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RECEIVED 04 April 2024

ACCEPTED 09 May 2024

PUBLISHED 24 May 2024

## CITATION

Li H, Liang Z, Wu L, Ke Y, Que H and Shi B (2024) The role of *Zip1* and *Zip3* in cadmium accumulation in Fujian oyster (*Crassostrea angulata*). *Front. Mar. Sci.* 11:1412127. doi: 10.3389/fmars.2024.1412127

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# The role of *Zip1* and *Zip3* in cadmium accumulation in Fujian oyster (*Crassostrea angulata*)

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Oysters are notably susceptible to accumulating substantial amounts of cadmium (Cd), leading to food safety concerns, yet the molecular mechanisms are not fully understood. Previous study identified a significant correlation between polymorphisms in the *Zip1* and *Zip3* of Fujian oyster (*Crassostrea angulata*) and the levels of Cd accumulation. *Zip1* and *Zip3*, belonging to the Zip family, are primarily responsible for the cellular transport of zinc and other metal ions. To delve deeper into their roles in Cd transport within oysters, this research cloned and analyzed the sequences of Fujian oyster *Zip1* and *Zip3*. The open reading frames for *Zip1* and *Zip3* were 960 and 978 bp, respectively, encoding proteins of 319 and 325 amino acids. Following Cd exposure, the expression levels of these genes in the mantle, gills, and other tissues initially increased significantly before decreasing, demonstrating a dose-dependent response. The lowest expression levels were observed in the adductor muscle, with *Zip1* reaching its highest expression in the mantle and *Zip3* in the visceral mass. *In vitro* RNAi experiments revealed that silencing *Zip1* led to a 1.26-fold decrease in new <sup>113</sup>Cd accumulation in the mantle, whereas silencing *Zip3* resulted in a 1.29-fold increase. Immunohistochemistry showed weak expression of *Zip1* and *Zip3* proteins in the connective tissue of the mantle. In summary, *Zip1* and *Zip3* in Fujian oyster can transport Cd as a substrate, and played different roles in the absorption and efflux of Cd.

## KEYWORDS

Fujian oyster, cadmium accumulation, *Zip1*, *Zip3*, expression analysis, RNAi, immunohistochemistry

## 1 Introduction

Oyster represents a significant aquaculture specie cultivated widely across the globe. According to 2022 FAO statistics, oyster cultivation reached a production of 6.06 million tons, accounting for 34.2% of the global mollusk farming volume (FAO, 2022). In recent years, rapid urbanization and industrialization in coastal areas have led to metal pollution,

posing substantial challenges to oyster farming. Oysters possess a remarkable capacity to accumulate metals, particularly cadmium (Cd), zinc (Zn), and copper (Cu) (Ke and Wang, 2001; Pan and Wang, 2009). The bioaccumulation factor of Cd can exceed 48,000 (Wu et al., 2013). After consumption, humans can absorb 78.3–87.8% of Cd from oysters, with the frequency and amount of consumption significantly correlating with Cd levels in human blood (Copes et al., 2008; He and Wang, 2013). Cd, a non-essential element for humans, can accumulate in the body through various pathways. Excessive intake may lead to acute poisoning and irreversible damage to the kidneys, liver, bones, and reproductive systems (Kumar and Sharma, 2019; Wang et al., 2021). Consequently, countries have established safety limits for Cd content in oysters (Satapathy et al., 2019). However, excessive safety limits of Cd are not uncommon. An investigation in India found an average Cd concentration of 10.6 µg/g dry weight in oyster soft tissues, 19 times higher than that of the surrounding water (Patra et al., 2021). In some areas of the South China Sea, the Cd concentration in oyster tissues ranged from 5.87 to 17.62 µg/g dry weight (Luo et al., 2018). The health risks posed by consuming oysters with excessive Cd content significantly limit the sustainable development of the oyster industry. Several scholars have made considerable progress in reducing metal content through genetic improvements by elucidating the molecular mechanisms of metal hyperaccumulation plant (Delhaize et al., 2004; Sasaki et al., 2004; Tang et al., 2017). Referring to these studies, exploring the molecular mechanisms controlling Cd accumulation in oysters and attenuating this capability through genetic improvements is of paramount importance.

Oysters, as typical Cd-accumulating organisms, have been studied for Cd accumulation kinetics and detoxification methods through isotope tracing and gel chromatography (Engel, 1999; Boisson et al., 2003; Mark et al., 2005). Most current researches focus on toxicodynamics, the impact of Cd exposure on gene or protein expression, and the estimation of the heritability of Cd accumulation. Toxicodynamic studies have partially explained the phenomenon of oyster Cd accumulation: oysters absorb Cd at a rate higher than other bivalves but excrete it more slowly (Ke and Wang, 2001; Pan and Wang, 2012). Although some scholars have investigated the molecular mechanisms behind oyster Cd accumulation, such as the relationship between Cd exposure and oyster gene polymorphisms (Meng et al., 2021), the specific roles of particular genes in Cd accumulation remain unknown. Thus, further exploration of the molecular genetic mechanisms is necessary.

Previous genome-wide association analyses in Fujian oyster identified a strong linkage between Cd accumulation traits and gene polymorphism on chromosome 3, where *Zip1* and *Zip3* were located (Wu et al., 2023). Prior research indicates *Zip1* is ubiquitously expressed in human tissues, serving as the primary Zn uptake transporter in K562 leukemia cells and prostate cells, transporting Zn from outside to inside the cytoplasm (Franklin et al., 2003; Zhao and Eide, 1996; Gaither and Eide, 2001). Besides Zn, *Zip1* has also been found to transport other metal ions, such as Cd, manganese, and iron, in yeast and Arabidopsis (Gitan et al., 1998; Korshunova et al., 1999; Connolly et al., 2002). However, in

oysters and scallops, only the expression correlation between *Zip1* and Cd exposure duration has been confirmed (Engel, 1999; Zhao et al., 2020). *Zip3* is related to Zn uptake in mammalian cells and is widely expressed in many tissue types (Kelleher and Lönnnerdal, 2005; Eide, 2006). Studies suggest that in cabbage varieties with high Cd levels, *Zip3* expression is higher than in those with low levels, hypothesizing that it may transport Cd from the roots to the shoots (Yu et al., 2017). Therefore, further exploration of *Zip1* and *Zip3*'s functions in Cd transport of oyster is warranted.

Fujian oyster is the most cultivated oyster specie in southern China. This study selected Fujian oyster as the research subject, cloning cDNA encoding *Zip1* and *Zip3* and analyzing their expression levels to explore their correlation with Cd accumulation. We then identified the Cd accumulation levels after *Zip1* and *Zip3* RNA interference (RNAi). Additionally, we attempted to explore the tissue and cellular localization of *Zip1* and *Zip3* in response to Cd stress. The results of this study provide a basis for researching the differential molecular mechanisms of oyster Cd accumulation and for breeding oyster strains with low Cd content.

## 2 Materials and methods

### 2.1 Oysters and Cd exposure

The oysters (*C. angulata*) used in the experiment were one-year-old, with an average weight of  $74 \pm 16$  g, and were sourced from an oyster farm near Xiaocheng Town, Lianjiang City, Fujian Province. Approximately 400 similarly sized and well-formed oysters were collected, brought back to the laboratory, cleaned, removed from their attachment, and domesticated for one week. The oysters were cultured in twelve 30 L polyethylene containers, with daily complete water changes. They were fed commercial *Chlorella* spp. powder at a rate of approximately 2% of their soft tissue dry weight per day.

After a 7-day acclimation, oysters were randomly divided into three groups. Each group was subjected to different exposure conditions: 0ppb, 2ppb, and 5ppb Cd<sup>2+</sup>. Stock solutions of 10 ppm Cd (CdCl<sub>2</sub>; MW:183.32 g/mol; Sigma-Aldrich) were prepared with Milli-Q-filtered water. After exposure, eight individuals per group were randomly sampled at 0 d, 1 d, 3 d, 7 d, and 15 d. Their soft tissues were rinsed several times with distilled water before dissecting for mantle, gills, adductor muscle, and visceral mass. Samples were partly designated for Cd concentration determination and partly in liquid nitrogen and stored at -80°C for gene expression pattern analysis.

### 2.2 *Zip1* and *Zip3* ORF clone

Total RNA from the oyster mantle was extracted using TRIzol<sup>®</sup> Reagent (Invitrogen, USA) (Ni et al., 2013). First-strand cDNA was synthesized using M-MuLV reverse transcriptase (Thermo Scientific, USA). *Zip1* and *Zip3* were amplified by PCR using primers Zip1-F and Zip1-R listed in Table 1, based on mRNA sequences for encoding

*Zip1* and *Zip3* selected from our lab's mantle transcriptome database. The PCR protocol was as follows: 1 cycle of predenaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, *Zip1* is 55.3°C for 30 s; *Zip3* is 52.5°C for 30 s, and 72°C for 1 min, followed by a 10 min extension at 72°C. All primer sequences are provided in Table 1. Nucleotide and protein sequences were characterized with DNAMAN software. Multiple sequence alignment was performed using Clustal W (University College Dublin, Dublin, Ireland) and MEGA 7.0's neighbor-joining (NJ) method, generating a phylogenetic tree.

## 2.3 Gene expression pattern analysis

### 2.3.1 Quantitative real-time PCR analysis

The expression levels of *Zip1* and *Zip3* in different tissues under Cd stress were detected using qRT-PCR. RNA extraction from Cd-exposed samples followed the method described in Section 2.2. Each 20 µL PCR reaction contained 10 µL 2×ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 4 µL of fivefold diluted cDNA, and 10 µM of gene-specific primers (Table 1). The amplification conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, with a final extension at 95°C for 15 s and 60°C for 60 s, ending with 95°C for 15 s. *EF1α* from Fujian oyster served as the reference gene. Each sample was analyzed in six biological replicates, with relative expression levels of *Zip1* and *Zip3* calculated according to the  $2^{-\Delta\Delta CT}$  method (Rao et al., 2013).

### 2.3.2 Cd content determination in oyster tissues

Cd content in oyster tissues was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS-7700, Agilent) (Luo et al., 2014; Weng and Wang, 2014). Oyster samples were dried at 80°C for 24 hours until constant weight, digested with nitric acid at room temperature for 12 hours, followed by hot digestion at 80°C for 12 hours until complete dissolution. After appropriate dilution,  $^{114}\text{Cd}$  concentration in oyster samples was measured by ICP-MS. A multi-element standard solution (Agilent) was used for ICP-MS calibration, with  $^{103}\text{Rh}$  as an internal standard to correct for instrumental drift and sensitivity changes. Quality control involved re-measuring a previously tested sample every twenty samples.

## 2.4 Immunohistochemistry assays

The localization of *Zip1* and *Zip3* within Fujian oyster tissues was determined via immunohistochemistry (Kingtong et al., 2007). Oysters subjected to 5ppb Cd stress were dissected on the third day of the experiment to collect mantle tissue, which was then fixed in 4% paraformaldehyde for 24 hours. Subsequently, the tissues underwent hydration in graded ethanol and dewaxing in xylene. The samples were incubated overnight at 4°C with polyclonal antibodies against *Zip1* and *Zip3* (1:20) (GL Biochem, China). The control group used pre-immune serum at the same

TABLE 1 Primers used in this study.

Primers	Sequence (5'-3')	Application
Zip1-F	ATGGTCGTGACCTCCGTG	cDNA cloning
Zip1-R	TCATGGCGTCGGCGG	
Zip1-qF	AGCAAAATCCAAGCGTCCTC	qRT-PCR
Zip1-qR	TCCGCTCCTTTCTGCTGTTA	
dsZip1-F	TAATACGACTCACTATAGGGAGGCCCTTATACCGTTTGCCT	RNAi
dsZip1-R	TAATACGACTCACTATAGGGTTGCCCTTTTATCCGTGTC	
Zip3-F	ATGAAATCCACCGCCAAAATTATT	cDNA cloning
Zip3-R	CTATTCACTGTGTCATGTA AAAA ACTCCACC	
Zip3-qF	CCAAGGACGCAATGACAGAC	qRT-PCR
Zip3-qR	GCACACAGAGTCGGGAGTAT	
dsZip3-F	TAATACGACTCACTATAGGGGAAATCCACC GCCAAAATTA	RNAi
dsZip3-R	TAATACGACTCACTATAGGGTTTCACGTTCCACCATTGTA	
M13-47	AGGGTTTTCCAGTCACG	cDNA cloning
M13-48	GAGCGGATAACAATTTACAC	
EF1α-F	ACCACCCTGGTGAGATCAAG	qRT-PCR
EF1α-R	ACGACGATCGCATTCTCTT	
pEGFP-N1-F	GCGACGTAACGGCCACAA	plasmid construction
pEGFP-N1-R	CGAACTCCAGCAGGACCATG	

concentration, followed by three 5-minute washes in 0.01M phosphate-buffered saline (PBS). Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (GL Biochem, China) was used as the secondary antibody (1:50) and incubated at room temperature for 1 hour before developing with DAB. A separate set of identical samples was stained with HE (Liu, 2022) and observed under a light microscope for comparison of tissue structures with those subjected to immunohistochemistry.

## 2.5 RNA interference

### 2.5.1 dsRNA synthesis

PCR was employed to amplify cDNA fragments of *Zip1* and *Zip3*, along with a GFP cDNA (612 bp) fragment from the pEGFP-N1 plasmid for *in vitro* transcription and dsRNA synthesis. Primers designed using siDirect 2.1 (University of Tokyo, Japan) included a T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') at the 5' end for dsRNA synthesis. Linear templates were amplified using these primers, followed by DNA precipitation with ethanol. T7 RNA Polymerase (Thermo Scientific, USA) was used for *in vitro* transcription to synthesize dsRNA, which was then stored at -80°C after electrophoresis verification and concentration measurement.

### 2.5.2 *Ex vivo* tissue culture and dsRNA interference

Following dsRNA preparation, tissue culture and RNAi were conducted according to the methods of *in vitro* culture of the mud crab (*Scylla paramamosain*) tissue and the clam (*Meretrix meretrix*) primary cells with slight modifications (You et al., 2012; Liu, 2022). Furthermore, based on qPCR results and existing studies on metal accumulation, the mantle is identified as the primary tissue for metal accumulation and target gene expression (Shi et al., 2019a). Hence, we have selected the mantle as the tissue material for our *in vitro* RNA interference experiments. Mantle tissue was excised under sterile conditions, washed thrice in sterile seawater containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (filtered through 0.22 µm), with each wash lasting 20, 60, and 100 minutes, respectively. Tissues were then cut into 1 mm<sup>3</sup> pieces and pre-cultured in modified sterile L15 medium (Gibco, USA) supplemented with 1 g/L glucose, 2.4 g/L HEPES, 12 g/L NaCl, 1.2 g/L NaHCO<sub>3</sub>, and 5 g/L yeast extract, along with the aforementioned antibiotics, in a 48-well cell culture plate (LABSELECT, Beijing) at 26°C for 60 minutes. After confirming no significant change in medium color, the medium was replaced.

The tissues were divided into three groups: *Zip1* interference, *Zip3* interference, and *Zip1+Zip3* interference, with each group including an EGFP dsRNA as a control. L15 medium containing the respective dsRNA (final concentration 1 µg/µL) was gently mixed into the culture plates. After 24 hours of RNAi, samples from the experimental and control groups were collected for Cd content determination. Additional corresponding dsRNA was then added to the remaining tissues to maintain RNAi efficiency, along with <sup>113</sup>Cd to a final concentration of 5 µg/L. After a further 24 hours of RNAi and <sup>113</sup>Cd exposure, mantle tissues were collected for qRT-PCR

analysis and <sup>113</sup>Cd content determination. Each group had three replicates.

### 2.5.3 <sup>113</sup>Cd concentration measurement

To assess the changes in <sup>113</sup>Cd accumulation in the mantle tissues following *Zip1* and *Zip3* interference, the concentrations of <sup>113</sup>Cd and <sup>111</sup>Cd were measured using the method described in section 2.3.2, and calculated following the approach by Zhong et al. (2020). The newly accumulated Cd in oyster tissues was calculated using the formula:

$$\text{new}[^{113}\text{Cd}] = ([\text{total Cd}]_{113} - [\text{total Cd}]_{111}) \times 12.22 \%$$

Here, [total Cd]<sub>111</sub> and [total Cd]<sub>113</sub> are the total Cd concentrations measured by ICP-MS when selecting <sup>111</sup>Cd and <sup>113</sup>Cd for analysis, respectively. 12.22% represents the natural abundance of <sup>113</sup>Cd. Thus, this formula allows for the calculation of newly accumulated Cd in oyster tissues.

## 2.6 Statistical analysis

All data are presented as mean ± SE (n=8 or 3). Statistical significance was determined through two-way ANOVA and paired sample T-tests. Graphs were created using GraphPad Prism 9.0 (San Diego, USA), with statistical significance set at *p* < 0.05.

## 3 Results

### 3.1 Sequence and phylogenetic analysis of *Zip1* and *Zip3*

The ORF of Fujian oyster *Zip1* was 960 bp (GenBank accession number: OR759776), encoding a protein of 319 amino acids with a predicted molecular weight of 35.7 kDa and an isoelectric point (pI) of 5.78. The predicted *Zip1* protein lacks a signal peptide but contains eight transmembrane helices located at amino acids 10–31, 43–65, 80–102, 160–182, 192–214, 223–245, 260–279, and 292–310 (Figure 1A). The ORF of *Zip3* was 978 bp long (GenBank accession number: OR759777), predicted to encode 325 amino acids, with a molecular weight of 35.9 kDa and a pI of 6.27, featuring eight α-helical transmembrane domains located at amino acids 10–31, 44–66, 81–103, 176–198, 203–225, 237–259, 269–289, and 302–321 (Figure 1B).

Phylogenetic trees constructed by comparing the amino acid sequences of *Zip1* and *Zip3* from a variety of published organisms revealed that Fujian oyster, like other animals, plants, and fungi, have eight transmembrane helices. All possess an extended sequence of 12 amino acids (underlined in blue) within the fourth transmembrane domain, characteristic of the Zip family, containing conserved histidine, serine, and glycine residues (highlighted in yellow). Notably, the serine has been replaced by methionine in mollusks, differentiating them from mammals. Additionally, *Zip3* features a conserved amino acid difference (marked in red) compared to *Zip1*, whose function and significance remain unclear. The evolutionary tree indicates that the Fujian oyster is

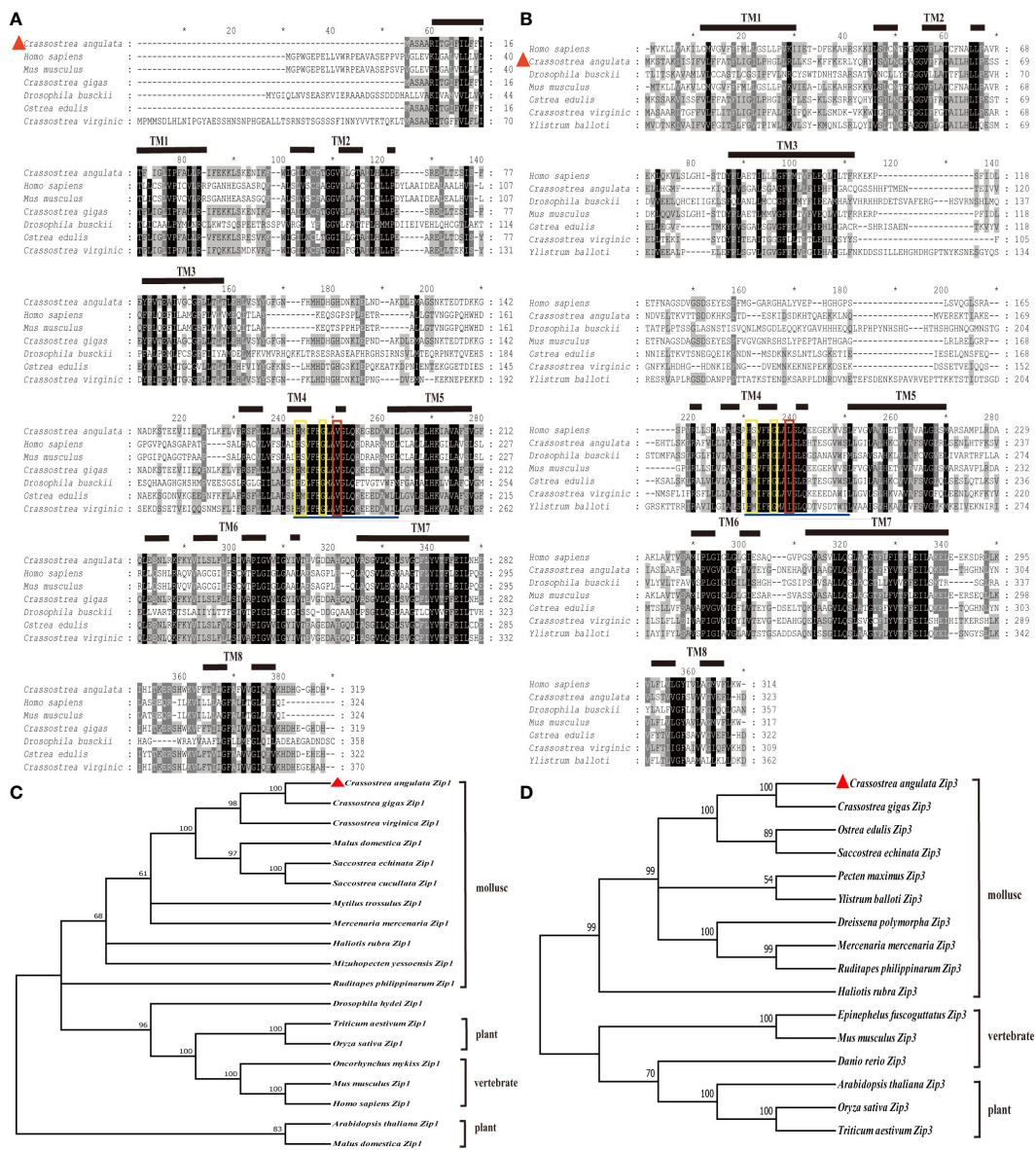


FIGURE 1

Illustrates the multiple sequence alignment and phylogenetic tree analysis of Zip1 and Zip3 with amino acid sequences from other species. Similar amino acids are highlighted, and the eight presumed transmembrane domains (TM) are denoted by solid and dashed lines. The fourth presumed transmembrane domain includes conserved histidine, serine or methionine, and glycine residues (highlighted in yellow boxes). (A) Multiple sequence alignment of Zip1 with amino acid sequences from various species. (B) Multiple sequence alignment of Zip3 with amino acid sequences from different species. (C) Phylogenetic tree analysis of Zip1. (D) Phylogenetic tree analysis of Zip3. Zip1 and Zip3 proteins were submitted to the GenBank database, with other GenBank accession numbers as follows: *Homo sapiens* (Zip1, NP\_001258888.1), *Mus musculus* (Zip1, NP\_038929.2), *Crassostrea gigas* (Zip1, XP\_011453296.2), *Drosophila busckii* (Zip1, ALC42177.1), *Crassostrea virginica* (Zip1, XP\_022330862.1), *Ostrea edulis* (Zip1, XP\_044873634.1), *Crassostrea gigas* (Zip1, XM\_011454994.3), *Crassostrea virginica* (Zip1, XM\_022475156.1), *Malus domestica* (Zip1, NM\_001294116.1), *Saccostrea echinata* (Zip1, XM\_061318195.1), *Saccostrea cucullata* (Zip1, XM\_062749567.1), *Mytilus trossulus* (Zip1, XM\_063579755.1), *Mercenaria mercenaria* (Zip1, XM\_045356306.2), *Haliotis rubra* (Zip1, XM\_046712745.1), *Mizuhopecten yessoensis* (Zip1, XM\_021487195.1), *Ruditapes philippinarum* (Zip1, XM\_060749981.1), *Drosophila hydei* (Zip1, XM\_030225087.1), *Triticum aestivum* (Zip1, XM\_044496491.1), *Oryza sativa* (Zip1, NM\_001397949.1), *Oncorhynchus mykiss* (Zip1, NM\_001124481.1), *Mus musculus* (Zip1, NM\_001411505.1), *Homo sapiens* (Zip1, NM\_001271957.2), *Arabidopsis thaliana* (Zip1, NM\_112111.3), *Malus domestica* (Zip1, NM\_001294116.1), *Homo sapiens* (Zip3, NP\_653165.2), *Drosophila busckii* (Zip3, ALC45898.1), *Mus musculus* (Zip3, NP\_001345827.2), *Ostrea edulis* (Zip3, XP\_048735000.2), *Crassostrea virginica* (Zip3, XP\_022330863.1), *Ylistrum balloti* (Zip3, XP\_060079049.1), *Crassostrea gigas* (Zip3, XM\_034467256.1), *Ostrea edulis* (Zip3, XM\_044879043.2), *Saccostrea echinata* (Zip3, XM\_061316256.1), *Pecten maximus* (Zip3, XM\_061316256.1), *Ylistrum balloti* (Zip3, XM\_060223066.1), *Dreissena polymorpha* (Zip3, XM\_052384496.1), *Mercenaria mercenaria* (Zip3, XM\_053532146.1), *Ruditapes philippinarum* (Zip3, XM\_060744456.1), *Haliotis rubra* (Zip3, XM\_046728897.1), *Epinephelus fuscoguttatus* (Zip3, XM\_049588280.1), *Mus musculus* (Zip3, NM\_001358898.2), *Danio rerio* (Zip3, NM\_001080619.1), *Arabidopsis thaliana* (Zip3, NM\_128786.4), *Oryza sativa* (Zip3, NM\_001419857.1), *Triticum aestivum* (Zip3, XM\_044469838.1). Multiple alignments were performed using Clustal W with default parameters. Identical and similar residues are displayed against black and grey backgrounds, respectively.  $\alpha$ -helices are represented by horizontal lines and dashed lines in the figures and red triangle symbol indicate the location of Zip1 and Zip3.

separately clustered with other bivalves, showing the highest similarity with the Pacific oyster (Figures 1C, D).

## 3.2 Gene expression pattern

### 3.2.1 Cd accumulation in oyster tissues

Cd content of Fujian oyster tissues (gills, mantle, adductor muscle, and visceral mass) was detected using ICP-MS before and after Cd stress (Figure 2). Before exposure, the average Cd content was in the order: visceral mass > gills > mantle > adductor muscle; after exposure, the average Cd content followed: gills > mantle > visceral mass > adductor muscle. As the exposure time and Cd dose increased, the accumulation of Cd in all tissues also increased, reaching the highest on the 15th day after exposure. In the gills of the oysters exposed to 5ppb, the Cd content on the 15th day was 138 times that of the control group.

### 3.2.2 Expression spectrum of *Zip1* and *Zip3* after Cd exposure

To analyze the expression patterns of *Zip1* and *Zip3* in oysters during Cd exposure, oysters were exposed to different concentrations of Cd. As shown in Figures 3A–D and 4A–D, at the same Cd concentration, the expression levels of *Zip1* and *Zip3* in all tissues generally first increased and then decreased over exposure time. Before Cd exposure, the highest expression of *Zip1* was in the gills, with the lowest in the adductor muscle. *Zip3* was most highly

expressed in the visceral mass and least in the gills. After Cd exposure, the highest expression of *Zip1* was in the mantle, while *Zip3* was most highly expressed in the visceral mass, with both showing the lowest expression in the adductor muscle. Within the same exposure time, the expression levels of *Zip1* and *Zip3* overall showed dose-dependency, increasing with the concentration of Cd exposure. In the 5ppb group, the expression of *Zip1* reached its peak on the 3rd day in the adductor muscle and visceral mass, while in the mantle and gill, it peaked on the 7th and 1st days, respectively, before gradually decreasing to control levels. In the gills, the expression of *Zip3* also peaked on the 3rd day, while in the mantle, it was highest on the 1st day, and in other tissues, it peaked on the 7th day.

## 3.3 Histological structure and tissue-cell localization of *Zip1* and *Zip3*

Further investigation into the localization of *Zip1* and *Zip3* within Fujian oyster tissues was conducted through immunohistochemistry. Location in the connective tissue was confirmed through comparison with a hematoxylin and eosin–stained mantle tissue section, which provided a more refined image of the target mantle structures (Figures 5A, B). No signal was detected in mantle tissue of control sections treated with preimmune sera (Figures 5C, D). *Zip1* (Figures 5E, F) and *Zip3* (Figures 5G, H) showed weak expression in the connective tissue of the mantle.

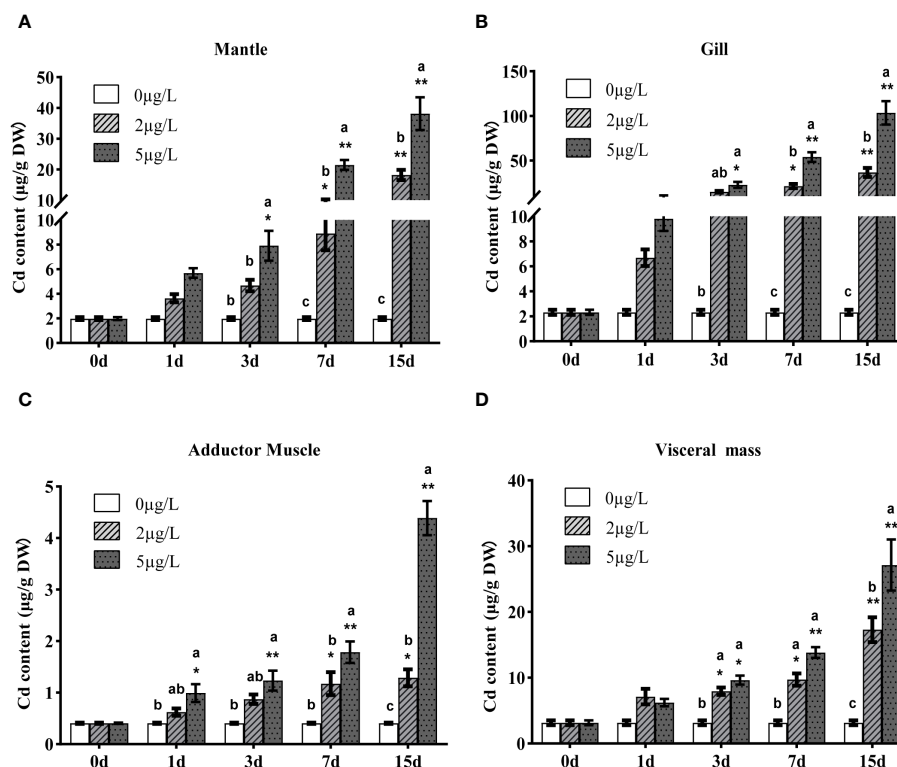


FIGURE 2

Accumulations of  $^{114}\text{Cd}$  in mantle (A), gills (B), adductor muscle (C) and visceral mass (D) of Fujian oyster after Cd exposure. Data are presented as mean  $\pm$  SE ( $n=8$ ). Different lowercase letters indicate significant differences between different Cd concentrations at the same exposure time ( $p<0.05$ ), while \* indicates significant differences between different exposure times at the same Cd level. (\* $p<0.05$ , \*\* $p<0.01$ ).

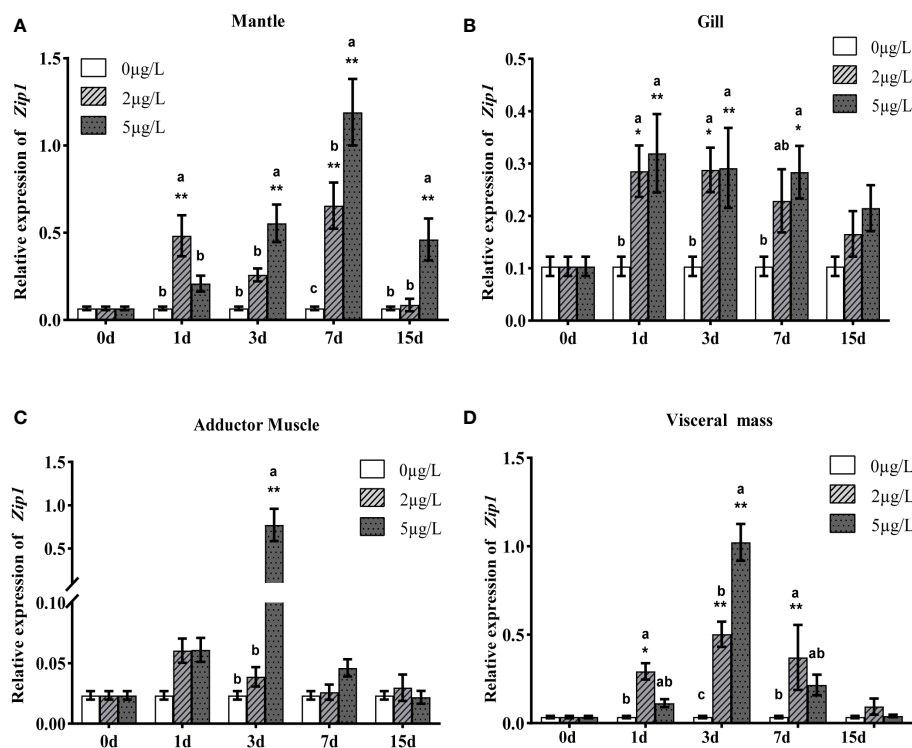


FIGURE 3

Gene expression analysis of *Zip1* in mantle (A), gills (B), adductor muscle (C) and visceral mass (D) of Fujian oyster. qRT-PCR data are represented as mean  $\pm$  SE (n=8). EF1 $\alpha$  was used as an internal reference to normalize *Zip1* expression levels. Different lowercase letters indicate significant differences between different Cd concentrations at the same exposure time ( $p < 0.05$ ), while \* indicates significant differences between different exposure times at the same Cd level. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

### 3.4 The impact of dsRNA-induced silencing of *Zip1* and *Zip3* on Cd accumulation

#### 3.4.1 mRNA expression levels after silencing *Zip1* and *Zip3*

We conducted individual as well as combined interference experiments on *Zip1* and *Zip3*. After 48 hours of interference, the expression levels of both *Zip1* and *Zip3* significantly decreased, as shown in Figure 6. Compared to the control group, silencing *Zip1* and *Zip3* for 48 hours resulted in a reduction of expression by 84% and 52%, respectively. Simultaneous silencing of *Zip1+Zip3* for 48 hours led to a reduction in their expression by 64% and 62%, respectively, demonstrating that the addition of dsRNA *in vitro* significantly reduces the expression levels of *Zip1* and *Zip3* in mantle cells.

#### 3.4.2 Cd content in mantle tissue after dsRNA-induced silencing of *Zip1* and *Zip3*

The data in Figure 7 revealed that, although Cd content significantly increased in both dsGFP and ds*Zip1* groups after silencing *Zip1* for 48 hours, but the Cd content in the dsGFP group was 1.26 times that of the ds*Zip1* group. Thus, *Zip1* silencing significantly reduced the accumulation of Cd in the mantle. After silencing *Zip3*, the Cd content in the ds*Zip3* group was 1.29 times that of the dsGFP group. The Cd content in the group with both

*Zip1* and *Zip3* silenced was 1.15 times that of the control group, slightly lower than the individual interference group.

## 4 Discussion

Our preliminary genome-wide association analysis of Cd accumulation traits in Fujian oyster revealed significant correlations between Cd accumulation and the polymorphisms of *Zip1* and *Zip3*, suggesting their potential involvement in the regulation of Cd accumulation in oysters. To date, research on *Zip1* and *Zip3* in aquatic animals has primarily focused on their role in Zn transport regulation in fish (Chen et al., 2020, 2020; Puar et al., 2020), crabs (Liu et al., 2023), and bivalves (Zhao et al., 2020; Meng et al., 2021). However, in bivalves, only the expression changes of *Zip1* and *Zip3* following Cd stress have been observed, with their involvement in the cellular transport of Cd in oysters remaining unclear. To further elucidate the function of *Zip1* and *Zip3* in bivalves and uncover their role in Cd transport, we cloned the cDNA of *Zip1* and *Zip3* from Fujian oyster. Multiple sequence alignments and phylogenetic analysis show that *Zip1* and *Zip3* are highly conserved and closely related to orthologues in other bivalve and gastropod species. Like other members of the Zip family, they contain eight  $\alpha$ -helical transmembrane domains and similar topologies, demonstrating strong conservation across animals, plants, and fungi (Eide, 2006).

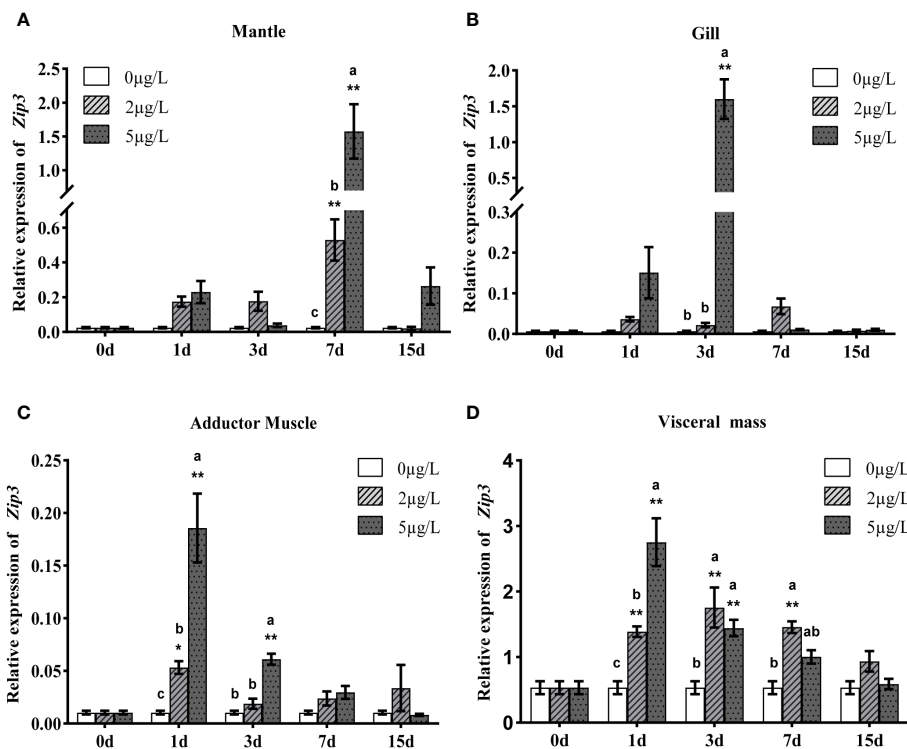


FIGURE 4

Analysis of *Zip3* gene expression in mantle (A), gills (B), adductor muscle (C) and visceral mass (D) of Fujian oyster, following the same presentation and statistical notation as *Zip1*. Different lowercase letters indicate significant differences between different Cd concentrations at the same exposure time ( $p < 0.05$ ), while \* indicates significant differences between different exposure times at the same Cd level. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

Between their TM3 and TM4 domains lies a His-rich loop region associated with metal-binding capacity. Studies have shown that, besides Zn, this region can also bind Cd and cobalt (Eide, 2006), indicating the potential of Fujian oyster *Zip1* and *Zip3* to bind Cd. Moreover, a conserved amino acid difference in TM4 between *Zip3* and *Zip1* suggests functional divergence.

In this study, as Cd exposure concentration and duration increased, oyster tissues exhibited similar Cd accumulation patterns, showing dose and time-dependent increases, peaking on the 15th day. Another study also reported significant increases in oyster Cd content after continuous exposure to various Cd concentrations for 96 hours (Moncaleano-Nino et al., 2017). Additionally, before Cd exposure, the highest Cd accumulation was in the visceral mass, followed by gills and least in the adductor muscle. After exposure, the highest accumulation shifted to the gills, then the mantle, with the visceral mass third. Previous research has shown that oyster tissues acquire Cd from both water and food, with increased waterborne Cd concentrations leading to rapid internal accumulation through the mantle and gills (Cao et al., 2018). The expression patterns under Cd stress indicated that *Zip1* and *Zip3* in the mantle, gills, adductor muscle, and visceral mass are rapidly upregulated by Cd stress, significantly increasing before gradually returning to control levels over time, showing dose and time-dependent changes. Similar Cd-induced expression of *Zip1* and *Zip3* has been observed in Pacific oyster, suggesting their potential role in regulating oyster Cd accumulation (Meng et al., 2021). Existing studies confirm that *Zip1* and *Zip3* expression in different

mouse cells is associated with Zn homeostasis and tightly regulated by Zn (Bogdanovic et al., 2022). In humans, high Zn levels activate the transcription factor RREB1, which downregulates *Zip1* (Zou et al., 2011) and upregulates *Zip3* (Franklin et al., 2014) to regulate cellular Zn homeostasis. Thus, Cd stress may activate transcription factors for oyster *Zip1* and *Zip3*, causing gene expression changes. Additionally, competition for binding sites between Zn and Cd in oyster cells may occur (Kandhol et al., 2023; Liu et al., 2023). During exposure, Cd may compete with Zn for ion channels, leading to changes in the homeostasis of Zn and thereby enhancing the expression of Zn transporters (Fontanili et al., 2016). Moreover, the significant expression of *Zip1* and *Zip3* in the mantle and gills, the primary Cd accumulation tissues, compared to lower accumulating tissues like the adductor muscle, and higher expression in individuals with greater accumulation (Wu et al., 2023) suggests their involvement in the transport and accumulation of Cd in oyster cells.

*Zip1* and *Zip3* show weak expression in the blood or nerve cells within the connective tissue of the oyster mantle. Studies in humans and mice have found *Zip1* expressed in almost all tissues, localized on the plasma membrane, whereas *Zip3*'s localization on the plasma membrane or intracellular compartments is influenced by Zn ion concentration (Franklin et al., 2003; Jeong and Eide, 2013; Bowers and Srari, 2018). The mantle, composed of inner and outer epithelia, connective tissue, muscle fibers, and secretory gland cells, acts as a filter-feeding tissue directly in contact with the external environment and is well-supplied with blood vessels, participating in oyster respiration and secretion, indicating the mantle serves as a



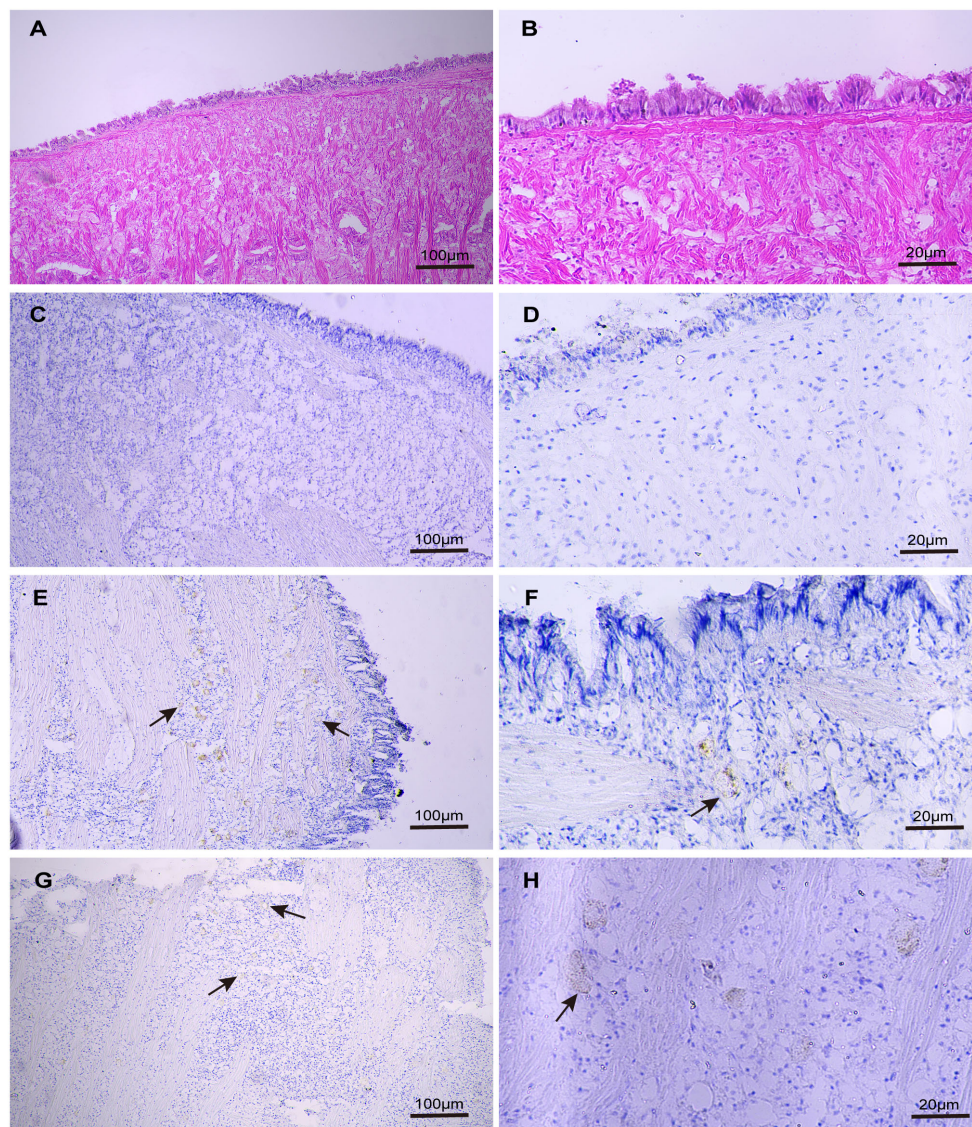


FIGURE 5

The distribution of Zip1 and Zip3 expression in the mantle was examined using optical microscopy images of immunoperoxidase-stained sections from paraffin-embedded Fujian oyster material exposed to 5ppb Cd for 3 days, counterstained with hematoxylin and eosin (H&E). Panels (A, B) display H&E-stained mantle sections from the same tissue not exposed to Cd. Panels (C, D) serve as negative controls for the mantle structure not exposed to Cd. Both Zip1 (E, F) and Zip3 (G, H) are weakly expressed in the connective tissue of the mantle.

primary barrier between the environment, hemolymph, and internal organs. Previous studies have shown that Cd stress in oysters activates the “hemocyte-neuron” neuroendocrine-immune axis in connective tissue and induces oyster hemocyte apoptosis to mediate neuroimmune regulation (Sokolova et al., 2004; Liu et al., 2017). Furthermore, hemocytes in connective tissue can store large amounts of metals like Zn, Cu, and Cd (Shi et al., 2019b). Based on the localization of *Zip1* and *Zip3* in the mantle’s connective tissue, it is speculated that ZIP transport proteins are involved in Cd absorption and accumulation in the mantle.

Cd is a non-essential element for biological systems, and its absorption and excretion processes require the utilization of channels for other essential metals. Studies in animals, plants, and fungi have shown that Cd can enter cells via transport channels for

Ca (Washko and Cousins, 1976; Min et al., 2008, 2015), Fe (Ragan, 1977; Flanagan et al., 1978), Zn (Waalkes and Perantoni, 1988; Dalton et al., 2005; Girijashanker et al., 2008) and Mn (Sun et al., 2019). The mantle is an important organ for oysters to accumulate metals (Shi et al., 2019a). In our study, disruption of *Zip1* led to a significant reduction in Cd accumulation in the oyster mantle compared to controls, indicating that *Zip1* transcription levels affect Cd accumulation in oyster mantles. *Zip1* has been confirmed to transport Zn from the extracellular environment into cells in humans, mice, and yeast (Zhao and Eide, 1996; Dufner-Beattie et al., 2003; Wang et al., 2004). Furthermore, research has indicated that in *Arabidopsis thaliana*, genome-wide bioinformatics screening revealed a significant upregulation of the expression of Zinc Transporter Protein 1 (*Zip1*) upon exposure to

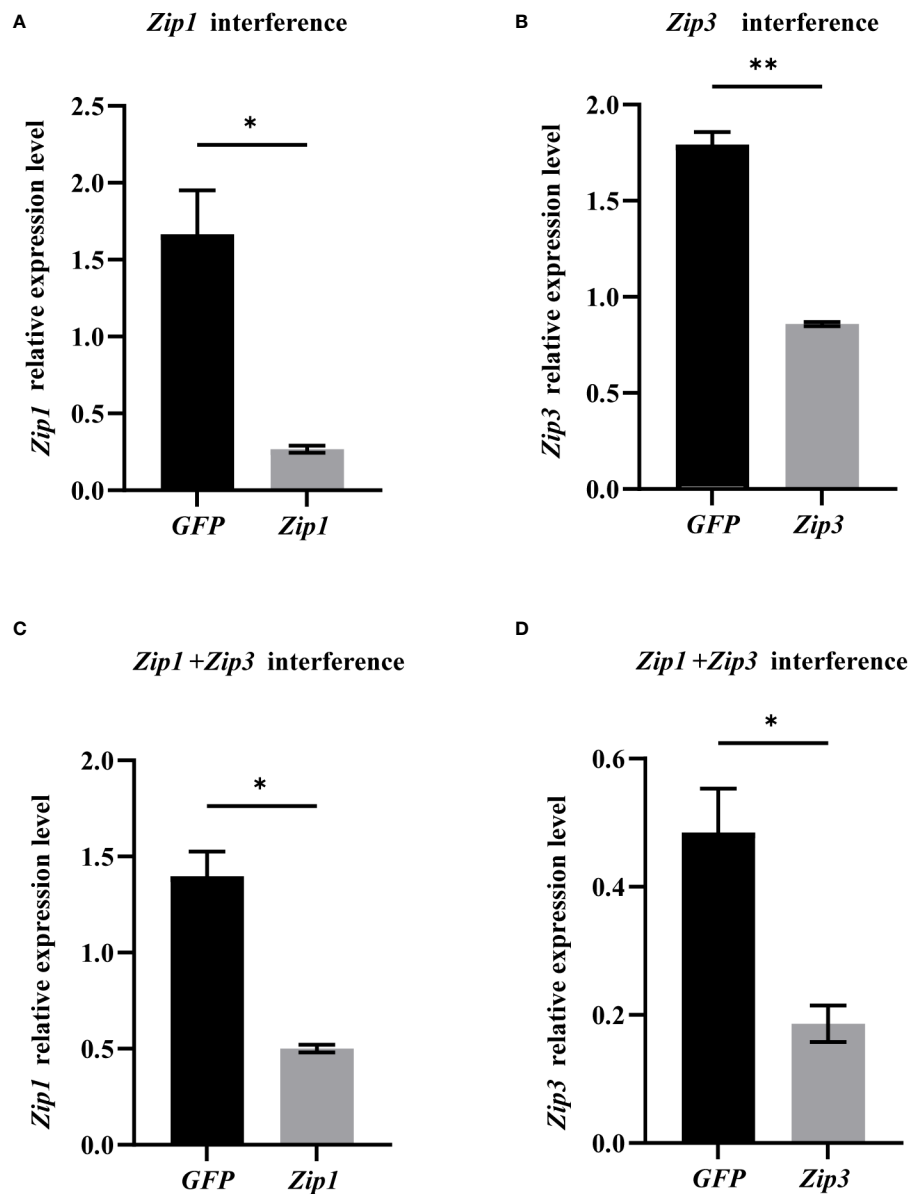


FIGURE 6

mRNA expression levels of *Zip1* and *Zip3* after gene silencing. (A) Silencing efficiency of *Zip1* after 48 hours. (B) Silencing efficiency of *Zip3* after 48 hours. (C) Silencing efficiency of *Zip1* after dual silencing of *Zip1* and *Zip3* for 48 hours. (D) Silencing efficiency of *Zip3* after dual silencing of *Zip1* and *Zip3* for 48 hours. The significant differences compared to controls are denoted with \*. (\* $p < 0.05$ , \*\* $p < 0.01$ ).

Cd. It is hypothesized that this may play a certain role in the uptake of Cd in plants (Zheng et al., 2018). Thus, we propose that *Zip1* in oyster cells is a crucial transport protein for Cd absorption. Silencing of *Zip1* reduced Cd absorption, leading to decreased Cd accumulation in the mantle. However, Cd accumulation still occurred in the mantle after *Zip1* silencing, suggesting that the interference was not completely blocking (efficiency of 84%) or that Cd might enter cells through other metal transport channels.

After disrupting *Zip3*, we observed a significant increase in Cd accumulation in the mantle. *Zip3* has been confirmed to have multiple Zn transport functions across the plasma membrane and intracellular compartments (Costello et al., 2011). Studies have shown that in Zn-deficient yeast, *Zrt3* can transport Zn stored in vacuoles to

the cytoplasm (Zhao and Eide, 1996). Additionally, in Zn-deficient mice, *Zip3* is localized on the surface of mammary epithelial cells; after Zn treatment, the protein relocates to intracellular compartments of secretory-type mammary epithelial cells, facilitating Zn transport from the cytoplasm into these compartments, which helps transfer Zn from maternal circulation to milk (Kelleher and Lönnnerdal, 2005). When oysters face metal stress, they can mitigate metal toxicity through enhanced excretion, metal chelation, and compartmentalization (Engel, 1983; Liu and Wang, 2016). Some studies on human and mouse Cd exposure have shown that absorbed Cd enters blood cells or lysosomes, forming Cd-metallothionein (MT) complexes, where Cd is released from MT and appears in the cytoplasm, altering the electrochemical gradient on

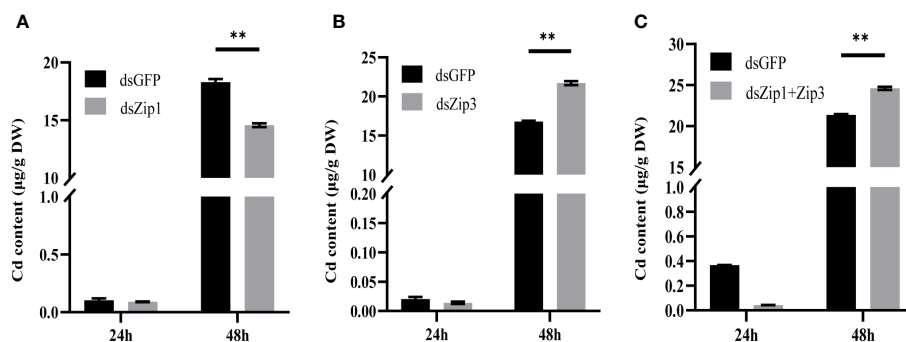


FIGURE 7

Cd content in oyster mantle tissue after gene silencing. Cd new accumulation in the mantle 24 hours after adding dsRNA (or dsGFP for the control group), followed by exposure to 5ppb  $^{115}\text{Cd}$  for 24 hours. (A) After adding dsZip1, (B) after adding dsZip3, (C) after adding both dsZip1 and dsZip3. The significant differences compared to controls are denoted with \*\*,  $p < 0.01$ .

membranes to facilitate Cd excretion through renal tubules with urine (Nordberg, 2009). In addition, numerous studies have investigated transport proteins involved in Cd ion transportation in plants. *A. thaliana* *AtABCG36/AtPDR8*, a member of the ABC transporter family, is localized to the cell membrane. It functions by transporting Cd from the cell interior to the exterior, reducing Cd toxicity in plants and correlating with lead tolerance (Kim et al., 2007; Park et al., 2012). Additionally, the three genes of the ABCC family, *AtABCC1*, *AtABCC2* and *AtABCC3*, sequester Cd by transporting it from the cytoplasm into vacuoles, thereby mitigating Cd's harmful effects on plants (Park et al., 2012; Brunetti et al., 2015). Based on the reported mechanisms of Cd efflux and the results of this experiment, we hypothesized that Cd efflux was blocked after *Zip3* interference, resulting in a significant increase in Cd content in the mantle. Therefore, based on the expression patterns, interference, and expression localization analysis of *Zip1* and *Zip3*, we hypothesize that they play pivotal roles in the accumulation of Cd in oysters. Further research is needed to evaluate the roles of *Zip1* and *Zip3* in intracellular and intercellular Cd transport in oysters.

## 5 Conclusion

Genetic improvements are highly beneficial for the sustainable development of the oyster aquaculture industry. This study has identified tissue-specific expressions of *Zip1* and *Zip3* in Fujian oyster. Results from RNAi and immunohistochemistry further confirm the critical roles of *Zip1* and *Zip3* in Cd accumulation in Fujian oyster. Altogether, these findings provide a basis for future research into the molecular mechanisms of Cd accumulation in Fujian oyster and the cultivation of low-Cd oyster strains.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number (s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, accession numbers: OR759776 and OR759777.

## Ethics statement

The animal study was approved by the Animal Care and Use Committee of the School of Aquatic Sciences, Jimei University (Animal Ethics Number 1067). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

HL: Writing – original draft, Writing – review & editing. ZL: Data curation, Software, Writing – review & editing. LW: Data curation, Software, Writing – review & editing. YK: Funding acquisition, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – review & editing. HQ: Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Visualization, Writing – review & editing. BS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

## Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was funded by the National Natural Science Foundation of China (grant number 32172949), the Natural Science Foundation of Fujian Province (grant number 2020J05135) and the Creation and Demonstration Application of Excellent New Germplasm of Fujian oyster (grant number FJHYF-L-2023-2).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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