Genome-wide profiling of DNA methylome and transcriptome reveals epigenetic regulation of *Urechis unicinctus* response to sulfide stress

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HIGHLIGHTS

- Sulfide stress induced a rise in the DNA methylation level of Urechis unicinctus.
- Sulfide metabolism and detoxification pathways were up-regulated by DNA methylation.
- DNA methylation regulated and enhanced the oxidative stress pathway.

GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT
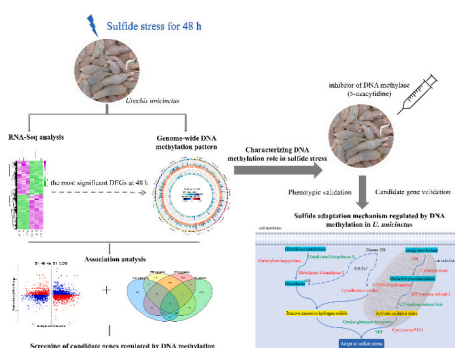
Sulfide is a well-known environmental pollutant that can have detrimental effects on most organisms. However, few metazoans living in sulfide-rich environments have developed mechanisms to tolerate and adapt to sulfide stress. Epigenetic mechanisms, including DNA methylation, have been shown to play a vital role in environmental stress adaptation. Nevertheless, the precise function of DNA methylation in biological sulfide adaptation remains unclear. *Urechis unicinctus*, a benthic organism inhabiting sulfide-rich intertidal environments, is an ideal model organism for studying adaptation to sulfide environments. In this study, we conducted a comprehensive analysis of the DNA methylome and transcriptome of *U. unicinctus* after exposure to 50 μM sulfide. The results revealed dynamic changes in the DNA methylation (5-methylcytosine) landscape in response to sulfide stress, with *U. unicinctus* exhibiting elevated DNA methylation levels following stress exposure. Integrating differentially expressed genes (DEGs) and differentially methylated regions (DMRs), we identified a crucial role of gene body methylation in predicting gene expression. Furthermore, using a DNA methyltransferase inhibitor, we validated the involvement of DNA methylation in the sulfide stress response and the gene regulatory network influenced by DNA methylation. The results indicated that by modulating DNA methylation levels during sulfide stress, the expression of glutathione S-transferase, glutamyl aminopeptidase, and cytochrome *c* oxidase could be up-

regulated, thereby facilitating the metabolism and detoxification of exogenous sulfides. Moreover, DNA methylation was found to regulate and enhance the oxidative phosphorylation pathway, including NADH dehydrogenase, isocitrate dehydrogenase, and ATP synthase. Additionally, DNA methylation influenced the regulation of Cytochrome P450 and macrophage migration inhibitory factor, both of which are closely associated with oxidative stress and stress resistance. Our findings not only emphasize the role of DNA methylation in sulfide adaptation but also provide novel insights into the potential mechanisms through which marine organisms adapt to environmental changes.

1. Introduction

Nowadays, there has been a noticeable increase in the levels of organic matter and other pollutants within the ocean, resulting in deterioration of sediment pollution (Boldrocchi et al., 2023; Strehse and Maser, 2020). H_2S, in particular, is a substance of concern, as it can be highly toxic in excessive amounts, although it serves as a crucial signaling molecule in small amounts. H_2S is widely recognized as an environmental pollutant (Chen et al., 2021; Dilek et al., 2020; Hao et al., 2021). It is high toxic to a wide range of organisms, as it inhibits the activity of cytochrome *c* oxidase at micromolar levels, affecting normal growth (Evans, 1967; Nicholls, 1975). Despite this, natural environments such as deep-sea hydrothermal vents, cold seeps, freshwater sulfide springs, and marine sediments contain sulfide in abundance (Tobler and Culumber, 2016). Remarkably, organisms belonging to various phyla have successfully colonized these toxic habitats, providing valuable resources for investigating the mechanisms underlying sulfide adaptation (Chen et al., 2023a; Kelley et al., 2016; Pfenninger et al., 2014; Sun et al., 2022; Zhu et al., 2021).

Epigenetics refers to changes in gene expression levels that are not caused by alterations in gene sequences (Feil and Fraga, 2012). Epigenetic modification is an adaptive mechanism through which organisms respond to environmental changes, especially in the absence genotype changes (Huang et al., 2021; Pfennig et al., 2010). This is particularly relevant for marine organisms that encounter rapid environmental changes and challenges (Fan et al., 2022; Wang et al., 2021, 2023). Observations have revealed that variations in fine-scale regulation of epigenetic modifications play a significant role in contributing to adaptive differences among populations of marine species (Kenkel et al., 2013; Li et al., 2018a; Place et al., 2012). With the escalation of global ocean pollution, marine organisms face significant selection pressure (Hu et al., 2021; Park et al., 2020; Reinardy et al., 2019). Some marine species populations can mitigate the adverse effects of environmental changes by utilizing epigenetic modifications in response to environmental stress (Yuan et al., 2013; Yang et al., 2023).

DNA methylation is a well-studied epigenetic process (Nasrullah et al., 2022). This process can occur at three different sites: CG, CHG, and CHH (where H represents A, C, or T) (Lämke and Bäurle, 2017). Both promoter and gene body DNA methylation can potentially impact gene expression. Methylation of the promoter region can hinder the binding of genes and transcription factors, while DNA methylation of the gene body region is closely linked to active gene transcription. It is worth noting that actively transcribed gene bodies often exhibit high methylation levels (Feng et al., 2010; Keller et al., 2016; Zemach et al., 2010). DNA methylation modifications play a crucial role not only in regulating the normal growth and development of organisms but also in their response to various abiotic or biotic stresses (Fan et al., 2022; Liang et al., 2019). Numerous extensive studies have consistently shown that DNA methylation is critical in alleviating the harmful effects of stressors on organisms. For example, DNA methylation plays a regulatory role in the phenotypic differentiation of Pacific oysters (*Crassostrea gigas)* inhabitants in distinct temperature environments (Wang et al., 2021, 2023). Additionally, DNA methylation serves as a key mediator of phenotypic plasticity in Pacific abalone (*Haliotis discus hannai*) populations from diverse regions and *Oreochromis niloticus* exposed to long-term cadmium exposure (Hu et al., 2021; Huang et al., 2021). Recently,

Kelley et al. (2021) discovered that over 80 % of DMRs in fish (*Poecilia mexicana*) residing in hydrogen sulfide-rich springs can be inherited across generations. However, the precise role of DNA methylation in sulfide adaptation of sulfide-tolerant organisms remains poorly understood based on current knowledge.

Sulfide is commonly found in intertidal sediments and can reach concentrations of up to 65 µM during low tides (Arp et al., 1992). *Urechis unicinctus*, a species of echiuran that inhabits intertidal sediments, has demonstrated remarkable sulfide tolerance and detoxification capabilities, making it a valuable model species for studying sulfide adaptation (Ma et al., 2011, 2012; Zhang et al., 2013, 2021; Liu et al., 2015; Liu et al., 2022). Although significant progress has been made in identifying the key enzymes involved in mitochondrial sulfide metabolism and signal transduction (Ma et al., 2011, 2012; Li et al., 2018b; Zhang et al., 2021), the regulatory mechanisms underlying sulfide adaptation in *U. unicinctus* remain unclear. Epigenetics is a regulatory mechanism that enables organisms respond to environmental stressors. However, the specific role of epigenetics in sulfide adaptation within *U. unicinctus* has not yet been thoroughly investigated. Therefore, it is crucial to investigate the epigenetic regulatory mechanisms that underlie sulfide adaptability in order to enhance our understanding of sulfide adaptation.

In this study, we utilized RNA-seq and whole-genome bisulfite sequencing (WGBS) data to investigate the potential role of genomic DNA methylation as an epigenetic mechanism in the response of *U. unicinctus* to sulfide stress. By analyzing the relationship between alterations in the transcriptome and dynamic methylation patterns, our goal was to deepen our understanding of the functional significance of epigenetic modifications in environmental adaptation. Our findings uncovered the specific molecular mechanisms and key gene pathways associated with DNA methylation during sulfide stress, providing novel insights into the adaptation of sulfide-tolerant organisms to sulfide-rich environments.

2. Materials and methods

2.1. Animals and sulfide treatment

Adult *U. unicinctus* with an average length of 13.5 ± 2.1 cm were obtained from the coast of Yantai (Shandong Province of China). After acquisition, the worms were temporarily held under aerated seawater conditions (20 °C, pH 8.0, salinity 30 PSU) for three days. Following this acclimation period, 18 healthy worms were randomly divided into three groups, each consisting of six worms. Each of these groups was carefully placed inside sealed aquariums containing 30 L of seawater. To maintain a consistent sulfide concentration of 50 µM, which is considered ecologically realistic (Arp et al., 1992), a sulfide stock solution comprised of 10 mM Na_2S and a pH level of 8.0 was periodically added every 2 h, as guided by sulfide concentration measurements determined using the methylene blue method (Cline and Richards, 1969). Prior to the addition of sulfide (serving as the control condition), as well as at the 48 h mark following the commencement of sulfide exposure, three representative worms were selected for sampling from each respective group, with one individual from each aquarium. Upon collection, the worms were immediately subjected to rapid freezing in liquid nitrogen to ensure the preservation of their biological composition. Subsequently, the frozen specimens were stored at −80 °C to maintain their structural

integrity until DNA extraction.

2.2. Genomic DNA extraction and whole-genome bisulfite sequencing (WGBS)

The hindgut samples from the S_0 and S_48 groups (with three biological replicates per group) were utilized for the extraction of total DNA. The Tissue DNA Kit from TIANGEN (China) was employed for DNA extraction, following the manufacturer's instructions. The genomic DNA from the hindgut tissues of each group was sent to Novogene Corporation (Tianjing, China) for WGBS. To obtain fragmented DNA of approximately 200–300 bp, 100 ng of genomic DNA was spiked with lambda DNA (0.5 ng) and sonicated using a Covaris S220 system. The resulting DNA fragments were then subjected to bisulfite treatment using the EZ DNA Methylation-GoldTM Kit (Zymo Research). Subsequently, the samples were sequenced using an Illumina platform (Illumina, CA, USA). The quality of the library was assessed using an Agilent Bioanalyzer 2100 system. Image analysis and base calling were performed using Illumina CASA V A pipeline, generating 150 bp paired-end reads.

2.3. Reads mapping to the reference genome and differentially methylated analysis

The Bismark software (version 0.16.3) was utilized to align bisulfite-treated reads to a reference genome (Krueger and Andrews, 2011). The reference genome underwent meticulous conversion to a bisulfite-treated version with C-to-T and G-to-A conversions implemented. The converted genome was then efficiently indexed using the bowtie2 algorithm (Langmead and Salzberg, 2012). The methylation extractor output (bismark_methylation_extractor, −-no_overlap) was converted to bigWig format for visualization purposes using the IGV browser. To assess the rate of non-conversion due to sodium bisulfite treatment, the percentage of cytosine successfully sequenced at the reference cytosine positions in genome was calculated (Wang et al., 2021). Differentially methylated regions (DMRs) were identified using the DSS software, and genes related to DMRs were defined as those whose gene body (from TSS to TES) or promoter region (2 kb upstream from the TSS) overlapped with DMRs (Feng et al., 2014; Park and Wu, 2016; Wu and Zhang, 2015).

2.4. GO and KEGG enrichment analysis of DMR-related genes

The gene ontology (GO) enrichment analysis of genes associated with DMRs was conducted using the GOseq R package (Young et al., 2010). This analysis was corrected for gene length bias. GO terms that exhibited a corrected *P*-value below 0.05 were considered significantly enriched by the genes related to DMRs. To assess the statistical enrichment of DMR-related genes in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we employed the KOBAS software (Mao et al., 2005). Through this analysis, we established the statistical significance of the enrichment of DMR-related genes within specific KEGG pathways.

2.5. Association between DNA methylation and gene expression

In our study, we incorporated RNA-seq data obtained from a previous investigation conducted by Liu et al. (2022) and aligned it with the WGBS data from our current study, using the same experimental materials. To investigate the relationship between DNA methylation and gene expression levels, we calculated the average methylation level and fragments per kilobase million (FPKM) value for each gene. Additionally, we classified the genes based on their expression levels to illustrate their corresponding methylation patterns (Wang et al., 2020).

2.6. 5-Azacytidine treatments, DNMT activity, 5mC levels, and survival rate determination

According to the pre-experiment on the optimal concentration of 5-azacytidine (Fig. A.1), each individual of *U. unicinctus* received an injection of 0.1 mL of 5-azacytidine (100 mM) into their coelomic fluid in the experimental group. In contrast, the control group received an injection of 0.1 mL of the 5-azacytidine solvent, which comprised 5 % glucose, propylene glycol, and tween 80 in a 13:6:1 ratio. After a period of 3 h, both the control (Sc_48) and experimental groups (Si_48) were subjected to a 48 h sulfide stress treatment. At the end of the treatment, hindgut samples from both the control group and the experimental group were collected. To measure the activity of DNA methyltransferases (DNMT) and the level of 5-methylcytosine (5mC), frozen hindgut samples from the control and experimental groups at 0 h and 48 h were analyzed using the DNMT ELISA Kit (HP-D203) and 5mC ELISA Kit (HP-A504), following the operation guidelines provided by Coibo Bio, China. The semi-lethal times of the experimental group that received sulfide stress and the inhibitor, the control group that experienced sulfide stress and received the solvent, and the blank control group that received the solvent without sulfide stress were determined. Each group comprised 12 individuals and sets up three biological repeats. Throughout the experimental period, the number of deceased individuals in each group of *U. unicinctus* was recorded at 12 h intervals. Deceased individuals, characterized by their lack of responsiveness to mechanical stimulation, were removed, and the survival rate was calculated.

2.7. qRT-PCR analysis

The methodology used for total RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR) analysis followed that of our previous study, with an annealing temperature set at 60 °C (Chen et al., 2023b). Each sample was analyzed in triplicate and *β-actin* served as the internal reference gene (Table A.1). To compare the expression levels, a two-sided *t*-test was conducted, considering a *P*-value <0.05 as statistically significant and a *P*-value <0.01 as statistically highly significant. The data were presented as mean ± standard error (M ± SE). To determine the significance of differences between the control and experimental groups, an independent samples t-test was performed using IBM SPSS 18.0.

2.8. Detection of individual gene methylation level changes

The extraction and assessment of DNA quality were conducted on the inhibitor-treated samples (Si_48) and control samples (Sc_48). The total DNA from three biological replicates at each time point was pooled equally and used for the examination of methylation levels. Polymerase chain reactions (PCRs) for CpG-rich regions were carried out on bisulfite-treated DNA using primers specific to the converted DNA sequence (Table A.2) (Su et al., 2023). The DNA fragments were then cloned into pMD™19 vector using T-Vector pMD™19 (Simple) Vector Kit (Takara Biomedical Technology, Beijing, China). Fifteen clones for each sample were sequenced to determine their methylation level. The BioAnalyzer software (http://quma.cdb.riken.jp/) was used to compare the sequencing outcomes before and after bisulfite modification and to generate methylation maps.

3. Results

3.1. Whole-genome bisulfite sequencing during sulfide stress in U. unicinctus

In our previous research, we found that there were the most significant DEGs ($|\log_2\text{FoldChange}| > 0.5$, $P < 0.05$) during 50 μM sulfide stress at 48 h compared with 0 h (5111 up-regulated genes and 4924

a

S_48vsS_0

b

c

d
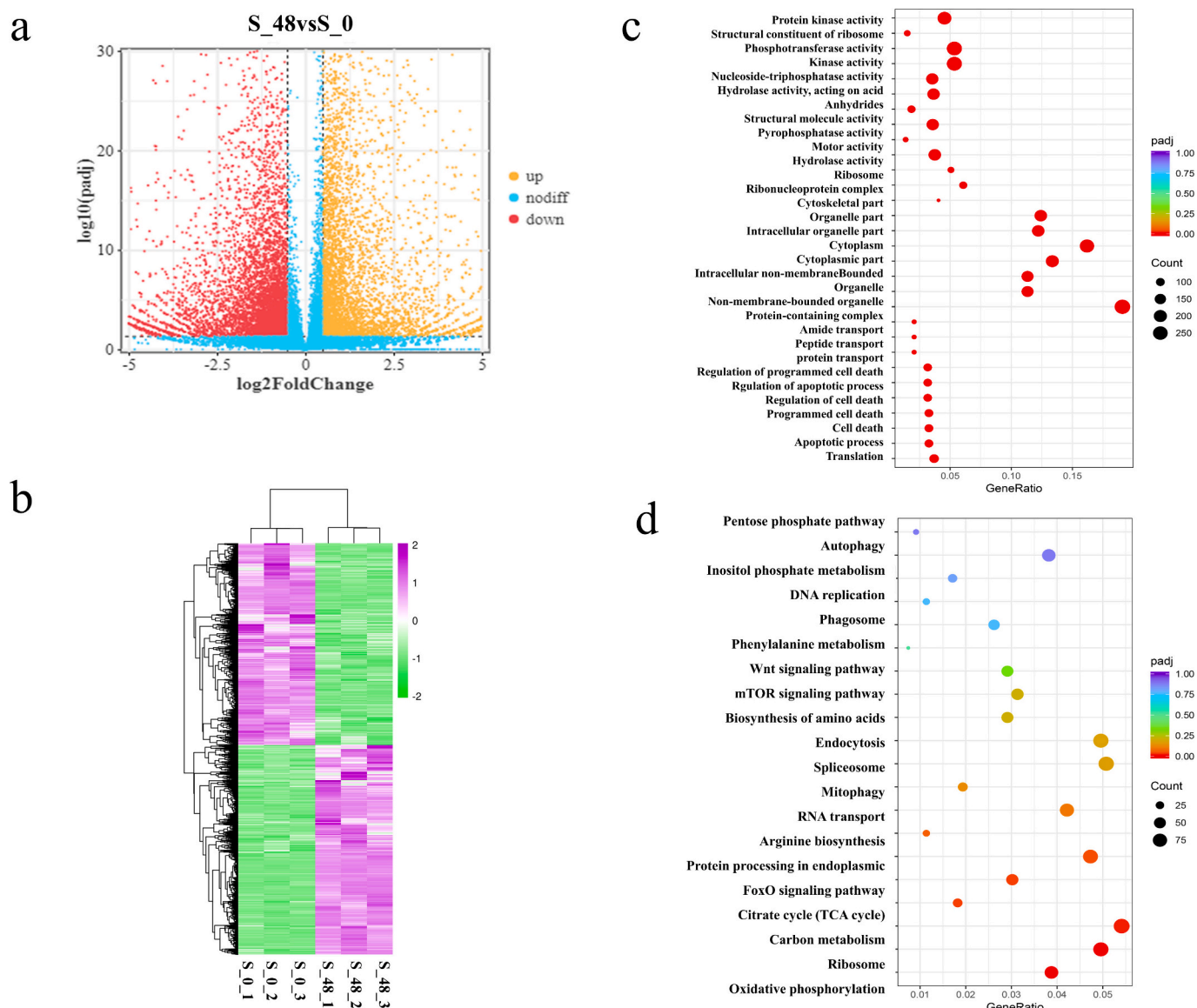
Fig. 1. Identification and verification of different expressed genes (DEGs) in *U. unicinctus* after 48 h sulfide stress.
(a) Volcano plot abundance of DEGs between S_48 and S_0 samples. |log$_2$FoldChange| >0.5, *P* <0.05. (b) Heatmap for DEGs between the two samples. Rows are genes, and columns are abalone derived from two samples. (c) The top enriched GO pathways, resulting from the DEGs between the two samples. (d) The top enriched KEGG pathways, resulting from the DEGs between the two samples.

down-regulated genes) (Fig. 1a-b; Table A.3). To better understand the functional implications of these DEGs, we conducted a GO enrichment analysis. The analysis revealed that the DEGs were predominantly enriched in several biological processes, including translation, apoptotic processes, and cell death (Fig. 1c; Table A.4). Additionally, we conducted a KEGG pathway analysis to identify enriched pathways among the DEGs. The result indicated that several pathways, such as oxidative phosphorylation, ribosome, and carbon metabolism, were significantly enriched (Fig. 1d).

To investigate the potential epigenetic mechanism during sulfide stress, WGBS was performed on S_0 and S_48 samples (three biological replicates per sample), resulting in methylation profiles at a single-base resolution. After trimming and filtering, WGBS generated an average of approximately 155 million and 138 million clean reads for S_0 samples and S_48 samples, respectively, with a conversion rate of at least 99.67 % for all samples. The detailed quality control data can be found in Table A.5. Mapping these reads to the *U. unicinctus* genome, S_0 and S_48 mapped to 72 million and 600 thousand and 72 and 400 thousand reads,

respectively. The unique mapping rate (%) of S_0 and S_48 was 46.83 % and 52.16 %, respectively (Table A.6). Moreover, 73.83 % to 78.88 % of cytosines in the entire genome were covered by at least one read (Table A.7). These results demonstrate the feasibility and accuracy of WGBS for profiling DNA methylation changes during sulfide stress in *U. unicinctus*.

3.2. Genome-wide DNA methylation pattern

To evaluate the overall methylation pattern in *U. unicinctus* under sulfide stress, the average methylation levels of two samples were examined. The S_0 and S_48 samples exhibited average methylation levels of 0.96 % and 1.07 %, respectively, which encompassed all cytosine sites in the genome. Notably, DNA methylation demonstrated a strong preference for CpG sites in both S_0 (4.51 %) and S_48 (5.02 %) samples, while exhibiting lower levels at non-CpG sites (CHG and CHH; H = A, T, and C) (CHG: 0.26 %, 0.28 %; CHH: 0.26 %, 0.28 %). The average methylation level of the S_48 samples was higher than that of
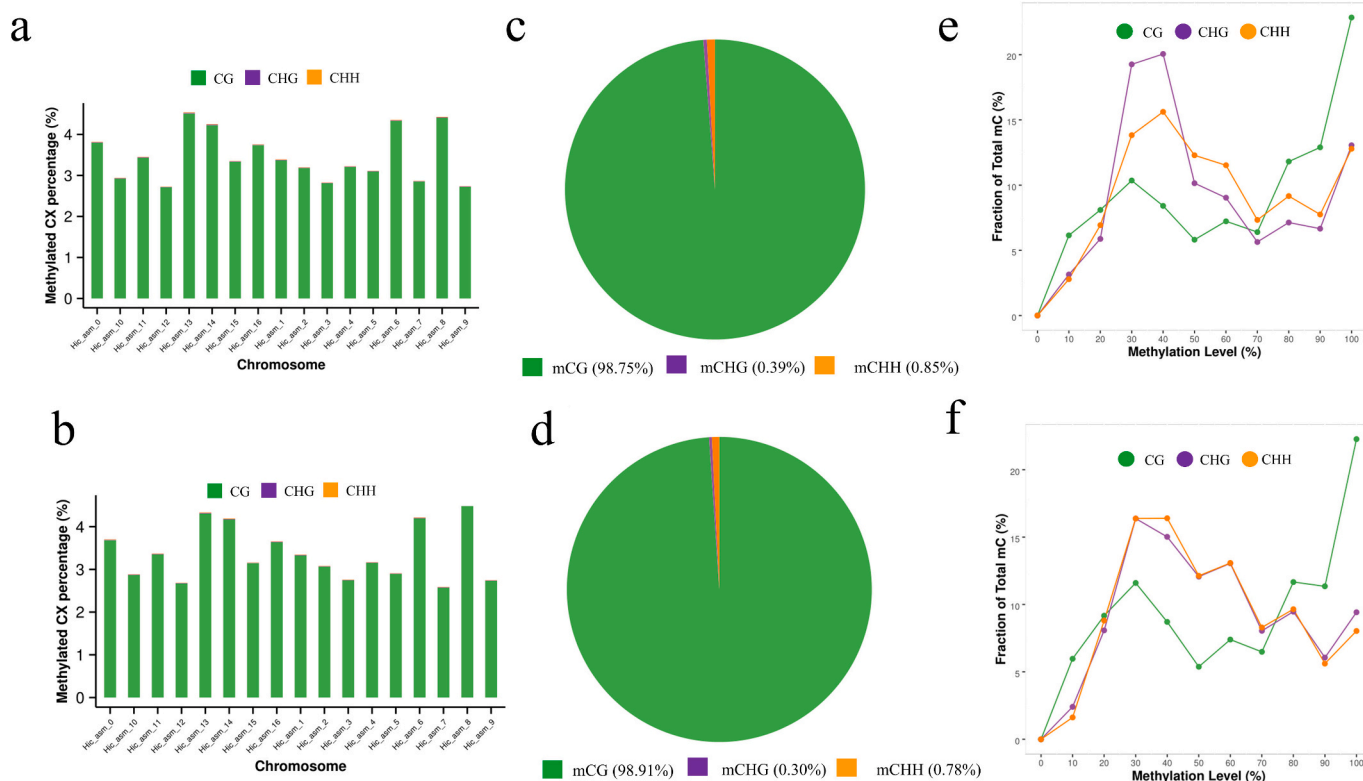
Fig. 2. The distribution of mC.

(a) Percentage map of environmental mC of each sequence at chromosome level in S_0. (b) Percentage map of environmental mC of each sequence at chromosome level in S_48. (c) The proportion distribution map of mC in S_0. (d) The proportion distribution map of mC in S_48. (e) Horizontal distribution map of methylation sites in S_0. (f) Horizontal distribution map of methylation sites in S_48.

the S_0 samples across CpG, CHG, and CHH sequence contexts (Table A.8).

Most of the mC sites on each chromosome in all samples were located in the CG sequence (Fig. 2a-b). In normal growth conditions, the mC site in the S_0 sample comprised of 98.75 % mCG, 0.39 % mCHG, and 0.86 % mCHH. After 50 μM sulfide stress for 48 h, the mC site in S_48 sample was composed of 98.92 % mCG, 0.30 % mCHG, and 0.78 % mCHH (Fig. 2c-d). There was no significant difference in the percentage of mC between the two samples under the three sequence environments after sulfide stress. Furthermore, when the methylation level reached 100 %, the mCpG exhibited the highest proportion of mC. On the other hand, non-CpG contexts (CHG/CHH) generally exhibited lower methylation levels, with mC reaching its peak proportion at methylation levels ranging from 30 % to 40 % (Fig. 2e-f). Principal component analysis (PCA) analysis was performed on the two samples in the three sequence environments, and the CpG sequence environment completely separated the two samples, with PC1 explaining 35.91 % of the difference (Fig. 3a). The mC in the CG sequence context displayed the highest methylation level in the entire gene sequence and various functional regions, especially in the exon and gene body regions. S_48 showed higher CpG methylation levels in all cases than S_0, whereas the non-CpG environment was not significantly different between the two samples (Fig. 3d-e; Fig. A.2). During sulfide stress in *U. unicinctus*, DNA methylation occurred in CpG, CHG, and CHH and demonstrated a strong preference for CpG. These methylation sites were widely distributed in the gene body region, with a certain number of sites located in the promoter and intron regions.

3.3. Analysis of the relationship between methylation and transcriptome

After exposure to sulfide stress, a total of 4481 DMRs were collectively identified (Table A.9). To gain insight into the functional implications of these DMRs, we performed enrichment analyses using GO and KEGG. Our GO analysis revealed that these DMRs were predominantly enriched in several biological processes, including oxidoreductase activity, disruption of cells of other organism, and endospore-forming forespore (Table A.10). In addition, our KEGG analysis indicated that several pathways, such as Metabolic pathways, ABC transporters, and ECM-receptor interaction were significantly enriched (Table A.11). During sulfide stress in *U. unicinctus*, DNA methylation occurs mostly in the CG sequence and is positively correlated with the read density of the transcriptome sequence (Fig. 4a-b). To further explore the potential regulatory effect of DNA methylation on gene expression in the CpG sequence environment, the transcriptional data from S_0 and S_48 samples were associated with DNA methylation data.

There were 1997 DMRs among the DEGs under the CpG sequence environment. The blue part in Fig. 4c represents the part where the DNA methylation level is positively correlated with the gene expression level, while the red part represents the opposite (Table A.12). The CpG sequence environment encompassing all genes was categorized into four grades based on their expression levels: none (FPKM ≤ 1), low ($1 \leq$ FPKM $\leq$ lower quartile value), medium (lower quartile value $\leq$ FPKM $\leq$ upper quartile value), or high (FPKM $\geq$ upper quartile value) (Table A.13). We calculated the methylation levels of the gene body as well as its upstream and downstream 2 kb regions in the CG sequence environment for genes with different expression levels. The results indicated that moderately expressed genes had higher methylation levels in the upstream 2 kb region of the gene body, while lowly expressed genes had lower methylation levels. Additionally, there were nonlinear positive correlations between gene expression and methylation levels in the gene body regions of both the S_0 and S_48 samples (Fig. 5a-b). These findings imply that DNA methylation may be associated with gene expression in *U. unicinctus* under the sulfide stress response.
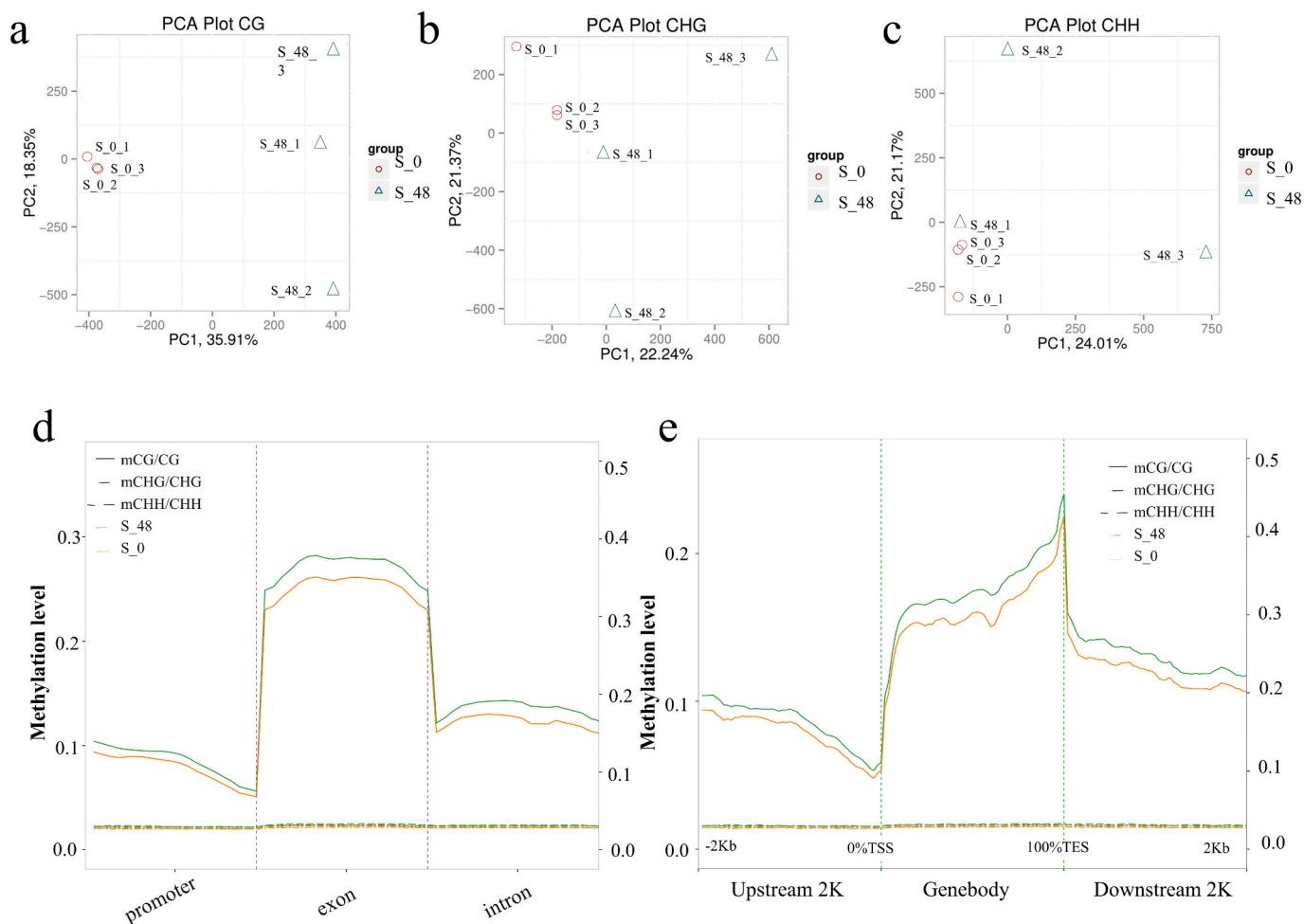
Fig. 3. Principal component analysis (PCA) and Horizontal methylation distribution of CG, CHG and CHH context.

(a) Principal component analysis (PCA) of S_0 between S_48 by CG context. (b) Principal component analysis (PCA) of S_0 between S_48 by CHG context. (c) Principal component analysis (PCA) of S_0 between S_48 by CHH context. (d) Methylation level distribution of S_0 and S_48 on gene functional elements under three context (CG, CHG, CHH). (e) Methylation level distribution of S_0 and S_48 at 2 K upstream and downstream of gene body under three context (CG, CHG, CHH). Abscissa represents different regions and ordinate represents methylation level. Each region of each gene is equally divided into 50 bin, and then the C site level of the corresponding bin in all regions is averaged. Different colors represent groups, and different line types represent different sequences context (CpG, CHG, CHH).

3.4. Screening of candidate genes regulated by DNA methylation in CpG

DNA methylation is a prevalent epigenetic modification that plays a role in regulating growth, development, and response to environmental stresses in organisms. Previous studies have demonstrated that DNA methylation levels in the gene body region are positively correlated with gene expression, while methylation levels in the promoter region are negatively correlated with gene expression (Zemach et al., 2010; Feng et al., 2010). Consequently, we focused our study on genes that exhibited DNA methylation changes, specifically in the gene body or promoter regions. In our study, we observed alterations in DNA methylation levels in the gene body regions of 645 DEGs. Among these genes, 53 % (343 out of 645) were identified as candidate genes regulated by DNA methylation. Notably, 58 % (200 out of 343) of these candidate genes were up-regulated (genes with up-regulated DNA methylation and expression levels). In the promoter region, we observed changes in DNA methylation levels in 304 DEGs. Among these genes, 51 % (155 out of 304) were identified as candidate genes regulated by DNA methylation. Within this group, 33 % (51 out of 155) were up-regulated (genes with down-regulated DNA methylation levels but up-regulated expression levels) (Fig. 5c-d). To further investigate the potential functions of the candidate genes regulated by DNA methylation, we conducted GO and KEGG enrichment analyses. Specifically, we focused on

the DMR hyper genes_DEG up genes and DMR hypo genes_DEG down genes in the gene body region, as well as the DMR_promoter hypo genes_DEG up genes and DMR_promoter hyper genes_DEG down genes in the promoter region.

During sulfide stress, we observed an enrichment of up-regulated metabolic pathways in gene body regions. Notably, stress-related oxidative phosphorylation and important signaling pathways, such as ABC transporters and glutathione metabolism, were significantly enriched (Fig. A.3). Moreover, a considerable number of pathways associated with substance metabolism were enriched, indicating their involvement in the response to sulfide stress. Additionally, in the promoter regions, there was significant up-regulation and enrichment of oxidative phosphorylation (Fig. A.4). This suggests that regulatory mechanisms in the promoter regions play a role in the activation of genes involved in oxidative phosphorylation during sulfide stress. To identify candidate genes regulated by DNA methylation in response to sulfide stress, we focused on the genes involved in these crucial signaling pathways. These seven genes were selected as potential sulfide stress genes subject to DNA methylation regulation (KEGG pathway in Table A.14).

In addition to conducting KEGG enrichment analysis, we identified genes that were not covered by this analysis. For genes regulated by DNA methylation in the gene body region, we focused on those with
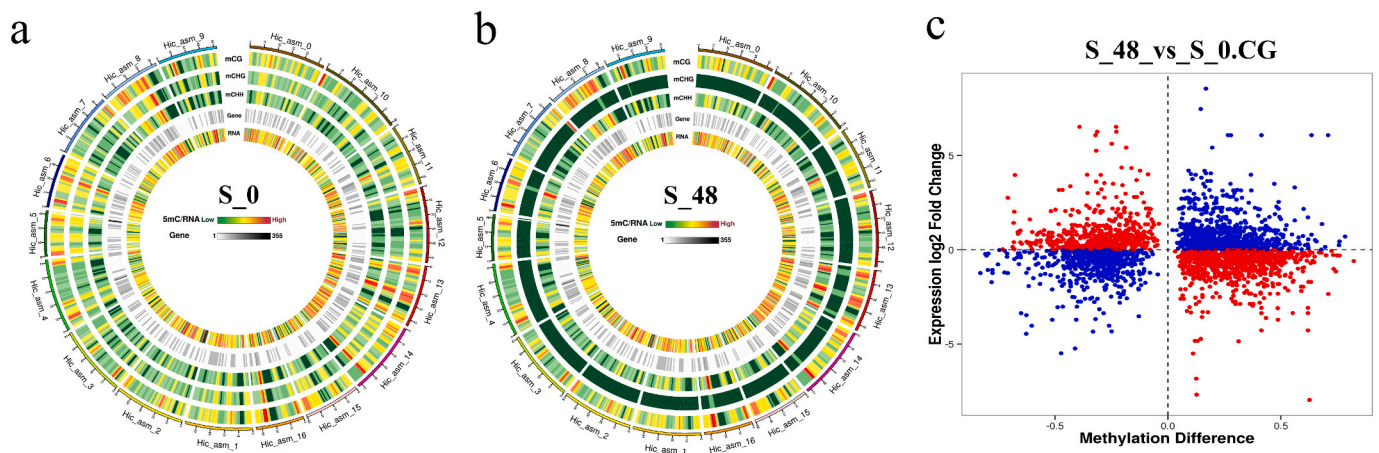
Fig. 4. Chromosomal reads density and methylation density Circos diagram, and relationship between DMR differential methylation levels and related transcriptome differential gene expression levels.

(a) Chromosomal reads density and methylation density Circos diagram of S_0. (b) Chromosomal reads density and methylation density Circos diagram of S_48. From outside to inside, it is expressed in turn: CG sequence environment methylation density, CHG sequence environment methylation density, CHH sequence environment methylation density, gene number density heat map; transcriptional sequence reads density heat map. (c) Relationship between DMR differential methylation levels and related transcriptome differential gene expression levels of S_48 VS S_0. Abscissa represents the difference of methylation level in DMR region, and ordinate represents the difference of expression level of DMR related genes ($\log_2$ (Foldchange)). The red dot represents the high expression level genes in the low methylation level DMR region related genes and the high methylation level DMR region related genes; the blue dot represents the low expression level genes in the low methylation level DMR region related genes and the high expression level genes in the high methylation level DMR region related genes.

increased of DNA methylation and expression levels. We selected twelve genes that were known to be involved in stress response and organized them into five categories based on their functions: oxidative stress (four genes), stress resistance (two genes), cell proliferation (three genes), apoptosis (one gene) and metabolic pathway (two genes). For genes regulated by DNA methylation in the promoter region, we focused on those with decreased DNA methylation levels and increased expression levels. Similar to our selection process for genes in the gene body region, we selected seven stress genes and divided them into three modules: oxidative stress (four genes), cell proliferation (one gene) and metabolic pathway (two genes) (Table A.14).

3.5. Verification of candidate genes regulated by DNA methylation

5-azacytidine is a widely used inhibitor of DNA methylase, which can significantly inhibit DNA methylation in organisms (Griffin et al., 2016). In this study, 5-azacytidine was employed to investigate the impact of DNA methylation on sulfide stress in *U. unicinctus*. Prior to sulfide stress, a dose of 0.1 mL of 100 mM 5-azacytidine was administered to the subjects, resulting in a significant inhibition of DNMTs and 5mC levels in *U. unicinctus* that persisted for at least 48 h (Fig. 6a-b). Semi-lethal times were assessed for the blank control, control, and experimental groups, with no mortality observed in the blank control group during the experiment. The control group exhibited a semi-lethal time of 126 h, whereas the experimental group displayed a semi-lethal time of 96 h (Fig. 6c). These findings indicate a marked decrease in sulfide tolerance of *U. unicinctus* following the administration of a DNA methyltransferase inhibitor.

After conducting a thorough analysis of the qRT-PCR data, we were able to identified genes that are regulated by DNA methylation in response to sulfide stress in both the gene body regions and promoter regions (Fig. 7; Table A.15). Following the administration of the DNA methylase inhibitor, we observed a significant downregulation of successfully validated candidate genes regulated by DNA methylation within the gene body regions. On the other hand, genes regulated by DNA methylation within the promoter regions showed significant upregulation. In the gene body region, several candidate genes within specific functional modules exhibited significant different expression patterns. In the oxidative stress module, cytochrome P450 and

cytochrome *c* oxidase subunit were significant down-regulated (P <0.01) (Fig. 7a). Moreover, genes involved in stress resistance and cell promotion, including isocitrate dehydrogenase, carboxylesterase, tyrosine-protein kinase, and transmembrane protein 45B, were significant differential expressed following inhibitor injection in *U. unicinctus* (Fig. 7b). Notably, candidate genes involved in the KEGG pathway module, such as NADH dehydrogenase, ATP synthase subunit d, cytochrome *c* oxidase, glutamyl aminopeptidase, glutathione transferase, and exocrin-like genes, were also significantly down-regulated (Fig. 7c). In the promoter region, the mRNA expression of macrophage migration inhibitory factor, glutathione S-transferase A, and cystine/glutamate transporter mRNA expression showed significant up-regulation after the injection of inhibitor compared to the control group (Fig. 7d). Additionally, the expression of ATP synthase subunit beta within the KEGG pathway module was significantly different (Fig. 7e).

We selected macrophage migration inhibitory factor (*MIF*) with significant differential expression among the successfully verified genes to compare alterations in DNA methylation levels between the control and the experimental groups. The distribution and statistical analysis of methylation sites within the CpG-enriched regions of the *MIF* promoter regions in the control and inhibitor-treated samples are depicted in Fig. A.5. The average rate of methylation in the CpG-enriched regions in the control group was 71.4 %. However, after administration of the inhibitor, the average rate of methylation sites decreased significantly to 41.9 %. This significant decrease in methylation site rate subsequent to inhibitor treatment resulted in the suppression of DNA methylation within the *MIF* promoter region, consequently leading to the up-regulation of gene expression. These findings were consistent with our previous quantitative gene results.

4. Discussion

The recent findings indicate that the environment can bring about alterations in the epigenetic markers, such as DNA methylation, which can facilitate organisms' ability to adapt to their surroundings. This phenomenon has significant implications for understanding the mechanisms of domestication, especially in the context of changing environments (Artemov et al., 2017; Flores et al., 2013; Lucibelli et al., 2022). Previous studies have demonstrated the high tolerance of
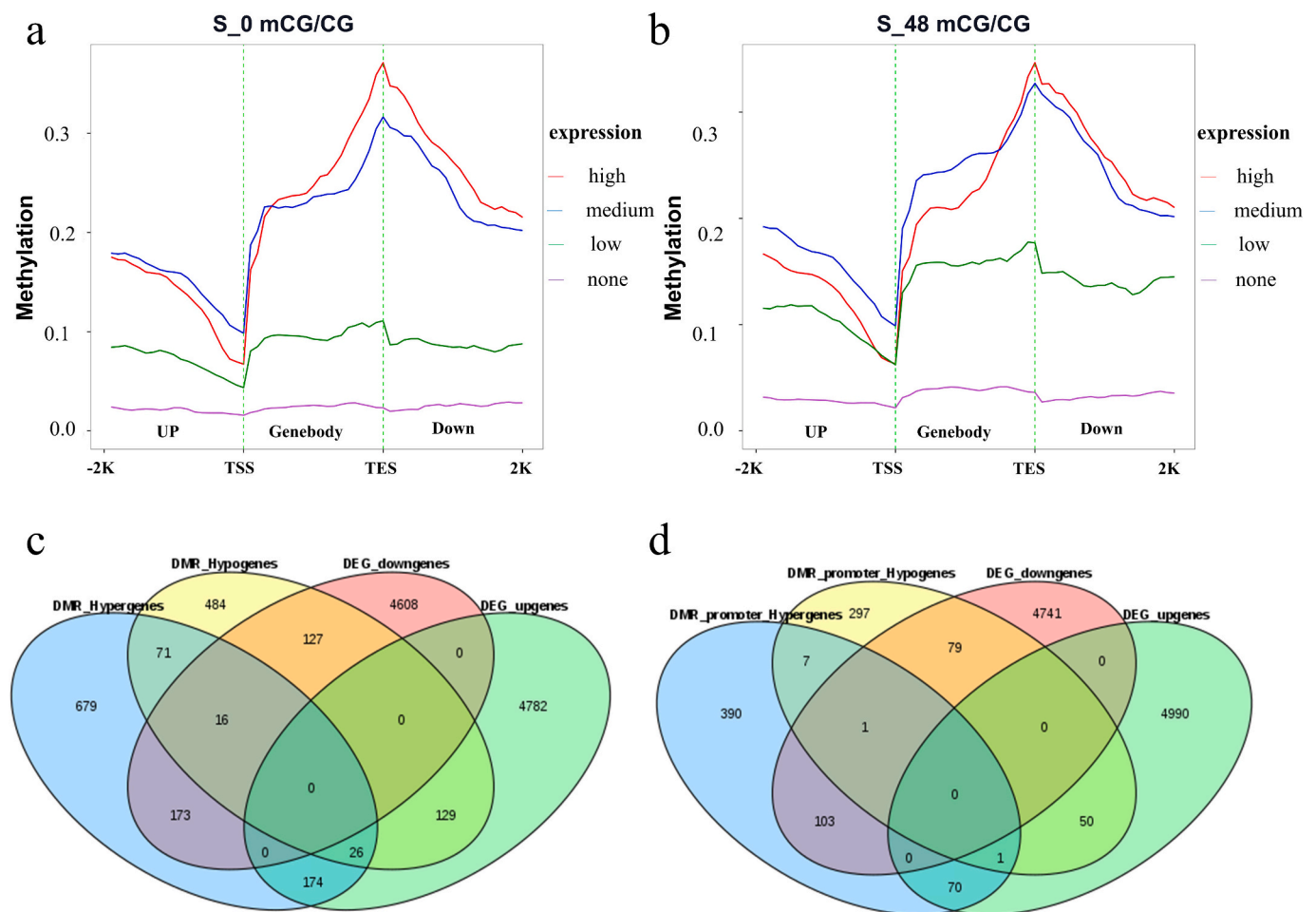
Fig. 5. Methylation level distribution of different expression levels of gene body and its upstream and downstream 2 kb in CG context, and overlapping gene Venn diagram of DMR and DEG.

(a) Methylation level distribution of different expression levels of gene body and its upstream and downstream 2 kb of S_0. (b) Methylation level distribution of different expression levels of gene body and its upstream and downstream 2 kb of S_48. Abscissa represents different regions, ordinate represents methylation level, and different colors represent different levels of expression (none, low, medium, high). Each region of each gene was equally divided into 50 bin, and then the C site level of the corresponding bin in all regions was averaged as the methylation level of the bin. (c) Overlapping gene Venn diagram of DMR and DEG in gene body. (d) Overlapping gene Venn diagram of DMR and DEG in promoter. DMR_Hyper genes: genes that gene body region is anchored by DMR with high methylation levels; DMR_promoter_Hyper genes: genes that promoter region is anchored by DMR with high methylation levels; DMR_Hypo genes: genes anchored by DMR with low methylation levels in the gene body region; DMR_promoter_Hypo genes: genes anchored by DMR with low methylation levels in the promoter region; DEG_Up genes: up regulated genes in transcriptome expression; DEG_Down genes: down regulated genes expressed in transcriptome.

U. unicincus to sulfide stress (Liu et al., 2015; Ma et al., 2012). In this study, we aimed to investigate the molecular mechanism underlying sulfide tolerance in *U. unicincus* by analyzing DNA methylation pattern using transcriptome and DNA methylation data. Furthermore, a DNA methyltransferase inhibitor was used to validate the crucial role of DNA methylation and the sulfide stress gene network regulated by DNA methylation in the process of sulfide adaptation in *U. unicinctus*. Through this approach, we identified several genes and metabolic pathways that appear to be regulated by DNA methylation in *U. unicinctus*, providing valuable insights into the mechanisms of adaptation of the organism. These findings contribute to our understanding of the molecular basis of sulfide stress response and tolerance in *U. unicinctus*. The identification of specific genes and pathways regulated by DNA methylation represents potential targets for further investigation and may have implications for studying similar adaptation mechanisms in other organisms confronting environmental challenges.

4.1. DNA methylation participates in regulating sulfide adaptation

In this study, the methylation landscape of *U. unicinctus* was

examined for the first time. The results indicate that the DNA methylation level in *U. unicinctus* is lower than that in vertebrates, with an average methylation level of approximately 5 % in the genome, and has a strong preference for CpG sequence environment. These results are consistent with previous reports on marine invertebrates such as the Pacific oyster (*Crassostrea gigas)*, Pacific Abalone (*Haliotis discus hannai*), and sea squirt (*Halocynthia roretzi*) (Huang et al., 2021; Wang et al., 2021; Zemach et al., 2010). In contrast, vertebrate genomes tend to be globally and highly methylated, with promoter DNA methylation being a crucial regulatory component of gene expression. However, in invertebrates, DNA methylation levels are generally low and predominantly occur in gene body regions (Jjingo et al., 2012; Keller et al., 2016).

In invertebrates, gene body methylation is a prominent form of genomic DNA methylation that likely functions in regulating gene expression. Invertebrate gene bodies exhibit a clear distinction between highly methylated and sparsely methylated genes, with the former being highly expressed and the latter being low-expressed (Suzuki et al., 2007; Sarda et al., 2012). In contrast, the genomes of most vertebrates are highly methylated, and the variation in methylation levels between
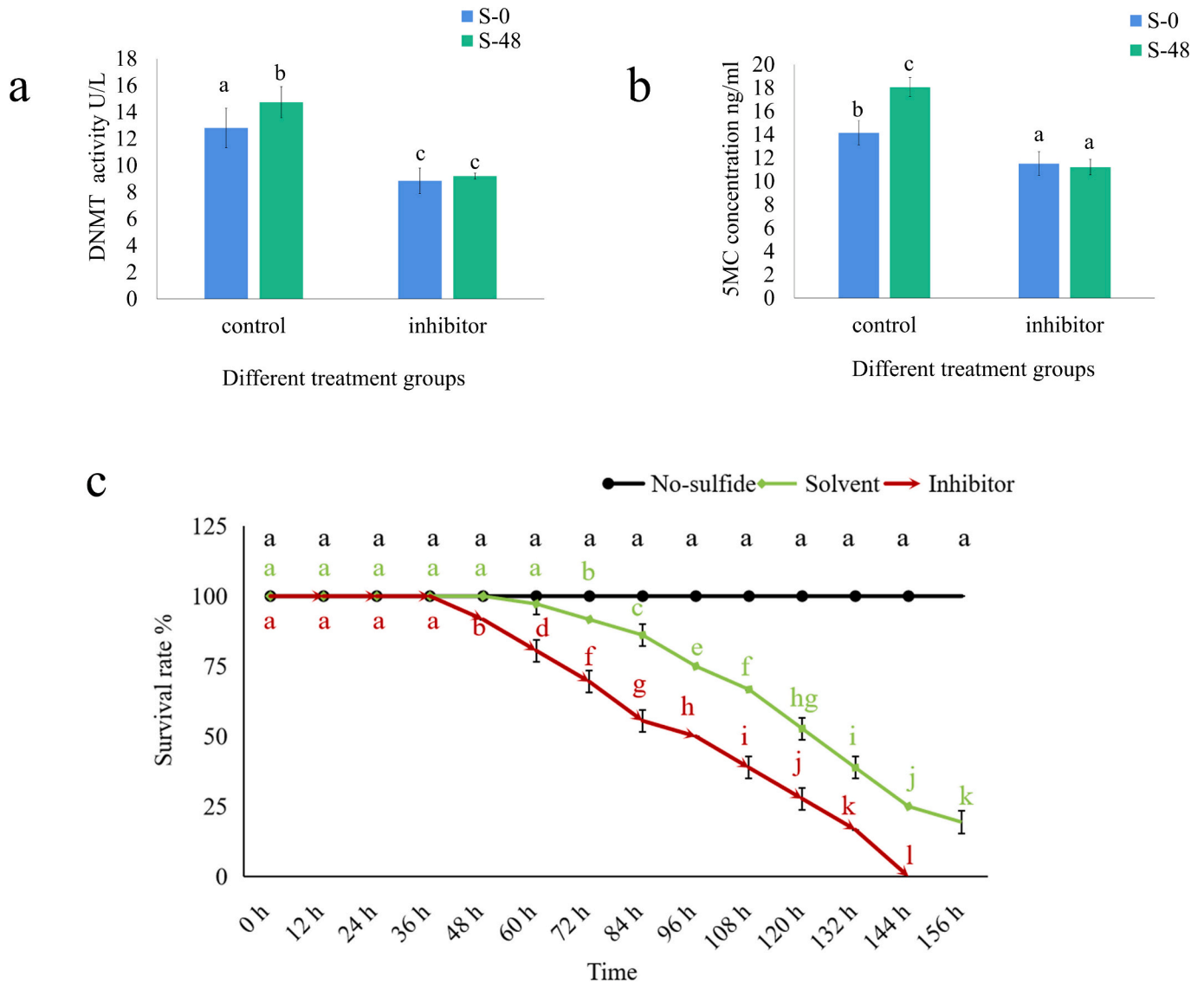
Fig. 6. The determination of sample DNA methyltransferase (DNMT) activity and DNA methylation (5mC) levels, as well as the measurement of semi-lethal times for each group.

(a) DNMT activity determination of S_0 and S_48 samples in control group and experimental group. (b) 5mC level determination of S_0 and S_48 samples in control group and experimental group. (c) The semi-lethal time of the experimental group (Inhibitor), subjected to sulfide stress and injected with the inhibitor, the control group (Solvent), subjected to sulfide stress and injected with the solvent, and the blank control group (No-sulfide), injected with the solvent without sulfide stress, were determined. Each bar represents mean ± standard deviation (SD). Independent samples *t*-test was used to perform statistical analysis. Different letters indicate significant differences ($P < 0.05$).

genes is relatively small (Jjingo et al., 2012). In vertebrates, promoter regions commonly display a bimodal distribution characterized by low methylation levels near ubiquitously expressed housekeeping genes, and hypermethylation in the proximity of tissue-specific genes. Notably, promoter methylation exhibits a negative correlation with gene expression (Antequera, 2003; Elango et al., 2009; Lou et al., 2014; Mendizabal et al., 2014; Saxonov et al., 2006). Gene body DNA methylation and promoter DNA methylation both play a role in regulating gene expression (Jones, 2012; Lou et al., 2014; Park et al., 2012).

The analysis of DNA methylation in *U. unicinctus* revealed an elevation in DNA methylation levels within the gene body region under sulfide stress conditions. This observation suggests a potential role of DNA methylation in facilitating sulfide adaptation processes. Regarding the relationship between methylation and expression in *U. unicinctus*, the study found a non-linear positive correlation between the methylation levels in gene regions and gene expression. The degree of methylation of highly and moderately expressed genes was the highest in the genome.

This observation is consistent with previous studies on oysters (Gavery and Roberts, 2013; Olson and Roberts, 2014), honeybees (Lyko et al., 2011), and mammals (Aran et al., 2011), suggesting that the association between hypermethylation and high expression in the genome is a common phenomenon.

Environmental factors can modify the methylation patterns of gene body, thereby achieving a balance between the expression of housekeeping genes and environmental response genes. This fine-tuned regulation of gene expression facilitates phenotypic plasticity in organisms, enabling them to adapt and thrive in their respective environments (Dixon et al., 2018). Heat stress has been shown to slightly increase the methylation levels of intertidal oysters and the colonial ascidian *Didemnum vexillum*. This suggests that acute heat shock induces DNA methylation to promote phenotypic plasticity (Hawes et al., 2018; Wang et al., 2021). Moreover, this phenotypic plasticity exhibits transgenerational heritability (Wang et al., 2023). The level of DNA methylation in yellow croaker (*Larimichthys crocea*) has been shown to
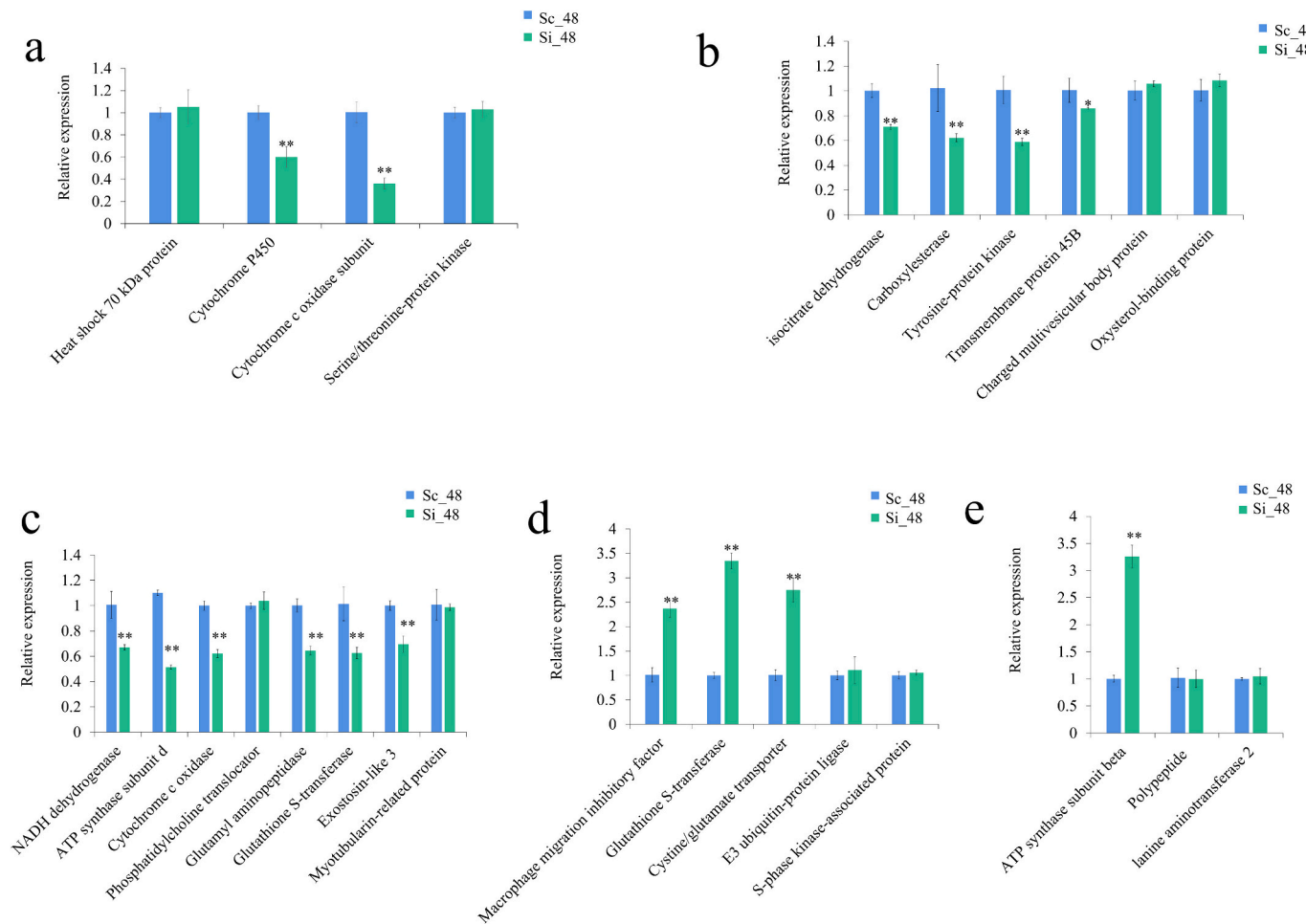
Fig. 7. Relative quantification expression of candidate genes regulated by DNA methylation in response to sulfide stress after inhibitor (Si_48)/solvent (Sc_48) injection.

(a) Relative quantification expression of candidate genes in the oxidative stress module of gene body regions. (b) Relative quantification expression of candidate genes in the Stress resistance, Cell proliferation, Apoptosis module of gene body regions. (c) Relative quantification expression of candidate genes in the KEGG pathway of gene body regions module. (d) Relative quantification expression of candidate genes in the oxidative stress module of promoter regions. (e) Relative quantification expression of candidate genes in the KEGG pathway in the promoter regions module. Each bar represents mean ± standard deviation (SD). Independent samples t-test was used to perform statistical analysis. $*P < 0.05$ between two groups, $**P < 0.01$ between two groups.

undergo dynamic changes in response to starvation stress (Zhang et al., 2019), whereas juvenile Nile tilapia (*Oreochromis niloticus*) increases their environmental adaptability by reducing DNA methylation levels under cadmium stress (Huang et al., 2021).

In this study, the level of DNA methylation increased in response to sulfide stress, particularly in the gene body regions, indicating that DNA methylation may play a role in regulating gene expression and promoting adaptation to sulfide stress in *U. unicinctus*. Furthermore, our results confirmed previous hypotheses, as the delivery of a DNA methyltransferase inhibitor to *U. unicinctus* demonstrated a positive correlation between increased DNA methylation levels and an augmented ability to withstand sulfide stress. To ensure the efficacy of the inhibitor, we assessed the methylation level of individual gene due to the non-specific nature of the DNA methyltransferase inhibitor that we used.

4.2. Primary mechanisms of DNA methylation regulation during sulfide stress

By integrating the DNA methylation and transcriptome data, we performed a comprehensive analysis to identify a specific set of genes in *U. unicinctus* that are regulated by DNA methylation under sulfide stress. To validate these candidate genes, a DNA methylation inhibitor was used, leading to the establishment of a gene network associated with

environmental adaptation controlled by DNA methylation in *U. unicinctus* during sulfide stress (Fig. 8). This network provides valuable insights into the molecular mechanisms responsible for the adaptation of *U. unicinctus* to sulfide stress. One notable finding is that *U. unicinctus* demonstrates enhanced tolerance to environmental sulfide concentrations, potentially achieved through the ability to maintain endogenous sulfide homeostasis despite continuous exposure to sulfide from the environment. The gene network identified in this study provides valuable insights into the molecular basis of sulfide stress adaptation in *U. unicinctus*. Additionally, these findings may have applications in environmental management and conservation.

Our study revealed two primary mechanisms of DNA methylation regulation in *U. unicinctus* that contribute to the maintenance of homeostasis during sulfide stress (Fig. 8). The first mechanism involves accelerating metabolism and detoxifying exogenous sulfides. It is widely recognized that genes involved in H₂S oxidation, such as sulfide: quinone oxidoreductase (SQR), persulfide dioxygenase (ETHE1), and sulfur transferase, play a crucial role in enzymatic H₂S detoxification in metazoans, as previously reported (Greenway et al., 2020; Hildebrandt and Grieshaber, 2008). The up-regulation of enzymes involved in glutathione metabolism aligns with the increased detoxification capability of hydrogen sulfide. This is because sulfur molecules, which are isolated by SQR, can transfer to glutathione (GSH) and bind to the thiol
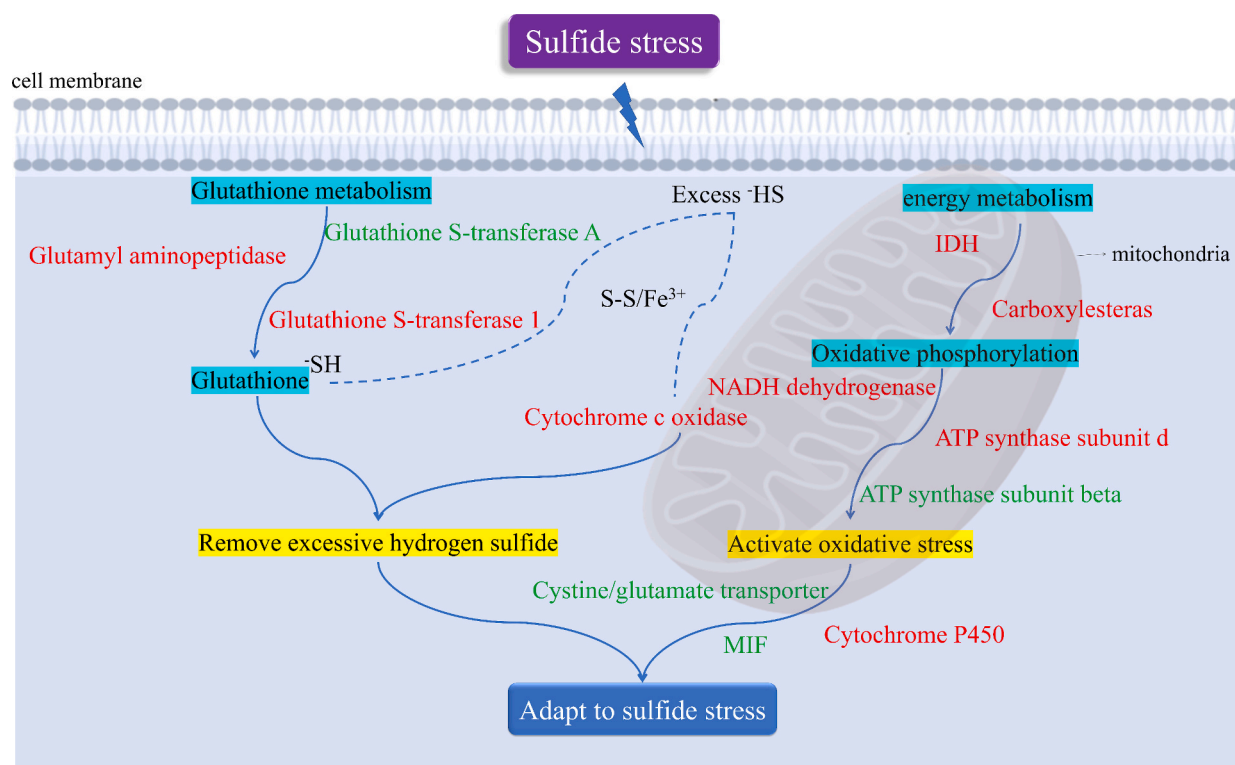
Fig. 8. Sulfide stress gene network of *U. unicinctus* regulated by DNA methylation. The red font represents the gene that up-regulates the level of 5mC in the gene body region, and the green font represents the gene that down regulates the level of 5mC in the promoter region during sulfide stress. The expression levels of all genes in the network diagram are up regulated during sulfide stress.

groups in intracellular GSH (Jackson et al., 2012; Truong et al., 2006; Smith and Abbanat, 1966). Although we did not find evidence of DNA methylation regulation of genes in the SQR pathway, our study demonstrated that genes involved in glutathione metabolism are up-regulated in expression through DNA methylation regulation, with a particular focus on glutathione S-transferases (GSTs). Specifically, GST1 and GSTA are regulated by the gene body region and promoter region, respectively. Additionally, the expression of glutamyl aminopeptidase, which acts as an exopeptidase, was significantly up-regulated. Its function involves cleaving N-terminal acidic residues from peptide substrates, thereby providing the glutamate required for glutathione synthesis (Wang et al., 1998).

The up-regulation of cytochrome *c* oxidase (*cox*) might serve as an adaptive response in sulfidic environments. The parallel evolution of *cox* genes in H_2S-tolerant fish has been considered as a critical adaptation to hazardous environments (Pfenninger et al., 2014). Our study revealed that *cox* is regulated by DNA methylation during sulfide stress, and its gene expression level is significantly up-regulated. Exposure to environmental H_2S is likely to cause deviations in stoichiometric balance. This can be attributed to the inhibition of COX and the transfer of electrons from SQR as well as complexes I and II to coenzyme Q (Lagoutte et al., 2010). Supplementary electron flow has been associated with oxidative stress, and the up-regulation of *cox* can alleviate bottlenecks in electron flow. Additionally, studies have reported that when the concentration of external hydrogen sulfide becomes excessively high and intracellular free hydrogen sulfide and sulfides cannot be promptly oxidized, hydrogen sulfide primarily binds to disulfide bonds or Fe^{3+} in COX of the respiratory chain, leading to the loss of electron transfer ability (Walewska et al., 2018).

The second mechanism involves enhancing the organism's ability to resist oxidative stress caused by sulfide pressure in the environment. When organisms are exposed to external pressures like sulfide stress, they typically activate energy metabolism pathways as a primary response. It is crucial to emphasize that H_2S has a profound ability to generate reactive oxygen species (ROS) and induce oxidative stress (Duan et al., 2019; Sun et al., 2022). Isocitrate dehydrogenase (IDH), a significant regulatory enzyme in the tricarboxylic acid (TCA) cycle, is significantly up-regulated during stress. IDH plays a role in numerous redox reactions and influences metabolic pathways involved in the production and consumption of ROS within organisms. Thus, IDH acts as an intermediary for transmitting stress signals (Noctor et al., 2015). Carboxylesterase (CXE), a hydrolytic enzyme with α/β-sheet hydrolase activity, is widely found in animals, plants, and microorganisms. It plays a crucial role in growth, development, and activation of the oxidative stress pathway during stress resistance (Rui et al., 2022).

Additionally, the activation of the oxidative phosphorylation pathway, a vital component of cellular energy production, leads to an increase in the expression of ATP synthase subunit d and ATP synthase subunit beta. Kelley et al. made a significant discovery that fish (*Poecilia mexicana*) adapted to sulfide-rich springs displayed a notable up-regulation of genes implicated in oxidative stress response, such as NADH dehydrogenase involved in the oxidative phosphorylation pathway, as compared to the fish surviving in sulfide-free habitats (Kelley et al., 2016). NADH dehydrogenase was also significantly up-regulated during sulfide stress in *U. unicinctus*. The remarkable up-regulation of these genes plays a crucial role in energy supply during sulfide stress in *U. unicinctus*.

Cytochrome P450 (CYP) enzymes, which are part of the hemoglobin superfamily, play a role in monooxygenase reactions of various endogenous and exogenous compounds in both mammals and plants. These enzymes are also used as biomarkers for assessing aquatic environmental pollution (Uno et al., 2012). Chronic exposure to hydrogen sulfide has been demonstrated to induce the activity of rat Cytochrome P450 enzymes, specifically CYP1A2 and CYP2B6 (Wang et al., 2013). Macrophage migration inhibitory factor (*MIF*) is a crucial regulator of innate immunity in organisms and plays a significant role in functions such as chemotactic factor activity and the regulation of systemic stress responses (Roger et al., 2003). The up-regulation of *CYP* and *MIF*, along

with oxidative stress-related genes, facilitates the adaptation of *U. unicinctus* to sulfide stress. In summary, DNA methylation up-regulates the energy metabolism and oxidative phosphorylation pathways during sulfide stress to maintain homeostasis in *U. unicinctus*.

5. Conclusions

In this study, we employed a comprehensive approach combining WGBS, transcriptome data, and DNA methylase inhibitor validation to elucidate the mechanism of sulfide tolerance in *U. unicinctus* under sulfide stress. Our findings provide further evidence of the involvement of DNA methylation in orchestrating sulfide stress tolerance phenotypes in *U. unicinctus*. Moreover, we inferred a gene network governed by DNA methylation, uncovering the potential mechanisms of sulfide stress tolerance. This study underscores the significant role of DNA methylation in sulfide stress and contributes to a better understanding of how epigenetic mechanisms are involved in the environmental stress response of marine invertebrates. Furthermore, our study sheds light on the potential significance of DNA methylation in facilitating the adaptation of marine invertebrates to changing environments. However, further investigation is needed to determine the duration of sulfide tolerance regulated by DNA methylation and its potential heritability.

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CRediT authorship contribution statement

Wenqing Zhang: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Long Zhang:** Methodology, Investigation, Data curation. **Yuxin Feng:** Data curation, Investigation, Methodology. **Dawei Lin:** Methodology, Data curation. **Zhi Yang:** Methodology, Data curation. **Zhifeng Zhang:** Supervision, Conceptualization. **Yubin Ma:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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.

Data availability

The genome-wide DNA methylation and transcriptome data presented in this study are deposited in the NCBI repository with the accession number PRJNA975095 and PRJNA752504, respectively.

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