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RESEARCH

Characterization of piRNAs in Diploid and Triploid Pacific Oyster Gonads: Exploring Their Potential Roles in Triploid Sterility

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Abstract

PIWI-interacting RNAs (piRNAs) are crucial for silencing transposable elements, germ cell development, and gametogenesis. Triploid Pacific oysters (*Crassostrea gigas*) are vital in the oyster aquaculture industry due to reduced fertility and rapid growth. This study integrates piRNA and mRNA expression analyses to elucidate their potential contributions to the sterility of triploid *C. gigas*. Bioinformatics analysis reveals a distinct U-bias at the 5′ terminal of oyster piRNAs. The abundance of piRNA clusters is reduced in triploid gonads compared to diploid gonads, particularly in sterile gonads, with a significant decrease in piRNA numbers. A specific piRNA cluster is annotated with the *PPP4R1* gene, which is downregulated in infertile female triploids and exhibits a negative correlation with three piRNAs within the cluster. Differential expression analysis identified 46 and 88 piRNAs in female and male comparison groups, respectively. In female sterile triploids, the expression of three target genes of differentially expressed piRNAs associated with cell division showed downregulation, suggesting the potential roles of piRNAs in the regulation of cell division-related genes, contributing to the gonad arrest observed in female triploid oysters. In male triploid oysters, piRNAs potentially interact with the target genes associated with spermatogenesis, including *TSSK4*, *SPAG17*, and *CCDC81*. This study provides a concise overview of piRNAs expression in oyster gonads, offering insights into the regulatory role of piRNAs in triploid sterility.

Keywords *Crassostrea gigas* · piRNA · Triplod · Sterility · piRNA-mRNA interaction

Introduction

PIWI-interacting RNAs (piRNAs) are a class of novel small non-coding RNAs, typically spanning 24 to 32 nucleotides, initially identified in mammalian germ cells (Aravin et al. 2006; Girard et al. 2006). The notable characteristics of piRNAs include a significant uracil (U) bias at the 5′ end position (1U-bais), a preference for adenine (A) at the tenth base (10A-bais), and 2′-*O*-methylation of the 3′ termini (Iwasaki et al. 2015). piRNAs originate from single-stranded precursors transcribed from discrete genomic loci known as piRNA clusters utilizing a Dicer-independent mechanism. These clusters encompass various transposons and transposon remnants, thereby ensuring genome integrity by silencing transposable elements (TEs) (Siomi et al. 2011). Some piRNAs also derive from 3′ untranslated regions (3′ UTRs) of protein-coding genes, regulating gene expression through complementary base pairing (Robine et al. 2009).

piRNAs specifically interact with PIWI proteins, members of the Argonaute protein family, forming the PIWI-piRNA complex known as the piRNA-induced silencing complex (piRISC) (Meister 2013). The primary function of the piRNA/PIWI pathway is to silence TEs and maintain genome integrity in the germline at both the transcriptional and post-transcriptional levels (Czech et al. 2018; Siomi et al. 2011). Post-transcriptional silencing of target transcripts in the cytoplasm is achieved by PIWI-mediated cleavage via ping-pong amplification (Czech and Hannon 2016). Transcriptional silencing mechanisms regulate target loci through PIWI-mediated DNA methylation or heterochromatin formation (Czech et al. 2018). Numerous studies highlight

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the critical role of the piRNA/PIWI pathway in germ cell development (Ku and Lin 2014). *Piwi* genes are specifically expressed in the testis of mice and regulate spermatogenesis (Yuan and Zhao 2017). Mutation in piRNA pathway genes results in spermatogenesis defects and male sterility (Chuma and Nakano 2013). Deletion of the MILI protein leads to the failure of piRNAs amplification from transposons, resulting in abnormal LINE1 retrotransposon activity and subsequent spermatogenic failure and infertility in mice (De Fazio et al. 2011). PIWI/piRNA complexes are also responsible for translational activation and mRNA degradation during spermiogenesis (Dai et al. 2020). In fly, mutations in *Piwi* genes result in dysregulation of germline stem cell self-renewal and division, ultimately causing infertility (Cox et al. 1998; Gonzalez et al. 2021; Wilson et al. 1996). piRNAs silenced telomeric retroelements (*HeT-A*, *TART*, and *TAHRE*) to maintenance telomere in *Drosophila* (Shpiz et al. 2011). Guo et al. (2018) suggested the presence of piRNAs in chicken germ cells, with piRNA-19128 playing an important role in regulating spermatogenesis by silencing the *KIT* gene. Although the role of piRNAs in gonadal development and germ cell differentiation has been widely investigated in model and vertebrate animals, their regulatory mechanisms in mollusks remain largely unexplored.

The Pacific oyster, *Crassostrea gigas*, is a widely distributed maricultural shellfish known for its high nutritional value and exceptional taste. However, diploid oysters exhibit a reduction in both flesh quality and growth rate during the spawning season. Triploid oysters are intentionally bred to establish sterile populations, thereby enhancing marketability during the reproductive season. Cultured triploid Pacific oysters have become integral to the global oyster aquaculture industry (Dégremont et al. 2016). However, triploid Pacific oysters are not entirely sterile. Histological studies in triploid *C. gigas* revealed that 25-59% of triploids contained numerous gametes (Hermabessiere et al. 2016; Jouaux et al. 2010; Yang et al. 2022b). Yang et al. (2022b) introduced a novel framework for classifying the gonadal development of triploids during the reproduction season. This framework included categorization into female, male, and hermaphroditic types. In female triploids, they identified numerous abnormal germ cells, termed β gonia. The development of oocytes in female triploids exhibited significant variability. In mature stage, female triploids with a substantial number of oocytes were designated as female α (F-3nα), whereas those with few or no oocytes were classified as female β (F-3nβ). In male triploids, although numerous immature spermatocytes were produced, the abundance of mature spermatozoa was markedly low, leading to significantly reduced fertility. Therefore, the mechanisms governing the fertility of triploids garner attention. Researchers have utilized various approaches, including histology, transcriptomics, proteomics, and epigenomics to investigate the sterility mechanisms of triploids (Chen et al. 2021; Sun et al. 2022). The results revealed complex and gender-specific sterility mechanisms in triploids. Xu et al. (2020) also found abnormal expression of the *Piwi-like* gene in the gonads of triploid Pacific oysters, suggesting the potential involvement of the piRNA/PIWI pathway in triploid sterility. However, the role of piRNAs in oyster gonadal development remains unknown.

In this study, we identified piRNAs and investigated their potential functions in the gonads of both fertile and sterile Pacific oysters. Differently expressed piRNAs were identified and the interactions between piRNA and mRNA were explored. The findings offered insights into the regulatory mechanisms of gonadal development and gametogenesis in triploid *C. gigas*.

Methods and Materials

Ethics Statement

The Pacific oysters utilized in this work were cultured animals, and all animal treatments adhered to the guidelines established by the Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201).

Sample Selection and Source of Sequencing Data

All diploid and triploid 2-year-old *C. gigas* specimens were collected from Rongcheng, Shandong province, China, in June 2021. Triploid oysters were generated by hybridizing female diploids and male tetraploids and ploidy was confirmed through flow cytometry (CytoFlex Beckman Coulter, US) using gill filaments. Individual gonad tissues were stored at - 80 ℃. Histological methods, as described by Yang et al. (2022b), were used to observe the gonadal development and determine sex. Raw data for small RNA (accession number: PRJNA791305) and RNA-seq (accession number: PRJNA690125) from the gonads of diploid and triploid Pacific oysters at the stage III (the maturation stage) were downloaded from GenBank (Supplementary Table S1) to identify piRNAs and analyze their expression levels.

Filtering and Annotation of Small RNA Sequencing Data

The small RNA raw data/reads were stored in FASTQ files. Quality analysis of the sequencing data was performed using FastQC (version 0.11.9, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads underwent filtering with Fastp (version 0.23.2) (Chen et al. 2018) to eliminate adapter sequences and low-quality reads ($Q \leq 20$). Additionally, we excluded reads failing outside the range of 18 to 35 nucleotides from the raw data to obtain clean

data. Subsequently, we summarized the length distribution of the clean data. The clean data were aligned to *C. gigas* reference genome (GCF_902806645.1_cgigas_uk_roslin_v1_genomic.fna) using Bowtie (version 2.4.5) (Langmead et al. 2009) without mismatch. The reads that successfully mapped to the genome were utilized for further analysis. Subsequently, the mapped reads were further processed by aligning to microRNAs (miRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) sequences. The miRNAs used in our study sourced from our published research (-v 0) (Chen et al. 2022) and invertebrate miRNAs (-v 2 --best --strata) in miRBase (Griffiths-Jones 2010). Mature miRNAs from various invertebrates, including *Strongylocentrotus purpuratus*, *Caenorhabditis elegans*, *Lottia gigantea*, *Nematostella vectensis*, *Haliotis rufescens*, *Melibe leonine*, and *Drosophila melanogaster*, were incorporated into our analysis. Other known small RNAs (-v 2 --best -strata) were obtained from the Rfam database (https://rfam.org/) (Gardner et al. 2009). The remaining unannotated sequences between 24 and 35 nt were used for subsequent analysis.

Prediction of piRNAs and piRNA Clusters in the Libraries of the Diploid and Triploid Pacific Oysters

We employed proTRAC (version 2.4.2) to predict piRNA clusters and piRNAs (Rosenkranz and Zischler 2012). Our predictions were based in the following criteria: more than 75% of the sequences were between 24 and 32 nt in length; more than 75% of reads with 1U or 10 A; more than 75% of sequence on the main strand (s). In our process, we selected the following parameters: -pimin 24 -pimax 32 -pisize 0.75 -1Tor10A 0.75 -pdens 0.01 -repeatmasker GCF_902806645.1_cgigas_uk_roslin_v1_rm.out -clhitsn 0 -clhits 0 -clsplit 0.1 -distr 1-90 -clsize 1000 -clstrand 0.75 -swsize 5000 -swincr 1000. The transposon annotation file was obtained from NCBI. Sequences that could be mapped to the piRNA clusters were predicted as piRNAs.

Quantification and Differential Expression of piRNAs

We identified differentially expressed (DE) piRNAs between three comparable group (F-2n vs. F-3nβ, F-3nα vs. F-3nβ, M-2n vs. M-3n) using DEseq2 method, which is based on the negative binomial distribution. The expression levels of predicted piRNAs were calculated and normalized using the reads per million mapped reads (RPM) algorithm: $RPM = N/M*10^6$, which N represented the number of unique piRNA reads and M referred to the total number of piRNA reads from each sample. This normalization method allows for a relative comparison of piRNAs expression levels across different samples, making it suitable for identifying DE piRNAs.

piRNA Target Prediction and Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

The target genes of DE piRNAs were predicted using the Miranda algorithm (v3.3a) (Enright et al. 2003), with the following parameters: -sc 150 -en -30 -strict -out. These target genes were compared with the differentially expressed genes (DEGs) identified in transcriptome data. Subsequently, GO and KEGG pathway enrichment analysis were conducted on the DE target genes. These analyses were carried out using the clusterProfiler package in R, which relied on the hypergeometric distribution assess enrichment.

RNA-seq Data Analysis

The raw RNA-seq reads were also saved in fastq files. FastQC (v0.11.9) was used to assess the quality of reads. Reads with a quality value below 20 were removed using Fastp (v0.23.2). The high-quality reads were mapped to the reference genome by Hisat2 (v2.2.1) with default parameters (Kim et al. 2019). We employed FeatureCounts (v2.0.1) to calculate genes expression levels (Liao et al. 2014), and then normalized these levels by the transcripts per million reads (TPM). Differential expression analysis was performed using DEseq2 with the same grouping method, and the standards were set at P-value < 0.05 and $|\log2 \text{ fold change (FC)}| \geq 1.5$.

qPCR Verification of DE piRNAs and DEGs

To validate the sequencing results of piRNAs and mRNAs in the gonads of oysters, we selected nine DE piRNAs and 15 DEGs for quantitative real-time PCR (qPCR). For piRNAs, small RNAs were extracted from gonads using RNAiso (TaKaRa, Japan). The reverse transcription reaction was performed in two steps, genomic DNA removal and first strand cDNA synthesis, using miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) from Vazyme (China). The first step involved mixing 2.0 μL of 5 × gDNA Wiper Mix, 1 μL of small RNA, and 7.0 μL of RNase-free water, followed by incubation at 42 °C for 2 min in a thermal cycler (Thermo Fisher Scientific). The reverse transcription reaction included the previous mixture, 1 μL of Stem-loop primer (2 μM), 2 μL of 10 × RT Mix, 2 μL of HiScript II Enzyme Mix, and 5 μL of RNase-free water. The reaction proceeded at 25 °C for 5 min, 50 °C for 15 min, and 85 °C for 5 min. Specific stem-loop primers and forward primers for the piRNAs and the internal reference were designed using miRNA Design (Version 1.01). The primers for qPCR can be found in Supplementary Table S2. The amplified reaction was performed on LightCycler 480 real-time PCR instrument (Roche, USA) with miRNA Universal SYBR qPCR Master Mix (Vazyme,

China) according to the manufacturer's instructions. U6 snRNA expression was used as the reference for normalizing expression levels.

For mRNAs, we extracted total RNA using RNA-easy Isolation Reagent (Vazyme, China). Total RNA was reverse-transcribed into first-strand cDNA using Evo M-MLV RT Mix Kit (AG, China) following the manufacturer's manual. The qPCR was implemented using SYBR Green Premix Pro Taq HS qPCR Kit (AG, China) on LightCycler 480 real-time PCR instrument (Roche, USA). The PCR reaction mixture (20 µL) consisted of 10 µL 2×SYBR Green Pro Taq HS Premix, 2 µL cDNA, 0.4 µL Primer F (10 µM), 0.4 µL Primer R (10 µM), and 7.2 µL RNase-free Water. The amplification conditions included denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and at 60 °C for 30 s. EF1α was utilized as the internal reference gene for normalization. Specific primers are detailed in Supplementary Table S2. For each qPCR reaction, three biological replicates were performed, and the relative expression levels of piRNAs and mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was carried out using one-way analysis of variance (ANOVA) for multiple comparisons and *T*-test in different groups.

Results

Prediction of piRNAs and piRNA Clusters in the Gonads of Diploid and Triploid Pacific Oysters

We totally obtained 181,786,773 raw reads from the fifteen datasets. After filtering out low-quality tags, adapters, reads shorter than 18 nt and longer than 35 nt, 168,142,879 clean reads remained. The length distribution of the clean data exhibited two peaks, one at 21-23 nt, corresponding to miRNAs, and another at 28-30 nt, indicative of the piRNA fraction (Fig. 1). For further analysis, sequences with length greater than 24 nt, corresponding to the typical length range of piRNAs, were chosen.

A total of 99,608 candidate piRNAs were identified in oyster gonads. Notably, these predicted piRNAs exhibited a pronounced bias towards uracil (U) at the 5′ end position (Fig. 2A). To analyze the genomic distribution of piRNAs, we mapped all putative piRNAs to transposons and gene regions (Fig. 2B). Within all unique piRNAs, there were more piRNAs derived from transposons compared to those from gene regions, including exons and introns. A substantial number of TE-piRNAs were found to be derived from class II transposons (DNA transposons) (Fig. 2B). Specifically, in the female groups, a significant reduction in the number of piRNAs was observed in sterile triploids (the chi-square test, $P < 0.05$). However, no significant difference was observed in the male group.

A total of 125 piRNA clusters were identified (Fig. 2C, Supplementary Table S4). In females, the number of piRNA clusters was notably reduced in triploid oysters, especially in infertile individuals. Similarly, a decrease in the number of piRNA clusters was observed in triploid males. Notably, cluster 99 contained the longest sequence located on chromosome NC_047567.1 (35,016,033-35,038,980), while cluster 87 had the shorted sequence on chromosome NC_047564.1 (52,475,460-52,476,696).

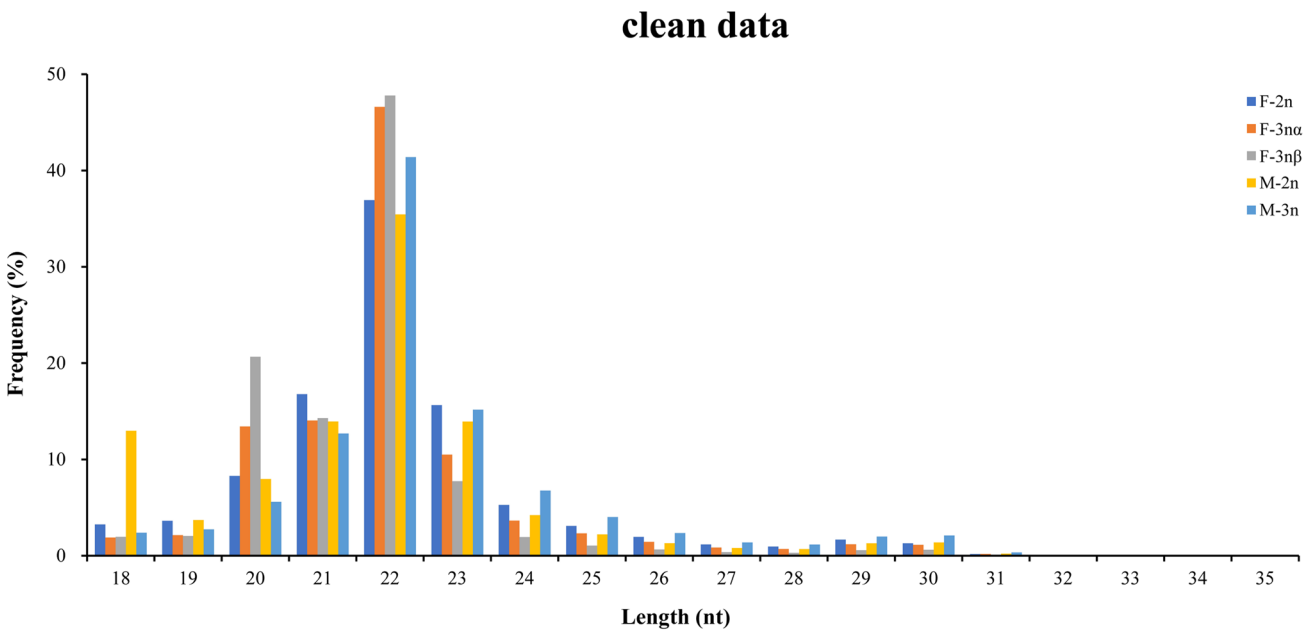


Fig. 1 Length distribution of small RNA clean data. Bar charts represent the percentage of small RNAs at different lengths

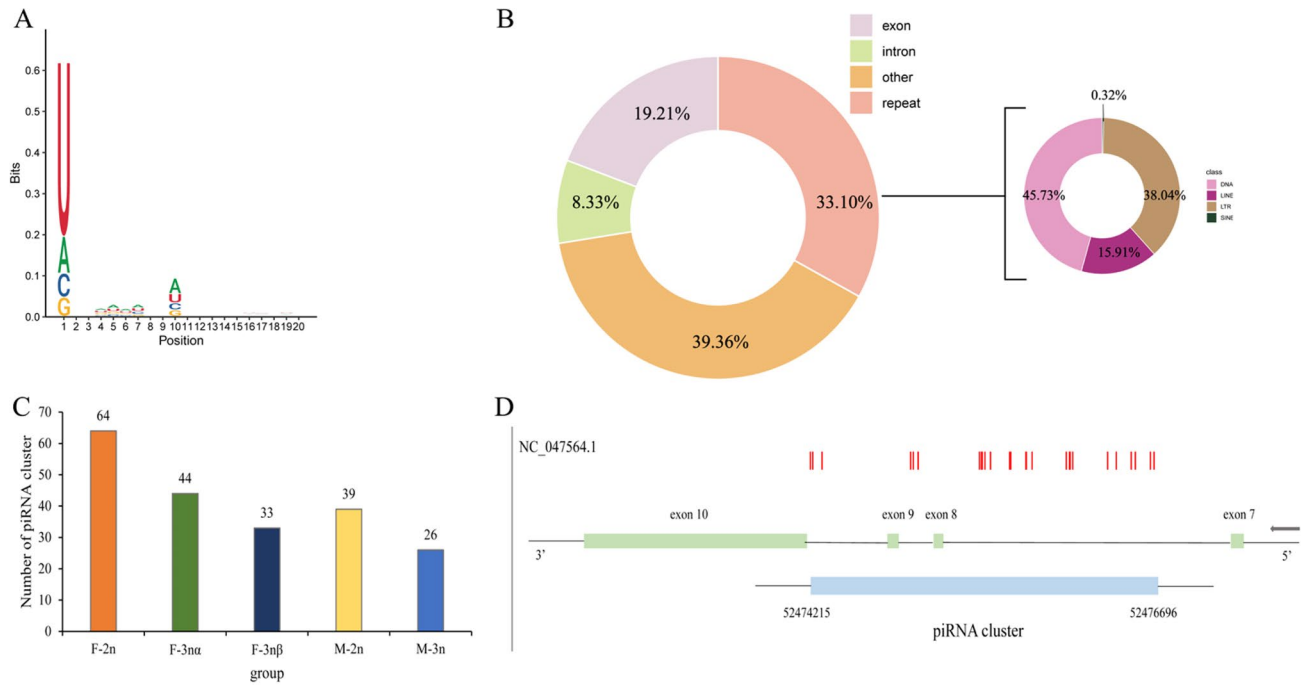


Fig. 2 Characteristics of piRNAs and piRNA clusters in diploid and triploid *C. gigas* gonads. **A** Nucleotide bias at virous positions of piRNAs in oysters. **B** Ratio of piRNAs mapped to transposable elements and genes. **C** Number of piRNA clusters in diploid and triploid oysters. **D** Structure of the piRNA cluster at the PPP4R1 loci. Red lines represent the piRNAs mapped to this region

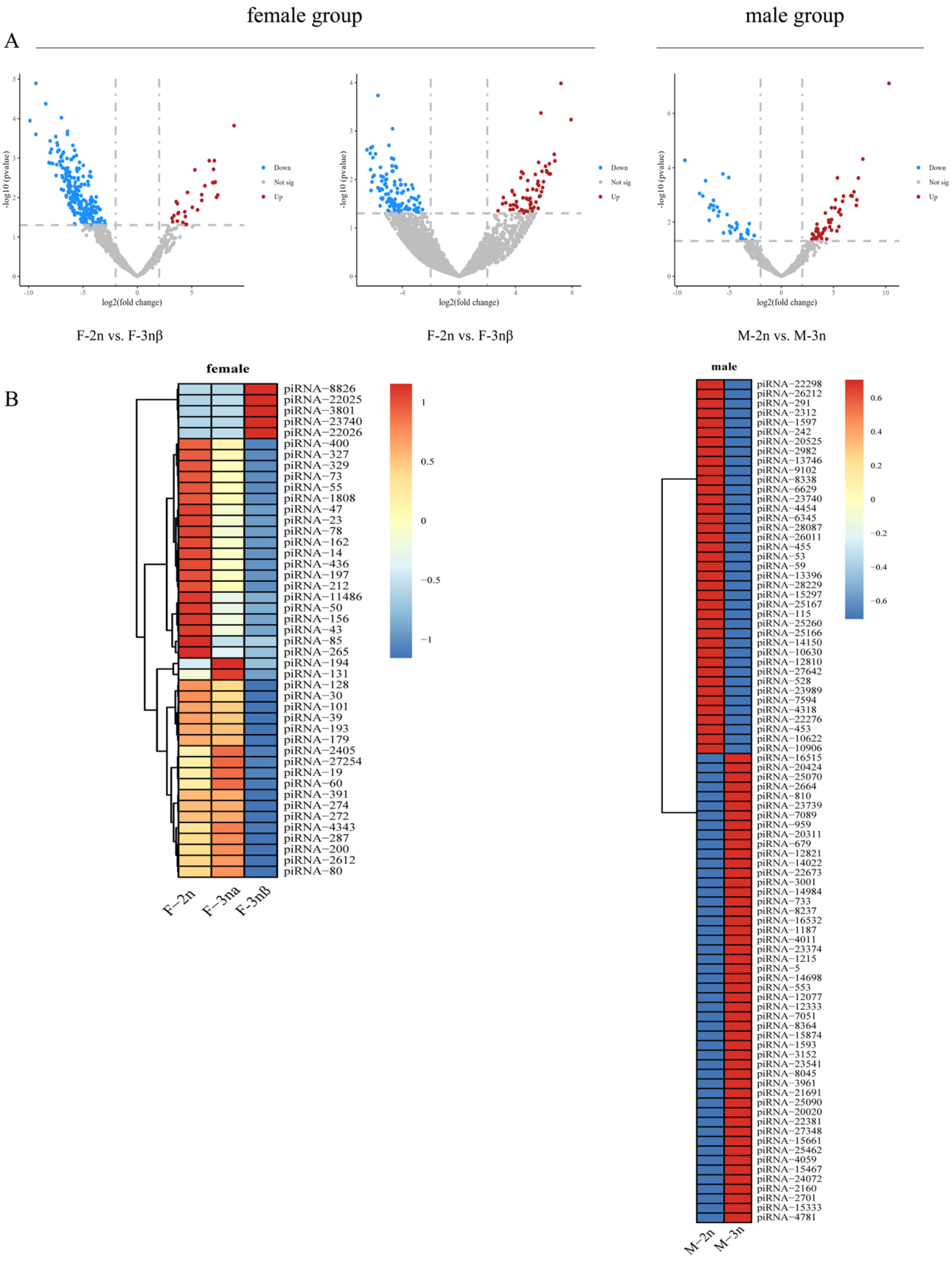
The majority of piRNA clusters were distributed on NC_047560.1 and NC_047561.1. Additionally, we found a large number of transposons and transposons residues, lncRNA loci, and protein-coding genes sites on piRNA clusters. One specific cluster (NC_047564.1; 52,474,215-52,476,696) was annotated with serine/threonine-protein phosphatase 4 regulatory subunit 1 (*PPP4R1*), located between the eighth and ninth exons of the *PPP4R1* gene (Fig. 2D). Meanwhile, the expression of *PPP4R1* was significantly downregulated in infertile triploid females compared to fertile females. Within this piRNA cluster, three piRNAs (piRNA-1, piRNA-25, and piRNA-540) were significantly upregulated in sterile female oysters (Supplementary Figure S1). These piRNAs displayed a negative correlation with *PPP4R1*.

Differential Expression Analysis of piRNAs Between Fertile and Sterile *C. gigas*

To further investigate the regulatory role of piRNAs in the triploid gonadal sterility, we selected piRNAs present in at least two libraries for subsequent analysis (Supplementary Table S3). Our analysis primarily compared fertile oysters with infertile oysters, with the main groups being F-2n vs. F-3nβ, F-3nα vs. F-3nβ, and M-2n vs. M-3n. The DEseq2 method was employed to analyze DE piRNAs, considering those with P-value < 0.05 and |log2 fold change (FC)| ≥ 2 as significantly differentially expressed. Specifically, in the female group, both the 2n and 3nα groups were fertile, whereas the 3nβ group was sterile. Therefore, we compared piRNA expression between 2n vs. 3nβ and 3nα vs. 3nβ, then identified the intersection of these group to screen for DE piRNAs. In total, 45 DE piRNAs were detected in the female comparison group, comprising 5 upregulated and 40 downregulated piRNAs in sterile oysters (Fig. 3A, B). In the male comparison group, 88 DE piRNAs were identified, including 49 upregulated and 39 downregulated piRNAs in sterile oysters (Fig. 3A, B).

Target Gene Prediction of Differentially Expressed piRNAs

To understand the function of differentially expressed piRNAs, we employed the Miranda algorithm to predict the target mRNAs of these DE piRNAs (Supplementary Table S5). Most of the DE piRNAs were found to correspond to multiple target genes, while only a few DE piRNAs corresponded to single target gene. Additionally, some target genes could be targeted by multiple DE piRNAs. In total, DE piRNAs in the female group predicted to target 1068 unique genes, while in the male group, 7542 unique genes were predicted as targets. We found that 47 target genes in females were differentially expressed, and 532 target genes were differentially expressed in males.


female group
male group
A
-log10 (pvalue)
log2(fold change)
Down
Not sig
Up
F-2n vs. F-3nβ
F-2n vs. F-3nβ
M-2n vs. M-3n
B
female
piRNA−8826
piRNA−22025
piRNA−3801
piRNA−23740
piRNA−22026
piRNA−400
piRNA−327
piRNA−329
piRNA−73
piRNA−55
piRNA−1808
piRNA−47
piRNA−23
piRNA−78
piRNA−162
piRNA−14
piRNA−436
piRNA−197
piRNA−212
piRNA−11486
piRNA−50
piRNA−156
piRNA−43
piRNA−85
piRNA−265
piRNA−194
piRNA−131
piRNA−128
piRNA−30
piRNA−101
piRNA−39
piRNA−193
piRNA−179
piRNA−2405
piRNA−27254
piRNA−19
piRNA−60
piRNA−391
piRNA−274
piRNA−272
piRNA−4343
piRNA−287
piRNA−200
piRNA−2612
piRNA−80
F−2n
F−3na
F-3nß
male
piRNA−22298
piRNA−26212
piRNA−291
piRNA−2312
piRNA−1597
piRNA−242
piRNA−20525
piRNA−2982
piRNA−13746
piRNA−9102
piRNA−8338
piRNA−6629
piRNA−23740
piRNA−4454
piRNA−6345
piRNA−28087
piRNA−26011
piRNA−455
piRNA−53
piRNA−59
piRNA−13396
piRNA−28229
piRNA−15297
piRNA−25167
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piRNA−25260
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piRNA−12810
piRNA−27642
piRNA−528
piRNA−23989
piRNA−7594
piRNA−4318
piRNA−22276
piRNA−453
piRNA−10622
piRNA−10906
piRNA−16515
piRNA−20424
piRNA−25070
piRNA−2664
piRNA−810
piRNA−23739
piRNA−7089
piRNA−959
piRNA−20311
piRNA−679
piRNA−12821
piRNA−14022
piRNA−22673
piRNA−3001
piRNA−14984
piRNA−733
piRNA−8237
piRNA−16532
piRNA−1187
piRNA−4011
piRNA−23374
piRNA−1215
piRNA−5
piRNA−14698
piRNA−553
piRNA−12077
piRNA−12333
piRNA−7051
piRNA−8364
piRNA−15874
piRNA−1593
piRNA−3152
piRNA−23541
piRNA−8045
piRNA−3961
piRNA−21691
piRNA−25090
piRNA−20020
piRNA−22381
piRNA−27348
piRNA−15661
piRNA−25462
piRNA−4059
piRNA−15467
piRNA−24072
piRNA−2160
piRNA−2701
piRNA−15333
piRNA−4781
M−2n
M−3n

◂**Fig. 3** Differentially expressed piRNAs between fertile and sterile oysters. **A** Volcano plot showing differentially expressed piRNAs in female and male groups. **B** Heatmap and hierarchical clustering of differentially expressed piRNAs in different groups. Each column represents a group, and each row represents a piRNA. Colors indicate piRNA expression levels, with red representing high expression and blue representing low expression

The reduced number of target genes and DE target genes in females might be due to the intersection of DE piRNAs.

GO and KEGG Pathway Enrichment Analysis

To gain deeper insights into the significance of DE piRNAs between fertile and sterile oysters, we preformed GO and KEGG enrichment analyses using DE target genes. In the female comparison group, the top 30 GO terms (including biological process, cell components, and molecular function) are shown in Fig. 4A. Differentially expressed target genes associated with biological processes mainly participated in sister chromatid cohesion, negative regulation of cell division, and regulation of DNA-templated DNA replication. Differentially expressed target genes were mostly associated with germ cell nucleus, mitotic spindle midzone, and mitotic spindle pole in cellular component. In the molecular function, differentially expressed target genes were mostly involved in Notch binding. In the male comparison group, the top 30 GO terms are displayed in Fig. 4A. Differentially expressed target genes in biological processes were mainly associated with positive regulation of protein maturation and positive regulation of protein processing. Regarding cellular component, differentially expressed target genes were predominantly linked to septate junction, microvillus membrane, and protein complex involved in cell adhesion. In the molecular function, differentially expressed target genes were mostly involved in laminin binding and Notch binding.

The KEGG enrichment analysis showed that most target genes were concentrated in the top 20 KEGG pathways. In females, differentially expressed target genes were mainly enriched in key pathway, such as cell cycle, DNA replication, nucleotide excision repair, meiosis, progesterone-mediated oocyte maturation, DNA repair, and recombination proteins (Fig. 4B). In males, differentially expressed target genes were mainly enriched in important pathways, such as adherens junction, cytoskeleton proteins, necroptosis, and glycosylphosphatidylinositol (GPI)-anchored proteins (Fig. 4B).

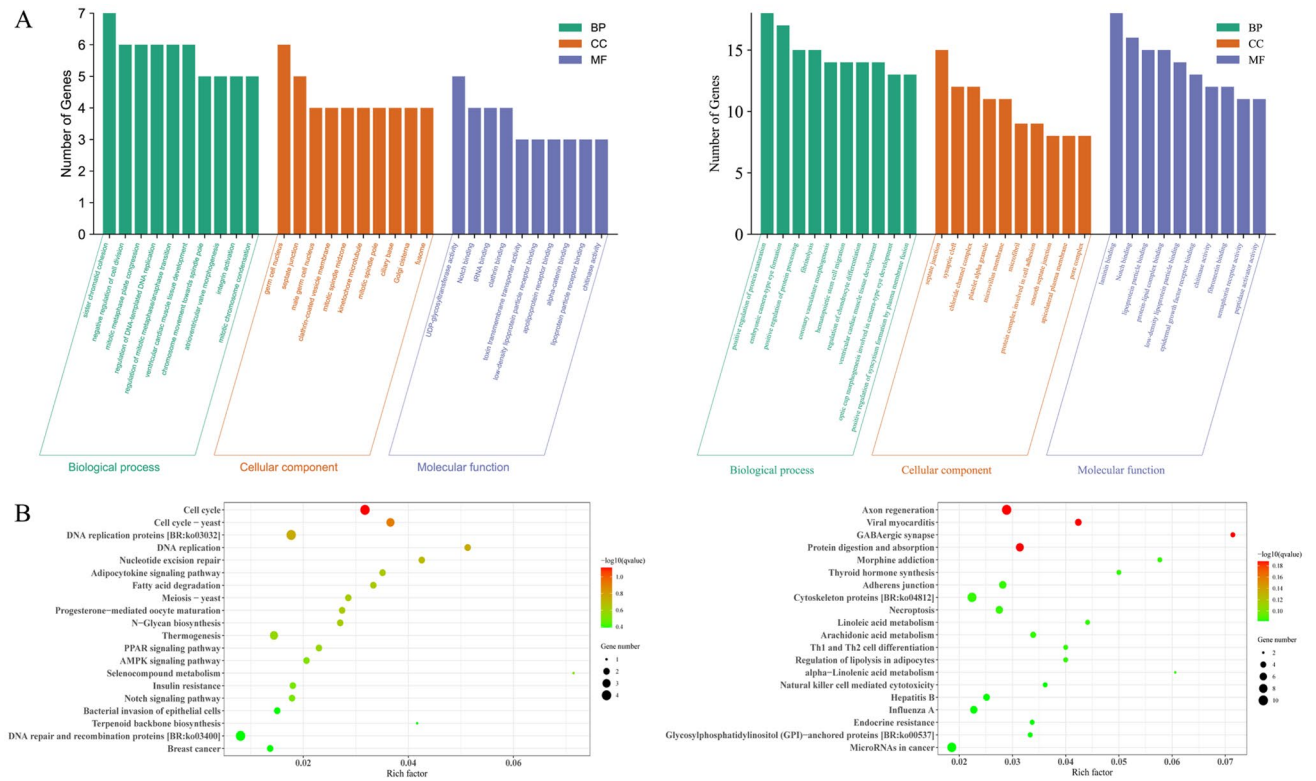

Fig. 4 Enrichment analysis of differentially expressed piRNA target genes. **A** GO distribution of DE target genes. **B** KEGG enrichment pathways of DE target genes in fertile vs. sterile comparisons. The left side represents the female comparison group and the right side represents the male comparison group

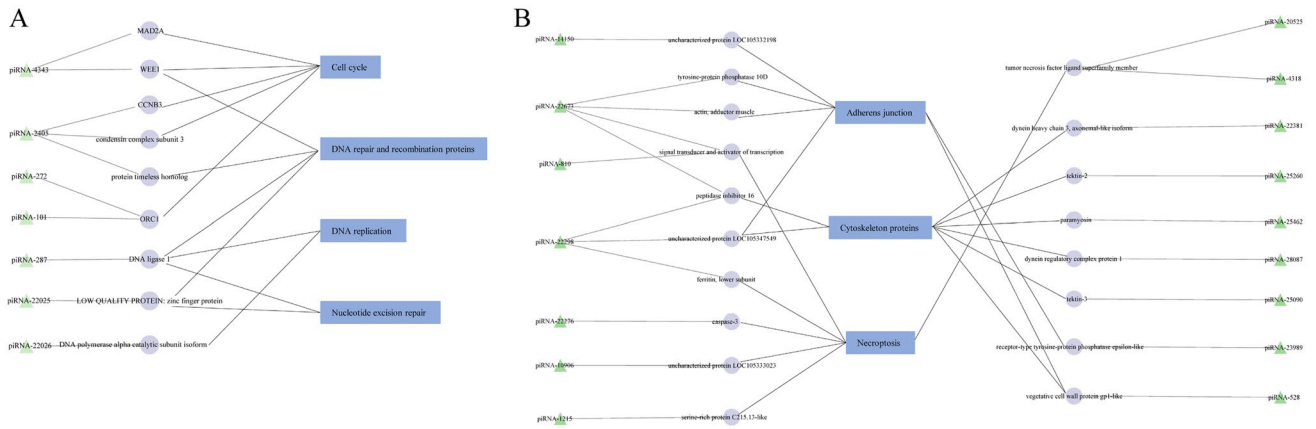

Fig. 5 Differentially expressed piRNAs and their target interactions in networks. **A** Interaction network of piRNAs and their target genes in the female group. **B** Interaction network of piRNAs and their target genes in the male group. The blue boxes indicate the KEGG pathway ($P < 0.05$)

DE piRNAs-DEGs Interaction Networks

To investigate the association between piRNAs and their target mRNAs in fertile and sterile oysters, we focused on the DE piRNAs-DEGs from pathways described above. Subsequently, we constructed interaction networks for these DE piRNAs and their DE target genes using Cytoscape. Our finding revealed a complex regulatory landscape, where a single piRNA could regulate the expression of multiple genes, and conversely, a single gene could be subject to the regulatory influence of multiple piRNAs (Fig. 5).

The qPCR Validation of the Expression of piRNAs and mRNAs

Nine DE piRNAs were selected for validation through qPCR (Fig. 6A). The expression levels of seven piRNAs (piRNA-30, piRNA-101, piRNA-2405, piRNA-156, piRNA-242, piRNA-53, piRNA-25260) were consistent with the results obtained from the high-throughput sequencing (Supplementary Table S6 and S7). Fifteen DEGs (*ORC1*, *CDC7*, *CCNB3*, *ASSP2*, *CUL4A*, *IWS1*, *SETD2*, *TSSK1*, *TSSK4*, *KIF9*, *SPATA17*, *SPAG17*, *CCDC81*, *tektin-2*, *QRICH2*) were also selected for validation using qPCR (Fig. 6B). The results were consistent with the high-throughput sequencing results, confirming the accuracy of the sequences (Supplementary Table S8 and S9).

Discussion

The sterility mechanism of triploid oysters is a highly complex process that remains poorly understood. piRNAs, recently discovered as a class of non-coding small RNAs, are extensively expressed in gonads and germline. These small RNAs recruit PIWI proteins to silence transposons in the germline, regulate histone modifications, and facilitate the formation of heterochromatins. We use a new perspective to explore this uncharted territory and seek to uncover the potential role of piRNAs in the regulation of gonadal development and gametogenesis in triploid oysters.

In this study, a typical piRNA length distribution of 28-30 nt was observed in the gonads of Pacific oysters, which was consistent with previous published study in mollusks (Jehn et al. 2018). Meanwhile, these piRNAs showed a strong U-bias at the 5′ end and A bias at position 10, typical characteristics of piRNAs (Ku and Lin 2014), suggesting that their synthesis in gonads might involve both primary biogenesis pathway and ping-pong cycle.

In *C. gigas*, we revealed the abundance of TEs and TE residues, along with protein-coding mRNAs and lncRNAs within the predicted clusters. piRNAs within the piRNA cluster were mainly concentrated at TE, gene, and lncRNA sites. Comparable to observations in mouse and fly systems, where piRNAs originate from transposons and transposon remnants, the 3′ UTRs of mRNAs, and lncRNAs (Klein et al. 2016; Wang and Lin 2021), our findings suggest that transposons, genes, and lncRNAs are sources of piRNAs in *C. gigas*. Meanwhile, potential roles for oyster piRNAs in transposon silencing, the regulation of gene expression, and interactions with lncRNAs (Han and Zamore 2014). Furthermore, in females, the lower number of piRNA clusters in infertile oysters compared to fertile controls implies a plausible direct association between piRNAs and gonadal sterility in triploid *C. gigas* individuals.

In our study, we identified a specific piRNA cluster annotated with *PPP4R1* gene, suggesting that the *PPP4R1* mRNA serves as the origin of this piRNA cluster. A comparable occurrence has been documented in mouse oocytes, where *PPP4R1* has been identified as a source of siRNAs

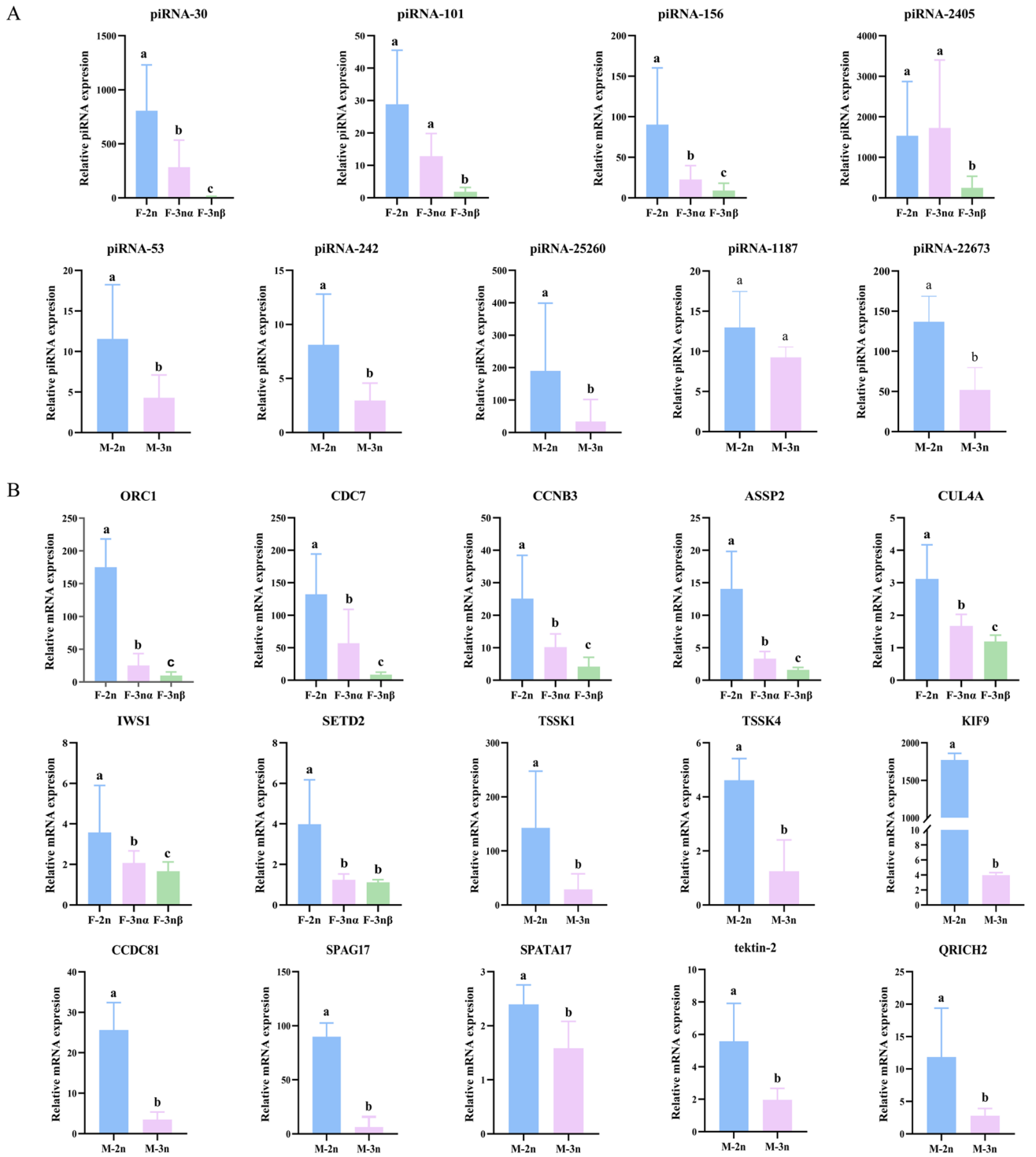

Fig. 6 qPCR validation of piRNAs **A** and mRNAs **B**. Significant differences are indicated by letters (a, b, and c). Bars represent standard error

(Watanabe et al. 2008). Serine/threonine-protein phosphatase 4 (PP4) was a highly conserved protein essential for regulating cell cycle progression, DNA damage repair, genomic stability, and other crucial cellular processes (Kavousi et al. 2023). In humans, DNA double-strand breaks (DSBs) trigger the phosphorylation of the histone H2A variant H2AX, leading to the production of γ-H2AX. The elimination of γ-H2AX at DNA damage foci was critical for effective DNA damage repair. *PPP4R1* specifically dephosphorylated γ-H2AX, facilitating the repair process

(Chowdhury et al. 2008). Moreover, PP4 ensured the proper assembly of microtubule-coupling outer kinetochore before the nuclear envelope decomposes, maintaining normal mitosis (Rocha et al. 2023). The significantly lower expression of *PPP4R1* in infertile female triploids compared to fertile females suggests the presence of a substantial number of DSBs during cell division in triploid females, potentially contributing to infertility. This finding aligned with the earlier discovery of abnormal cyclin gene expression in triploids (Yang et al. 2022a), indicating aberrant cell division during triploid gametogenesis. Importantly, we found that three piRNAs in the cluster were negatively correlated with the expression of *PPP4R1*, indicating that piRNAs likely regulated *PPP4R1* expression at the transcriptional and post-transcriptional levels. These piRNAs might cleave *PPP4R1* mRNA through imperfect base pairing in the 3′ UTR (van Wolfswinkel 2023), or they could facilitate mRNA degradation by mediating CAF1 or CCR4-NOT degradation complexes at the post-transcriptional level (Ramat and Simonelig 2021). Additionally, these piRNAs also downregulated *PPP4R1* gene expression at the transcriptional level through epigenetic mechanisms (Czech et al. 2018).

The analysis of predicted piRNAs in diploid and triploid Pacific oysters revealed a significant number of DE piRNAs. Notably, a majority of these DE piRNAs displayed significant downregulation in triploid individuals. To enhance the robustness of these findings, stem-loop qPCR was employed for the validation of piRNA expression associated with gonadal sterility. The qPCR results aligned with the trends observed in the sequencing data, bolstering confidence in the reliability of the sequencing results for subsequent investigations. In prior investigations of hybrid male sterility, a notable decrease in pachytene piRNA expression was observed in infertile cattle. This reduction was considered a potential contributor to infertility (Zhang et al. 2020). These findings illuminate the potential roles of piRNAs in the gonads of *C. gigas*, suggesting their crucial regulatory functions in oyster gonadal development and gametogenesis. The downregulation of piRNAs in triploid individuals suggests their involvement in the complex mechanisms underlying gonadal sterility in triploid Pacific oysters.

KEGG enrichment analysis of the differentially expressed target genes of DE piRNAs revealed several pathways related to gonadal development and gametogenesis. In the female group, KEGG analysis of DE target genes showed significantly enriched in pathways related to cell cycle, DNA replication, nucleotide excision repair, AMPK signaling, and Notch signaling pathways, as well as DNA repair and recombination proteins, which have been strongly implicated in triploid sterility in previous studies (Yang at el. 2024; Zhang at el. 2024). The previous research observed the downregulation of genes like cyclin B in sterile oysters, resulting in mitotic stasis and abnormal germ cells (Yang et al. 2022a), providing a mechanistic link to cell cycle and DNA replication. This suggests that piRNAs may indeed play a role in regulating these critical processes during gonadal development and oogenesis. In the male group, KEGG analysis revealed that the differentially expressed target genes were associated with pathways such as adherens junction, cytoskeleton proteins, and necroptosis, which are crucial for germ cell differentiation and apoptosis in males. It has been found that the adherens junction is closely related to spermatogenesis. Dysregulation of the adherens junction leads to premature release of sperm from the Sertoli, resulting in male sterility (El-Shehawi et al. 2020; Lui et al. 2003). The cytoskeleton played a crucial role in male fertility by providing structural support and enabling key spermatogenic processes through dynamic regulation of germ cell and Sertoli cell functions (Dunleavy et al. 2019). Absence of cytoskeletal proteins leaded to meiotic arrest or abnormal sperm morphology, ultimately resulting in male infertility. In both human and mouse, necroptosis of spermatocytes has been demonstrated to diminish spermatogenic capacity (Hasani et al. 2022; Sun et al. 2023). Overall, these results highlight the potential regulatory roles of piRNAs in the gonadal development and gametogenesis of triploid oysters, providing valuable insights into the molecular mechanisms underlying gonadal sterility in triploids.

In females, the expression of three target genes associated with cell division showed downregulation in F-3nβ. The *CDC7* gene, potentially regulated by piRNA-156, displayed significantly low reduced in sterile triploid oysters. *CDC7*, a serine/threonine kinase, is activated during the G1/S phase of the cell cycle and plays essential roles in DNA replication. Loss of *CDC7* in mouse embryonic stem cells leads to cell cycle arrest at the G1/S boundary, triggering checkpoint responses and p53-dependent cell death (Kim et al. 2019). Herein, we hypothesized that piRNA-156 and its potentially target gene *CDC7* may exert influence over germ cell mitosis and DNA replication. Another intriguing interaction involved piRNA-2405 with the Cyclin B3 (*CCNB3*) gene, a member of the highly conserved cyclin family that acts as a positive regulator of cyclin-dependent kinase 1 (cdc2). *CCNB3* is associated with cdc2 to form maturation-promoting factor (MPF), crucial for regulating the G1/S and G2/M phase transitions. Mutations in cyclin B can result in female infertility in fly and mouse (Jacobs et al. 1998; Karasu et al. 2019). We deduce that piRNA-2405 may play a role in controlling the cell cycle and germ cell division through *CCNB3* regulation in oysters. Additionally, we identified piRNA-156 targeting the *WEE1* gene, a mitotic inhibitor with serine and tyrosine phosphorylation activities, ensuring proper DNA damage response pathways and regulating the normal G2/M phase of the cell cycle. Inhibition of *WEE1* results in extensive DNA damage and cell death (Elbæk et al. 2020;

Khan et al. 2022). The downregulation of *WEE1* in sterile triploid oysters indicated that piRNAs may be involved in its regulation, ultimately affecting germ cell division. These findings highlight the potential roles of piRNAs in the regulation of cell division-related genes, contributing to the gonad arrest observed in female triploid oysters.

In males, the downregulation of target genes, including *TSSK4*, *SPAG17*, *CCDC81*, and *KIF9*, suggests potential roles of piRNAs in spermatogenesis. Two interesting pairs involve piRNA-25260 and piRNA-28229 with *TSSK4* (testis-specific serine kinase 4), known for its crucial role in spermatogenesis and spermiogenesis. A *TSSK4* knockout mouse model demonstrated defective sperm morphology, leading to reduced sperm motility, subfertility, and infertility (Wang et al. 2015). In *C. gigas*, piRNAs may downregulate the *TSSK4* gene in male triploid oysters, potentially impacting spermatogenesis and contributing to male infertility. Another significant finding involves the potential interaction of piRNA-22673 with *SPAG17* (sperm-associated antigen 17), which encodes a protein found in the axonemes of cells with a microtubule organization known as "9 + 2." Knockout mice lacking *SPAG17* exhibit male infertility due to a significant spermatogenesis defect, which halts spermatogenesis at the spermatid stage (Kazarian et al. 2018). The downregulation of *SPAG17* in male triploid oysters suggested its involvement in spermatogenesis. CCDC81, a recently identified centrosomal protein, is likely to play a significant role in spermatogenesis (Burroughs et al. 2017; Firat-Karalar et al. 2014). Potentially regulated by three piRNAs, *CCDC81* exhibited significant downregulation in male triploid oysters, indicating its potential importance in this process. Collectively, these findings suggest that piRNAs potentially interact with these target genes in male triploid oysters, thereby impairing spermatogenesis, sperm morphology, and motility, ultimately leading to reduced fertility in male triploid oysters.

Conclusion

In this study, we successfully identified piRNAs in the gonads of both diploid and triploid oysters. Through a comprehensive analysis of piRNAs and mRNAs, we uncovered the potential regulatory role of piRNAs in triploid oyster sterility by modulating the expression of target genes associated with cell division, and sperm morphology and motility in females and males, respectively. The insights gained from this research contribute valuable knowledge for future investigations into the underlying mechanisms governing gonadal development in oysters.

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Author Contribution Yaru Zhou designed the experiment, analyzed the data, organized the data and wrote the manuscript. Hong Yu designed the experiment and revised the manuscript. Lingfeng Kong, Shikai Liu and Chengxun Xu analyzed the data. All authors have read and approved the final version of the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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