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Transcriptomic analyses of *Pinctada fucata martensii* responses under stress of titanium dioxide nanoparticles

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Titanium dioxide nanoparticles (TiO_2-NPs) released into the environment is becoming more prevalent due to their increased usage, marine TiO_2-NPs contamination is escalating concerns in coastal areas. To understand the potential impact of TiO_2-NPs on transcript changes in pearl oyster (*Pinctada fucata martensii*), transcriptome analysis on the gill tissues of pearl oysters was conducted after 14-day TiO_2-NPs exposure and 7-day brief recovery. A total of 911 differentially expressed genes (DEGs) were identified between the control group (TC) and the experimental group (TE) exposed to 14-day TiO_2-NPs. Gene ontology (GO) analyses of the DEGs demonstrated their substantial enrichments in functions related to "hydrolase activity", "oxidoreductase activity", and "DNA integration". The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses of the DEGs indicated enrichment in several pathways, including "ubiquitin-mediated protein hydrolysis", "ECM-receptor interactions", "NOD-like receptor signaling pathway", "Toll-like receptor", and "FOXO signaling pathway". This suggests that exposure to TiO_2-NPs intensifies oxidative stress and apoptosis in pearls oysters, leading to negative effects such as disrupted protein homeostasis, decreased biomineralization activity, reduced neuronal excitability, weakened immune response, and reduced cellular metabolism. Transcriptome analysis identified 844 DEGs between the TE and recovery group (TR), which underwent a 7-day brief recovery period. GO analyses of the DEGs demonstrated their substantial enrichments in functions related to "DNA integration", "obsolete oxidation-reduction process", and "proteolysis". KEGG pathways analyses of the DEGs indicated enrichment in several pathways, including "lysine degradation", "glycine, serine, and threonine metabolism", and "NOD-like receptor signaling pathway". The findings indicated that although pearl oysters showed only slight relief after 7 days of brief recovery, they

continued to experience negative effects from TiO_2-NP exposure. Our findings shed light on the complex responses of pearl oysters to TiO_2-NPs stress and offer valuable theoretical insights into the toxicological impact of TiO_2-NPs on pearl oysters.

KEYWORDS

TiO_2-NPs, *Pinctada fucata martensii*, transcriptomics, protein homeostasis, oxidative stress

1 Introduction

Titanium dioxide nanoparticles (TiO_2-NPs) demonstrate remarkable UV-absorbing properties, outstanding chemical stability, high opacity, and excellent resistance to fading. These unique characteristics make TiO_2-NPs an essential component in numerous consumer goods, including sunblocks, cosmetics, pharmaceuticals, coatings, fabrics, polymers, paper, and wood treatments (Aitken et al., 2006). TiO_2-NPs can enter environments via production, transportation, usage, and disposal stages, thereby posing risks to diverse aquatic organisms and their ecosystem (Shi et al., 2016; Carbuloni et al., 2020). High-concentration TiO_2-NPs have been detected in coastal waters, with even greater accumulations observed in sediments (Garner and Keller, 2014). Based on model computations, the TiO_2-NP concentration in surface waters and wastewater varies from dozens to several hundred micrograms per liter (Sun et al., 2016). Concentrations have been recorded to be 900 μg/L in the surface water near French Mediterranean beaches (Labille et al., 2020). The titanium dioxide levels were found exceeding the 1.85 ± 0.55 ng/g detection limit by analyzing the bivalve shellfish from northern China coastal cities, indicating notable accumulation (Li and Gao, 2014). Increased levels of TiO_2-NPs pose significant threats to various marine organisms, leading to increased concerns about the ecological and physiological impact of TiO_2-NPs. A growing body of evidence has demonstrated that TiO_2-NPs can lead to various adverse effects, including reduced fertilization success (Gallo et al., 2016), decreased metabolism (Johari et al., 2013; Muller et al., 2014), delayed embryonic development (Libralato et al., 2013), impaired immune response (Ringwood et al., 2009; Rocha et al., 2014), intestinal microbiome disorders (Chen et al., 2022b; Duan et al., 2023; Li et al., 2024a), and DNA damage (Hu et al., 2017).

RNA sequencing (RNA-seq) is used to identify gene expression patterns under environmental stress. This method is crucial for examining the overall functional arrangement of genes and investigating the control mechanisms of biological metabolic pathways. In recent years, transcriptomics technology has been extensively employed in toxicological studies of aquatic organisms exposed to pollutants. By examining changes in gene expression under toxic stress, this technology can reveal the intrinsic toxicity of pollutants in aquatic organisms at the transcriptional level, facilitating the prediction and prevention of pollutant toxicity in aquatic organisms and the environments (Sharon et al., 2013; Liu et al., 2020). Banni et al. (2016) examined the impact of TiO_2-NPs and 2,3,7, 8-tetrachlorodibenzo-p-dioxins on genes related to the digestive glands of *Mytilus galloprovincialis* using a combination of transcriptomics and immunohistochemistry. Zhou et al. (2024) used transcriptomic methods to discover that TiO_2-NP exposure triggers lipid metabolism disorders, immune defense disruption, and oxidative stresses in *Acipenser schrenckii*. These findings underscore the effectiveness of transcriptomics in providing a comprehensive analysis of the impact of TiO_2-NPs on aquatic organisms.

Pinctada fucata martensii, native to southern China, Southeast Asia, Australia, India, and Japan (Yang et al., 2019, 2023), are filter-feeding mollusks renowned for their high economic values, contributing near 90% of the world's marine pearl production (He et al., 2020; Wu et al., 2022). Raft and pile cultures are the primary cultural approaches of offshore pearl oysters (Yang et al., 2017). Nevertheless, escalating nearshore marine pollution has recently placed pearl oysters under significant threat. TiO_2-NPs are prone to condensation and deposition on the surface of sediments. The gills of marine bivalves are the primary organs exposed to waterborne pollutants and serve as major detoxification organs in these species, rendering gene expression in this organ highly prone to alterations (Brandts et al., 2018). This study used RNA-Seq analysis of gill tissue to identify the potential genes and pathways affected by TiO_2-NPs in pearl oysters after 14-day exposure and 7-day brief recovery, providing a theoretical basis of TiO_2-NPs' effects on pearl oysters.

2 Materials and methods

2.1 Exposure of pearl oysters to TiO_2-NPs

The anatase form of TiO_2-NPs, approximately 5–10 nm in size, was obtained from Shanghai McLean Biochemical Technology (Shanghai, China). TiO_2-NPs were initially added to filtered seawater and homogenized for 20 min using an LC-JY92-TTN

ultrasonic homogenizer (65 W; Lichen Bonsi Instrument Technology, China) to prepare 1 g/L nanoparticle suspension. The suspension was subsequently diluted with seawater to a final TiO_2-NPs concentration of 5 mg/L.

The pearl oysters with intact and uniform-sized shells (shell width with 26.81 ± 2.53 mm) were selected from a fast-growing, black-selective line of pearl oysters (Deng et al., 2013), sourced from Liusha Bay, China. The pearl oysters were cleaned to remove biofoulings and cultured in cylindrical barrels (300 L) for 7 days. Then the pearl oysters were exposed to 5 mg/L TiO_2-NPs for 14 days (treatment phase) and subsequently cultured in TiO_2-NP-free seawater for 7 days (recovery phase). Pearl oysters were fed unicellular algae (morning: 20,000 cells/mL *Chlorella*; evening: 10,000 cells/mL *Platymonas subcordiformis*) during the study period. The experiment comprised three parallel groups, each consisting of 30 pearl oysters. The water in the barrels was daily refreshed, and TiO_2-NPs suspension was added to each barrel maintain a constant TiO_2-NPs concentration of 5mg/L. On days 0, 14, and 21, we randomly selected 2 pearl oysters from each parallel group, designated the control (TC), experimental (TE), and recovery (TR). Gill tissues were clipped and frozen in liquid nitrogen and then stored at −80°C for subsequent analyses. No dead pearl oysters were found during the exposure and recovery periods.

2.2 RNA extraction and library construction

RNA extraction and purification from the gill tissue were conducted utilizing TRIzol (Invitrogen, CA, USA) following instructions from the manufacturer. The purity and quantity of total RNA was evaluated utilizing a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). Oligo(dT) magnetic beads (Dynabeads Oligo, No. 25-61005, Thermo Fisher, USA) were utilized in two consecutive purification steps to specifically capture mRNA containing PolyA tails. NEBNext® Magnesium RNA Fragmentation technology (product number E6150S, USA) was used to fragment the captured mRNA at a high temperature of 94°C for 5 to 7 min. Subsequently, the DNA-RNA complex double strands into pure DNA double strands. Simultaneously, a dUTP solution (product number R0133, Thermo Fisher, California, USA) was added to smooth the ends of the double-stranded DNA. Subsequently, A bases were added to each end to and used magnetic beads for fragment size screening and purification. The UDG enzyme (NEB, product number m0280, Massachusetts, USA) was then used to digest the double-stranded DNA, followed by PCR amplification. Thus, a library was constructed with a 300 bp ± 50 bp fragment size. In accordance with the standard process provided by LC Bio-Technology Co., Ltd. (Hangzhou, China), Illumina Novaseq™ 6000 was utilized for dual-end PE150 sequencing.

2.3 Bioinformatics analyses of RNA-seq

Cutadapt ver. 1.9 was utilized to discard adapter sequences, polyA sequences, polyG sequences, and other low-quality reads. FastQC ver. 0.11.9 was used to verify the GC content and Q20/30 content of the quality-controlled reads. HISAT2 ver. 2.0.4 was utilized to align the reads to the reference genome (Zheng et al., 2023). StringTie ver. 2.1.6 was employed for gene transcription or assembly using FPKM quantification. The reads were assembled with StringTie ver. 1.3.4d, using default parameters. The gffcompare ver. 0.9.8 was selected for comprehensive transcriptomic reconstruction. The R package EdgeR was then utilized to examine the difference between sample genes, defining significant difference as twice difference ratio > or < 0.5 times and p value < 0.05 (Robinson et al., 2010). Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) gene enrichments analyses were performed utilizing DAVID software. Raw transcriptome sequences data have been deposited at Genome Sequence Archive (GSA) under accession CRA017826.

2.4 Gene expression validation

Eight DEGs were randomly selected for qRT-PCR experiments to determine whether the results of RNA-seq data were reliable. β-Actin was used as the internal reference gene. Primers were designed using Primer Premier 5.0 software as shown in Supplementary Table 1. The total mixture of 10 μL of each sample was reacted by the following procedure: denaturation at 95°C for 5 min, followed by 40 amplification cycles (95°C for 10 s, 60°C for 15 s for annealing, and 72°C for 15 s for extension). Expression levels of different genes were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3 Results

3.1 Transcriptome sequencing

Illumina sequencing generated a significant number of raw reads (758,415,850), which were processed to yield 737,017,614 high-quality clean reads following quality control (QC). The GC contents of these reads ranged from 38.5% to 40%, with Q30 and Q20 quality scores ranging from 96.93% to 97.52% and 99.97% to 99.98%, respectively, as detailed in Table 1. Subsequently, a substantial proportion of the genes (61.95%–67.44%) were aligned successfully with the reference genome. This alignment rate highlights the suitability and reliability of sequencing data for follow-up analytical procedures.

3.2 Detection of differentially expressed genes at different experimental periods

Further differential expression analyses revealed 911 DEGs between the TC and the TE, with 390 downregulated and 521 upregulated in the TC in comparison to the TE (Figure 1). Among these, Cytochrome P450 4A25 (PIN0130055), toll-like receptor 13 (PIN0061698), DNA damage-inducible protein GADD45 gamma

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TABLE 1 Summary of sequencing quality and annotation results.

Sample	Raw Data	Valid Data	Valid Ratio (reads)	Q20%	Q30%	GC content%	Mapped reads
TC1	32386114	31605872	97.59	99.97	97.12	39.50	20590581 (65.15%)
TC2	40495812	39388364	97.27	99.98	97.31	39.50	26443594 (67.14%)
TC3	41246484	40113990	97.25	99.97	97.27	39.50	26235833 (65.40%)
TC4	36327138	35354150	97.32	99.97	97.22	39.50	21902326 (61.95%)
TC5	38432986	37181954	96.74	99.98	97.52	39.50	24313435 (65.39%)
TC6	38887062	37821322	97.26	99.97	97.23	39.50	24316076 (64.29%)
TE1	38779560	37672548	97.15	99.97	97.24	38.50	23417337 (62.16%)
TE2	43661260	42422590	97.16	99.97	97.23	39.50	26907831 (63.43%)
TE3	36839476	35906184	97.47	99.97	97.38	39.50	22550883 (62.81%)
TE4	45824350	44640396	97.42	99.97	97.15	40.00	28952056 (64.86%)
TE5	45630492	44422588	97.35	99.97	97.14	39.00	29365397 (66.10%)
TE6	40980192	39882088	97.32	99.97	97.14	39.50	25996906 (65.18%)
TR1	43830504	42419362	96.78	99.98	97.30	39.50	27699419 (65.30%)
TR2	43413570	42196960	97.20	99.97	96.93	39.50	27080947 (64.18%)
TR3	47130618	45804880	97.19	99.97	97.19	39.00	30198648 (65.93%)
TR4	52324586	50979916	97.43	99.97	97.14	39.50	33672004 (66.05%)
TR5	46114218	44446490	96.38	99.98	97.32	39.50	29762848 (66.96%)
TR6	46111428	44757960	97.06	99.98	97.36	39.50	30185390 (67.44%)

(PIN0103192), and several other genes expression exhibited significant upregulation, as illustrated in Figure 2. Conversely, pyridoxal 5'-phosphate synthase subunit SNZERR (PIN0051258), gamma-glutamyltranspeptidase 1-like isoform X1 (PIN0020608), DNA damage-inducible protein GADD45 gamma (PIN0103192), ATP-binding cassette sub-family B member 6 (PIN0010578), and other genes expression exhibited significant downregulation. Furthermore, we identified 844 DEGs between the TE and the TR, including 517 downregulated and 327 upregulated in TE in comparison to the TR (Figure 1). Similarly, gamma-glutamyltranspeptidase 1-like isoform X1 (PIN0020608), ATP-binding cassette (ABC) sub-family B member 6 (PIN0010578), and several other genes expression exhibited significant upregulation after a short recovery. Conversely, genes, such as toll-like receptor 3 (PIN0120448) and heat shock 70 kDa protein 12B (PIN0060012), were substantially downregulated (Figure 3). These significantly altered genes are sensitive to TiO_2 NP-induced stress and may play an important role in physiological processes.

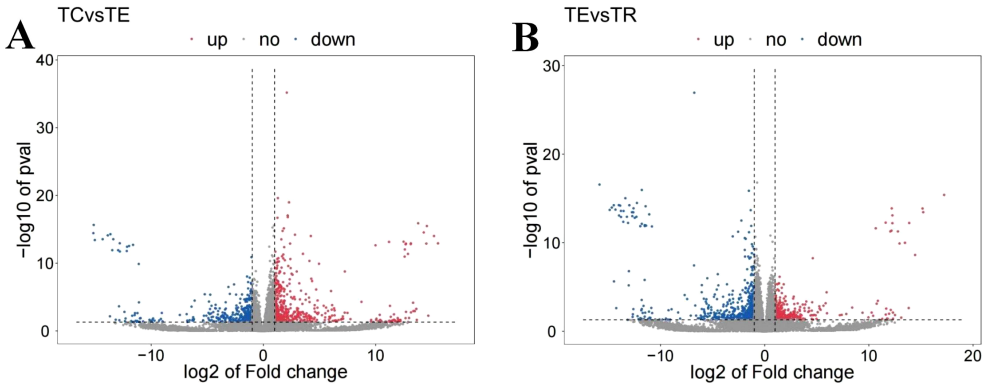

FIGURE 1
(A) Differential gene expression analyses between TC and TE. **(B)** Differential gene expression analyses between TE and TR. Red indicates the upregulated gene expression level, blue indicates the downregulated gene expression level, and grey indicated insignificant differential expression.

FIGURE 2
A heatmap showing the differential gene expression analyses between TC and TE.

3.3 GO analyses

GO enrichments analyses of the DEGs annotated 3373 and 2577 DEGs across molecular functions, cellular components, and biological process in the TC and TE groups and TE and TR groups, respectively. We selected the 30 entries with the highest enrichment levels for bar charts plotting (Figure 4A). Among them, the most annotated DEGs in the TC and TE groups were “metal ion binding”, “DNA binding”, “ATP binding”, “deoxyribonucleotide biosynthetic process”, “DNA recombination”, “mitotic spindle assembly”, “RNA-dependent DNA biosynthetic process”, “DNA integration”, and “nucleus”. In the TE and TR groups, the most annotated DEGs were “biological process”, “proteolysis”, “zinc ion binding”, “metal ion binding”, “obsolete oxidation-reduction process”, “DNA integration”, “DNA binding”, and “integral component of membrane” (Figure 4B).

3.4 KEGG enrichments analyses

KEGG enrichments analyses demonstrated the enrichments of a total of 369 DEGs in 129 signaling pathways in the TC and TE groups (Figure 5A). Notably, these pathways included significant enrichment in “vitamin B6 metabolism”, “glycine, serine, and threonine metabolism”, “biosynthesis of unsaturated fatty acids”, “ubiquitin-mediated protein hydrolysis”. Furthermore, the “ABC transporter”, and “ECM-receptor interactions”, “NOD-like receptor signaling pathway”, “Toll-like receptor”, “neuroactive ligand-

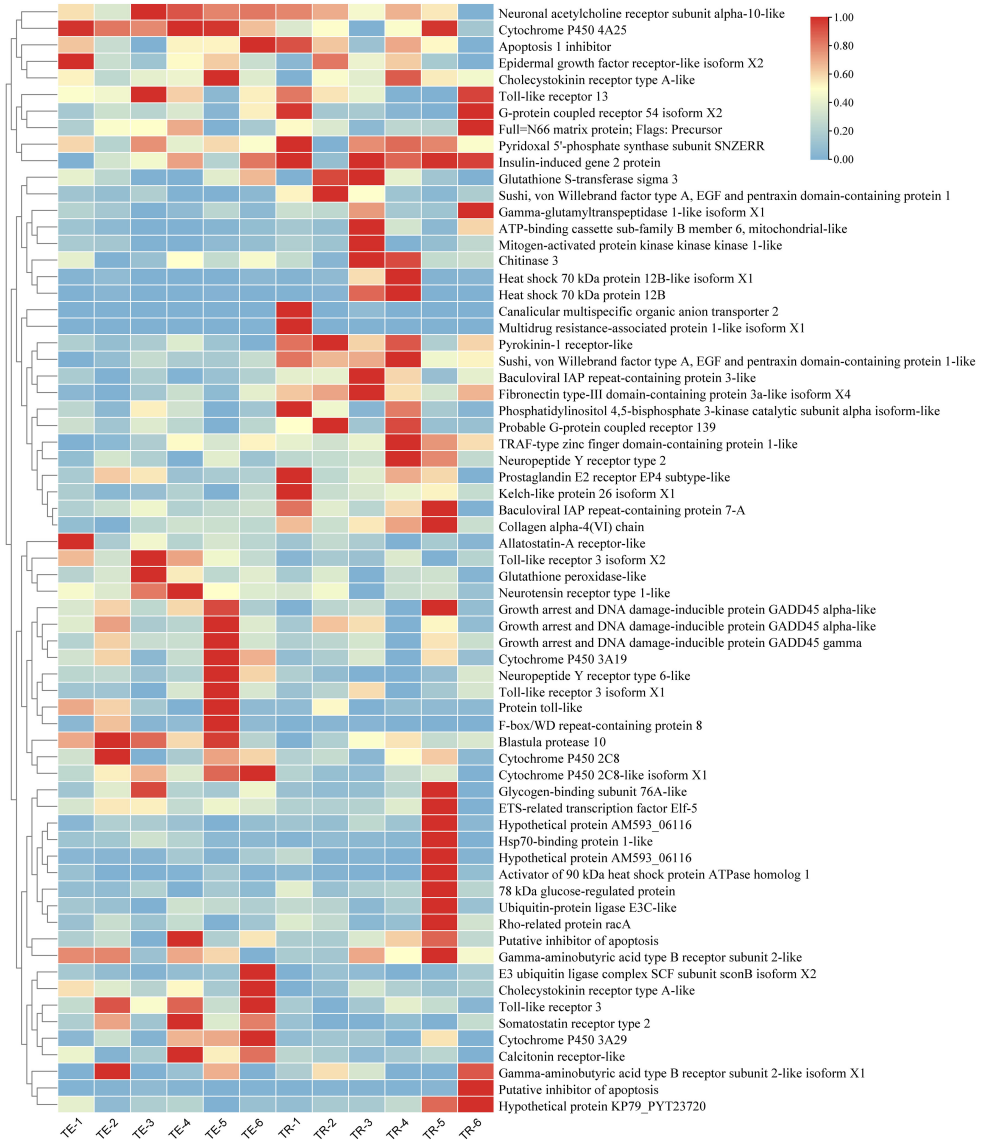

FIGURE 3
A heatmap showing the differential gene expression analyses between TE and TR.

receptor interaction signaling pathway", "lysosomal pathway", "phagosomal pathway", "FOXO signaling pathway", and "glutathione metabolite" were also identified to be associated with pearl oysters' responses to TiO_2-NPs exposure. Conversely, a total of 284 DEGs in the TE and TR groups were enriched in 102 signaling pathways (Figure 5B). Notably, significant enrichment was observed in "lysine degradation", "glycine, serine, and threonine metabolism", "NOD-like receptor signaling pathway", and other pathways.

3.5 qRT-PCR validation

To assess the accuracy of the RNA-seq results, 8 DEGs were randomly selected for qRT-PCR in each of the TC vs TE and TE vs TR groups. qRT-PCR selective gene expression trends were consistent with DEG analyses, suggesting that the transcriptome data were reliable (Figure 6).

4 Discussion

The survival of aquatic organisms is significantly affected by salinity, oxygen content, temperature, and pollutants in the water (Valavanidis et al., 2006). In particular, environmental chemicals pose various toxic threats to aquatic organisms, such as DNA damage, oxidative stress, inflammation, apoptosis, and cell death (Lim et al., 2022; Wang et al., 2023). To withstand complex and variable living environments, intertidal bivalves have developed highly intricate adaptive mechanisms (Falfushynska et al., 2020). Considerable research interest has recently been focused on the potential impacts of TiO_2-NPs on marine bivalves (Abdel-Latif

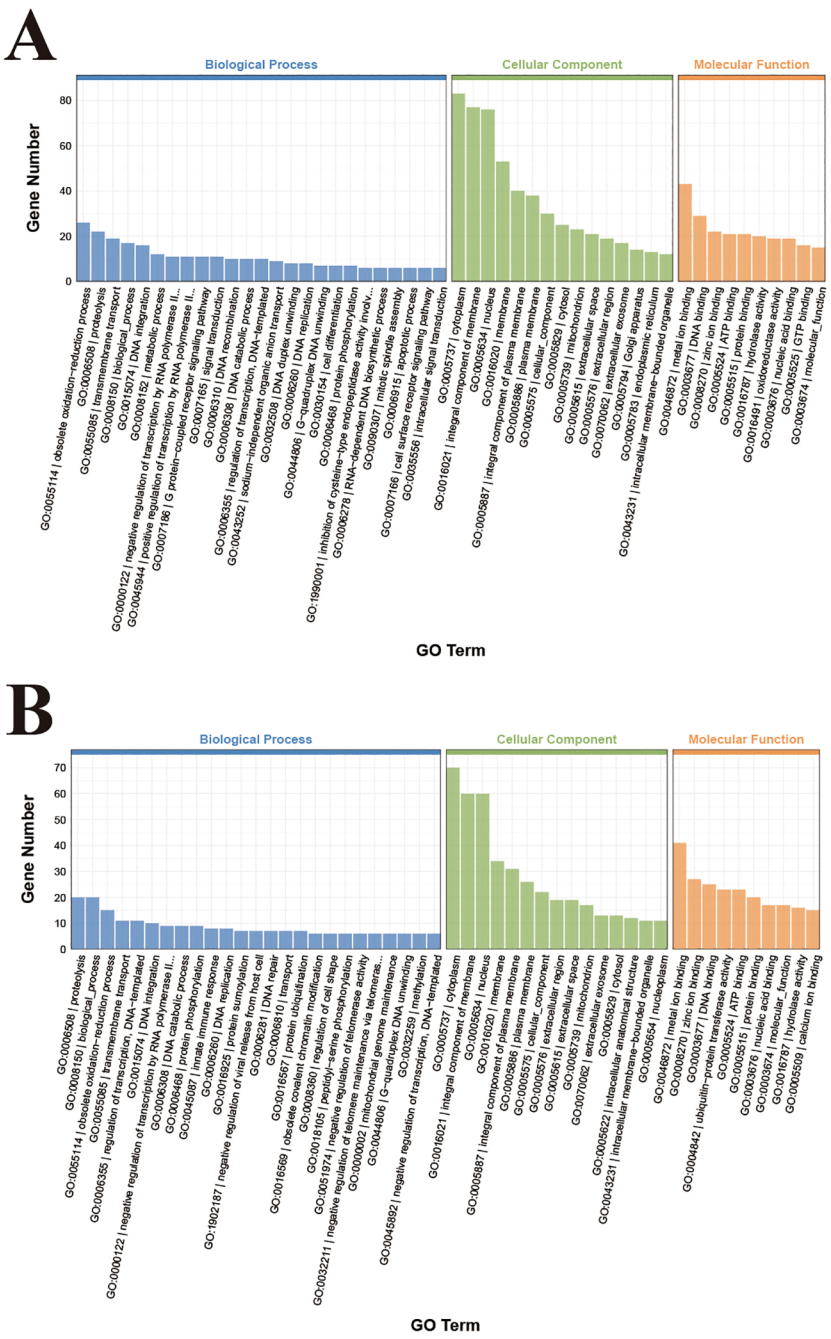

FIGURE 4
GO terms enrichment analysis of DEGs in TC and TE **(A)**, TE and TR **(B)**.

et al., 2020; Li et al., 2021). This study revealed the molecular response mechanisms of pearl oysters after exposure to TiO_2-NPs, providing crucial insights into evaluating the potential toxic impacts of TiO_2-NPs on these organisms.

4.1 TiO_2-NPs cause of oxidative stress

TiO_2-NPs function as prooxidants that can enter cell membrane, inducing mitochondrial damage (Barmo et al., 2013). It triggers excessive cellular production of reactive oxygen species (ROS), leading to compromised membrane permeability (Girardello et al., 2016). Under such conditions, living beings may experience oxidative stress due to excessive ROS production when the cellular antioxidant defense system becomes overwhelmed (Ray et al., 2012). To mitigate the harmful impacts of ROS, numerous animals can employ antioxidant defensing mechanisms, including non-enzymatic components (such as carotenoids and glutathione) and enzymatic components (such as glutathione peroxidase and superoxide dismutase) (Yang et al., 2017). We detected the

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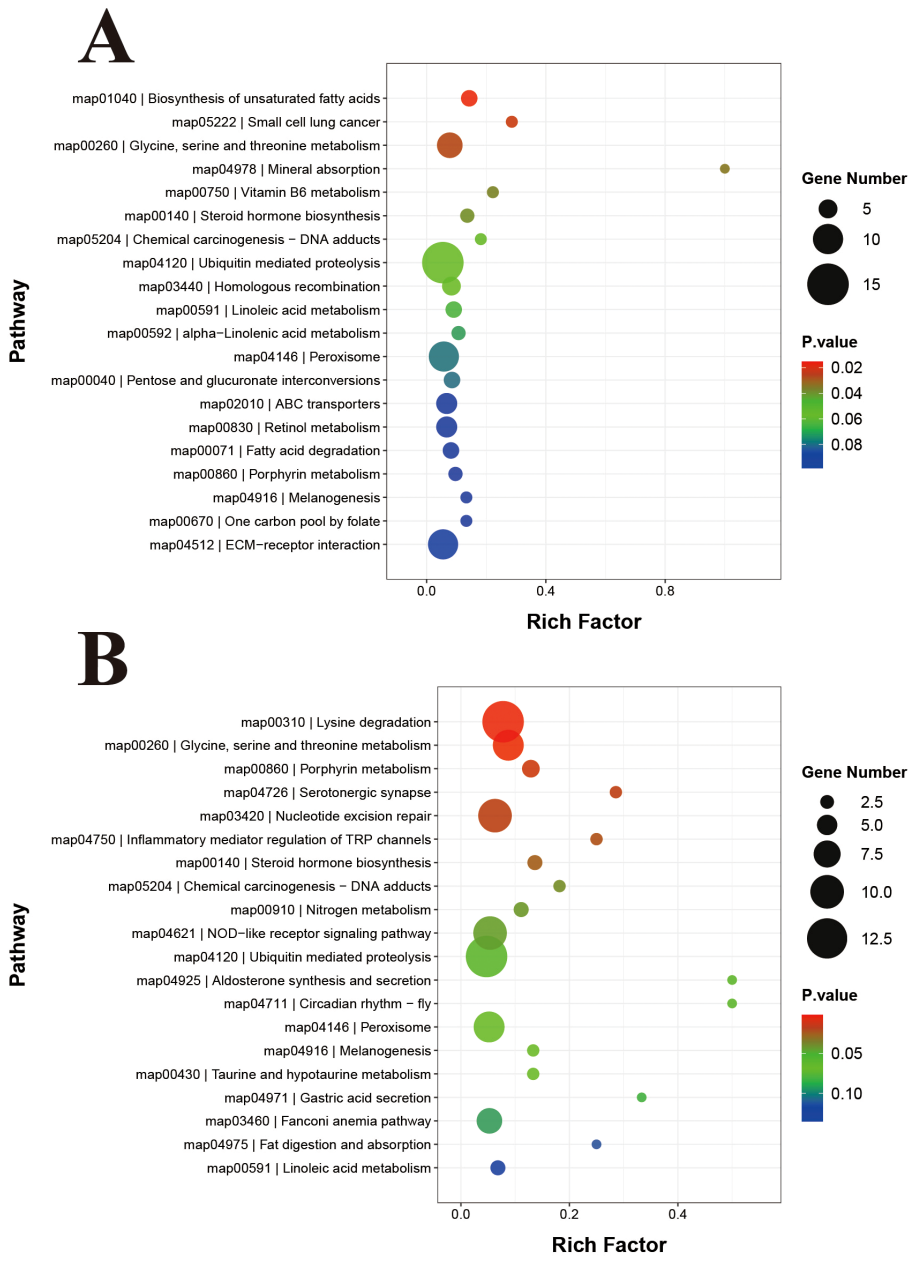

FIGURE 5
KEGG pathway enrichment analysis of DEGs in TC and TE **(A)**, TE and TR **(B)**.

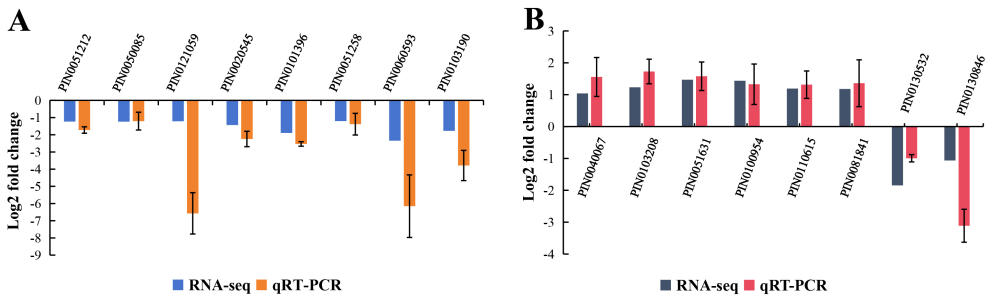

FIGURE 6
Validation of RNA-seq data by qRT-PCR in TC and TE groups **(A)**, TE and TR groups **(B)**.

substantial enrichment of the DEGs in the glutathione metabolism pathway under TiO_2-NP stress. Genes encoding glutathione peroxidase-like (PIN0101396), gamma-glutamyltranspeptidase 1-like isoform X1 (PIN0020608), glutathione S-transferase sigma 3 (PIN0052777), microsomal glutathione S-transferase 2 (PIN0060908), and aminopeptidase N (PIN0020097) were substantially downregulated. According to these results, pearl oysters experience oxidative stress under TiO_2-NP stress, being consistent with our prior finding (Li et al., 2024b).

Here, we observed notable enrichment of vitamin B6 metabolism in the KEGG pathway enrichment. Specifically, we found that the pyridoxal 5'-phosphate synthase subunit SNZERR (PIN0051258), which is involved in vitamin B6 metabolism, was downregulated in response to oxidative stress. Pyridoxal-5'-phosphate (PLP) acts as the physiologically active coenzyme of vitamin B6, potentially playing pivotal roles in antioxidation (Schneider et al., 2000). PLP can directly scavenge free radicals for lipid peroxidation inhibition. Alternatively, PLP acts indirectly as a coenzyme of the glutathione antioxidant defense system (Liu et al., 2013). Studies have indicated that deficiencies in vitamin B6 can weaken antioxidant defensing mechanisms and increase oxidative stresses (Danielyan and Chailyan, 2020). This study demonstrated that TiO_2-NPs exposure induced downregulation of pyridoxal 5'-phosphate synthase subunit SNZERR (PIN0051258), which is involved in vitamin B6 metabolism, thereby affecting vitamin B6 synthesis and increasing oxidative stress in pearl oysters.

Aquatic species depend on the multi-xenobiotic resistance (MXR) mechanism as a key defensing mechanism against environmental toxins, minimizing intracellular accumulation of toxins and potential damage (Kurelec, 1995), which is mediated by the ABC transporter (Kingtong et al., 2007). The relationship between ABC transporters and nanoparticles has gained increasing attention (Yin et al., 2023). Kingtong et al. (2007) demonstrated the significant roles of ABC transporters in the detoxification metabolic mechanism of *Saccostrea forskali* after exposure to tributyltin. Research on mussel gill cells has demonstrated the upregulation of Abcb1 by silver NPs, aiding in silver NPs detoxification (Katsumiti et al., 2015). Bhagat et al. (2022) reported that polystyrene and metal oxide nanoparticles disrupt ABC transporters' functionality in zebrafish embryos, resulting in increased aluminum and cerium ion toxicity. Similarly, Georgantzopoulou et al. (2016) found that the MXR system could be inhibited by silver nanoparticles in *Daphnia magna* larvae, leading to increased calcineurin accumulation. After TiO_2-NPs exposure, significant downregulation was observed in genes linked to the ABC transporters pathway, including ABC sub-family B member 6, mitochondrial-like (PIN0010578), multidrug resistance-associated protein 1-like isoform X1 (PIN0080856), and canalicular multispecific organic anion transporter 2 (PIN0080857). This indicates that TiO_2-NPs inhibit the activity of ABC transporter protein activity, thereby worsening oxidative stress and particle accumulation in pearl oysters. After a brief 7-day recovery period, genes associated with ABC transporters and glutathione metabolism pathways exhibited significant upregulation, indicating an increase in oxidative stress in pearl oysters.

Cytochrome P450, a class of monooxygenase, is crucial for the syntheses and metabolisms of various hormones, such as glucocorticoids and mineralocorticoids, which are essential for stress response (Urlacher and Girhard, 2012). Increased P450 production in aquatic organisms typically indicates they are experiencing stress (Ibrahim et al., 2021). For instance, elevated transcription of the cytochrome P450 gene was observed in the kidney, gill, and liver tissues of Nile tilapia following exposure to *Aeromonas hydrophila* (Hal and El-Barbary, 2020). An upregulation was observed in genes such as cytochrome P450 4A25 (PIN0130055), cytochrome P450 2C8 (PIN0120270), cytochrome P450 3A19 (PIN0100308), cytochrome P450 2C8-like isoform X1 (PIN0140634), and cytochrome P450 3A29 (PIN0100307) following 14-day TiO_2-NPs exposure, indicating that TiO_2-NPs exposure induces oxidative stress in pearl oysters. After a brief recovery period, the related cytochrome P450 gene expression was downregulated compared with that of the TE group, which also indicates that brief recovery can alleviate stress in pearl oysters.

4.2 TiO_2-NPs changes in immune activities

Exposure to TiO_2-NPs can adversely affect bivalves' immune system (Wei et al., 2023). Bivalves primarily depend on innate immunity due to their lack acquired immune system (Jiang et al., 2017). Microbial recognition by innate immunity is mediated by pattern recognition receptors, which bind to pathogen-associated molecular patterns, the conserved microbial molecules. Toll-like receptors detect microorganisms in extracellular cellular lumens and phagosomes, whereas the Nod-like family of receptors recognize intracellular pathogens, triggering transcriptional responses and activating inflammatory vesicles (Chamaillard et al., 2003; Inohara et al., 2005). Innate immune receptors interact with specific microbial ligands to generate signaling pathways triggering host transcriptional response linked to inflammation. Research has demonstrated that bacterial lysis in phagosomes is crucial for acquired and innate immune response development (Herskovits et al., 2007). Phagosome maturation involves regulatory interactions with other membrane organelles, such as regenerating endosomes, late endosomes, and lysosomes. Furthermore, the lysosomes-phagosomes fusion can release toxic compounds that effectively kill and fragment major bacteria (Luzio et al., 2007). Lysosomes play pivotal roles in various physiological functions, such as immune responses, energy metabolism, cell survival, cell membrane repair, and cellular homeostasis, most of which rely on lysosomes' protease activities (Martínez-Fábregas et al., 2018). We detected substantial upregulation of the toll-like receptor 13 (PIN0061698), toll-like receptor 3 isoform X2 (PIN0061696), and toll-like receptor 3 isoform X1 (PIN0061693) genes after exposure to TiO_2-NPs. Furthermore, the expression of some genes in the NOD-like receptor signaling pathway, lysosome pathway, and phagosome pathway was substantially upregulated, indicating that TiO_2-NPs exposure activates the immune system of pearl oysters and improves their immune ability to resist the invasion of TiO_2-NPs, being consistent with our prior finding (Li

et al., 2024a, b). We observed substantial downregulation of the toll-like receptor 3 (PIN0120448) and protein toll-like (PIN0071163) genes after a brief recovery period. In addition, the NOD-like receptor signaling, lysosome, and phagosome pathways remained substantially upregulated compared with the TE group, indicating that brief recovery may alleviate immune stress in pearl oysters.

4.3 TiO_2-NPs induces apoptosis

Apoptosis is critical for the immune response of mollusks (Zhang et al., 2022). The FOXO signaling pathway is associated with apoptosis, DNA repair, and several cellular pathways (Chen et al., 2022a). In aquaculture, there is increasing research interest in investigating the changes in the FOXO pathway caused by environmental stress. For instance, studies have demonstrated that acute hypoxia induces changes in the FOXO signaling pathway in *Hypophthalmichthys nobilis* (Chen et al., 2021) and *Oncorhynchus mykiss* (Wu et al., 2023). Additionally, Ma et al. (2023) revealed that high-temperature stress mitigates cellular apoptosis by regulating the expressions of FOXO target genes related to apoptosis, thereby mediating liver injury in *Brachymystax lenok tsinlingensis*. After exposure to TiO_2-NPs, the expressions of genes related to the FoxO signaling pathway were substantially downregulated, including epidermal growth factor receptor-like isoform X2 (PIN0140090), growth arrest and DNA damage-inducible protein GADD45 gamma (PIN0103192), growth arrest and DNA damage-inducible protein GADD45 alpha-like (PIN0103191, PIN0103190), growth arrest and DNA-damage-inducible protein GADD45 gamma (PIN0103188), and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform-like (PIN0101618). This indicates that exposure to TiO_2-NPs inhibits the FOXO pathway and induces apoptosis. Gadd45 α is known to be activated in response to DNA damage (Salvador et al., 2013). Martin-Folgar et al. (2022) discovered that exposure of *Danio rerio* to plastic particles at a concentration of 0.1 ppm resulted in upregulation of GADD450 expression, whereas exposures to 0.5 and 3 ppm led to downregulation of GADD450 gene expression. Consistent with observations of Martin-Folgar et al. (2022), we also observed a significant downregulation of Gadd45 expression in pearl oysters after 14 days of TiO_2-NPs exposure, which indicates that TiO_2-NPs may have impacted the DNA repair mechanism of pearl oysters by affecting the overall repair capacity of DNA, potentially leading to apoptosis.

Inhibitors of apoptosis proteins (IAP) protect aquatic organisms from the detrimental impacts of atmospheric exposure, thereby extending their survival time (Zhou et al., 2021). A significant upregulation of IAPs in oysters exposed to high temperatures, air, and low salinity has been demonstrated previously (Zhang et al., 2012). Under hypoxia in pearl oysters (Yang et al., 2023) and manila clams (Nie et al., 2020), the expressions of IAPs were substantially upregulated to activate their apoptotic systems in response to survival conditions. Nevertheless, baculovirus IAP repeat sequence protein 3-like (PIN0060593), baculovirus IAP repeat sequence protein 7-A (PIN0060592), and putative apoptosis inhibitory factors (PIN0070159, PIN0070593) were found to be downregulated under the conditions of TiO_2-NPs exposure, leading to apoptosis and affecting cell viability and proliferation.

4.4 Energy supply and energy expenditure

Bivalves are particularly vulnerable to nanoparticle toxicity in aquatic environments because of their filter-feeding strategy and sessile lifestyle. Nanoparticles, which are often agglomerated, are trapped in their gills and can enter the digestive system through endocytosis, leading to harmful effects (Gornati et al., 2016; Li et al., 2021). Nanomicroplastics have been demonstrated to disrupt the digestive system of shellfish, impairing nutrient absorption and consequently affecting shellfish health (Cole et al., 2020; Cappello et al., 2021). Furthermore, *Mytilus edulis* could absorb 100 nm microplastic particles, which affect the filtration activity of these organisms, produce pseudofaeces, and consume energy (Wegner et al., 2012).

Gluconeogenesis and glycolysis are critical metabolic pathways involved in carbohydrate synthesis and catabolism (Jitrapakdee, 2012). Studies have demonstrated that shellfish maintain energy balance under stress conditions by boosting glycolysis (Sun et al., 2021; Hu et al., 2022). During pathogen infections, the host requires increased energy to fight and survive the infection. However, relying solely on sugar metabolism is insufficient to support the higher energy requirements of the host due to its limited carbohydrate content. Proteins and lipids serve as crucial alternative energy sources to meet the increased energy demands of the host. Lipids contribute significantly to animal adaptation to adverse environments, in addition to supporting normal growth and development by providing energy (Chen et al., 2013; Liu et al., 2016). Studies have demonstrated that zebrafish experience increased glucose consumption and energy supply deficits after exposure to NPs (Chen et al., 2020). In *Crassostrea gigas* larvae, stimulation by OsHV-1 infection resulted in large-scale mortality, likely attributed to reduced oyster feeding activity and significant disruption of lipid metabolism within the organisms (Young et al., 2017). After exposure to TiO_2-NPs, KEGG secondary pathways analysis revealed significant enrichment in pathways related to carbohydrate metabolism. Specifically, the expression of most related genes within the glycolytic pathways, pentose and glucuronate interconversion pathway, citrate cycle pathway, and nucleotide and amino sugars metabolism pathway was substantially downregulated. Furthermore, key genes associated with energy metabolisms, such as glycogen-binding subunit 76A-like (PIN0121059), 78 kDa glucose-regulated protein (PIN0111091), and insulin-induced gene 2 protein (PIN0010512) were substantially downregulated. This indicates a reduction in sugar and carbohydrate supply to the organism from pearl oysters exposed to TiO_2-NPs. Most genes in the KEGG secondary pathways associated with amino acid metabolism, such as methionine and cysteine metabolism pathways, threonine, serine, and glycine metabolism pathways, lysine degradation pathway, and arginine and proline metabolism pathways, were substantially downregulated. Conversely, most genes involved in pathways

associated with lipid metabolism, such as steroid hormone biosynthesis, unsaturated fatty acids biosynthesis, linoleic acid metabolism, and fatty acid degradation pathways, were substantially upregulated. This indicates that under TiO_2-NPs stress, lipid metabolism becomes the primary energy supply for pearl oysters. We speculate that sugars, carbohydrates, and proteins may primarily supply energy during the pre-exposure phase of TiO_2-NPs, and then switch to lipids during the post-exposure phase of the exposure. However, further studies are required to confirm this hypothesis. After a brief recovery period, secondary pathways related to glycan biosynthesis and metabolism, lipid metabolism, amino acid metabolism, and nucleotide metabolism exhibited significant upregulation, revealing the restored energy supply in pearl oysters after alleviating the stress induced by TiO_2-NPs.

Under TiO_2-NPs stress, the downregulated DEGs substantially enriched the GO terms associated with "mitotic spindle assembly", "deoxyribonucleotide biosynthetic process", "RNA-dependent DNA biosynthetic process", "DNA recombination", and "DNA integration". Extended exposure to TiO_2-NPs may cause cellular injury, potentially disrupting metabolic activities within cells. This suppression of cellular processes is hypothesized to be a strategic response to conserve energy under TiO_2-NPs-induced stress.

4.5 TiO_2-NPs disrupt protein homeostasis

Ensuring protein equilibrium is essential for maintaining physiological function, as it involves a dynamic balance between synthesis and degradation. The accumulation of damaged or misfolded proteins within cells can be toxic and disrupt normal cellular processes (Sherman and Goldberg, 2001). The HSP70 family members are crucial for maintaining protein homeostasis by facilitating newly produced and denatured peptides' folding into native conformation as well as the refolding of proteins forming aggregates within cells (Fu et al., 2014; Artigaud et al., 2015). Overexpression of HSP70 genes is often pivotal for enhancing resistance to various stresses, a common phenomenon observed among aquatic organisms exposed to pollutants (Ivanina et al., 2009). For instance, the proliferation of the HSP70 gene family and the overexpression of the HSP70 gene are believed to significantly contribute to the capability of oysters' high-temperature withstanding (Ivanina et al., 2009). Distinct amplification of HSP70 was identified in bivalve shellfish, such as the *Crassostrea hongkongensis* (Brandts et al., 2018). Conversely, HSP70 downregulation has been reported in *Mytilus galloprovincialis* after exposure to heat stress and cadmium (Izagirre et al., 2014). Transcript levels of hsp70 in the gill tissues of *Mytilus galloprovincialis* were observed to decrease after simultaneous exposure to nanoplastics and carbamazepine (Brandts et al., 2018). In our experiments, we observed substantial downregulation of the heat shock 70 kDa protein 12B (PIN0060012), heat shock 70 kDa protein 12B-like isoform X4 (PIN0131632), and HSP70-binding protein 1-like (PIN0050777) genes. However, inactivating these gene transcripts does not necessarily indicate low HSP70 protein levels. High HSP70 level has been demonstrated to cause transcription factors inactivation through a negative feedback mechanism (Pinsino et al., 2017). HSP70 proteins aid in the lysosomal degradation of aggregated proteins, using either molecular chaperone-assisted autophagy or the ubiquitin-proteasome system (Rokutan, 2000). Based on reports, animals can regulate misfolded peptides breakdown through the ubiquitin-proteasome mechanism (Hampton, 2002). Ubiquitin genes activation is stimulated to break down unrepairable proteins in a heat-sensitive abalone lineage (Chen et al., 2019). Whiteleg shrimp (*Penaeus vannamei*) responds to stress by upregulating ubiquitin gene expression after a short-term microplastic exposure (Han et al., 2021). After exposure to TiO_2-NPs, substantial decreased expression of 13 genes, such as ubiquitin-protein ligase E3C-like (PIN0100476) and apoptosis 1 inhibitor (PIN0060591) was observed, indicating that the downregulation of genes involved in the ubiquitin-mediated protein hydrolysis pathway could affect the ability of pearl oyster to remove misfolded proteins, thereby resulting in cytotoxicity. Heat shock 70 kDa protein 12B (PIN0060012) and heat shock 70 kDa protein 12B-like isoform X1 (PIN0081248) remained downregulated following a short-term recovery period of 7 days. In addition, although seven genes in the ubiquitin-mediated proteolysis pathway were upregulated, six genes remained downregulated, indicating that the short-term recovery period was insufficient to restore pearl oysters to normal levels. These findings reveal that TiO_2-NPs can significantly affect protein homeostasis in pearl oysters.

4.6 TiO_2- NPs disrupt neuronal excitability

In invertebrates, neurons play crucial roles in receiving and processing environmental information. Furthermore, neurons positively regulate synaptic structural plasticity and function, enhancing adaptive capacity under stressful conditions (Konstantinos et al., 2012). In *Limulus polyphemus*, neurons contribute to osmotic stress tolerance and adaptation through several processes, such as depolarization of membrane potentials, modulation of burst frequencies, and alteration of spiking patterns (David and Sidney, 1981). However, prolonged stress can lead to irreversible neuronal damage (Samokhvalov et al., 2008). For instance, TiO_2-NP stress downregulates related genes in pearl oyster neurons, leading to decreased neuronal excitability and inhibited neuronal activity (Yang et al., 2023). Furthermore, Guan et al. (2018) discovered that high-concentration TiO_2-NPs induced substantial neurotoxic effects on *Tegillarca granosa*. In addition, NPs are potentially neurotoxic for mammals and teleosts (Sheng et al., 2016; Ze et al., 2016). Neurofunctions are directly linked to the signaling pathway of neuroactive ligand-receptor interactions (Duan et al., 2016). Neuronal function is affected by neuroactive ligands that bind to intracellular receptors, which in turn can bind with transcription factors and regulate gene expressions (Xu et al., 2012). Disruption of genes associated with neuroactive ligand-receptor interaction results in compromised memory functions (Papassotiropoulos and de Quervain, 2015). Wei et al. (2020) reported that silica nanoparticles cause developmental neurotoxicity in zebrafish embryos by disrupting the signaling pathway of neuroactive ligand-receptor interactions. Similarly, Gainey and Greenberg (2003) observed that neurotransmitters that are both excitatory, such as acetylcholine, and inhibitory, such as gamma-aminobutyric acid, are important for

mollusks' neural signal transduction. Previous studies have highlighted the vital roles of neurotransmitter receptors in responding to changes in neurotransmitters (Mao et al., 2017). Consequently, the downregulation of genes encoding these receptors demonstrates that TiO_2-NPs exposure may disrupt various physiological processes regulated by neurotransmitters (Guan et al., 2018). Here, we identified a total of 23 DEGs significantly associated with pathways related to "neuroactive ligand-receptor interaction." Following 14-day TiO_2-NPs exposure, there was an indication of inhibited neuronal activity, marked by substantial downregulation of various neuron-related genes, such as activator of 90 kDa heat shock protein ATPase homolog 1 (PIN0090777), gastrin/cholecystokinin type B receptor-like (PIN2170002), ETS-related transcription factor Elf-5 (PIN0050085), prostaglandin E2 receptor EP4 subtype-like (PIN0100254), cholecystokinin receptor type A-like (PIN0071117), neuronal acetylcholine receptor subunit alpha-10-like (PIN0051212), hypothetical protein KP79_PYT23720 (PIN0110990), pyrokinin-1 receptor-like (PIN0101895), G-protein coupled receptor 54 isoform X2 (PIN0010507), neuropeptide Y receptor type 2 (PIN0030378), probable G-protein coupled receptor 139 (PIN0070411), gamma-aminobutyric acid type B receptor subunit 2-like (PIN0020047), and gamma-aminobutyric acid type B receptor subunit 2-like isoform X1 (PIN0051226). These genes are known to play crucial roles in regulating nervous system processes, such as synaptic transmission, strength, and maturation. After 7 days of recovery, a total of 10 DEGs were significantly associated with pathways related to "neuroactive ligand-receptor interaction," indicating that the transient recovery period restored neuronal activity. Furthermore, seven neuronal genes, including somatostatin receptor type 2 (PIN0101576), allatostatin-A receptor-like (PIN0111909), calcitonin receptor-like (PIN0010366), blastula protease-10 (PIN0091326), cholecystokinin receptor type A-like (PIN0080212), neuropeptide Y receptor type 6-like (PIN0120125), and neurotensin receptor type 1-like (PIN0090157), remained significantly downregulated. This indicates that even after 7 days of recovery, the neuronal activity of pearl oysters could not return to normal levels.

4.7 Impact of TiO_2-NPs on biomineralization activity

Biomineralization is a biological process by which organisms control and promote mineral substances precipitation, such as pearls and shells, through biological mechanisms (Yang et al., 2021). Increased phenanthrene levels, seawater acidification, temperature changes, hypoxia, and pollution can impact the expressions of genes related to shell and pearl formation in economic pearl oysters (Jafari et al., 2023; Yang et al., 2023). Du et al. (2017) discovered that chitin plays a pivotal role as the primary organic matrix in the formation of nacre in the shell of *Pinctada fucata martensii*. The researchers also hypothesized that genes encoding chitinase could have had substantial effects on the biomineralization process (Du et al., 2017). After exposure to TiO_2-NPs, the expression levels of chitinase 1 (PIN0102750), chitinase 3 (PIN0101472), and chitinase-3-like protein 1 (PIN0140499) were substantially downregulated. Studies have demonstrated that proteins containing VWA domains, particularly collagen-related VWA-containing proteins (VWAP), are critical for the organic matrix of nacre and are essential for the formation of nacreous shells in *Pinctada fucata martensii* (Du et al., 2017). A protein discovered in the shell of Pteria sterna, referred to as N66, has been isolated and investigated, indicating that it exhibits activity similar to carbonic anhydrase and can generate crystalline forms of calcium carbonate under laboratory conditions (Rivera-Perez et al., 2019). The shell matrix serves as a complex system that facilitates interactions between proteins and minerals, proteins and proteins, as well as the epithelium and minerals (Marin et al., 2012), being critical for the biomineralization process (Sedanza et al., 2022). Here, the expressions of von Willebrand factor type A (PIN0110615), Full=N66 matrix protein (PIN0020545), and larval shell matrix protein 3 (PIN0090029) genes associated with pearl oyster biomineralization were substantially downregulated in the TE group compared with the TC group following exposure to TiO_2-NPs. This indicates that TiO_2-NPs negatively affected the biomineralization capacity of pearl oysters. This finding is consistent with our previous study, which demonstrated that TiO_2-NP stress damages pearl oyster microstructure (Li et al., 2024a). Moreover, research has indicated that the kelch-like gene plays a role in the prismatic shell layer formation in pearl oysters (Yang et al., 2022). Shell formation involves a co-expression network of extracellular matrix (ECM)-related proteins (Du et al., 2017). The ECM comprises structural and functional macromolecules, such as fibers (e.g., collagen, fibronectin) (Mariman and Wang, 2010). After 7 days of recovery, the biomineralization-related genes kelch-like protein 26 isoform X1 (PIN0081045), von Willebrand factor type A (PIN0110615, PIN0053245), fibronectin type-III domain-containing protein 3a-like isoform X4 (PIN0081841), and collagen alpha-4(VI) chain (PIN0111490) were observed upregulated, indicating that a short recovery period restored some biomineralization capacity of pearl oysters, being consistent with our prior finding (Li et al., 2024b).

5 Conclusion

In this study, transcriptomic analysis was conducted to assess the effects of TiO_2-NPs stress on gene expression in pearl oyster. A total of 911 and 844 DEGs were identified in the TC vs TE groups and the TE vs TR groups, respectively. These findings indicate that exposure to TiO_2-NPs conditions disrupts protein homeostasis, reduces biomineralization, decreases neuronal excitability, promotes apoptosis, inhibits immune responses, and induces oxidative stress. These findings help to provided a significant reference to evaluate the threat of TiO_2-NPs to pearl oysters in the general ecological environment. Nevertheless, future studies are required for a better assessment of these issues, such as whether a longer recovery period can restore their normal levels, the potential interaction of TiO_2-NPs with variable environments (such as water temperature, salinity, and the presence of other pollutants), more comprehensive understanding of the systemic effects of TiO_2-NPs, and how TiO_2-NPs affect pearl oysters populations and their role in the ecosystem.

Data availability statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA017826) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

FL: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JL: Data curation, Methodology, Writing – original draft. ZG: Data curation, Writing – original draft. CY: Conceptualization, Writing – original draft, Writing – review & editing. LS: Data curation, Writing – original draft. YL: Conceptualization, Writing – original draft. YD: Funding acquisition, Writing – review & editing.

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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.

The reviewer [YB] declared a past co-authorship with the author [YD] to the handling editor.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1462589/full#supplementary-material

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