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Physiological role of CYP17A1-like in cadmium detoxification and its transcriptional regulation in the Pacific oyster, *Crassostrea gigas*

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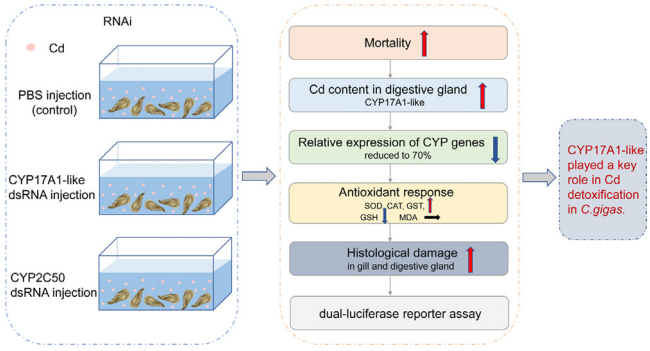
HIGHLIGHTS

- Knockdown of CYP17A1-like led to significant increase of mortality and Cd content.
- Oxidative stress and tissue damage were observed after interference CYP17A1-like.
- CYP17A1-like might play a critical role in detoxification of Cd in *C. gigas*.
- MTF-1 is essential for transcriptional regulation of CYP17A1-like in *C. gigas*.

GRAPHICAL ABSTRACT



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ABSTRACT

Cadmium (Cd) is one of the most harmful heavy metals due to its persistence and bioaccumulation through the food chains, posing health risks to human. Oysters can bioaccumulate and tolerate high concentrations of Cd, providing a great model for studying molecular mechanism of Cd detoxification. In a previous study, we identified two CYP genes, *CYP17A1-like* and *CYP2C50*, that were potentially involved in Cd detoxification in the Pacific oyster, *Crassostrea gigas*. In this work, we performed further investigations on their physiological roles in Cd detoxification through RNA interference (RNAi). After injection of double-stranded RNA (dsRNA) into the adductor muscle of oysters followed by Cd exposure for 7 days, we observed that the expressions of *CYP17A1-like* and *CYP2C50* in interference group were significantly suppressed on day 3 compared with control group injected with PBS. Moreover, the mortality rate and Cd content in the CYP17A1-like dsRNA interference group (dsCYP17A1-like) was significantly higher than those of the control on day 3. Furthermore, the activities of antioxidant enzymes, including SOD, CAT, GST, were significantly increased in dsCYP17A1-like group, while were not changed in dsCYP2C50 group. More significant tissue damage was observed in gill and digestive gland of oysters in RNAi group than control group, demonstrating the critical role of CYP17A1-like in Cd detoxification. Dual luciferase reporter assay revealed three core regulatory elements of MTF-1 within promoter region of *CYP17A1-like*, suggesting the potential transcriptional regulation of *CYP17A1-like* by MTF-1 in oysters. This work demonstrated a critical role of CYP17A1-like in Cd detoxification in *C. gigas* and provided a new perspective toward unravelling detoxification mechanisms of bivalves under heavy metal stress.

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1. Introduction

Heavy metal pollution in coastal environments is getting worse owing to fossil fuel combustion and industrial effluents (Peng et al., 2009; Wang et al., 2018). Among heavy metal pollutants, cadmium (Cd) is one of the most toxic elements in the aquatic environment, which ranks eighth in the priority list of top 20 hazardous substances (Meng et al., 2017). Cadmium can be bioaccumulated through the food chains, posing health risks to aquatic organisms and human consumers (Meng et al., 2017; Yao et al., 2020). This element can interact with the sulfhydryl groups of organic compounds (e.g., amino acids and proteins), changing their activity and conformation (Giguere et al., 2003; Park et al., 2001). Cadmium can influence mitochondrion, reproductive system, immune system of aquatic invertebrates and cause physiological responses (Li et al., 2018; Meng et al., 2017; Thijssen et al., 2007; Wang et al., 2018).

Bivalves, such as oysters, are popular seafood widely distributed in coastal and estuaries, which are hyperaccumulators of metals, and are usually used as bio-monitor for surveillance of metal pollution. Understanding of mechanisms underlying hyperaccumulation of heavy metals in bivalves is of importance for providing solutions into concerns of environment pollution and seafood safety. In a previous study, we revealed the potential involvement of Cytochrome P450 (CYP) in detoxification of Cd in the oyster (Tian et al., 2021). The CYP superfamily contains a group of heme-thiolate proteins, which are found in all domains of life. The CYPs play critical roles not only in the metabolism of endogenous compounds and exogenous substances but also in the phase I detoxification systems in vertebrates, invertebrates, and plants (Lu et al., 2019; Meunier et al., 2004; Nebert and Russell, 2002; Nelson et al., 2013). Among the CYPs, members of CYP1 family are of major interest due to their roles in metabolizing procarcinogens and environmental pollutants such as PAH and metals (Korashy and El-Kadi, 2005; Shimada and Fujii-Kuriyama, 2004).

With extensive expression profiling of all the CYP members based on meta-analysis of transcriptome sequencing data and verification using quantitative real-time PCR in the Pacific oyster, we identified *CYP17A1-like* and *CYP2C50* that were potentially involved in Cd detoxification in the oysters (Tian et al., 2021). CYP17 catalyze both steroid-17α-hydroxylase and steroid-17,20-lyase, and is a typical membrane-bound bifunctional monooxygenase (Yang et al., 2006). It plays a vital role in steroid metabolism, converting progesterone and pregnenolone into 17α-products with multiple functions (Gonzalez et al., 2018). Besides, the xenobiotic detoxification roles of the CYP17 and CYP2 homologous genes have also been reported in various organisms (de Toledo-Silva et al., 2008). Luchmann et al. (2015) reported that *CYP17* was up-regulated in response to the chemical challenges such as phenanthrene and sewage. Members of CYP2C subfamily encode enzymes that are important in the metabolism of many drugs, detoxification of potential carcinogens or the bioactivation of some environmental procarcinogens (Danielson, 2002; Fujita and Kamataki, 2001). *CYP2C50* is a lineage-specific gene identified in the *C. gigas*, which has a close evolutionary relationship with *CYP2C14* that was up-regulated after exposure to domoic acid-producing *Pseudo-nitzschia* in Queen Scallop (Ventoso et al., 2019). In oysters, how these two candidate CYPs are involved in metal detoxification requires further investigations with additional lines of evidence.

RNA interference (RNAi) is a widely used reverse genetic tool for determining the function of uncharacterized gene via introducing dsRNA molecules into organisms or cells to inhibit the expression of genes of interest (Fire, 1999; Fire et al., 1998). In recent years, RNAi has also been used for gene function analysis in aquatic bivalves, such as Pacific oyster (Fabioux et al., 2009), pearl oyster (Funabara et al., 2014), Zhikong scallop (Wang et al., 2019) and clam (Wang et al., 2020), in gastropods, such as great pond snail (Korneev et al., 2002), and in crustaceans, such as Chinese mitten crab (Ma et al., 2016).

In this study, we used RNAi to knockdown the expression of the two candidate CYP genes to further clarify their functional roles in the detoxification system induced by Cd exposure. The CYP expression levels, content of accumulated Cd, activity of antioxidant system and histopathological damage were compared between the interference group and the control group. Furthermore, we performed a dual-luciferase reporter assay and identified three core regulatory elements for MTF-1 within promoter region of *CYP17A1-like* gene. This work demonstrated a critical role of CYP17A1-like in Cd detoxification in *C. gigas* and provided a new perspective toward unravelling detoxification mechanisms of bivalves under heavy metal stress.

2. Materials and methods

2.1. Experiment animals

The oysters used in this work were one-year-old healthy Pacific oysters (77.46 ± 0.91 mm in shell height, 44.35 ± 0.57 mm in shell length, 21.09 ± 0.31 mm in shell width, and 39.92 ± 1.13 g in body weight) collected from an oyster farm in Weihai, China, in July 2020. The shells of oysters were drilled with a small hole near the adductor muscle, and were acclimated in tanks (490 × 350 × 250 mm) containing aerated seawater (salinity 30‰, pH 8.1) at 23 ± 1 °C for 5 days in laboratory before experiment. During the acclimation period, the seawater was changed (100%) once a day. We fed an averaged 0.075 g of concentrated algae fluid (~3×10^8 algae cells) for each oyster every other day as described in previous study (Tian et al., 2021). For each tank, the algae density was about 2.52×10^5 cells/mL.

2.2. Synthesis of double-stranded RNA (dsRNA)

Based on the *CYP17A1-like* (GenBank accession LOC105337486) and *CYP2C50* (GenBank accession LOC105333812) cDNA sequence, two fragments of nucleotide sequences from position 1033 to 1331 of *CYP17A1-like*, and 1022 to 1459 of *CYP2C50* were used to design the target site of the dsRNA for RNAi (Table 1). The 299 bp fragment of *CYP17A1-like* and 438 bp fragment of *CYP2C50* were amplified using cDNA that was reversely transcribed from total RNA extracted from digestive gland. The purified PCR products were sub-cloned to pGMT-easy vector (Promega, USA) and sequenced to validate sense and antisense recombined plasmids. The linearized recombined plasmids were

Table 1
Primers used in this study.

Primer	Nucleotide sequence (5′-3′)	Application
CYP17A1-like_Forward	ACATTACCGCAGGAGCATA	RNAi
CYP17A1-like_Reverse	CCAGACGTTAGGAATGATCAC	RNAi
CYP2C50_Forward	AGCGGGAACTGAAACCACG	RNAi
CYP2C50_Reverse	GCGAGCGAATCTCCTAAGCAC	RNAi
CYP17A1-like_Forward	GGTAATTGGACGGAACGAGAA	qRT-PCR
CYP17A1-like_Reverse	GGTTGTGGATTGTGAAGTGGC	qRT-PCR
CYP2C50_Forward	TTCCAGAGCCTACCAAATTCCAAC	qRT-PCR
CYP2C50_Reverse	GCGAGCGAATCTCCTAAGCAC	qRT-PCR
EF_Forward	AGTCACCAAGGCTGCACAGAAAG	qRT-PCR
EF_Reverse	TCCGACGTATTTCTTTGCGATGT	qRT-PCR
PGL3-basic-CYP-2127_Forward	TACCGAGCTCTTACGCGTGCTAGC ATGATCAACAGTACAACGCA	Construction of report plasmids
PGL3-basic-CYP-1671_Forward	TACCGAGCTCTTACGCGTGCT AGCTGTGTGACTTCTTGCCCTTC	Construction of report plasmids
PGL3-basic-CYP-1062_Forward	TACCGAGCTCTTACGCGTGCT AGCATGTGGCACTAATACGCTTC	Construction of report plasmids
PGL3-basic-CYP-603_Forward	TACCGAGCTCTTACGCGTGCT AGCCGGCACGTAGACTTTAGTGT	Construction of report plasmids
PGL3-basic-CYP-258_Forward	TACCGAGCTCTTACGCGTGCT AGCTCTGGATTAGCTGCATCAAT	Construction of report plasmids
PGL3-basic-CYP_Reverse	CAACAGTACCGGAATGCCAAG CTTCTCACGCAGAACATCCACT	Construction of report plasmids
pcDNA3.1(+)-MTF-1_Forward	ATAGGGAGACCCAAGCTGGCT AGCATGGACGACACCAGCAATGG	Construction of report plasmids
pcDNA3.1(+)-MTF-1_Reverse	GGATCCGAGCTCGGTACCAAG CTTTTAAGTAGTGTTTATCGCAT	Construction of report plasmids

transcribed to produce dsRNA using T7 RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's instructions. The quality of dsRNA was assessed by running 1.5% agarose gel electrophoresis, and the concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

2.3. Cadmium exposure experiment

A total of 126 oysters were randomly divided into three groups, CYP17A1-like dsRNA interference group (indicated by dsCYP17A1-like hereafter), CYP2C50 dsRNA interference group (indicated by dsCYP2C50 hereafter) and phosphate buffer saline (PBS) group. These three groups were cultured under 200 μg/L Cd exposure environment and were maintained with same practice during experiment including cadmium exposure, water changing and feeding. For Cd exposure, the working solution was prepared by dissolving 0.618 g $CdCl_2 \cdot 2.5H_2O$ (99%) into 30 mL sterile water. The realistic concentration of Cd in the tanks we measured was 201.18 ± 2.28 μg/L ($n = 3$). For the oysters in interference groups (i.e., dsCYP17A1-like and dsCYP2C50 group), each oyster was injected with 45 μL dsRNA (1 μg/μL) referring to other previous studies (Funabara et al., 2014; Choi et al., 2013; Wang et al., 2020). For the oysters in PBS group, each oyster was injected with 45 μL PBS buffer (pH = 7.4). The composition of PBS including NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4. The concentration of Cd used for exposure was determined based on previous studies which was below lethal level but sufficient to alter the antioxidant system of shellfish (Pan, 2015). The dsRNA and PBS were injected into the adductor muscle using a micro syringe. The dsRNA and PBS were injected at beginning of the experiment on day 1 (1d), and were further injected on day 2 (2d), day 4 (4d) and day 6 (6d). Six oysters randomly selected from each group were sampled at the beginning of the experiment (0 h), and on 1d, 3d, 5d and 7d post-injection. The gill and digestive gland were dissected, flash-frozen in liquid nitrogen and stored at −80 °C freezer until use.

2.4. Measurement of cadmium content in digestive gland

To measure Cd content, 50 mg digestive gland were thoroughly digested in concentrated HNO_3 and H_2O_2, followed by boiling. The Cd content was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using an Agilent 720-ES (Agilent Technologies, USA) after cooling. The detection sensitivity for Cd was ~0.001% ppm (mg/L). The correlation coefficient of standard curve reached 99.9% ensuring the accuracy of the cadmium content. The operating conditions of Agilent 720-ES were set as follows: RF Power was 1.20 KW; Plasma flow was 15.0 L/min; Auxiliary flow was 1.50 L/min; Nebulizer flow was 0.75 L/min; Sample uptake delay was 15 s; Instr stabilization delay was 15 s; Replicate read time was 2 s; Replicates were 3 times.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA of the digestive gland was extracted using TRIzol® reagent in accordance with the manufacturer's protocol (Invitrogen, USA). The RNA quality was measured via Nanodrop 2000 (Thermo Scientific, USA) at A260 and A280. The RNA integrity was assessed via electrophoresis in a 1.2% agarose gel. cDNA synthesis was performed using 1 μg total RNA as template by PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) following instructions of the manufacturer (Takara, Japan). The synthesized cDNA samples were stored at −30 °C prior to use.

The primers used for qRT-PCR were provided in Table 1 and the elongation factor gene (EF) was chosen as the internal standard due to EF has been widely used as internal reference genes in many expression studies of the *Crassostrea gigas* and has high efficiency (Du et al., 2013; Yu et al., 2017; Meng et al., 2018). The qRT-PCR was performed using a LightCycler480 II Real-Time Detection System (Roche, Germany) in a total volume of 10 μL reaction mix, containing 5 μL of SYBR Green PCR Master Mix (QIAGEN, Germany), 2 μL of 1:10 diluted cDNA, 0.7 μL each of the forward and reverse primers and 1.6 μL of RNase-free water. The PCR program were set as: PCR initial heat activation (95 °C for 2 min), amplification and quantification program by 40 cycles (95 °C for 5 s and 60 °C for 10 s), melting curve (95 °C for 1 min, 62 °C for 30 s and 95 °C continues) and finally a cooling step to 40 °C. The relative expression levels of genes were calculated according to the comparative $2^{-\Delta\Delta Ct}$ method.

2.6. Analyses of enzyme activity and oxidative damage

Digestive gland (100 mg) dissected from oysters were washed twice with cold PBS, and homogenized in 900 μL PBS on ice using a hand-held electric tissue homogenizer IKA T10 basic (IKA, Germany). The homogenate was then centrifuged at 4000 × rpm for 10 min at 4 °C to precipitate insoluble tissue. The supernatant was collected and assayed for detecting the activities of total-superoxide dismutase (T-SOD), catalase (CAT) and glutathione-S-transferase (GST), and the contents of reduced glutathione (GSH) and malondialdehyde (MDA) following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The protein concentration was measured via Nanodrop 2000 (Thermo Scientific, USA) which does not require any reagent and is simple to operate and easy to recycle. All assays were performed in a SYNERGY H1 microplate reader (BioTek, USA).

2.7. Histological observation

Based on the number of injections and the total dose, we inferred that the maximum damage caused by interference was on the last day of the experiment, so the day 7 was selected as the observation of tissue damage. The gill and digestive gland of three oysters from each group at 0 h and 7 d were sampled and fixed overnight in Bouin's fixative. After 24 h, the Bouin's fixative was replaced with 70% alcohol solution for several times until the tissue was colorless or light yellow. The samples removed from Bouin's fixative were packed into the embedding box, and the HistoCore Pearl (Leica, Germany) was used for ethanol dehydration, xylene transparency, paraffin infiltration and finally embedded in paraffin blocks. The paraffin blocks were then trimmed and sliced into 5 μm thick slices. The histological slices were stained with Haematoxylin-Eosin. All slices were visualized using light microscope (Olympus DP80, Japan).

2.8. Dual-luciferase reporter assay

Based on the results of RNAi showing the critical involvement of *CYP17A1-like* gene expression in the Cd detoxification, we just predicted the transcription factor binding sites of *CYP17A1-like*. The transcription factor binding sites in the promoter region of *CYP17A1-like* were predicted via JASPAR (http://jaspar.genereg.net/#opennewwindow). The progressive 5′ deletion fragments were amplified from a pair of primers containing two restriction enzyme cutting sites, *Nhe*I and *Hin*dIII. The purified products were subcloned into the linearized pGL3-basic vector (Promega, USA) and verified by DNA sequencing. The ORF sequence of metal regulation transcription factor-1 (MTF-1) was cloned into the pcDNA3.1(+) expression vector (Invitrogen, USA) with the MTF-1-F/R primers containing the NheI and HindIII restriction sites. The plasmids for transfection were prepared from overnight bacterial cultures using Endo Free Plasmid Mini Kit (D6950, Omega Bio-tek) according to the manufacturer's protocol. HEK293 cells were grown in DMEM high-glucose medium (Hyclone, USA) supplemented with 5% fetal bovine serum (FBS) at 37 °C in a humidified incubator under a 5% CO_2 atmosphere. At 24 h prior to transfection, the cells were plated into 24-well plates (1×10^5 cells/well) in fresh medium. Then, 500 ng of *CYP17A1-like* promoter construct of different lengths in pGL3-basic, 250 ng of pcDNA3.1-MTF-1 and 100 ng of pRL-TK (to normalize transfection efficiency) containing Renilla luciferase were transiently co-transfected

into the cells in 750 μL of serum-free medium using Xfect™ Polymer (Takara, Japan). The transfection medium was replaced with fresh medium after 4 h of treatment, cells were collected at 48 h after transfection, and luciferase activity assays were carried out using a Dual-Luciferase kit (Promega, USA).

3. Results

3.1. Mortality during experiment

The number of deaths in each group within 7 days after four-time injection was quite different. As shown in Fig. 1, the numbers of deaths in the dsCYP17A1-like and dsCYP2C50 groups injected with dsRNA were significantly higher than the group injected with PBS ($P < 0.05$). Specifically, the mortality rates of dsCYP17A1-like, dsCYP2C50 and PBS group on day 7 were 50%, 46.5% and 23.8%, respectively.

3.2. Cd content in digestive gland

During the 7-day RNAi experiment under cadmium exposure, the content of Cd accumulated in the digestive gland showed a significant increasing trend (Fig. 2). The average Cd content of three injection groups under Cd exposure on day 3 and day 7 reached 38.6 and 119.3 mg/kg, respectively, which was 5.2- and 16.2-fold higher than those without Cd treatment and RNAi samples (0 h), indicating a significant accumulating pattern. On day 3, the Cd content of the dsCYP17A1-like group was significantly higher than that of the PBS injection group ($P < 0.05$), while the dsCYP2C50 group was not significantly different from the PBS injection group. Interestingly, no significant difference in the Cd content was observed among the three groups on day 7 (Fig. 2).

3.3. Expression of CYP genes altered by RNAi treatment

The mRNA expression levels of *CYP17A1-like* and *CYP2C50* in the digestive gland were significantly altered by RNAi (Fig. 3). For *CYP17A1-like*, the expression level in dsCYP17A1-like group was significantly reduced by 49% on day 1. Moreover, this knockdown effect reached peak on day 3, with knockdown rate of expression being 70% ($P < 0.05$). Notably, the expression levels showed no significant difference between experimental group and control on day 7 (Fig. 3A). Similarly, the expression level of *CYP2C50* was significantly reduced by 62% on day 1, and the knockdown effect reached its peak on day 3 which was reduced by 70% ($P < 0.05$) (Fig. 3B).

3.4. Effect on antioxidant activity by RNAi

The activity of T-SOD, CAT, GST, and the content of GSH in digestive gland after injection with dsRNA and PBS under Cd exposure was determined to assess the effect of RNAi on antioxidant system (Fig. 4). The T-SOD and CAT activities in three groups were firstly increased and then decreased during the experiment. At each specific time-point, the activity T-SOD and CAT in dsCYP17A1-like group was significantly higher than PBS group, and peaked on day 3. Unlike the dsCYP17A1-like group, the T-SOD activity in dsCYP2C50 group showed no significant difference on day 1 and was significantly lower than control group on day 3 ($P < 0.05$). Whereas the CAT activity in dsCYP2C50 group showed no significant variation throughout the experiment compared to control ($P > 0.05$). The GST activity in three groups were decreased first, then gradually increased (Fig. 4C). The GST activity in dsCYP17A1-like group was significantly induced and reached peak on day 1, then returned to the control group on day 3. Further, the activity was significantly re-induced on day 7. While the GST activity in dsCYP2C50 group was significantly higher than the control group until day 7. GSH was considered as the main defense against metal detoxification due to the cysteine thiol group. The content of GSH did not change significantly in the early period until day 7. On the day 7, the GSH content of CYP17A1-like group and CYP2C50 group decreased significantly compared with control ($P < 0.01$). The greater the content of GSH decreased, the greater the GST activity was, which corresponded to the significant increase in GST activity on day 7 (Fig. 4D). What's more, the content of MDA in digestive gland after injection with dsRNA and PBS under Cd exposure was also determined to assess the effect of RNAi on oxidative damage. As shown in Fig. 4E, there was no significant difference in the content of MDA among three groups ($P > 0.05$), which was in accordance with the increase of antioxidant enzyme activity.

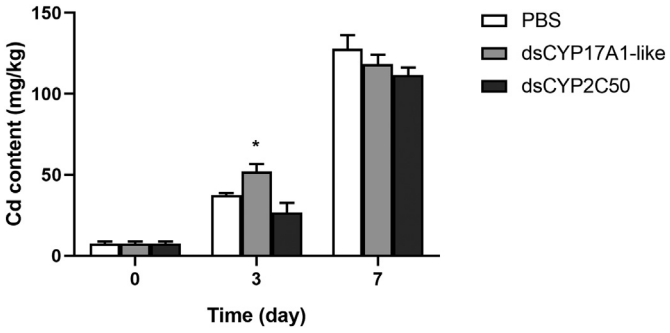

Fig. 2. Cd content in digestive gland of *C. gigas* with RNA interference under Cd exposure experiment. Asterisk (*) indicates significant differences with control (mean ± SE; $n = 3$, ANOVA, $0.01 < P < 0.05$).

3.5. Analysis of histopathological alterations

To evaluate the effects of CYP interference, histopathological alterations were observed in both gill and digestive gland of the oyster under Cd exposure (Fig. 5 and Fig. 6). In the blank control group (0 h), the gill of oyster was intact, arranged regularly and there was no swelling or damage in the gill filaments (Fig. 5A). After 7-day RNAi under Cd exposure, the gill filaments in the PBS group were arranged regularly, only a few gill filaments showed vacuoles, lateral cilia loss and swelling tips of gill filaments (Fig. 5B). While the gill in dsCYP17A1-like group obviously showed the broken gill filaments, the scattered arrangement, a large number of fall-off cilia and the swelling tips of the gill filaments (Fig. 5C). In addition to the above damages observed in the dsCYP17A1-like group, a large number of vacuoles were observed in the gill filaments in dsCYP2C50 group (Fig. 5D).

In the blank control group, the outline of the digestive tubules was complete, the structure of connective tissues was normal, the interstitial tissue of inner wall was in a "star" structure, and there was no obvious tissue necrosis or damage (Fig. 6A). After 7-day RNAi under Cd exposure, the digestive tubules in the PBS group were partially degraded, showing enlarged digestive tract lumen, disintegration of some digested cells, separation of inner wall interstitial tissue from epithelial cells, and

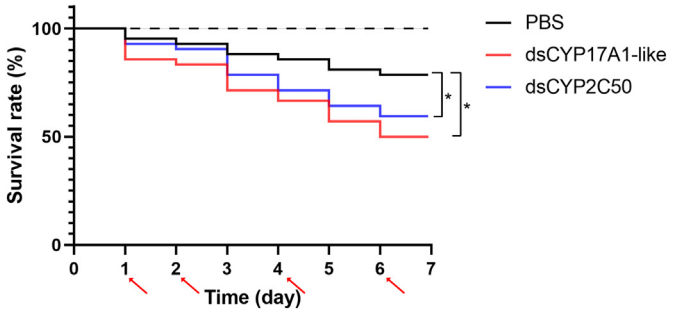

Fig. 1. Survival analysis of *Crassostrea gigas* with RNA interference under Cd exposure experiment. The red arrows denoted the time-points for injection of dsRNA and PBS in interference group and control group, respectively. Asterisk (*) indicates significant differences with control ($0.01 < P < 0.05$). The dotted line represents the survival rate of oysters under cadmium stress without injection.

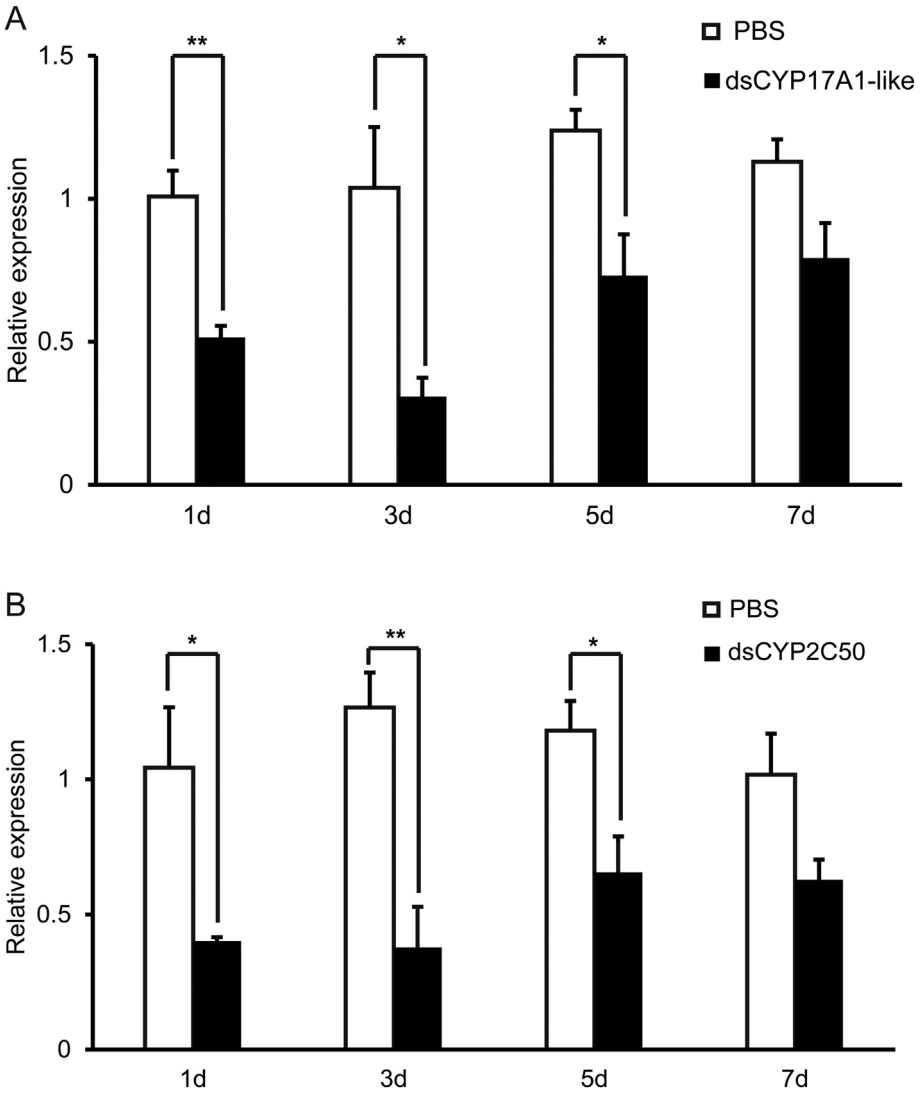

Fig. 3. Relative expression of *CYP17A1-like* and *CYP2C50* in the digestive gland of *C. gigas* post-injection of sterile PBS, CYP17A1-like dsRNA and CYP2C50 dsRNA under Cd exposure. Values are shown as mean ± SE ($n = 6$). Significant difference compared with the control expression level was marked with asterisks (* $0.01 < P < 0.05$; ** $P < 0.01$).

the overall damage was minor (Fig. 6B). In contrast, in dsCYP17A1-like group, a large number of digestive cells were collapsed, epithelial cells were damaged, the digestive tubes were collapsed as a whole, resulting in blurred outlines and vacuoles in the tube (Fig. 6C). The dsCYP2C50 group showed similar tissue damages with dsCYP17A1-like group with a less severe damage level (Fig. 6D).

3.6. Analysis of core regulatory element within promoter region of CYP17A1-like

As revealed by RNAi, expression of *CYP17A1-like* was highly associated with Cd detoxification in the oysters. We, therefore, further investigated transcriptional regulation of *CYP17A1-like* gene by determining its core promoter elements involved in transcription. The transcription activity of the putative promoter region of *CYP17A1-like* gene were evaluated by dual-luciferase reporter assay. As shown in Fig. 7, the results showed that the activity of all truncated promoter fragments containing transcriptional binding sites were significantly higher than control (pGL3-basic, plasmid without inserted promoter fragment). Deletion of the *CYP17A1-like* promoter to position −2127, position −1671 and position −1062 did not abolish the promoter activity. Deletion of the *CYP17A1-like* promoter at −603 still exhibited higher promoter activities, but such activity was eliminated when the promoter was deleted at −258 (Fig. 7). Therefore, we concluded that three key MTF-1 binding sites located from −258 to −603 of *CYP17A1-like* promoter would be critical elements for MTF-1 transcription factor mediated transcription regulation.

4. Discussion

Heavy metals are a major class of pollutants released into the aquatic ecosystems due to human activities, and cadmium is considered as one of the most toxic elements found in all the ecosystem. Oysters are a group of bivalves that are tolerant to high levels of heavy metals, while how they act to maintain detoxification process at physiological and molecular level remains largely unexplored. The detoxification of heavy metals is a complex process. Therefore, oysters can be used as ideal materials to illustrate the detoxification mechanism of heavy metals, which will provide valuable information on environmental surveillance and production of healthy seafood. To characterize the physiological role of CYPs, herein we used RNAi to knockdown the expression of two CYPs with critical roles in Cd detoxification in *C. gigas*. From our previous study, we observed tissue-specific pattern of cadmium accumulation in gill and digestive gland under cadmium treatment. The cadmium content in the gill increased constantly, while the cadmium content in the digestive gland decreased after

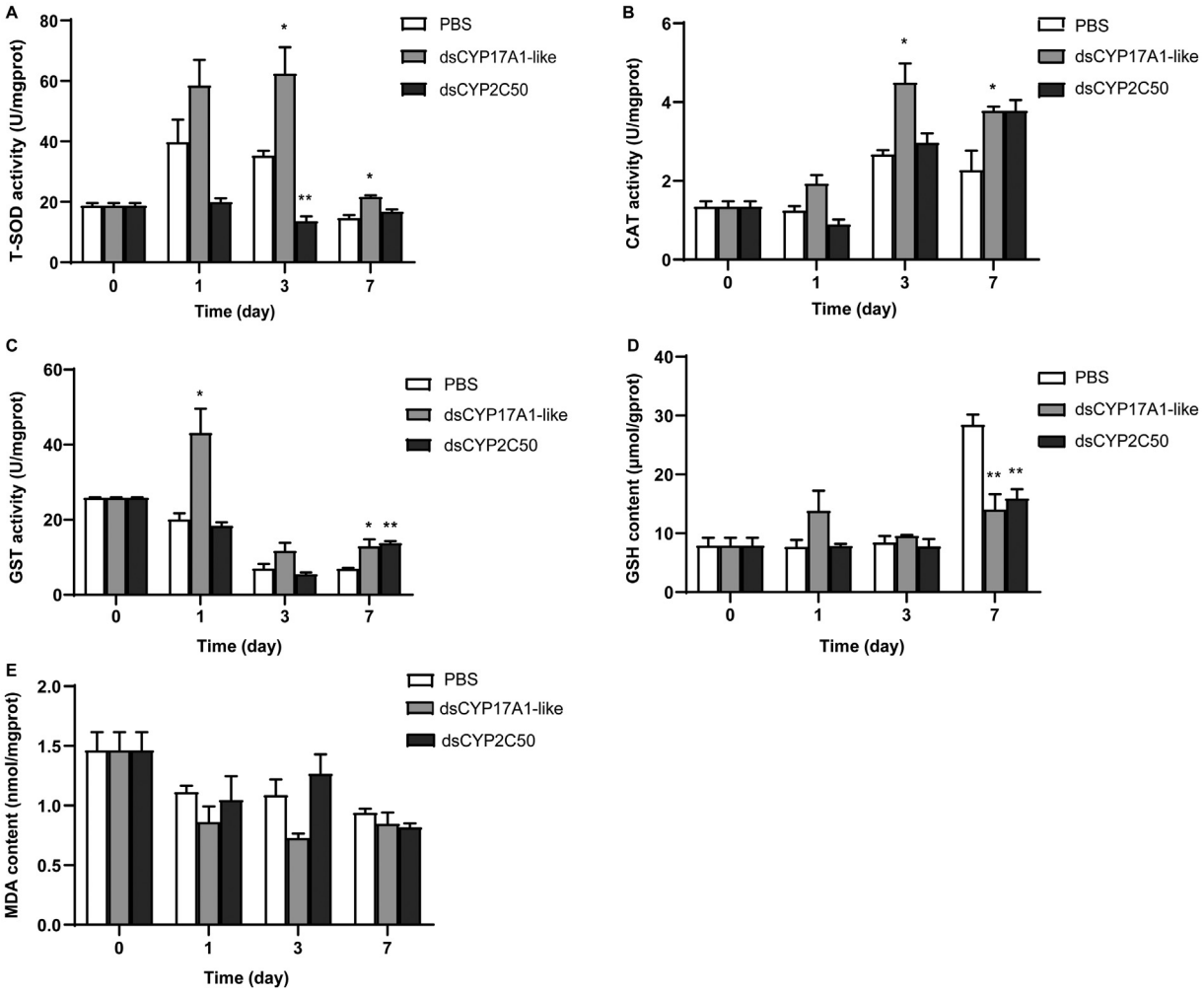

Fig. 4. The enzyme activity of T-SOD, CAT and GST and the content of GSH and MDA in the digestive gland of *C. gigas* post injection of PBS, CYP17A1-like dsRNA and CYP2C50 dsRNA under Cd exposure. Values are shown as mean ± SE (n = 3). Asterisks indicate significant difference compared with the control (* $0.01 < P < 0.05$; ** $P < 0.01$).

peaking on day 7, indicating that the digestive gland could be the potential site for Cd detoxification (Tian et al., 2021). Therefore, we performed further analysis of gene expression and enzyme activity with digestive gland, while investigated the histopathological alterations in both tissues.

It was generally believed that transcription knockdown to be 70% with dsRNA treatment would be effective for RNAi treatment (Jiang et al., 2006). In the present study, the relative expression levels of both *CYP17A1-like* and *CYP2C50* mRNA were significantly suppressed by 70% on day 3 ($P < 0.05$) (Fig. 3), suggesting the effectiveness of RNA interference achieved in our study. Similar knockdown efficiency was achieved in the clam for 63% down-regulation of the AHR gene after 3-day dsRNA injection (Wang et al., 2020), 77% down-regulation of the MTF-1 gene after injection (Meng et al., 2015), and 80% down-regulation of the Dmrt gene after dsRNA injection (Ma et al., 2016). Consistent with the expression levels, the cadmium content of dsCYP17A1-like group was significantly higher than control, while the cadmium content of dsCYP2C50 group showed no significant difference compared with control (Fig. 2), which suggested that knockdown of *CYP17A1-like* led to increased level of the accumulated cadmium, indicating the critical role of CYP17A1-like in the process of cadmium detoxification in the oysters. However, no significant difference in cadmium content among the three groups was observed on day 7 of the experiment, which could reflect the gradual return of CYP gene expression levels to normal from day 3 to day 7, which could be due to the injection interval between the first two injections (24 h) and the last two injections (48 h). In addition, although the mortality of oysters injected with PBS was with a high value of 23.8%, the mortality rate of CYP17A1-like group was also significantly higher than PBS group, which also indicated that CYP17A1-like exerted important functions upon cadmium stress in the oysters (Fig. 1). Previous studies have shown that there was no mortality observed in *Crassostrea gigas* treated with the same concentration of cadmium without injection during the experiment (Tian et al., 2021). Therefore, the mortality of PBS group observed in this work could due to the injection process combined with constant cadmium exposure. Notably, dsCYP17A1-like group was also intramuscular injection combined with cadmium stress under the same conditions, and its mortality was significantly higher than PBS group, indicating the significant effects of RNAi on the mortality.

The production of reactive oxygen species (ROS) upon metal stress was one of the main mechanisms causing metal-induced toxicity. When the rate of ROS generation exceeds the inherent antioxidant defense capabilities, metals will cause oxidative stress (Ercal et al., 2001; Valavanidis et al., 2006). Therefore, bivalves have developed a complex antioxidant mechanism to counteract oxidative stress, including antioxidant enzymes such as SOD, CAT, and GST, and non-enzymatic antioxidants GSH. Therefore, effects of RNAi treatments on the representative oxidation indicators were tested in this study.

Among antioxidant enzymes, SOD-CAT is considered as the first line of defense against oxidative stress. SOD is a metal-specific enzyme that first interacts with active oxygen free radicals in the organism. It can remove superoxide anions in the body to produce H_2O_2 and O_2, thereby, it

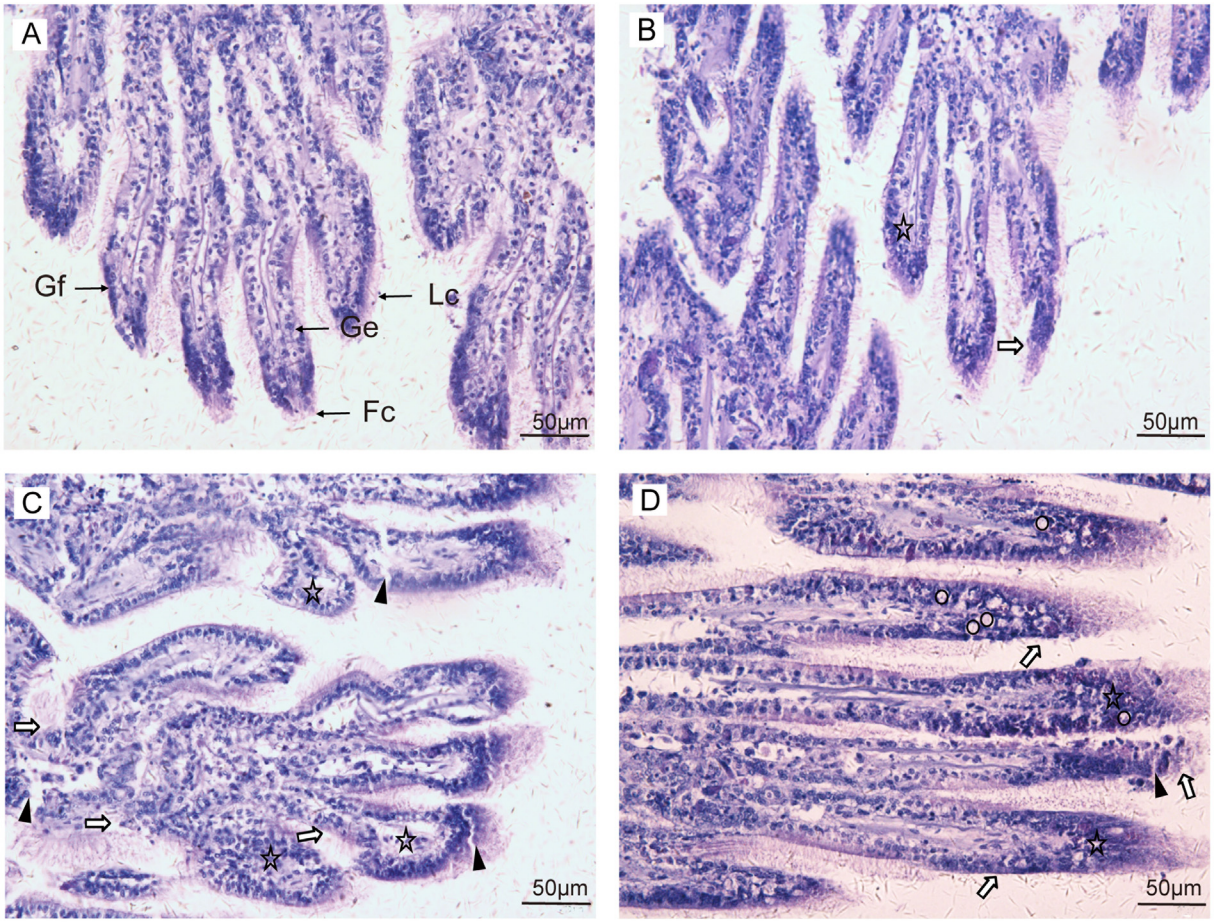

Fig. 5. Histological observation of gill of *C. gigas* exposed to Cd for 7 days after RNAi treatment. Frontal cilia, lateral cilia, gill epithelium and gill filaments are abbreviated as Fc, Lc, Ge and Gf. The arrow represents shed cilia; pentacle represents hypertrophy of gill filaments; triangle represents injured epithelial; circle represents vacuolization. A: the control group without Cd treatment and injection; B: PBS injection group treated with Cd for 7 days; C: CYP17A1-like dsRNA injection group treated with Cd for 7 days; D: CYP2C50 dsRNA injection group treated with Cd for 7 days.

is not only an antioxidant enzyme, but also a source of H_2O_2 (Fridovich, 1974). CAT is a ubiquitous oxidoreductase antioxidant enzyme, which can decompose hydrogen peroxide molecules into water and oxygen molecules to eliminate the toxicity of H_2O_2 (Livingstone, 2001). Therefore, the enzyme activity of SOD was coordinated with the activity of CAT. In this work, the activity of SOD and CAT in dsCYP17A1-like group was significantly higher than the PBS group on day 3 (Fig. 4A, B), which indicated that the oxidative stress in vivo was formed due to the generation of ROS that further activated the antioxidant defense system to eliminate ROS. However, with the prolonged exposure time, the activity of the two enzymes began to decrease gradually, indicating that the oysters were damaged by RNAi and Cd stress. In addition, the higher enzyme activity in the dsCYP17A1-like group than that in the PBS group indicated that the oxidative stress caused by RNAi treatment was greater, as well as the damage to tissues.

As a major phase II detoxification enzyme widely distributed in organisms, GST has the functions of eliminating peroxides and detoxification in organisms (Jozefczak et al., 2012; van der Oost et al., 2003). It can catalyze the coupling of some foreign harmful substances or endogenous electrophilic groups with the sulfhydryl group on GSH, thereby making it easy to be decomposed out of the body. In this study, the GST activity in the dsCYP17A1-like group was increased significantly on day 1, indicating that the interference of *CYP17A1-like* lead to greater oxidative stress and thus induced the antioxidant defense of GST. However, unlike previous studies, the induction of GST activity was earlier than SOD and CAT, which indicated that compensatory adaptation mechanisms may exist among different biochemical biomarkers. In previous studies, it has been reported that when SOD and CAT were depleted, the increase of GST activity may be a compensatory adaptive mechanism to neutralize the increased level of ROS (Fernandez et al., 2010; Regoli and Principato, 1995).

GSH participates in protecting cell membranes from lipid peroxidation by scavenging oxygen free radicals in organisms. Due to its thiol group, GSH also plays a key role in the complexation and detoxification of heavy metals (Bouzahouane et al., 2018; Drira et al., 2017). It is reported that GSH accounts for more than 90% of the total non-protein sulfur in oysters, which may be associated with defense against metals (Wang et al., 2018). The amount of GSH is an important factor to measure the antioxidant capacity of the organism. As can be seen from Fig. 4D, the GSH content in the control group on day 7 was significantly higher than that on day 0, indicating that cadmium stress caused oxidative stress, which was consistent with the previous report that this concentration was sufficient to cause oxidative stress (Pan, 2015). In this study, the content of GSH did not change significantly before day 7 and GSH content decreased significantly on day 7 when compared to PBS group (Fig. 4D), indicating that GSH consumption could occur to reduce the occurrence of lipid peroxidation and that the degree of oxidative stress was greater in the interference group. What's more, the consumption of GSH was associated with the increase of Cd content in the digestive gland on day 7.

MDA is the product of lipid peroxidation reaction, and it is an index to measure the degree of oxidative stress in the body. When heavy metals are excessively enriched in the body, antioxidant enzymes are inhibited, and the excess ROS produced by the body cannot be

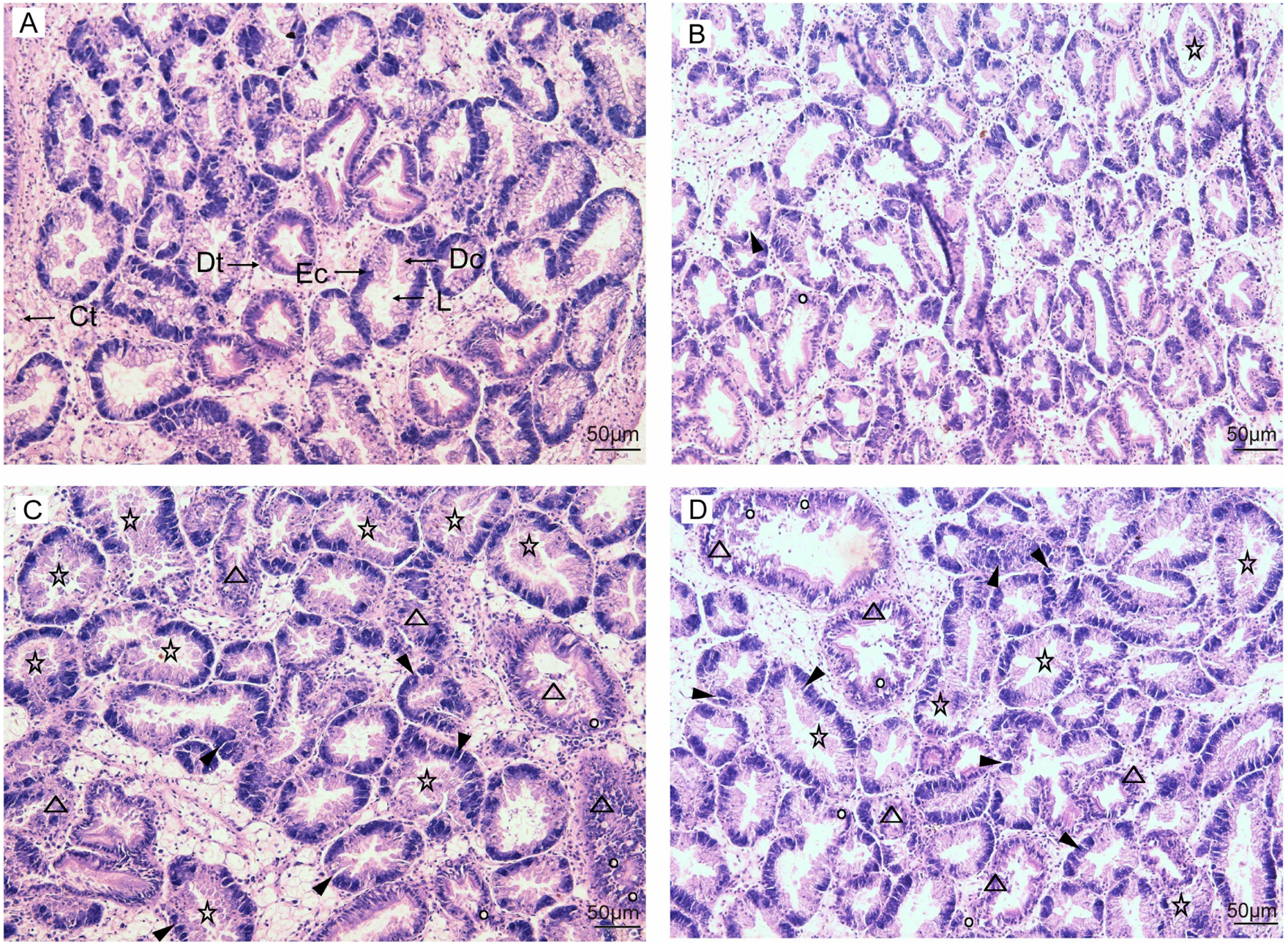

Fig. 6. Histological observation of digestive gland of *C. gigas* exposed to Cd for 7 days after RNAi treatment. Connective tissue, digestive tubules, epithelial cells, digestive cells and lumen are abbreviated as Ct, Dt, Ec, Dc and L. The pentacle represents digestive cell shedding; black triangle represents epithelial injury; circle represents vacuolization; white triangle represents digestive tube disintegrates. A: the control group without Cd treatment and injection; B: PBS injection group treated with Cd for 7 days; C: CYP17A1-like dsRNA injection group treated with Cd for 7 days; D: CYP2C50 dsRNA injection group treated with Cd for 7 days.

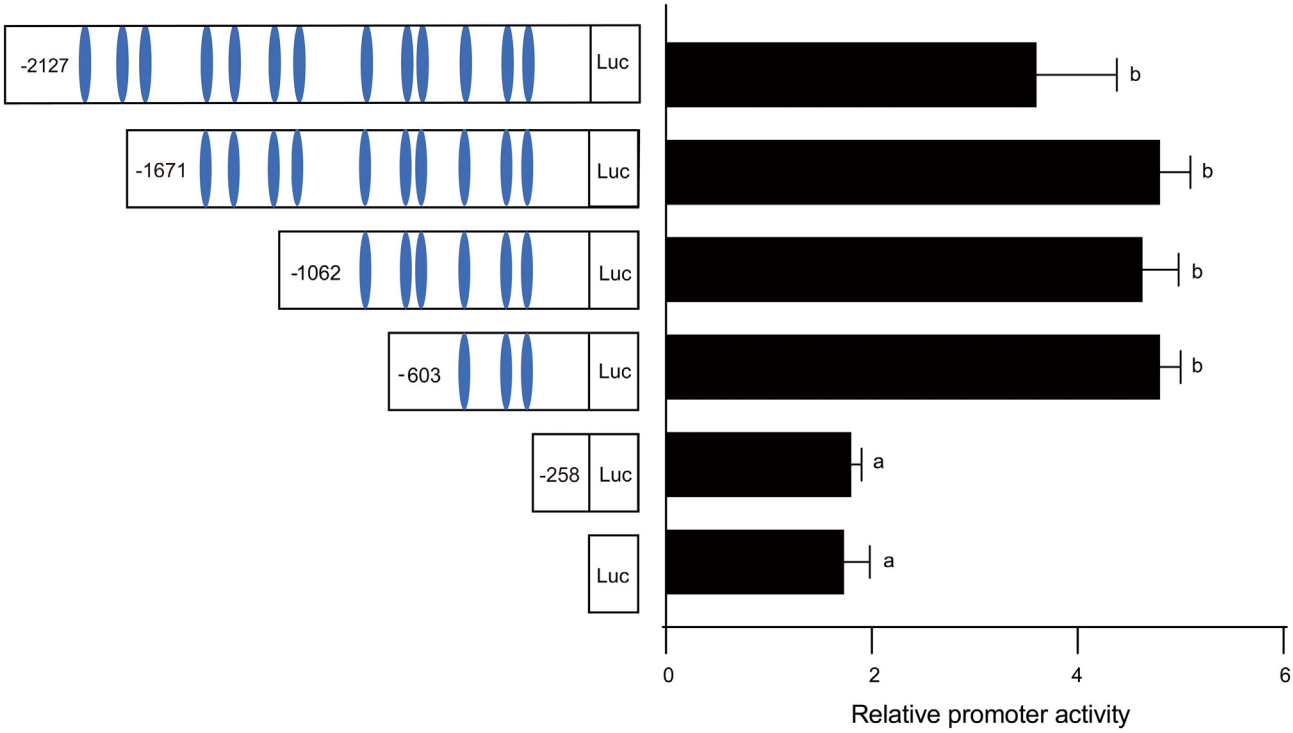

Fig. 7. Analysis of regulatory core element within promoter region of *CYP17A1-like* gene. Left panel denotes schematic representation of the 5′-deletion constructs and the positions of the putative MTF-1 binding sites, and right panel represents the relative promoter activities of *CYP17A1-like*. The different letters on the error bars represent significant differences (mean ± SD; n = 3, ANOVA, $P < 0.05$).

completely eliminated, causing oxidative damage to the body, increasing the degree of lipid peroxidation, and ultimately leading to an increase in the content of lipid peroxidation product MDA. In this work, there was no significant difference in MDA levels among three groups, which was related to the induction of antioxidant enzyme activity. In addition, oxidative damage is not only reflected in altering the MDA content, but also in causing DNA damage and protein metabolism (Wang et al., 2018). Therefore, it does not mean that no oxidative damage was occurred during the experiment. Taken together, we found that interfering the expression of *CYP17A1-like* would lead to the increased level of oxidative stress upon heavy metal Cd stress.

Tissue damage caused by Cd stress was directly observed through histological examination. We chose gill and digestive gland for analyses because they were well-recognized as active sites of metal accumulation, oxy-radical generation, and enzyme biotransformation (Meng et al., 2018). Our results showed that tissue damage of the gill and digestive gland in the interference group was more serious than that in the PBS group, which was roughly reflected in the damage or disintegration of digestive tract cells, enlargement of the digestive tract lumen, swelling of gill filaments, loss of cilia and vacuoles (Fig. 5 and Fig. 6). These damages have been observed in many bivalves such as *Crassostrea gigas*, *Mytilus edulis*, *Patinopecten yessoensis* and *Ruditapes philippinarum* when they were stressed by various pollutants (Romero-Geraldo Rde et al., 2016; Sheir et al., 2010; Sikdokur et al., 2020). Therefore, interference of *CYP17A1-like* aggravated the tissue damage under cadmium stress, suggesting the important role it played in detoxification of cadmium.

As the critical role of *CYP17A1-like* gene expression in the Cd detoxification, we further investigated its transcriptional regulation using dual-luciferase reporter assay. The MTF-1 functions as the cellular metal sensor that coordinates the expression of genes involved in metal efflux and storage, as well as those that protect against metal toxicity and oxidative stress (Gunther et al., 2012). When cells are treated with metals, MTF-1 responds to changes in intracellular zinc and other heavy-metals (e.g., cadmium and copper), which is translocated from the cytosol to the nucleus upon metal-occupancy. Subsequently, MTF-1 binds to metal response elements (MREs) through its zinc finger in the promoter/enhancer regions of the target genes, resulting in an increased rate of transcription (Lichten et al., 2011; Wang et al., 2018). Metal-inducible genes regulated by MTF-1 include the Metallothioneins (MTs), zinc transporter (ZnT1) and glutamate-cysteine ligase heavy chain ($cGCS_{hc}$) (Meng et al., 2015). In present study, the expression level of MTF-1 increased significantly after cadmium stress, indicating that it may be activated by cadmium (Supplementary Fig. 1). we further identified 13 transcription factor binding sites in promoter regions of *CYP17A1-like* gene, and revealed the transcription regulatory relationship between CYP and MTF-1. The dual-fluorescence reporting assay showed that three MTF-1 binding sites located at the −603 site were critical for the transcription of *CYP17A1-like* in *C. gigas* (Fig. 7). Together, we showed that MTF-1 was a strong transcriptional regulator of the *CYP17A1-like* gene, whose expression was highly associated with detoxification process in *C. gigas*. Based on the results of this study, we hypothesized the detoxification process of CYP17A1-like mediated by MTF-1 under cadmium stress (Fig. 8). When cadmium enters the cell, it can bind to the sulfhydryl group of CYP17A1-like to form a compound that can be catalyzed by the phase II detoxification enzyme or water-soluble complexes to be directly exported outside of the cell. Meanwhile, cadmium can activate MTF-1, which is translocated from the cytoplasm to the nucleus, thus binding to the transcriptional binding site of *CYP17A1-like* and regulating its expression level. It was reported that Pb induced fatty acid β-oxidation and CYP450 catalyzed ω-oxidation due to increased metabolic expenditure for detoxification (Meng et al., 2018). Therefore, it is likely that cadmium affects the biosynthesis of steroid-like compound that could cause some endocrine disruption and affect other vital pathways in the oyster metabolism for detoxification. Certainly, it is undeniable that there are other transcription factors that regulate the expression of *CYP17A1-like* and other associated genes that orchestrate to participate in metal detoxification, which deserves future investigations.

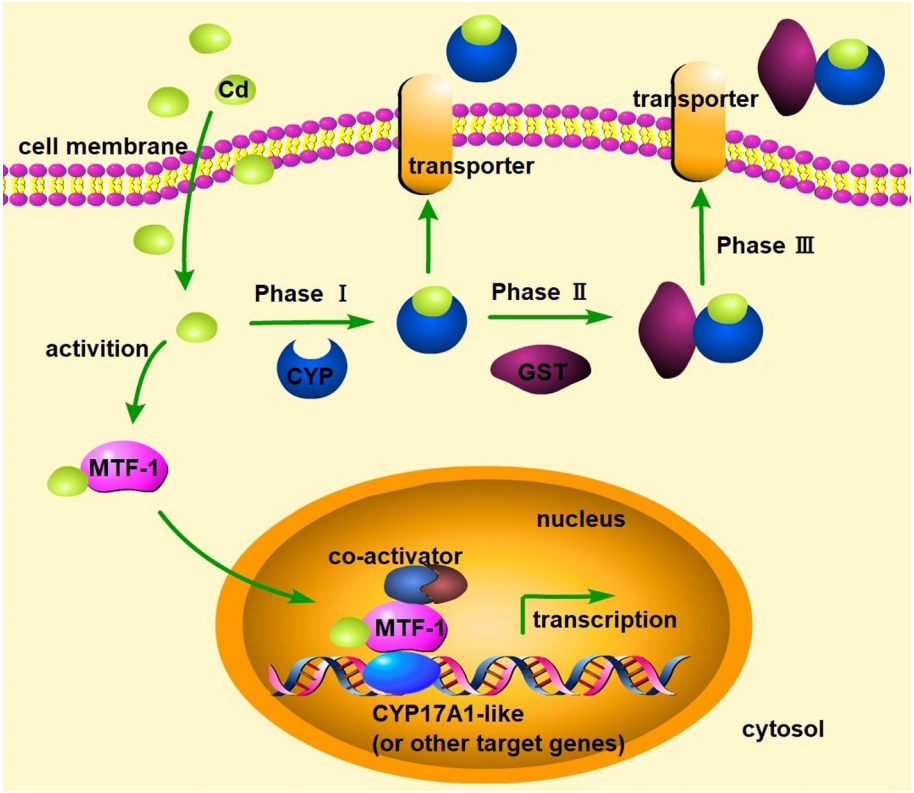

Fig. 8. The proposed detoxification process of CYP17A1-like regulated by MTF-1 under cadmium stress.

5. Conclusions

In this work, we determined the potential involvement of *CYP17A1-like* gene in detoxification of Cd with RNAi experiment by examining the accumulated cadmium content, alteration of antioxidant enzyme activity and histopathological damage to tissues. Furthermore, we identified the MTF-1 binding sites in the promoter region of *CYP17A1-like* as a core regulatory element for its transcriptional regulation. This work demonstrated a critical role of CYP17A1-like in Cd detoxification in *C. gigas* and provided a new perspective toward unravelling detoxification mechanisms of bivalves under heavy metal stress.

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CRediT authorship contribution statement

Jing Tian: Investigation, Data curation, Methodology, Writing – original draft. **Yongjing Li:** Methodology. **Huiru Fu:** Methodology. **Liting Ren:** Methodology. **Yameng He:** Data curation. **Shangyu Zhai:** Data curation. **Ben Yang:** Data curation. **Qi Li:** Supervision, Resources. **Nannan Liu:** Data curation. **Shikai Liu:** Supervision, Project administration, Data curation, Writing – review & editing.

Declaration of competing interest

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

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