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The roles of thyroid hormone receptor and T3 in metamorphosis of *Haliotis diversicolor**

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Abstract Thyroid hormone is a kind of important hormone which regulates metamorphosis. Its role is well described in amphibian metamorphosis. Thyroid hormones (T3 and T4) have also been demonstrated to play a role in metamorphosis of marine invertebrates. However, the mechanism of thyroid hormone in metamorphosis of marine invertebrates remains unknown. A homolog of vertebrate thyroid hormone receptor (TR) was cloned and identified in abalone *Haliotis diversicolor* and was named *HdTR*. The mRNA expressions of *HdTR*, thyroid peroxidase (*TPO*), thyroid peroxidase 1 (*TPO1*), idothyronine deiodinase III (*IDIII*) and integrin alpha-V (*ITGAV*) had significant difference in metamorphosis of *H. diversicolor*. Metamorphosis rate and mortality rate were significantly different in *HdTR* RNAi experiment and T3 inducing experiment. In RNAi experiment, *ITGAV* and *CCND1* (cyclin D1) expression of dsRNA *HdTR* exposing group were significantly lower than those of blank control and negative control. But *CTNNB* (catenin beta) expression of dsRNA *HdTR* exposing group was higher than that those of blank control and negative control. *ERK* (extracellular signal regulated kinases) and *PI3K* (phosphoinositide-3-kinase) had no significant difference in RNAi experiment. Moreover, *ITGAV* of 1 μmol/L T3 group was significantly lower than that of 0 μmol/L T3 group, *PI3K* expression of 10 μmol/L T3 group was higher than that of 0 μmol/L T3 group, and the other genes expression had no significant difference in T3 inducing experiment. The data of genes expression suggested that *CCND1* might be an effector gene of TR genomic action, while *CTNNB* might be regulated by unliganded TR. *CCND1* and *CTNNB* may be involved in cell proliferation of metamorphosis. T3 might regulate the expression level of *PI3K* via nongenomic way. These results shed light on the mechanism of thyroid hormone in abalone metamorphosis.

Keyword: thyroid hormone receptor; thyroid hormone (TH) (T3); abalone; metamorphosis

1 INTRODUCTION

Extant metazoans have a wide variety of life cycles. In most marine invertebrates, hatching produces a larva. The larva continues to grow and develop to varying degrees. There appear dramatic morphological, physiological, behavioral and ecological transformations between the larvae and the juvenile (Bishop et al., 2006; Holstein and Laudet, 2014), which is defined metamorphosis. The juvenile grows and becomes an adult after experiencing sexual maturation.

Transitions between different states of development, physiology, and life history are typically mediated by hormones (Bishop et al., 2006; Flatt et al., 2006). Apart from insects and amphibians, our understanding of the molecular cascade controlling metamorphosis is very limited. In amphilbians and insects, the production of hormones (thyroid hormone in amphibians, ecdysone and juvenile hormone in

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insects) is controlled by the nerve system, as well as growth and environmental signals. These hormones control a complex tissue-specific gene regulatory network that drives metamorphosis (Holstein and Laudet, 2014).

Many studies have demonstrated that thyroid hormones (THs) regulate development, metamorphosis, and other life history transitions in marine invertebrates. For example, a TH derivative, TRIAC, induces metamorphosis in the *Cephalochordate amphioxus* (Paris et al., 2008). In echinoids, the TH thyroxine (T4) accelerates development to metamorphosis (Heyland and Hodin, 2004; Heyland and Moroz, 2005; Heyland et al., 2006a, b). Larvae of two species of abalone, *Haliotis discus discus* and *H. gigantea* metamorphosed strongly in response to T4. The metamorphic response to T4 was comparable to the response to gammer amino butyric acid (GABA), which is a high-efficiency inducer of abalone metamorphosis (Fukazawa et al., 2001).

In mammals, THs (T4 and T3) are produced in the thyroid gland, and thyroid peroxidase (TPO) is the primary enzyme of THs biosynthesis (Degroot and Niepomniszcze, 1977; Kimura et al., 1987). In the target tissue, idothyronine deiodinases (ID) convert T4 to T3 (Flatt et al., 2006; Orozco et al., 2012). Cellular actions of THs may be initiated by two different ways. THs bind thyroid hormone receptors (TRs) and regulate gene expression depending on thyroid hormone response elements (TREs). This process happens in the cell nucleus and is termed genomic mechanism (Cheng et al., 2010). Moreover, via the integrin receptor, THs can regulate signal pathways, such as extracellular signal regulated kinases (ERK) (Lei et al., 2008; Lin et al., 2008; Cheng et al., 2010) or phosphatidylinositol 3-kinase (PI3K) (Lei et al., 2004; Cheng et al., 2010). Activation of ERK or PI3K involves TRs resident in cytoplasm (Cheng et al., 2010). This process is termed nogenomic mechanism. TRs play an important role in both ways, and therefore the biological functions of THs are mediated largely through TRs.

Vertebrate's TRs regulate growth, development and metabolism by binding either as monomers, homodimers or form heterodimers with RXRs (Muñoz et al., 1993). However, the function of TRs in invertebrates is still unknown. TRs have been reported in limited invertebrate groups. For example, a TR has been characterized in amphioxus (Paris et al., 2008). As well, an oyster TR ortholog has been identified (Huang et al., 2015). Moreover, there are TR orthologs in several species of trematode (Bertrand et al., 2004; Wu et al., 2006, 2007; Pakharukova et al., 2014). However, there are few reports about the role of TRs in marine invertebrate metamorphosis.

The spiral-cleaving embryos of abalone develop into trochophore larvae that undergo torsion and become free-swimming, lecithotrophic (nonfeeding) veligers (Williams et al., 2009). Then, 3 to 4 days later, *H. diversicolor* veligers become competent and are able to be induced by signals released by diatoms, mucus of adult, or biofilm to settle and metamorphose (Bryan and Qian, 1998; Li et al., 2006). TH has been shown to induce settlement and metamorphosis in two species of abalones (Fukazawa et al., 2001). *H. diversicolor* is commercially important in the southeast coast of China as a primary cultured species. In this study, the homolog of TRs from *H. diversicolor* was cloned and its mRNA expression profiles in metamorphosis and RNAi experiments were determined. In order to analyze abalone TRs function in metamorphosis, the expression profiles of TR-related genes were showed in RNAi experiments and T3 inducing experiments. The understanding of TRs in metamorphosis will enhance our knowledge about life history of marine invertebrate, and contribute to seeding production of abalone.

2 MATERIAL AND METHOD

2.1 Larval sample

Mature *H. diversicolor* and larval culture were carried out at Hongyun Aquaculture Co. Ltd. Abalone larvae were obtained as described in our previous paper (Wang et al., 2016). In brief, fertilized eggs and larvae were cultured in sand-filtered seawater at 23–25°C. The larvae developed into veliger at 48 hours post fertilization (hpf), competent larva stage at 72 hpf, and post larva stage at 96 hpf. Six broods of larvae from different parents were collected into liquid nitrogen at veliger stage (48 hpf), competent larva stage (72 hpf) and post larva stage (96 hpf). Each brood larvae were derived from gametes contributions from at least three males and three females. Some competent larvae were used in dsRNA exposure assay and T3 inducing assay. When larva develops two eyespots, it is competent for metamorphosis, and becomes competent larva. When 80% larvae developed their eyespots, we consider these larvae as competent.

Table 1 Oligonucleotide primers used in RACE, qRT-PCR and dsRNA generation

Primer name	Nucleotide sequence (5′→3′)	Gene name	Accession No.	Purpose
5′TR-out	GGTAGCAGCGTCGGAACACACAA	HdTR	KT023066	5′RACE
5′TR-inner	CCCGATGTATCCCGTGACAGAGA	HdTR	KT023066	5′RACE
3′TR-out	CGAAAATCGCCCGCACCAAC	HdTR	KT023066	3′RACE
3′TR-inner	AACCAGGGAGTTTGGAGCAGAATAGT	HdTR	KT023066	3′RACE
dsTR-F	ACAGGTCGCTGATAGCAGATATTTGTG	HdTR	KT023066	dsRNA generation
dsTR-FT7	*TAATACGACTCACTATAGGG*ACAGGTCGCTGATAGCAGATATTTGTG	HdTR	KT023066	dsRNA generation
dsTR-R	GCAATGTAACGCTTGTATGCTCCC	HdTR	KT023066	dsRNA generation
dsTR-RT7	*TAATACGACTCACTATAGGG*GCAATGTAACGCTTGTATGCTCCC	HdTR	KT023066	dsRNA generation
dsGFP-F	CGACGTAAACGGCCACAAGT	GFP	pEGFP-N1	dsRNA generation
dsGFP-FT7	*TAATACGACTCACTATAGGG*CGACGTAAACGGCCACAAGT	GFP	pEGFP-N1	dsRNA generation
dsGFP-R	TTCTTGTACAGCTCGTCCATGC	GFP	pEGFP-N1	dsRNA generation
dsGFP-RT7	*TAATACGACTCACTATAGGG*TTCTTGTACAGCTCGTCCATGC	GFP	pEGFP-N1	dsRNA generation
TR-F	ACCTGACCAATGGACCAGAG	HdTR	KT023066	Target gene
TR-R	CGACCCTCACACACTTCTGA	HdTR	KT023066	Target gene
IαV-F	TTTGCTTTCGAGCTTTCCAT'	ITGAV	JU071958	Target gene
IαV-R	GGACCACAGTGAACGGATCT	ITGAV	JU071958	Target gene
TPO-F	CGAGGCACTGAAGAAAGGAG	TPO	GT866571	Target gene
TPO-R	ATGAAAGCGTTTCGCTGAAT	TPO	GT866571	Target gene
TPO-1-F	CCTTCATTCGTCCGGAAATA	TPO-1	JU072637	Target gene
TPO-1-R	ACGTCGGTTGGAAACGTAAC	TPO-1	JU072637	Target gene
D3-F	GTGGTCTCCACGGAAAGTGT	IDIII	JU072393.1	Target gene
D3-R	GGCCCTTGTCTAGGACCTTC	IDIII	JU072393.1	Target gene
β-catenin-F	AGACAGATAGCACCTTCAGAACACG	CTNNB	JU071282.1	Target gene
β-catenin-R	CTATGGAAACCAGGAAAGCAAACTC	CTNNB	JU071282.1	Target gene
cyclin D1-F	CGCCGATTCTGCTGTCTCAC	CCND1	JU071279.1	Target gene
cyclin D1-R	TTAACACCATCCTTACATTCCATTC	CCND1	JU071279.1	Target gene
PI3K-F	TGCTGATGCATGTCTGTCAA	PI3K	KX245017.1	Target gene
PI3K-R	CGGGAAACTCTCAAACCAGA	PI3K	KX245017.1	Target gene
YB1-F	AAGTTCTAGCAACGAGGGTCA	YB1	JN997407	Reference gene
YB1-R	GGTATTTCTTTGGGTTGTTCTTC	YB1	JN997407	Reference gene

The sequence of T7 promoter is italic.

2.2 Isolation of total RNA and synthesis of cDNA

Total RNA was extracted as described in our published peer-reviewed article (Wang et al., 2016). The total RNA was examined by agarose gel electrophoresis and ultraviolet spectrophotometer to ascertain its integrity and quantity. Then the total RNA was treated with RQ1 RNase-Free Dnase (Promega, Beijing, China) to remove potential trace amount of contaminating DNA. The cDNA was synthesized with 2 μg total RNA+0.5 μmol/L random primer+M-MLV reverse transcriptase, that was described in our published peer-reviewed article (Wang et al., 2016).

2.3 Molecular cloning of *HdTR*

BLAST analysis of our transcriptome of *H. diversicolor* larvae revealed that a partial sequence was homologous to known sequences of TR. Gene special primers were designed based on the partial sequence (Table 1). RACE was performed to obtain the full length sequence of *HdTR*. SMART RACE Kit (Clontech, Mountain View, CA, USA) was used to get the 5′ and 3′ end according to its instructions. Expected PCR products were isolated by agarose gel electrophoresis and purified with a Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified DNA was ligated into the T/A cloning vector

pMD19-T (TaKaRa, Dalian) to construct a recombinant vector, which was transfected into *E. coli* JM109 competent cells. The positive clones were sent to Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China) to sequence using ABI 3730 automated sequencers (Applied Biosystems, Foster City, CA, USA).

Isoelectric point and molecular weight were computed using 'Compute pI/Mw tool' (http://cn.expasy.org/tools/pi_tool.html). Potential N-glycosylation and phosphorylation sites were analyzed with NetNGlyc1.0 Server (http://www.cbs.dtu.dk/). Protein multiple-alignments were performed with the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The phylogenetic tree of TRs was constructed using maximum likelihood method with MEGA7 program (http://www.megasoftware.net/). Retinoic acid receptor of *H. diversicolor* was used as an outgroup.

2.4 dsRNA generation

The fragment of *HdTR* (521–1 056 bp of KT023066) was added T7 promoter sequence via PCR using dsTR-FT7 and dsTR-R primers or using dsTR-F and dsTR-RT7 primers. A 657-bp fragment of the green fluorescent protein (GFP) gene from the pEGFP-N1 vector was also added T7 promoter sequences by eithere dsGFP-FT7 and dsGFP-R or dsGFP-F and dsGFP-RT7. The sequences of primers were showed in Table 1. The italics at 5′ ends are T7 promoter sequence.

After purification and sequencing, the DNA fragments of *HdTR* and *GFP* were transcribed to single-stranded RNA (ssRNA) by T7 phage RNA polymerases (Promega, Fitchburg, USA). The DNA templates were degraded by DNase I (Promega, Fitchburg, USA). After purified, the sense and antisense cRNA strands were used to produce dsRNA, which was monitored by agarose gel electrophoresis and spectrophotometrically. The dsRNA of *HdTR* were used to silence *HdTR*. GFP dsRNA was served as the control. The details of dsRNA generation were described in our previous paper (Wang et al., 2016).

2.5 dsRNA exposure assay

Competent larvae were collected by 100 μm screens, and washed with 0.2 μm filtered seawater (FSW) into 50 mL beakers. The *HdTR* dsRNA/GFP dsRNA was added in a 50-mL beaker with a final concentration of 5 μg/mL. The beaker with *HdTR* dsRNA/GFP dsRNA was regarded as RNAi group / negative control (NC) group. The beaker without any dsRNA was regarded as blank control (BC) group. After 2 h, larvae were transferred into a 1-L beaker, which was covered by benthic diatom. Larvae in beaker were collected after 10 h transferred. Approximately 50 larvae were used to count the number of metamorphosed and dead larvae. Metamorphosis is defined as losing cilia and forming juvenile shell. Death is defined as deterioration of the outer cuticle and inactivity (Bryan and Qian, 1998). Larvae of different development stages were put into liquid nitrogen for RNA isolation then real time qRT-PCR assay. The expression level of *HdTR*, *TPO*, *DIII* and effector genes (*CCND1*, *CTNNB*, *ERK* and *PI3K*) of THs were detected by qRT-PCR. The sequences of these genes were selected from transcriptomes (Huang et al., 2012) and ESTs (Jiang et al., 2011) of *H. diversicolor*. All sequences of transcritptomes and ESTs were aligned with Nr and Swissprot database by BLAST. The BLAST results showed that the sequences of our manuscript had the highest scores and lowest e-value with *TPO*, *IDIII*, *CCND1*, *CTNNB*, *ERK* and *PI3K* of other species, respectively. We had amplified these sequences by PCR and verified by Sanger's sequencing method. There were six replicate beakers of each treatment. After normality and homogeneity, data were analyzed with a one-way ANOVA and Fisher's LSD test to compare difference among treatments.

2.6 The assay of T3 inducing metamorphosis

Competent larvae were used in the inducing assay. A stock solution of T3 was prepared in FSW at a concentration of 100 μmol/L. Competent larvae were exposed in different T3 assay concentrations of 10, 1 and 0.1 μmol/L. The FSW without adding T3 was regarded as blank control. In detail, larvae collected by screening were put into 1-L beaker covered by benthic diatom with different T3 concentration. After 12 h, larvae were collected for metamorphosis rate counting and the sample collections of real time quantitative PCR (qRT-PCR) and whole mount in situ hybridization (WISH), which were similar to description in dsRNA exposure assay.

2.7 qRT-PCR

The detection of gene expression war carried out according to our previous report (Li et al., 2012; Wang et al., 2016). Briefly, total RNA was extracted from the samples with TRIZOL Reagent (Invitrogen,

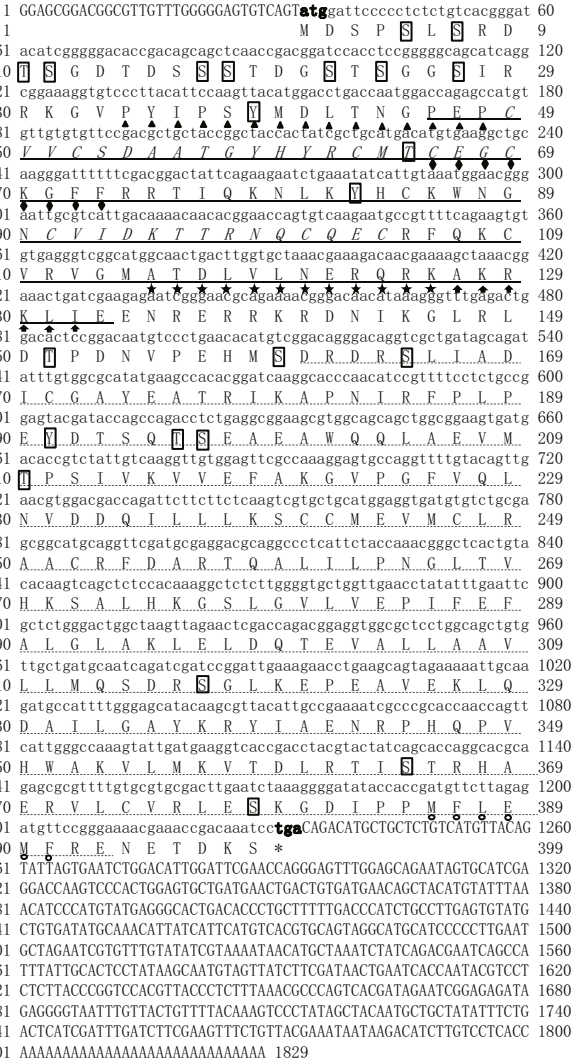


Fig.1 The characters of *HdTR* cDNA sequence and its encoding protein sequence

The "*" is terminator, and the initiation codon, termination codon and tail signal is in bold. The box is on behalf of phosphorylation site. Underline and dashed part respectively indicate DNA binding domain (DBD domain) (46aa–133aa) and ligand binding domain (LBD domain) (161aa–393aa). Zinc finger structure is in italic. N-terminal signature sequence (NTSS, 34aa- 48aa) P-box (66aa–73aa), T-box (115aa–126aa), A-box (127aa–132aa) and AF2 (385aa–391aa) are labeled by triangle, rhombus, star and arrow, respectively.

USA), and cDNA was generated by M-MLV (Promega, Fitchburg, USA). qRT-PCR was performed with an ABI 7500 real-time system by SYBR Green. Y-box binding protein gene (YB-1) was used as a reference, because of its constant expression in development (Huang et al., 2012). The comparative threshold cycle method was used to calculate the relative concentration. Six sample from different treatments were tested (n=6) as larval replicates, and each sample was assayed in triplicate for technical replicates according ABI qRT-PCR guide. The normalized CT values were analyzed by One-way ANOVA and Duncan variant significance tests. The genes and primers used in quantitative PCR were listed in Table 1.

3 RESULT

3.1 Identification and characterization of a full-length HdTR cDNA from abalone

The full-length cDNA of *HdTR* is 1 829 bp, with 33 bp 5′ un-translated region (UTR) and 596 bp 3′ UTR (Fig.1). The open reading frame is comprised of 1 200 bp, which encodes a protein (HdTR) of 399 amino acid residues. The calculated molecular mass of HdTR is 44.93 kDa, and the calculated isoelectric point is 8.40. Like typical nuclear receptor, HdTR contains a DBD and LBD (Fig.1). Alignment of HdTR and other TR sequences showed that DBD of HdTR is the most conserved domain, which contains two zinc finger motifs (Figs.1, 2). P-box sequence is highly conserved, which determines the specificity of target DNA binding. The T-box and A-box are also conserved. NTSS sequence of HdTR is PYIPSYMDLTNGPEP, and is conserved in non-chordate. While in chordates, three amino acids are deleted generating a sequence of GYIPSYL(D/E)KDE(P/Q/L) (Fig.2). The analysis of phylogenetic relationship indicated that TRs were divided into two branches, one comprising vertebrate, and the other one containing invertebrate. *H. diversicolor* and three bivalve species formed a cluster, and a starfish and acorn worm formed another one. The two clusters and a cluster of three amphioxus species formed the invertebrate branch, which corresponded with the evolutionary relationships among these invertebrate species (Fig.3).

3.2 The expressions of *HdTR*, *TPO*, *TPO-1*, *IDIII* and *ITGAV* in metamorphosis

The relative expression level of *HdTR* to *YB1* is showed in Fig.4. There was a significant difference of *HdTR* expression the three in the detected three stages (P=9.86E-11). *HdTR* expression of competent larva was significant lower than veliger (P=0.042 25), and higher than post larva (P=0.000 391). *TPO* expression level rapidly declined from veliger stage to competent larva stage (P=0.026 52), but there was no significant difference between competent larva and post-larva (P=0.252 9). *TPO-1* had only significant difference between competent larva and post-larva (P=0.026 16). *IDIII* and *ITGAV* of post-larva were significantly

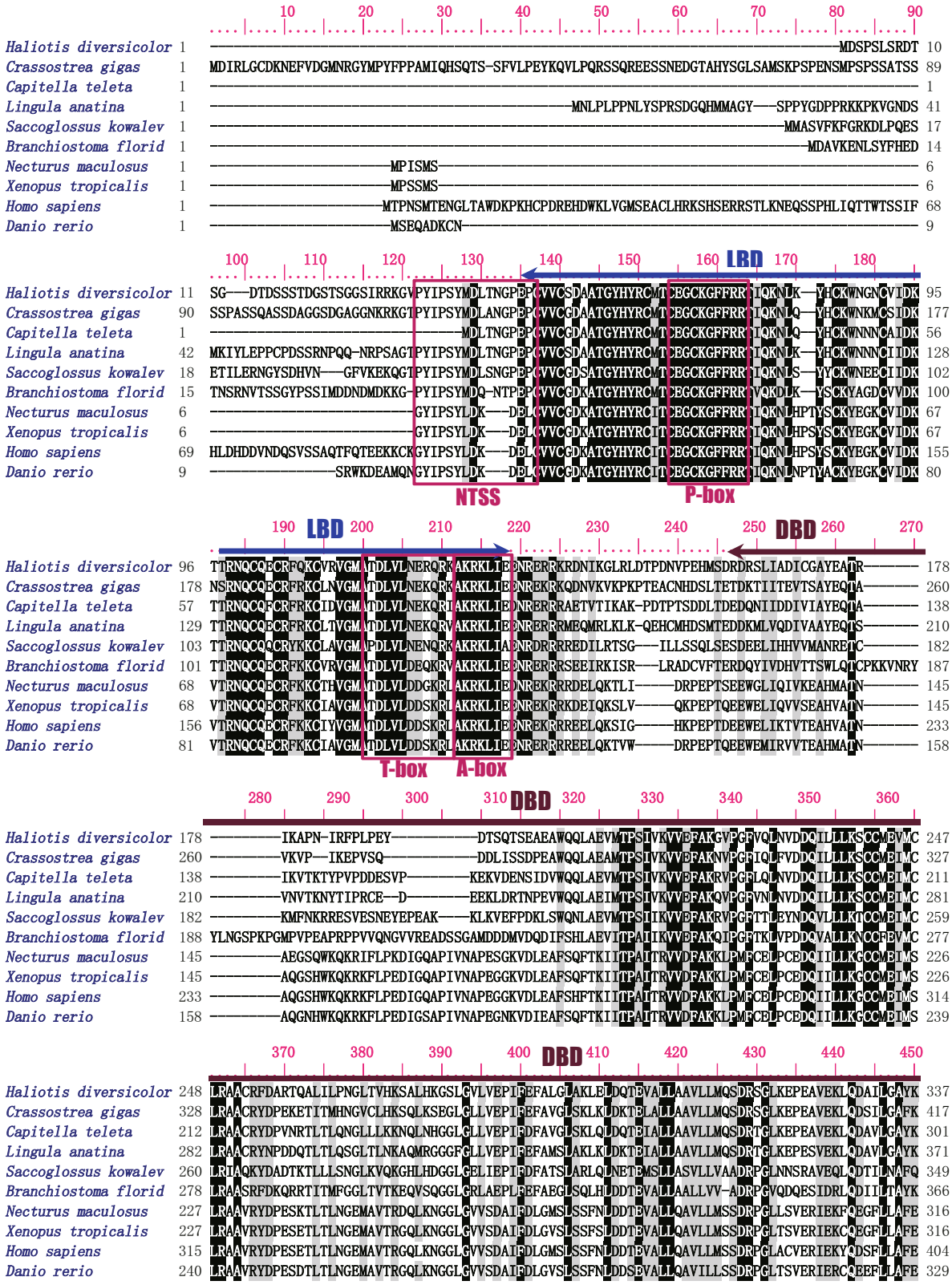
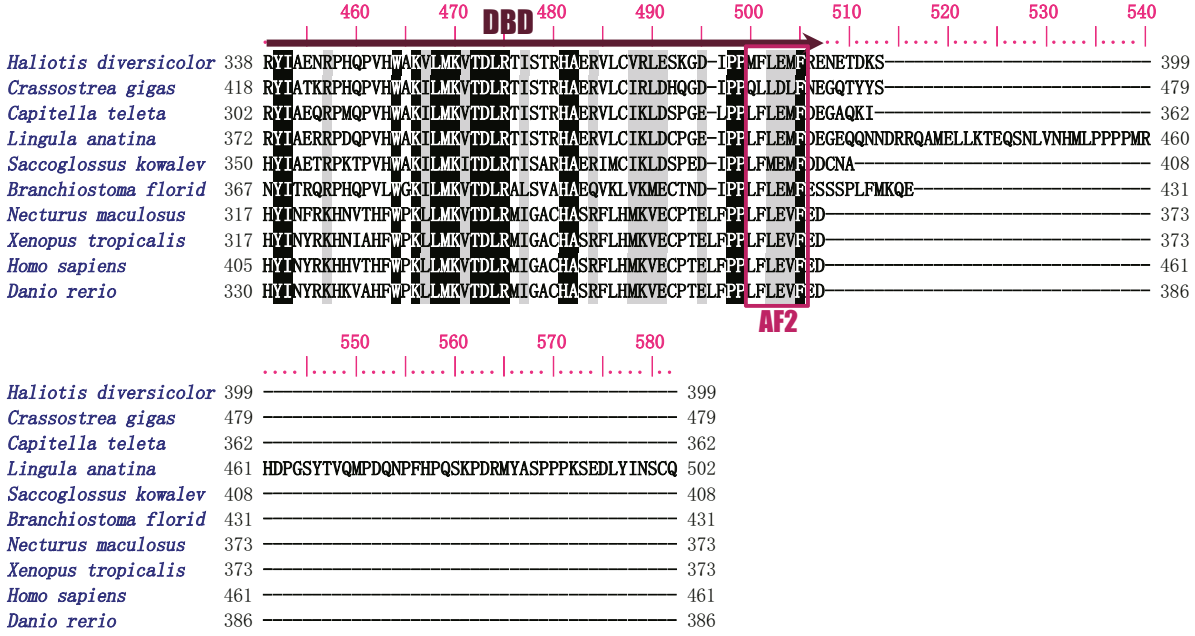


Fig.2 Multiple sequence alignment of HdTR amino acid sequence with other known TR proteins

The identity and similar amino acid residues are highlighted in black and gray, respectively. LDB domain is labeled by blue line and DBD by purple line. The conserved NTSS, P-box, T-box, A-box and AF2 are framed in red. The accession numbers of TR protein sequences are: *Haliotis diversicolor* (ALM96676.1), *Crassostrea gigas* (AKE80988.1), *Capitella teleta* (ELT99607.1), *Lingula anatine* (XP_013406710.1), *Saccoglossus kowalevskii* (XP_006822150.1), *Branchiostoma floridae* (ABS11249.1), *Necturus maculosus* (AAO62999.1), *Xenopus tropicalis* (ADL74876.1), *Homo sapiens* (NP_000452.2), *Danio rerio* (NP_571415.1).

To be continued

Fig.2 Continued



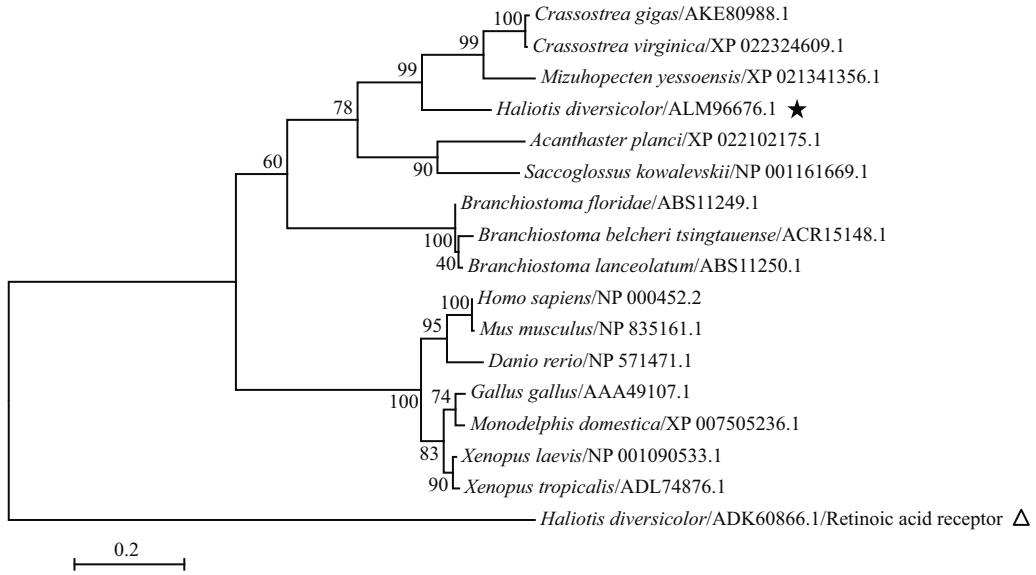


Fig.3 Phylogenetic tree of *Hd*TR and other known TR

Retinoic acid receptor of *H. diversicolor*, marked with triangle, is used as an outgroup. *Hd*TR is marked with star. The GenBank accession No. of each TR is shown with its species name.

higher than those of veliger and competent larva (P=0.000 92 and 0.041 33 respectively).

3.3 Effect of dsRNA exposure on metamorphosis rate and genes expression

After dsRNA exposure, the metamorphosis rate of RNAi group was significantly lower than that of BC and NC (P=0.042 64) (Fig.5a). The mortality rate of RNAi group was significantly higher than that of BC and NC (P=0.001 0) (Fig.5a). There were no significant differences of metamorphosis rate and mortality rate between BC and NC (P=0.069 99, P=0.072 29 respectively).

The exposure of *HdTR* dsRNA significantly reduced the expression level of *HdTR* (P=0.043 79) (Fig.5b). *HdTR* expression dropped from 1.39 of NC group to 0.41 of RNAi group (P=0.014 67), and was inhibited 70.26%. The *HdTR* expression of BC was

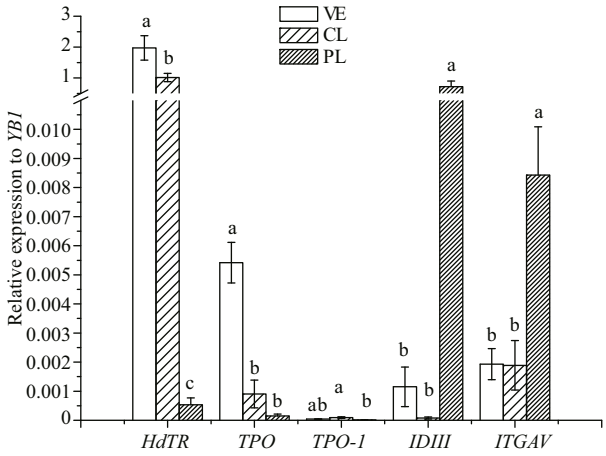


Fig.4 The relative expressions of *HdTR*, *TPO*, *TPO-1*, *IDIII* and *ITGAV* were detected during metamorphosis

YB1 served as reference gene. Each bar represents the mean value from six samples (n=6) with the standard error (SE). Three stages: veliger (VE), competent larvae (CL) and post larvae (PL) were detected by qPCR. The bars of each gene, if marked with different lowercase, indicate they are significantly different. The exact P values are described in the result section.

0.97, and 57.38% of *HdTR* expression was inhibited with RNAi group (P=0.029 97). There were no significant expression differences of *TPO*, *TPO-1* and *IDIII*, which encode the key enzymes of thyroid synthesis and conversion (Fig.5b).

In order to determine the signaling pathway of *HdTR*, genes of genomic signaling pathway and nongenomic signaling pathway were detected by qRT-PCR. In nongenomic signaling pathway, *ITGAV* of RNAi group was significantly lower than that of BC and NC (P=0.042 23, Fig.5b). The expression levels of other genes had no significant changes in the exposure of dsRNA *HdTR* (P=0.874 6, P=0.856 0, Fig.5b). In genomic signaling pathway, *CTNNB* of dsRNA *HdTR* was higher than that of BC and NC (P=0.039 62). *CCND1* of RNAi group were lower than that of BC and NC (P=0.034 76, Fig.5b).

3.4 Effect of T3 inducing on metamorphosis rate and genes expression

T3 had a significant effect on metamorphosis rate and mortality (P=0.037 697 and 0.028 466 respectively) (Fig.6a). The metamorphosis rate of 1 μmol/L T3 was higher than the other concentrations of T3 (P=0.037 697), while the mortality of 1 μmol/L T3 was lower than the other concentration of T3 (P=0.028 466). But the metamorphosis rate and mortality rate of 10 μmol/L T3 and 0.1 μmol/L T3 had no significant difference compared to that of 0 μmol/L T3 (P=0.333 704, 0.606 512; and 0.672 621, 0.290 906 respectively).

There was no significant difference of TPO/TPO-1 expression in different T3 concentrations (P=0.170 1 and 0.910 5, respectively) (Fig.6b). IDIII expression of 0 μmol/L T3 had no significant difference than other T3 concentrations (P=0.328 3, 0.115 5 and 0.524 2, respectively). In nongenomic signaling pathway, *ITGAV* expression of 1 μmol/L T3 and 0.1 μmol/L T3 was lower than that of 0 μmol/L T3 (P=0.036 678 and 0.033 265 respectively) (Fig.6b). But *PI3K* expression of 10 μmol/L T3 was higher than that of 0 μmol/L T3 (P=0.046 908). *ERK* expressions of different T3 concentrations had no significant difference (P=0.236 5). In genomic signaling pathway, *HdTR* expressions of 1 μmol/L T3 and *CCND1* expressions of 0.1 μmol/L T3 were higher than that of 0 μmol/L T3 (P=0.032 877 and 0.046 99, respectively) (Fig.6b). *CTNNB* expressions had no significant difference in different T3 concentrations (P=0.304 3).

4 DISCUSSION

Nuclear receptors are exclusive to multicellular metazoans. As a member of nuclear receptor (NR) gene family, TR was previously believed to be an innovation of chordates as no TR orthologs have been identified in the genomes of insects and nematodes (Bertrand et al., 2004). The presence of TR homologues in several invertebrates demonstrated that the TR orthologue genes are present outside of chordates. They were considered to originate in a common ancestor of bilateria (Wu et al., 2007). Although the N-terminal of NR is highly divergent, there is a conserved NTSS (N-terminal signature sequence), which is TR-specific. TR has two highly conserved domains, which are DBD and LBD (Wu et al., 2007). The TRs of flatworm *Schistosoma mansoni*are highly conserved not only in sequence similarity, but also in gene organization, protein-protein interaction and in DNA-binding ability. This suggests that the sequence and function of TRs are highly conserved between invertebrate animals (but not Porifera or Cnidaria) and vertebrate animals (Wu et al., 2007). *HdTR* has conserved sequence, including NTSS, DBD and LBD. This suggests that HdTR may have similar function of vertebrate TR.

THs are small, lipophilic signaling molecules built from tyrosine and iodine. THs primary source of marine invertebrate is marine phytoplankton, although

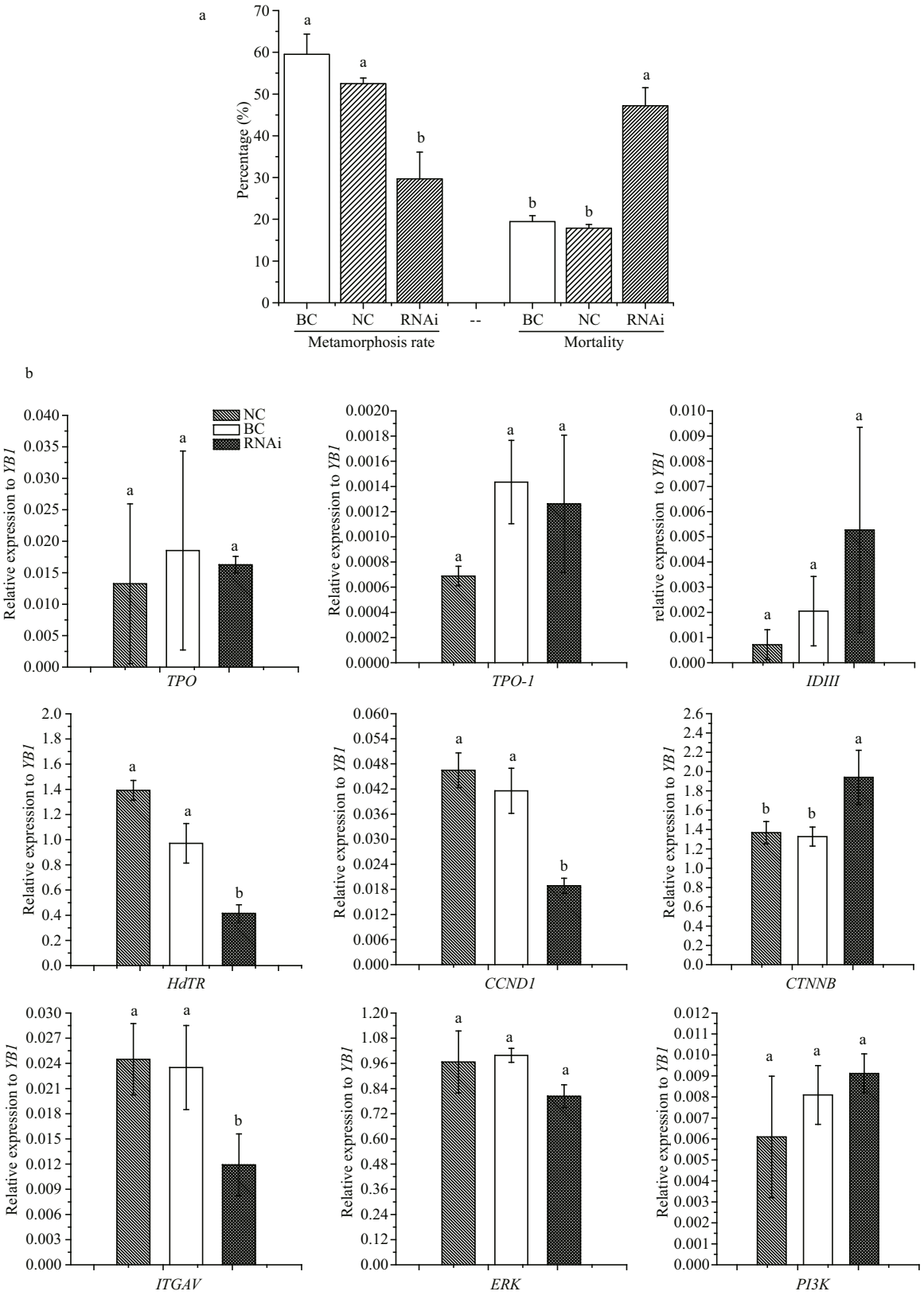


Fig.5 Metamorphosis rate, mortality and genes expressions in dsRNA exposure

Each bar represents the mean value from six samples (n=6) with the standard error (SE). The bars of metamorphosis rate or mortality in (a), if marked with different lowercase, indicate they are significantly different. The bars of each gene in (b), if marked with different lowercase, indicate they are significantly different. The exact P values are described in the result section.

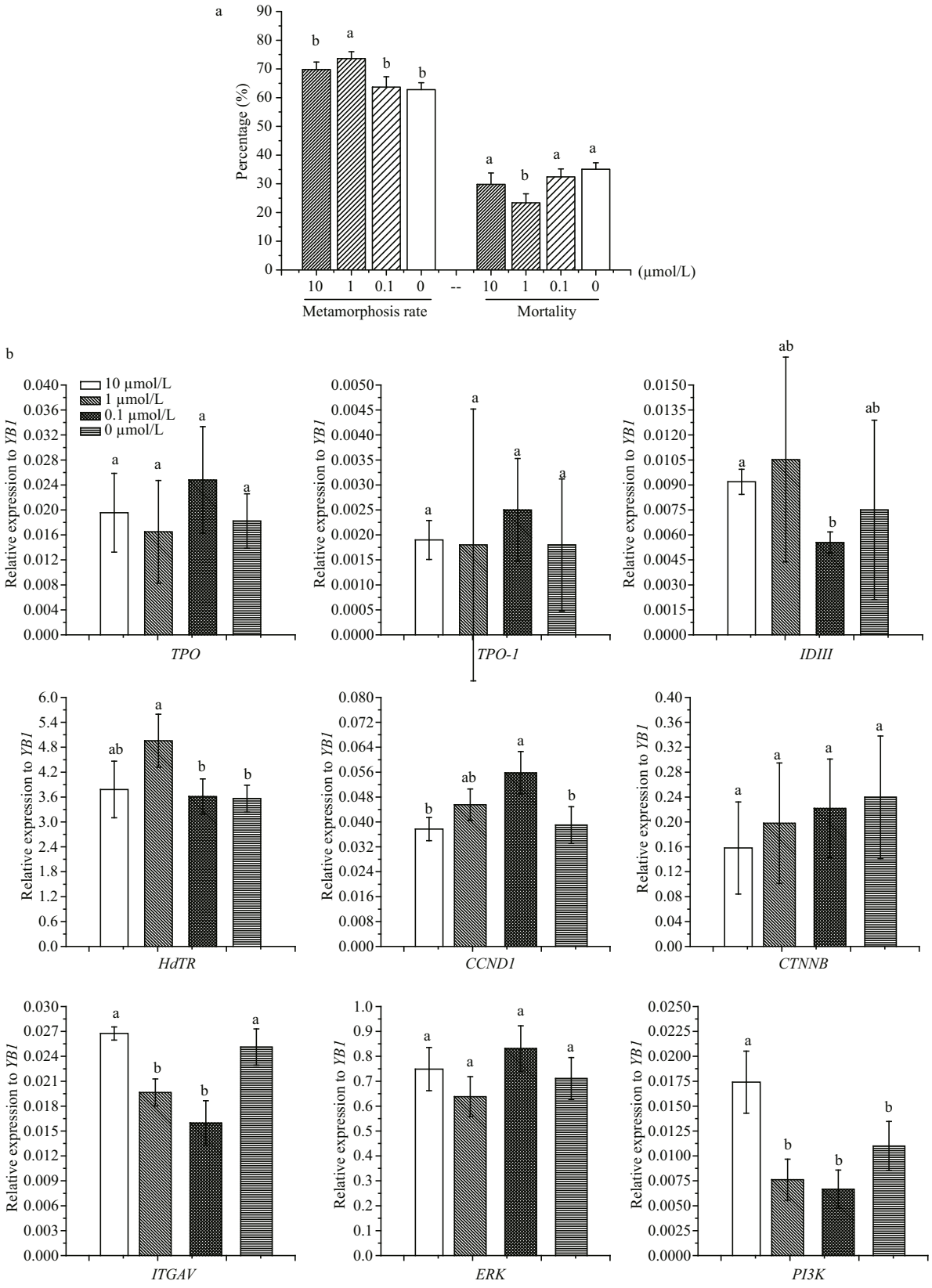


Fig.6 Metamorphosis rate, mortality and genes expressions in T3 exposure

Each bar represents the mean value from six samples (n=6) with the standard error (SE). The bars of metamorphosis rate or mortality in (a), if marked with different lowercase, indicate they are significantly different. The bars of each gene in (b), if marked with different lowercase, indicate they are significantly different. The exact P values are described in the result section.

some larvae may synthesize THs (Heyland and Moroz, 2005). THs may be transferred through the food chain and work as a cross-kingdom hormonal signaling (Heyland and Moroz, 2005). Heyland and Hodin (2004) had shown that THs can act via exogenous routes as environmental messengers in echinoderm larvae. Abalone larvae (*H. discus discus* and *H. gigantea*) were induced metamorphosis by THs at the concentration of 1 μmol/L (Fukazawa et al., 2001). Our findings are similar to this paper. The T3 inducing abalone metamorphosis is similar to the case in echinoderm larvae. T3 may take a message of metamorphosis, which means environment is suitable for metamorphosis of abalone larvae.

TPO is a critical enzyme in THs of biosynthesis. The presence of TPO suggests that abalone larvae can synthesize THs from incorporated iodine, as echinoderm larvae (Heyland et al., 2006a, b). As abalone larvae are lecithotrophic during swimming larvae stage, and there is no significant concentration of THs in seawater, the exogenous source of THs may not exist before metamorphosis. The higher expression level of *TPO* and *TPO-1* in veliger or competent larvae suggests that there are higher THs before metamorphosis. As THs accelerate development to metamorphosis in echinoderm larvae (Johnson 1998; Heyland and Hodin 2004), it suggests that endogenous THs of abalone larvae may be involved in metamorphosis. When competent larvae settle down substances which induce abalone larval metamorphosis, such as coralline algae, diatoms films or mucus trails (Fukazawa et al., 2001), these microenvironments may apply exogenous THs. These exogenous THs may accelerate metamorphosis (Fukazawa et al., 2001).

There was a high expression level of *HdTR* in swimming larvae stage and a rapid decline at post larva stage. When the high expression of *HdTR* was reduced by RNAi at competent larvae stage, there appeared lower metamorphosis rate and higher mortality rate. Moreover, the expression pattern of *HdTR* was similar to that of *TPO* and *TPO-1*. This means that *HdTR* expression level has positive correlation with endogenous THs. And the significant increase of *HdTR* expression is associated with increase of metamorphosis rate in 1 μmol/L T3. These data suggest that *HdTR* is involved in inducing metamorphosis of THs.

It is well known that TR binding THs can regulate gene expression with TREs. It is termed genomic action of THs. *CCND1* and *CTNNB* are effective genes of genomic signaling pathway (Cheng et al., 2010). The expression of these four genes had a significant difference in *HdTR* RNAi, but their expression patterns of 0.1 μmol/L T3 were different. Only *CCND1* had a higher expression in 0.1 μmol/L T3 than that in control (0 μmol/L T3). The expressions of *CTNNB* in 0.1 μmol/L T3 had no significant difference compared to that in 0 μmol/L T3. This result showed that TR genomic action may only mediate the expression of *CCND1*. *CCND1* encodes the cyclin D1 protein, which is required for progression through the G1 phase of the cell cycle (Baldin et al., 1993). *CCND1* is an early target in hepatocyte proliferation of rat induced by the THs (Pibiri et al., 2001), and TR deficient mice exhibit a significant delay in liver cell proliferation (Pascual and Aranda, 2013). These results are similar to our findings. Abalone larvae need develop juvenile/adult structures (such as gill, foot, and digestive system) in metamorphosis (Bishop, 2006; Heyland and Moroz, 2006). The cell proliferation is exuberant in the organogenesis. The up-regulated expression of *CCND1* suggests that the effect of TR genomic action may mainly show in cell proliferation during metamorphosis.

The transcriptional activity of the TRs is modulated by T3, but there is also activity in the unliganded state (Bernal and Morte, 2013). The unliganded TRs interact with the corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor) and may repress or enhance gene expression (Cheng et al., 2010). The unliganded TRs are responsible for changes of many THs responsive genes in mouse liver (Yen et al., 2003). Meanwhile, the unliganded TRs influence the timing and control certain aspects of amphibian pre-metamorphosis (Sato et al., 2007). The expression changes of *CTNNB* in *HdTR* RNAi may be due to the activity of the unliganded TRs. *CTNNB* encodes β-catenin, a structural component of cell adhesion complexes, which regulates actin filament assembly to regulate cellular functions (Gottardi and Gumbiner, 2001). In addition, β-catenin also functions as a co-activator of transcription factors, and is involved in cell proliferation, survival, and migration (Moon et al., 2002). Our data suggested that *CTNNB* may play a role in new structure development of metamorphosis by unliganded HdTR.

T3 and other THs can also act via nongenomic signaling. These effects are mediated via membrane receptors, such as integrin αvβ3 (Bergh et al., 2005).

ITGAV expression in 0.1 μmol/L T3 was lower than that in 0 μmol/L T3. This suggests that T3 may have a negative regulation on the expression of *ITGAV*. Via the integrin αvβ3, T3 stimulates ERK through phospholipase C (PLC) and protein kinase C (PKC). The signaling process is based on protein phosphorylation. The mRNA expression of ERK was not significantly different. The results did not suggest that integrin αvβ3 signal pathway does not work. However, we had not detected the protein activity of ERK and PI3K. As the cascade effect of αvβ3 is dependent on the protein activity of molecular components in the signal pathway, we are not sure that integrin αvβ3 signal pathway works, neither.

The Na,K-ATPcase activity stimulated by T3 nongenomic effect requires PI3K. This means that PI3K is involved in another nongenomic signal pathways of T3 (Lei et al., 2004). The expression level of *PI3K* had a significant difference in T3 inducing, but no in *HdTR* RNAi. This means *PI3K* were affected by T3. Our data is consistent with these previous papers.

Metamorphosis is a life-history transition that involves radical changes in habitat, morphology, and physiology (Bishop et al., 2006). For abalone, the swimming larvae change to creeping juveniles. Then the larval velum is lost. Meanwhile, the juvenile gill, foot and secondary shell appear. The cell differentiation and apoptosis are involved in these processes. Moreover, metamorphosis is tightly regulated by hormones and a variety of environmental signals (Bishop et al., 2006). Many kinds of chemical and biological materials are used to induce metamorphosis of abalone larvae (Roberts et al., 2001). This suggests that there are massive signal pathways involving abalone metamorphosis and there are complicated interactions among these pathways. However, there are short of protein tools and materials in pathway analysis of abalone metamorphosis. We only try to analyze the role of TR depending on gene expression data. Our data showed that *HdTR* is involved in cell proliferation of metamorphosis by TR genomic action or unliganded TR effect, and energy metabolism by unliganded TR effect. T3 may induce some THs nongenomic effect, but we have no evidence in protein level.

5 CONCLUSION

In summary, there is a significant decrease of metamorphosis rate after competent larvae were exposed to dsRNA of *HdTR*, and a significant increase in 1 μmol/L T3 inducing. The results suggested that HdTR and T3 play roles in abalone metamorphosis. The different expression patterns of thyroid hormone effector genes (CCND1, CTNNB, GSK, STAT1 and PI3K) in RNAi of *HdTR* and T3 inducing suggested that HdTR and T3 regulate metamorphosis by different ways. HdTR may be involved in cell proliferation of metamorphosis by TR genomic action or unliganded TR effect, and energy metabolism by unliganded TR effect in metamorphosis. T3 may induce some THs nongenomic effect in metamorphosis.

6 DATA AVAILABILITY STATEMENT

HdTR had been deposited in the GenBank database under accession number KT023066.1.

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