors such as the STAT5, cAMP-responsive element-binding protein (CREB) and DAF-16 (FoxO homologous protein in *Caenorhabditis elegans*), and function to regulate organism growth. In vertebrates, growth hormone induces the expression of *TPH1* gene through activation of STAT5 transcription factor, then 5-HT stimulates adult perinatal β-cell proliferation (Moon et al., 2020). Moreover, calcium and cAMP signals synergistically mediate glucose-stimulated *TPH1* transcription in rat β-cells by activating the binding between CREB and *TPH1* promoter. The activated *TPH1* up-regulated the expression of genes that are related to islet function and augmented glucose-stimulated insulin secretion (Zhang et al., 2017). In *C. elegans*, expression of *TPH1* in ADF neurons is modulated by DAF-16 through the activities of TGF-β signaling pathway to regulate growth and development arrest (Estevez et al., 2006). Excepted that a number of transcription factors have also been identified to regulate the transcription of *TPH* gene, such as CDP/Cut (CUTL, CUX) (Teerawatanasuk et al., 1999), NF-Y and SP1 (Côté et al., 2002), UNC-86 (Sze et al., 2002), MSX-1 and MSX-2 (Brown et al., 2005). How the variations in promoter region of *TPH* influenced the transcriptional activity of it and affected the growth rate of organisms need further study.

The Pacific oyster, *Crassostrea gigas*, is one of the most widely cultivated mollusks (Zhu et al., 2016). Through successive selection breeding for over a decade, our group produced a fast-growing variety of *C. gigas* known as "Haida No. 1", which shows superior growth performance over wild oysters (Li et al., 2011). The molecular markers associated with growth traits based on genetic variations have been extensively studied in oysters (Li et al., 2018; Wang, 2017), which is a better choice to improve the breeding efficiency. In this work, we identified the TPH in *C. gigas* (*CgTPH*), and compared its expression profiles and genetic variations between "Haida No. 1" and wild oysters. We identified four genotypes (-904CC, -522CC, −272TT, -262CC) and two haplotypes (CCCC and TCTC) that could be used as markers for growth studies and molecular breeding. Further investigations revealed that a single-base mutation at core transcription regulatory region was critical for binding with transcription factors FoxO and CUX to transcriptionally regulate *CgTPH* expression and growth in *C. gigas*. This work will be of great significance for further elucidating the role of 5-HT system in regulation of growth in mollusks and provides a candidate molecular marker for molecular breeding of growth with other production and performance traits in *C. gigas*.

2. Materials and methods

2.1. Identification of TPH in C. gigas

The TPH in *C. gigas* was identified based on the latest genome assembly in NCBI database (NCBI genome assembly: cgigas_uk_roslin_v1, Accession: GCA_902806645.1, Release date: 14-Feb-2020). The amino acid sequences of TPH from other representative organisms, including mammals (*Homo sapiens*, *Pan troglodytes*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Ovis aries*, *Delphinapterus leucas*), birds (*Gallus gallus*, *Zonotrichia aibicollis*), reptiles (*Chelonia mydas*, *Alligator sinensis*), amphibians (*Xenopus tropicalis*, *Nanorana parkeri*), fishes (*Salmo salar*, *Oncorhynchus mykiss*, *Danio rerio*), echinoderms (*Strongylocentrotus purpuratus*, *Acanthaster planci*), mollusks (*Crassostrea virginica*, *Mizuhopecte yessoensis*, *Biomphalaria glabrata*), arthropods (*Galendromus occidentalis*, *Limulus polyphemus*, *Drosophila melanogaster*), nematodes (*Necator americanus*, *Aenorhabditis elegans*) and platyhelminth (*Schistosoma haematobium*) were collected from the NCBI database for phylogenetic analysis. Sequence alignment was performed by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the phylogenetic tree was performed on MEGA (version 10.0). The parameters within the best amino acid substitution of JTT + G were input to MEGA to construct phylogenetic topologies using maximum likelihood (ML) with 1000 bootstrap replicates for the evaluation of their branch supports.

2.2. Experiment animals and sample collection

A total of 100 one-year-old Pacific oysters from "Haida No.1" and wild oysters, respectively, were used in this study. The oysters were bred and cultured with same practice in an oyster farm in Sanggou bay in Rongcheng (37.1°N, 122.5°E, Shandong, China). The growth traits including shell height, shell length, shell width, and total weight were measured and weighed before tissue sampling (Fig. S1). The adductor muscle was dissected from each of 30 individuals from the two groups and kept at −80 °C until DNA isolation. Various tissues including labial palp, digestive gland, heart, hemocytes, adductor muscle, visceral ganglion, gill, and mantle were dissected from 9 individuals from each of the two groups, respectively. Equal amount of tissue dissected from three oysters was pooled as one sample, resulting in three biological replicates for "Haida No.1" and wild groups, respectively. Tissues were flash frozen in liquid nitrogen and then transferred to −80 °C until RNA extraction.

2.3. Total RNA isolation and synthesis of cDNA

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality was confirmed by running 1% agarose gel electrophoresis with 10 X loading buffer (Takara, Japan). RNA concentration and purity were measured at optical density (OD)260 / (OD)280 with a NanoDrop 2000 (Thermo Fisher Scientific) spectrophotometer. PrimeScript™ Reverse Transcription Kit (Takara, Japan) was used to synthesize first-strand cDNA according to the manufacturer's instructions. The cDNA was preserved at −30 °C freezer.

2.4. Real-time PCR and data analysis

Specific primers for RT-PCR (Table S1) were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA), and their specificity were detected by conventional PCR and melting curve analysis. Elongation factor 1-α (EF1-α) was used as an internal control to normalize gene expression by RT-PCR (Du et al., 2013). The amplification was performed on the LightCycle®480 real-time PCR instrument (Roche, Switzerland) using QuantiNova SYBR® Green RT-PCR Kit (Qiagen, Germany). The 10 μL qRT-PCR reaction contained 5 μL 2 X SYBR Green PCR Master Mix, 0.7 μL of each primer, and 3.6 μL diluted cDNA. Cycling parameters were 95 °C for 5 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The IBM SPSS Statistics 25 was used to analyze the significant difference between the mean. All the data was analyzed using one-way ANOVA followed by a multiple comparison, $P < 0.05$ was considered as statistical significance.

2.5. In situ hybridization (ISH)

The whole soft body of 6-month-old Pacific oysters was used for ISH to determine tissue expression of *CgTPH*. The samples were fixed in 4%

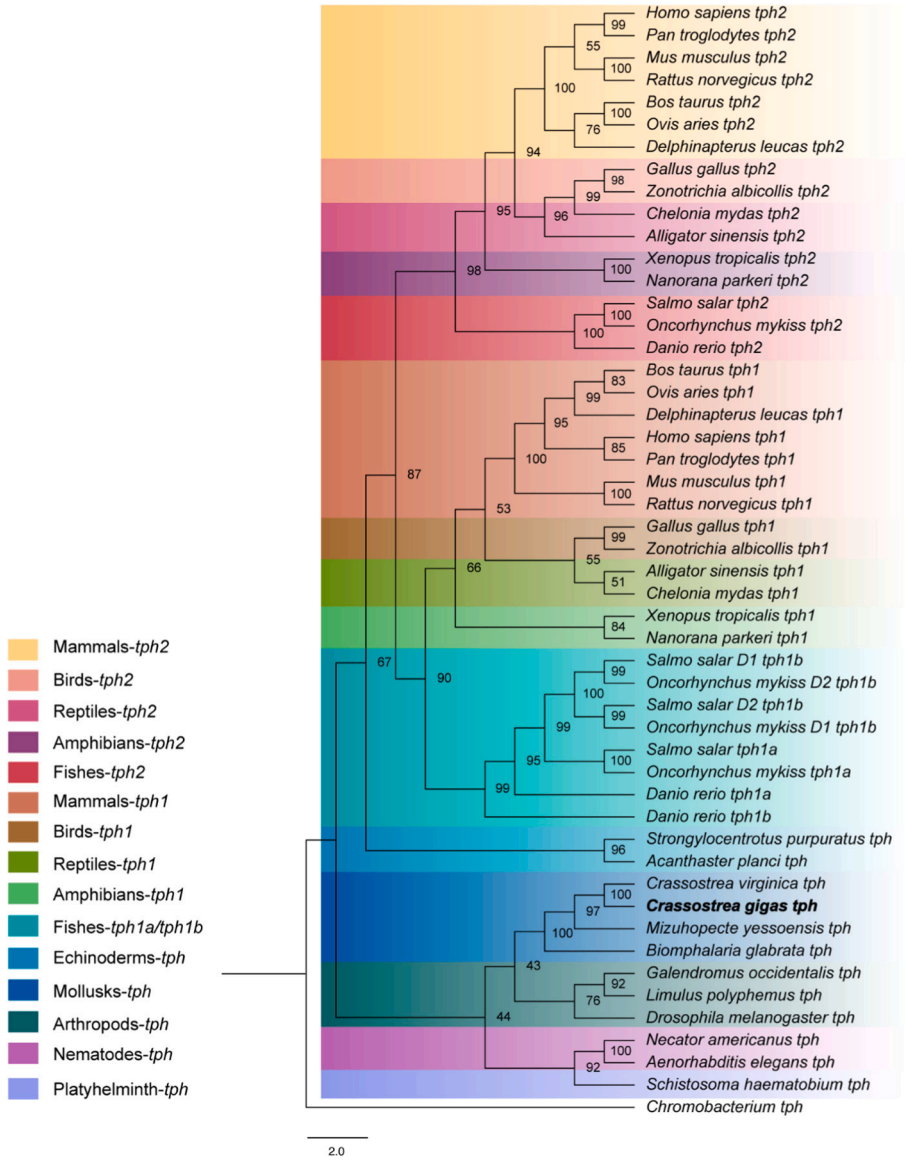

Fig. 1. Phylogenetic analyses of *TPH* genes in vertebrates and invertebrates. Numbers on the branches are bootstrap values from 1000 replications generated in MEGA X.

paraformaldehyde at 4 °C, and then dehydrated and embedded in paraffin to make 5 μm paraffin sections. The probes of sense and antisense digoxigenin-labeled RNA strands were transcribed *in vitro* using an RNA labeling kit (Roche, Switzerland) from the plasmid DNA containing *CgTPH*. To guarantee the authenticity of results in ISH experiment, negative control (sense probe of *CgTPH*) was also set up. ISH was performed according to the protocol as previously described (Li et al., 2021). All the ISH images were acquired with a Zeiss Axio Scope A1 microscope equipped with an AxioCam MRc5 digital camera.

2.6. DNA extraction, amplification and target sequencing of CgTPH regulation region

The genomic DNA was extracted from the adductor muscles of "Haida No.1" and wild oysters using proteinase K and phenol/chloroform method (Park and Kijima, 2002). The RNA in the genomic DNA was dismissed by RNase A (Takara, Japan). The integrity of genomic DNA was checked with 1% agarose gel electrophoresis, and the concentration and purity were measured by NanoDrop 2000 spectrophotometer.

A sequence of 1246 bp 5′ upstream of *CgTPH* ATG codon was extracted for target amplification and sequencing. Specific PCR primers were designed as listed in Table S1. PCR amplification of the fragment was conducted using the 2 X Taq Plus Master Mix II (Vazyme, China), and the thermocycling program of the PCR was set as follows: predenaturation at 95 °C for 3 min, 95 °C for 15 s, 60 °C for 20 s, 72 °C for 60 s, 36 cycles; 72 °C for 5 min. The PCR products were purified by Gel Extraction Kit D2500 (Omega Bio-tek, China) followed by sequencing using an ABI 3730 Genetic Analyzer (Applied Biosystems).

2.7. Sequence feature and genetic variation analyses of CgTPH regulation region

The core regulatory elements were analyzed using the Patch System (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi). The possible transcription start site was predicted using the NNPP database (http://www.fruitfly.org/seq_tools/promoter.html). The SNPs within regulatory region of *CgTPH* were detected and their allele and genotype frequencies were determined in 30 individuals of "Haida No.1" and wild oysters, respectively. The Hardy-Weinberg Equilibrium (HWE) tests were performed in each of the two groups.

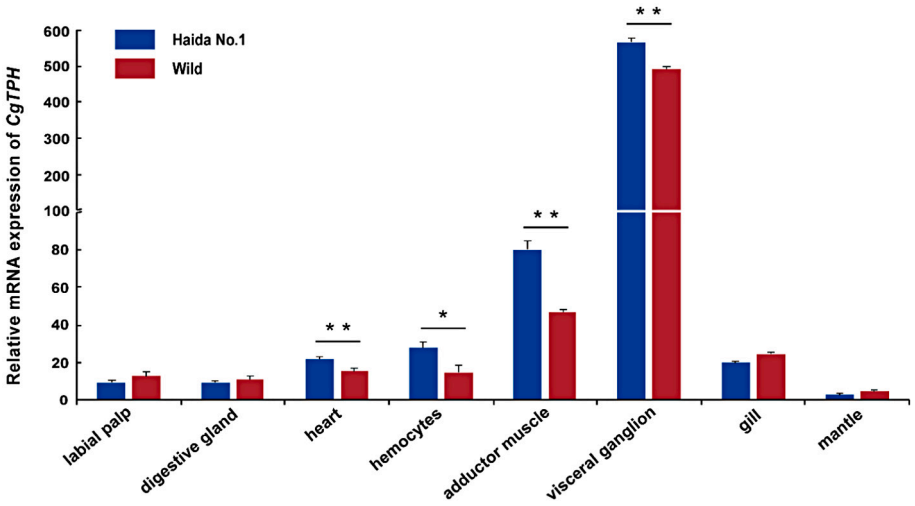

Fig. 2. Expression of *TPH* gene in various tissues between "Haida No. 1" and wild oysters. *CgTPH* was ubiquitously expressed in tissues of labial palp, digestive gland, heart, hemocytes, adductor muscle, visceral ganglion, gill and mantle. Higher expression of *TPH* gene in the heart, hemocytes, adductor muscle and visceral ganglion was observed in the fast-growing "Haida No. 1" compared with the wild oysters. Vertical bars represent the mean ± SD ($n = 3$). *$P < 0.05$.

The linkage disequilibrium (LD) test and haplotype analysis were conducted according to the genotyping results. The data was analyzed using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). A *P* value less than 0.05 was accepted as statistical significance. Based on the SNPs in 5′ upstream regions of *CgTPH* in two groups, the putative transcription factor binding sites were predicted using JASPAR database (http://jaspar.genereg.net) (Fornes et al., 2020).

2.8. Plasmids construction and site-directed mutagenesis

The *CgTPH* 5′-deletion constructs (−1627 ~ +141, −1129 ~ +141, −691 ~ +141, −403 ~ +141, −179 ~ +141 bp) were obtained by PCR, using specific primer (Table S1) with pGL3-basic vector (Promega, USA) homologous sequences. The amplified fragments were subcloned into the pGL3-basic vector using ClonExpress® II One Step Cloning Kit (Vazyme, China). The reporter plasmids containing mutations (Haida No.1 -272 T and wild -272C) of the *CgTPH* were generated using the Mut Express® II Fast Mutagenesis Kit V2 (Vazyme, China). Primers for the construction of mutational reporter plasmids were listed in Table S1. The transcriptional factors FoxO and CUX were subcloned into pcDNA3.1(+) vector for expression (Invitrogen, USA) using the ClonExpress® II One Step Cloning Kit. Primers for the construction of expression plasmids were shown in Table S1. Plasmids used in transfection experiments were extracted using EndoFree Midi Plasmid Kit (TIANGEN, China). The constructs and mutations were confirmed by direct sequencing.

2.9. Cell culture, transient transfections and dual-luciferase reporter assays

Prior to transfection, 293 T cells were grown in DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1 X penicillin-streptomycin solution (Sangon, China) with 5% CO_2 at 37 °C. Before transfection, 1×10^5 cells/well were seeded into 24-well cell culture plates. Then, 500 ng of *CgTPH* promoter 5′-deletion constructs or mutations in pGL3-basic, 250 ng FoxO-pcDNA3.1 or CUX-pcDNA3.1 expression plasmid, and 100 ng of pRL-TK (to normalize transfection efficiency) containing Renilla luciferase were transfected into the cells using EXfect® 2000 Transfection Reagent (Vazyme, China). Four hours after transfection, the medium was aspirated and replaced with complete growth medium (DEME basic medium containing 10% FBS and 1 X penicillin-streptomycin solution). Cells were washed with PBS 48 h after transfection and lysed in 100 μL luciferase lysis buffer. Firefly luciferase and Renilla luciferase reading were detected using the Dual-Luciferase Reporter Assay System (Promega, USA) and Synergy™ H1 (BioTek, USA). The statistical significance was set as *P* value less than 0.05.

3. Results

3.1. Identification and expression profiles of TPH in C. gigas

Only one copy of *TPH* gene (*CgTPH*, LOC105336312) was identified in *C. gigas* based on the latest genome assembly in NCBI database (NCBI genome assembly: cgigas_uk_roslin_v1, Accession: GCA_902806645.1, Release date: 14-Feb-2020). Phylogenetic analyses showed that TPH was generally divided into two main clades with two *TPH* copies (*tph1* and *tph2*) in vertebrates, while only one copy in invertebrates (Fig. 1). The invertebrate TPH were further split into *Protostomia* and *Deuterostomia*. The *CgTPH* had a higher level of homology with its counterparts from other mollusks including *Crassostrea virginica*, *Mizuhopecte yessoensis* and *Biomphalaria glabrata*. Moreover, the molluscan TPHs had a relatively closer homology with the TPHs from arthropods such as *Galendromus occidentalis*, *Limulus polyphemus* and *Drosophila melanogaster*.

The *CgTPH* was ubiquitously expressed in various tissues, including labial palp, digestive gland, heart, hemocytes, adductor muscle, visceral ganglion, gill and mantle (Fig. 2). Apparently, its expression level was significantly higher in visceral ganglion than any other tissues ($P < 0.05$). Notably, higher expression of *TPH* gene was observed in fast-growing "Haida No. 1" than wild oysters, in various tissues, including heart, hemocytes, adductor muscle and visceral ganglion ($P < 0.05$). *In situ* hybridization further showed that the expression of *CgTPH* was distributed among various tissues including mantle, visceral ganglion, heart, intestine, digestive gland and labial palp (Fig. 3). Apparently, expression of *CgTPH* was highly enriched in the visceral ganglion compared with other tissues, which is consistent with the real-time PCR results.

3.2. Genetic variations within the 5′ upstream regulatory region of CgTPH

Nucleotide variations within the promoter and 5′ untranslated regions of *CgTPH* were analyzed as shown in Fig. 4. A sequence of 1246 bp upstream of *CgTPH* gene was retrieved for analyses of sequence features and genetic variations. The putative transcription start site (TSS) was identified at −568 bp upstream of the ATG codon. A few regulatory elements were predicted, including five TATA boxes and three CAAT

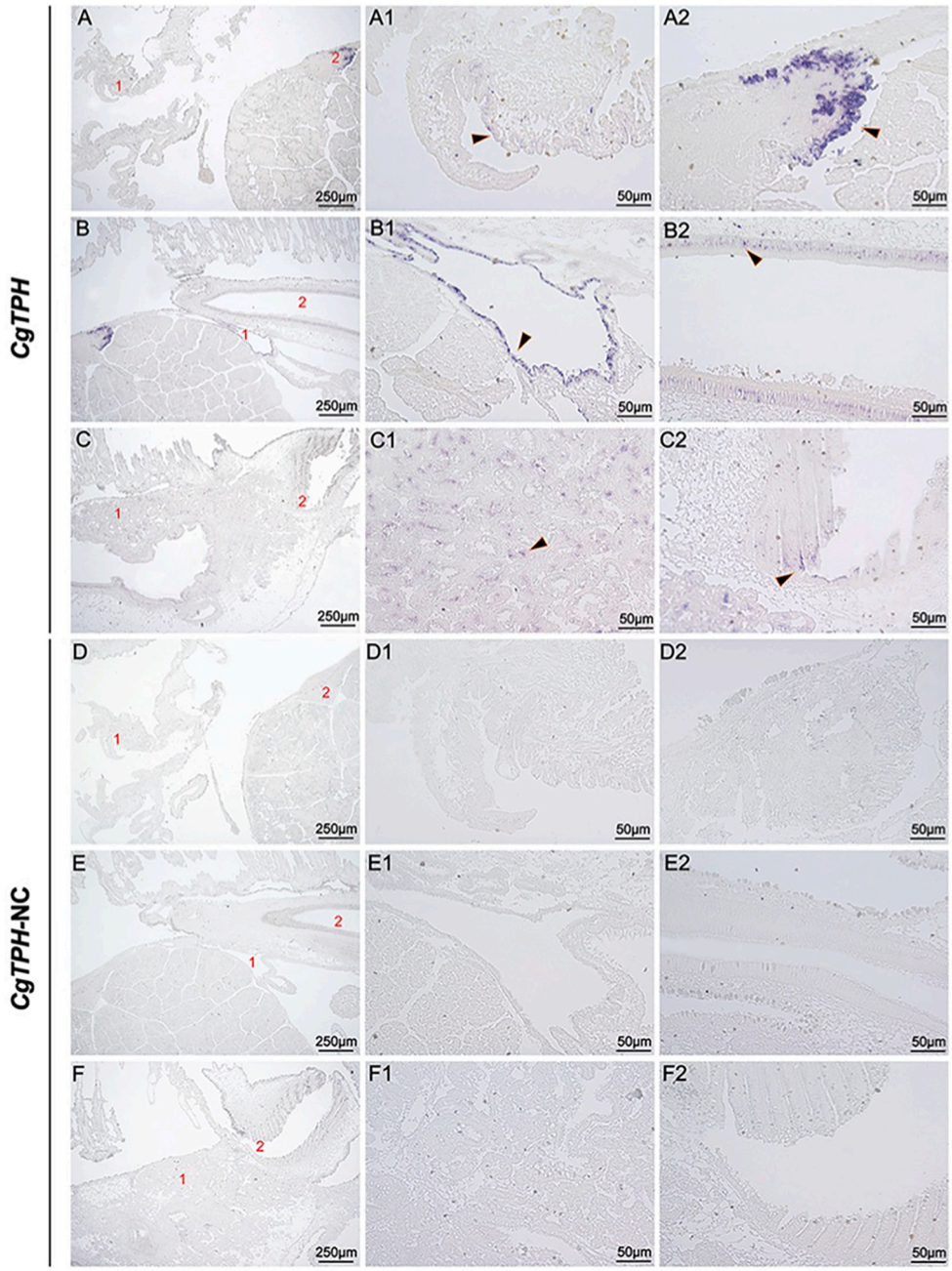

Fig. 3. *In situ* hybridization of *TPH* in *C. gigas*. *TPH* positive signals (A-C) and negative controls (D-F). *TPH* was mainly expressed in mantle (A1), visceral ganglion (A2), heart (B1), intestine (B2), digestive gland (C1) and labial palp (C2). Arrows indicated dense distribution of *TPH* positive signals.

boxes (Fig. 4). A total of 15 SNPs were identified from this region, including −1097A/T, −1091T/A, −904T/C, −891T/A, −838A/C, −828G/A, −522A/C, −272C/T, −266G/C, −264C/T, −262C/G, −258T/C, −216G/T, −205C/G and − 141T/A. Prediction of the transcription factors binding to the SNP sites allowed for identification of FoxO, CUX, MSX, UNC-86, NFIX and NFIC that were potentially critical for transcriptional regulation of *CgTPH*.

3.3. Genetic polymorphism of TPH gene is highly associated with growth of C. gigas

The association of the 15 SNPs with growth of *C. gigas* was further investigated by examining their allele frequencies in 30 oysters randomly sampled from the "Haida No. 1" population and wild population, respectively. The frequencies of allele and genotype at the 15 SNP loci were summarized in Table S2. Of which, allele and genotype frequencies of eight SNPs located at −1097, −1091, −904, −891, −828, −522, −272 and − 262 bp were significantly different between the two populations ($P < 0.05$). The Hardy-Weinberg equilibrium (HWE) further analyzed with goodness-of-fit χ^2-test revealed that four SNPs at −1097, −1091, −891, −828 bp were in HWE ($P > 0.05$) in both populations. The other four SNPs at −904, −522, −272, −262 bp were in HWE ($P > 0.05$) in wild population, whereas deviating from the HWE ($P < 0.05$) in "Haida No. 1" population. Moreover, the numbers of individuals with genotype −904 CC, −522 CC, −272 TT and − 262 CC in "Haida No. 1" were significantly higher than those in the wild oysters ($P < 0.05$) (Table 1).

The linkage disequilibrium (LD) between two loci were measured by D' value, which were divided into three cases, including completely independent ($D' = 0$), complete LD ($D' = 1$) and a certain degree of LD (0

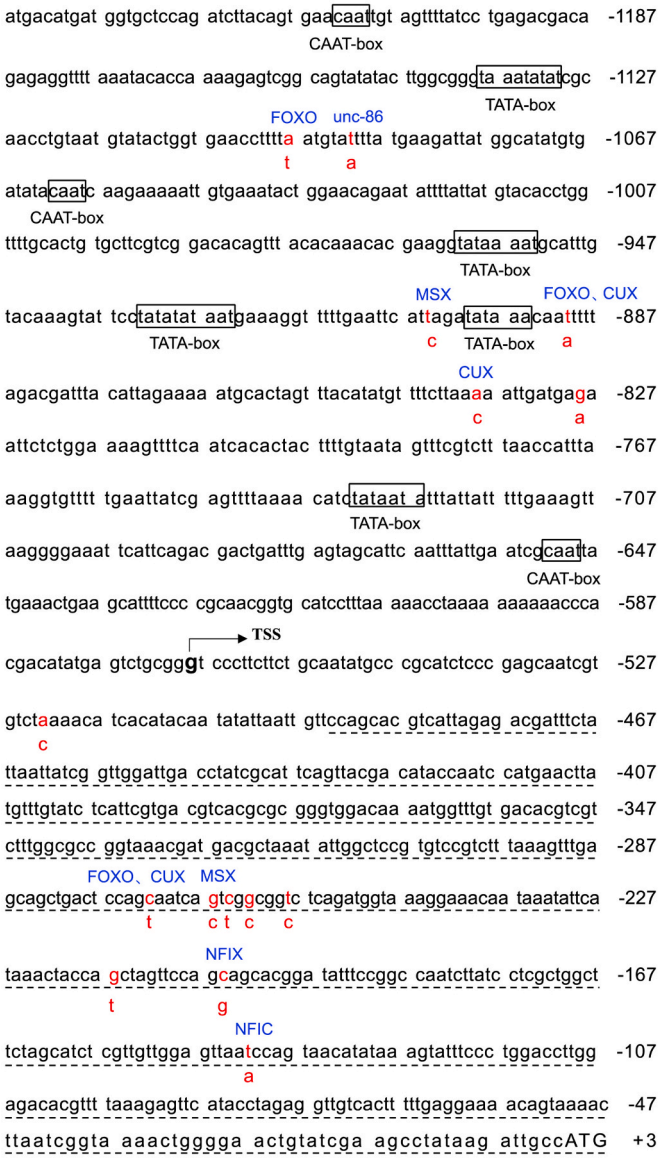

Fig. 4. Sequence features and genetic variations in the regulatory region of *CgTPH*. The negative numbers on the right indicated upstream sequence relative to the translation start codon. The putative transcription start site (TSS) was represented at curved arrow. The predicted TATA-box and CAAT-box were in highlighted boxes. The nucleotide polymorphisms were highlighted in red, and the variants were listed below. The putative transcriptional factors binding to specific regions were highlighted in blue. The putative 5' UTR region was indicated by the dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$< D' < 1$). Further pair-locus LD test revealed that all 15 loci were in strong LD with a pairwise $D' > 0.85$ (Fig. 5). The four SNPs associated with growth of *C. gigas* showed distinct linkage, the SNP at −904 T/C was in strong linkage with SNP at −522C/A and SNP at −262C/G. The SNP at −522C/A was strongly correlated with SNP -262C/G, but was with independent linkage with SNP -272C/T. The SNP -272C/T and SNP -262C/G were in complete linkage. The four SNPs had various degrees of linkage, and the LD between different loci in one gene usually forms different haplotypes. Haplotype analysis showed that these four SNPs could form eight haplotypes with frequency greater than 0.01, and the frequencies of haplotypes, CCCC and TCTC, in "Haida No. 1" were significantly higher than those in wild oysters ($P < 0.01$) (Table. 2).

Table 1
Allele and genotype frequencies of four SNPs associated with growth of *C. gigas*.

SNPs	Genotype/allele	Haida No. 1	Wild	χ2 (*P*)
-904T/C	Allele			8.391($P <$ 0.01)
	T	42	53	
	C	14	3	
	Genotypes			6.140($P <$ 0.05)
	T/T	18	25	
	T/C	6	3	
	C/C	4	0	
	HWE test Pearson's χ2 (*P*)	5.143($P <$ 0.05)	0.090($P >$ 0.05)	
-522C/A	Allele			9.666($P <$ 0.01)
	C	24	9	
	A	32	47	
	Genotype			6.747($P <$ 0.05)
	C/C	8	2	
	C/A	8	5	
	A/A	12	21	
	HWE test Pearson's χ2 (*P*)	4.861($P <$ 0.05)	3.200($P >$ 0.05)	
-272C/T	Allele			7.213($P <$ 0.01)
	C	37	49	
	T	19	7	
	Genotype			6.585($P <$ 0.05)
	C/C	17	22	
	C/T	3	5	
	T/T	8	1	
	HWE test Pearson's χ2 (*P*)	16.216($P <$ 0.01)	0.945($P >$ 0.05)	
-262C/G	Allele			18.327($P <$ 0.01)
	C	54	38	
	G	2	18	
	Genotype			17.567(P < 0.01)
	C/C	27	14	
	C/G	0	10	
	G/G	1	4	
	HWE test Pearson's χ2 (*P*)	28($P < 0.01$)	0.920($P >$ 0.05)	

3.4. SNP -272C/T and SNP -262C/G are located in the core regulatory region of CgTPH

To determine how the genetic variations within regulatory regions of *CgTPH* affect its transcription, the 5′ flanking sequence of *CgTPH* was truncated into fragments with different lengths and subcloned into the pGL3-basic vector and transfected into 293 T cells. The results showed that the transcriptional activity was significantly decreased ($P < 0.05$) with deletion of *CgTPH* 5′ flanking fragment (Fig. 6). The transcriptional activity of *TPH* was decreased by 86.86% after deletion of −403 ~ −179 bp fragment. While deletion of other fragments also resulted in decrease of transcription activity ($P < 0.05$), the degree was less than that after the deletion of −403 ~ −179 bp fragment. Therefore, we reasoned that the core transcriptional regulatory region would be located at −403 ~ −179 bp upstream of *CgTPH*. Notably, two SNPs (−272C/T and − 262C/G) associated with growth were located in this region, suggesting the critical roles in affecting the transcription activity of *CgTPH* gene.

3.5. SNP -272C/T is critical for different binding efficiency of FoxO and CUX between "Haida No. 1" and wild oysters

The prediction of transcription factor binding sites found that potential binding sites of transcription factors FoxO and CUX were detected at SNP -272C/T, while no binding site of any transcription factor was

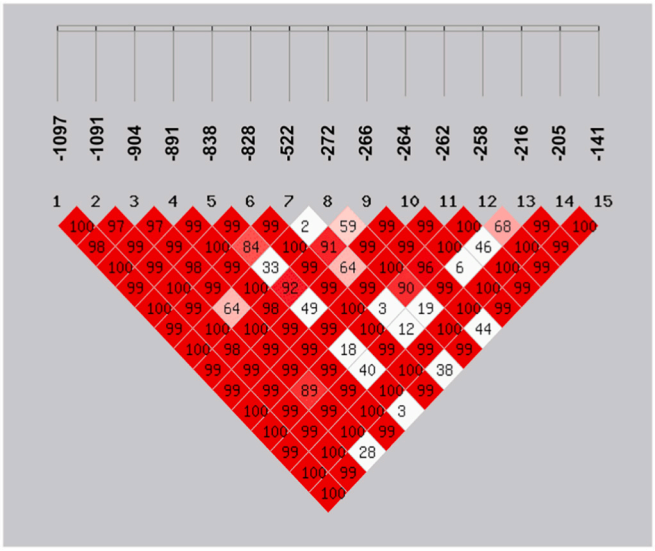

Fig. 5. Assessment of linkage disequilibrium among *CgTPH* polymorphic sites. The colour scheme is: white (D = 0), pink (0 < D < 1), red (D = 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Haplotype analysis of four SNPs associated with growth between "Haida No. 1" and wild oysters.

Haplotype	Haida No. 1 (frequency)	Wild (frequency)	χ2 (*P*)	Odds Ratio [95%CI]
CCCC*	12.96 (0.231)	0.02(0.000)	13.647 (0.000222)	748.941 [45.921–12,214.705]
TCTC*	6.74(0.120)	0.00(0.000)	7.169 (0.007432)	/
TATC	11.22 (0.200)	6.01(0.107)	1.868 (0.171717)	2.087 [0.716–6.082]
CCTC	1.04(0.019)	0.99(0.018)	0.001 (0.979781)	1.045 [0.107–10.236]
TACG*	2.00(0.036)	16.02 (0.286)	12.995 (0.000315)	0.092 [0.020–0.425]
TACC	18.78 (0.335)	24.98 (0.446)	1.443 (0.229675)	0.626 [0.291–1.346]
TCCC	3.26(0.058)	6.00(0.107)	0.882 (0.347522)	0.515 [0.127–2.096]
CCCG	0.00(0.000)	1.98(0.035)	2.019 (0.155286)	/

detected at SNP -262C/G. The reporter plasmids containing "Haida No. 1" allele -272 T and wild allele -272C, and the FoxO-pcDNA3.1 and CUX-pcDNA3.1 expression plasmid were co-transfected into 293 T cells for luciferase relative activity detection. Dual-luciferase reporter assay analysis suggested that the transcription activity of "Haida No. 1" allele -272 T was significantly higher than that of wild allele -272C ($P < 0.01$) (Fig. 7A and B). Two transcription factors FoxO and CUX could transcriptionally regulate *TPH* gene through directly binding with the specific regulatory element. The binding efficiency of both transcription factors with "Haida No. 1" allele -272 T was superior to that of wild allele -272C, while CUX showed a stronger effect than FoxO (Fig. 7A).

4. Discussion

As the rate-limiting enzyme of 5-HT biosynthesis, TPH has been known to play indispensable roles in growth regulation in vertebrates, but remains largely unknown in invertebrates such as mollusks. In present study, we identified *CgTPH* and compared its expression and nucleotide variations between a selectively bred fast-growing variety "Haida No. 1" and wild oysters. Phylogenetic analysis verified clear evolutionary relationship of *CgTPH* with counterparts from other invertebrates and vertebrates. Expression profiling and *in situ* hybridization showed that *CgTPH* was highly expressed in the nerve-rich tissues such as visceral ganglia. Notably, *CgTPH* was expressed at significantly higher levels in fast-growing "Haida No.1" than wild oysters. Association and haplotype analyses of genetic variations within 5′ upstream regulatory region of *CgTPH* identified four SNPs (located at −904, −522, −272, −262 bp) and two haplotypes (CCCC and TCTC) that were significantly associated with growth rate of *C. gigas*. Luciferase reporter assay analysis revealed the core transcription regulatory region located at −403 ~ −179 bp of *CgTPH*, in which a critical SNP -272C/T was significantly associated with *CgTPH* transcription activity. Further analysis revealed that the difference of transcription activity was due to the distinct binding efficiency with two transcription factors, FoxO and CUX. Together, we identified a novel SNP in the regulatory region of *CgTPH* that was critical for its transcription regulation by FoxO and CUX conferring the essential roles of 5-HT in growth regulation of *C. gigas*.

TPH belongs to the superfamily of aromatic amino acid hydroxylase (AAAHs), which also include phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH) (Fitzpatrick, 2004). It is relatively well documented that *TPH* genes could be clearly divided into two main clades (*tph1* and *tph2*) in vertebrates. TPH1 and TPH2 showed obvious differences in the tertiary structures, which may determine their distinct catalytic or substrate specificity (Walther et al., 2003). In most teleosts, *tph1* was duplicated into *tph1a* and *tph1b* due to the teleost-specific genome duplication (Xu et al., 2019). However, comprehensive investigation on the copy number and evolutionary status of the *TPH* in invertebrates is unavailable at present. In this study, we identified only one copy of *TPH* in the genome of *C. gigas*. Phylogenetic analysis revealed clear evolutionary relationships among vertebrates and invertebrates. Systematic evolution analyses found that *TPH* of mollusks have a relative closer homology with arthropods, implying that the function of 5-HT in mollusks may be similar to that of 5-HT in arthropods, which can regulate gonadal development (Vaca and Alfaro, 2000), growth (Campos et al., 2019) and immunity (Bao et al., 2010).

The TPH play indispensable role in growth of animals through regulating the synthesis of 5-HT. Previous studies reported that 5-HT play roles in regulating growth and development in a large number of species, such as *M. musculus* (Alenina et al., 2009), *D. melanogaster* (Kaplan et al., 2008), *C. elegans* (Estevez et al., 2006), *D. magna* (Rivetti et al., 2018) and *Aedes aegypti* (Ling and Raikhel, 2018). In this study, we observed higher expression of *TPH* gene in the fast-growing "Haida No. 1" compared with the wild oysters, implying its association with growth in oysters. TPH was the rate-limiting enzyme of 5-HT, a neurotransmitter participates in regulation of neuroendocrine system. Previous studies reported that 5-HT stimulated secretion of insulin in β cells, and cooperatively regulated the energy metabolism and growth (Cataldo Bascuñan et al., 2019). In addition, 5-HT also regulated the secretion of growth hormone (Collu et al., 1972), and the level of ghrelin to control eating behavior and energy substrate utilization (Currie et al., 2010). In this study, we observed that visceral ganglion was the vital tissue of *TPH* gene expression in *C. gigas*, suggesting that TPH played an indispensable role in regulation of neuroendocrine system. In addition, *CgTPH* was broadly expressed in adductor muscle, gill, digestive system, heart and hemocytes, suggesting that 5-HT system may be involved in the exercise and feeding behavior (Carroll and Catapane, 2007; Twarog, 1960), food digestion and absorption (Gershon, 2013), immunity and stress response (Dyachuk, 2016; Smith and Hill, 1986).

Genetic variations in the regulatory region of a genome can directly affect gene expression and may eventually lead to changes in the phenotype of organisms (Kwon et al., 2018). To explore the molecular mechanism of 5-HT system involved in growth regulation of *C. gigas*, the 5′ upstream sequence of *CgTPH* was further analyzed. Sequence analyses showed that the basic cis-acting elements, including TATA-box and CAAT-box, were present, as observed in most gene promoters (Basi et al.,

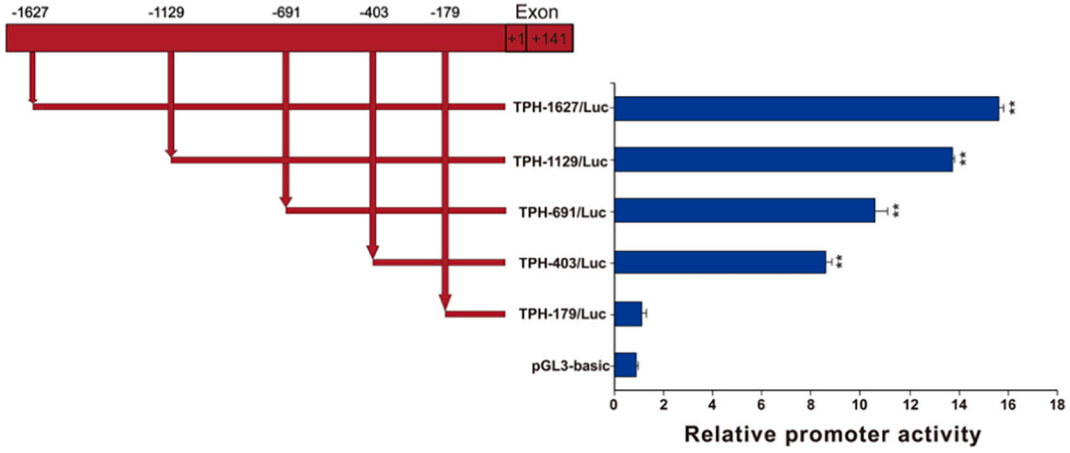

Fig. 6. The core transcriptional regulatory region of *CgTPH* was identified at −403 ~ −179 bp. The reporter plasmids containing 5′-deletion constructs of *CgTPH* were transfected into 293 T cells and luciferase activities were measured. The luciferase activity of each construct was compared with RL-TK transcription activity. The data was shown as mean ± SD from three wells. $^{**}P < 0.01$ as compared to RL-TK transcription activity.

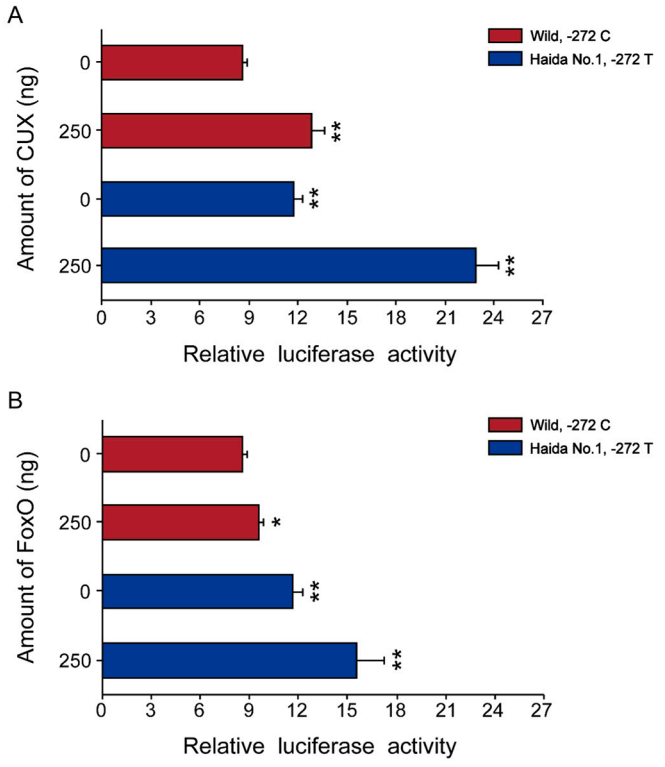

Fig. 7. Allelic effects of −272C/T on transcription activity for FoxO and CUX transcription factors. Luciferase activities were measured at 48 h after co-transfection of reporter plasmids with allele T in "Haida No.1" and allele C in wild oyster and FoxO or CUX cDNA expression plasmid into 293 T cells. The luciferase activity of each construct was compared with RL-TK transcription activity. The data was shown as mean ± SD from three wells. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to RL-TK transcription activity.

1993; Faniello et al., 1999). In addition, we examined the genetic variations of *TPH* regulatory region between "Haida No.1" with wild oysters, allowing for identification of 15 SNPs and the transcription factors such as FoxO and CUX predicted at the polymorphic sites. Notably, frequencies of alleles and genotypes of the four SNPs (located at −904, −522, −272, −262 bp) were significantly different between "Haida No.1" and wild oysters. Moreover, the comprehensive LD and haplotype analysis of SNPs revealed that frequencies of two haplotypes CCCC and TCTC were significantly higher in "Haida No.1" than that in wild oysters ($P < 0.01$). The genotypes associated with traits can be used as markers for trait selection (Wang et al., 2018; Yang et al., 2014). In this study, these four genotypes and two haplotypes in regulatory region of *CgTPH* will be useful for marker-assisted selection breeding in *C. gigas*.

cis-Acting elements (promoters, enhancers and silencing elements) and trans-acting factors (universal transcription factors, activators, suppressors and coordinators) play critical roles in regulating gene expression (Maston et al., 2006). To further verify the regulatory effect of genetic variations in 5′ upstream region of *CgTPH* on *TPH* expression, we identified the core regulatory region (−403 ~ −179 bp) of *CgTPH* and found that the two SNPs (located at −272, −262 bp) associated with growth were located within this region. Single-based mutations in the regulatory elements can alter the affinity of binding with trans-acting factors to regulate gene expression (Nagore et al., 2013). The binding transcription factors FoxO and CUX were predicted at SNP -272C/T, while no any binding transcription factor was detected at SNP -262C/G. The FoxO are a subgroup of the Forkhead family of transcription factors. It is involved in insulin signaling pathways and controls a wide range of biochemical processes, including cellular growth, proliferation, differentiation, embryonic development, metabolism, cell cycle arrest and protection from stress (Barthel et al., 2005). In *C. elegans*, the transcription factor *daf-16* (*FoxO* homologous gene) was reported to regulate the transcription of *TPH* and the synthesis of serotonin (Accili and Arden, 2004; Estevez et al., 2006). The CUX transcription factors belong to a family of homeodomain proteins and play an important role in regulating cell differentiation, growth and development (Nepveu, 2001). Previous studies reported that inactivation of *Cux-1* by gene targeting in the mouse led to growth retardation, while overexpression of *Cux-1* gene resulted in multiple organ proliferation and organ enlargement (Ellis et al., 2001; Ledford et al., 2002). The CUX is also involved in regulation of cell cycle process, especially in the G1/S transition phase (Sansregret et al., 2006). Furthermore, the expression patterns of *Cux-1* and *Cux-2* have been proved to be closely related to the development of inter-neurons (Cubelos et al., 2008). In this study, we showed that the relative transcription activity of "Haida No.1" allele -272 T was significantly higher than that of allele -272C in wild oysters. We speculated that the two transcription factors FoxO and CUX could transcriptionally regulate *TPH* gene through directly binding with the specific regulatory element, while the binding efficiency of both transcription factors was different between allele -272 T and wild type allele -272C.

5. Conclusions

We identified the TPH in *C. gigas* (*CgTPH*), and compared its expression profiles and genetic variations between "Haida No. 1" and wild oysters. Four genotypes (-904CC, -522CC, −272TT, -262CC) and two haplotypes (CCCC and TCTC) were identified in the regulatory region of *CgTPH* that could be used as markers for growth studies and molecular breeding. Further investigations revealed that a single-base mutation at core transcription regulatory region was critical for binding with transcription factors FoxO and CUX to transcriptionally regulate *CgTPH* expression and growth in *C. gigas*. This work is of great significance for elucidating the role of 5-HT system in regulation of growth in mollusks and provides a marker for molecular breeding of growth with other production and performance traits in *C. gigas*.

Declaration of Competing Interest

All authors declare that they have no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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