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Examination of the roles of *Foxl2* and *Dmrt1* in sex differentiation and gonadal development of oysters by using RNA interference

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ABSTRACT

FOXL2 and DMRT1 are transcription factors that play critical roles in sex differentiation and gonadal development in model organisms for both vertebrates and invertebrates. However, studies on *Foxl2* and *Dmrt1* have been restricted to levels of gene expression and localization in adult molluscs because of limitations to experimental conditions. Here, non-invasive RNA interference was conducted in order to investigate the roles of *Foxl2* and *Dmrt1* in sex differentiation and gonadal development in the Pacific oyster *Crassostrea gigas*. The results showed that feeding oysters with dsRNA of *Foxl2* could knockdown gene expression corresponding to *Foxl2* by 62% - 82% and led to a decrease in gonadal coverage area, hindering development in female gonads. Similar knockdown results (58% - 70%) were obtained in male oysters subjected to *Dmrt1* RNAi, causing 54% of the gonads to failing to differentiate. These findings for *Foxl2* and *Dmrt1* in *C. gigas* together with the regulation of *Esr* expression indicated that both genes are involved in gonadal development through an estrogen signaling pathway. The inhibitory effects of DMRT1 on *Foxl2* supported a hypothesis of antagonism between *Foxl2* and *Dmrt1* in the sex-determining pathway in oysters. The results of this study have deepened the understanding of the *Foxl2* and *Dmrt1* functioning underlying bivalve gonadal development and sex differentiation, thus providing useful data for the control of sex in aquaculture.

1. Introduction

Sexual differentiation refers to the expression process of sex-cytodifferentiation genes controlled by sex-determining genes (Sánchez, 2008). *Foxl2* (winged halix/forkhead transcription factor gene 2 gene) and *Dmrt1* (doublesex and mab-3 related transcription factor 1 gene), as downstream genes of the sex-related cascade, play pivotal roles in regulating the process of sexual differentiation and gonadal development (Matson and Zarkower, 2012; Raymond et al., 2000). The FOXL2 gene is specifically expressed in eyelid and adult ovarian granulosa cells as a member of the winged halix/forkhead transcription factor (Alam et al., 2008; Oshima et al., 2008). In vertebrates, FOXL2, β-Catenin and WNT4 are indispensable for promoting and maintaining ovarian development while inhibiting the expression of *Sox9* (Uhlenhaut et al., 2009). For instance, abnormal oocyte growth and infertility were observed in XX mice with a deficiency of *Foxl2* (Ottolenghi et al., 2005). Moreover, *Foxl2* was confimed to have a critial role in sex determination in goats (Boulanger et al., 2014). In fact, FOXL2 can interact with estrogen receptors (ESRs) to produce a specific signal that indirectly determines the sex of vertebrates by acting on gonad-specific enhancer mediated testis SOX9 expression, then inhibiting the expression of *Sox9* (Georges et al., 2014; Uhlenhaut et al., 2009). In invertebrates, the expression of *Foxl2* that is orthologous to vertebrates has no prominent sexual dimorphism. RNA expression and in situ hybridization showed that *Foxl2* was involved in sex differentiation and ovarian development of bivalves (Li et al., 2018; Yue et al., 2021; Zhang et al., 2014). *Foxl2* knockdown in freshwater mussel increased the expression of *Wnt4*, which had an antagonistic relationship with *Foxl2* during ovarian development (Wang et al., 2020). These results indicate that *Foxl2* is not only involved in female gonadal differentiation, but also maintains its differentiation throughout adult life. The DMRT1 gene, which encodes a protein with a conserved DNA-binding DM domain, is a well-described and important gene demanded for male sexual development in a wide range of invertebrates (e.g., flies and worms) and vertebrates (e.g., birds and human) (Matson and Zarkower, 2012; Raymond et al., 2000). Recent evidence shows that *Dmrt*1 is involved in the maintenance of testicular function and gametic formation in zebrafish and silkworms (Guo et al., 2004; Kasahara et al., 2021). Moreover,

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Dmrt1 is required for testicular differentiation following sex determination, but is dispensable for ovarian development in a knockout mouse (Raymond et al., 2000). DMRT activates the expression of testicular specific genes *Sox8* and *Sox9*, inhibiting the expression of ovarian specific genes *Wnt4* and r-spondin-1 signaling protein estrogen receptor, which plays a fundamental role in the sex differentiation of vertebrates (Kim et al., 2007a; Lindeman et al., 2021; Matson and Zarkower, 2012; Murphy et al., 2010; Wu et al., 2020). The functions of *Foxl2* and *Dmrt1* have been widely reported in model organisms of vertebrates and invertebrates, but studies in molluscs have been restricted to gene expression and localization.

The animal kingdom possesses a wealth of mechanisms via which sex is decided. This is no more evident than among oysters, for which no clear mechanism of sex determination in bivalves. The Pacific oyster, *C. gigas*, is a sequential hermaphrodite species (encountering sex changes at some point in their lifespan) without secondary sexual characteristics (Broquard et al., 2019) and represents one of the major farmed shellfish in the world. Reproduction and sex control are vital in aquaculture, but battle with limited knowledge on sex determination and differentiation in most bivalves. Potential downstream gene cascades for sex determination and differentiation have been identified, including female sexual differentiation genes *β-catenin* and *Foxl2*, as well as male sex-determining gene *DSX/Dmrt1* in oysters (Naimi et al., 2009; Yue et al., 2018; Zhang et al., 2014). *Foxl2* may be related to sex differentiation and gonadal development, as expression is increased in gametogenesis cycle of adult male and female oysters (Yue et al., 2021). Through transcriptional analysis, two DMRT family genes (*Dmrt1* and *Dmrta2*) were identified in oysters (Zhang et al., 2015), among which *Dmrt1* was related to sex determination and embryonic development, and *Dmrta2* was involved in larval development and neural development (Zhang et al., 2015). Currently, most studies are limited to the quantification and localization of *Foxl2* and *Dmrt1*. Therefore, the functional verification of these two genes in gonadal development is essential.

RNA interference (RNAi) is a cellular response that introduces double stranded RNA (dsRNA) into cells to cause the degradation of a specific gene mRNA (Fire et al., 1998; Mello and Conte, 2004), which is a form of post transcriptional silencing. At present, three methods of transporting dsRNA, including microinjection (Qiao et al., 2021), feeding (Feng et al., 2019; Payton et al., 2017) and immersion (Baum et al., 2007) have been reported, with feeding first found in the *Caenorhabditis elegans* (Kamath et al., 2001). Bacterial feeding is extensively applied in the study of insect functional genes (Turner et al., 2006), and has also been reported in small invertebrates such as planarians (Newmark et al., 2003), sponges (Rivera et al., 2011) and microcrustaceans (Sarathi et al., 2008). RNAi is classically studied by injecting dsRNA into soft tissues in molluscs, but the approach requires anesthesia and can cause wound and physiological pressure (Payton et al., 2017). Injecting method is frequently limited to a small number of individuals and short duration since the expensive consuming. Recently, Payton et al. (2017) improved the approach by performing microalgae as the carrier of *Escherichia coli*, a dsRNA producer, and applied it to the study of functional genes of molluscs. Subsequently, Feng et al. (2019) applied this approach to explore the functional gene of shell biosynthesis and pigmentation in oysters. Therefore, this method is a promising tool for elucidating the functional genes of filter feeding animals.

Here, a feeding-based RNAi experiment was conducted to characterize sex differentiation and gonadal development in Pacific oysters. Genes encoding FOXL2 and DMRT1 that are potentially critical for sex differentiation and gonadal development were selected as the targets for RNAi. The expression of target genes, state of gonadal development and the expression of other sex-related genes were described under RNAi. These data provide evidence for the function of *Foxl2* and *Dmrt1* in sex differentiation and gonadal development of bivalves, and provide useful data for sex control in bivalves in aquaculture.

2. Materials and methods

2.1. Animals

The oysters (*C. gigas*) were collected from Sanggou Bay in Yantai, Shandong, China in January 2021. The oysters (shell height, 64.5 ± 7.7 mm) were reared in six polyethylene tanks with 50 oysters each tank and placed in 50 L aerated sand-filtered seawater at a temperature 18 ± 1 °C. The oysters were raised and fed according to Feng et al. (2019) for 7 days before the experiment.

2.2. Construction of vectors and expression of dsRNA

L4440 vector with two convergent T7 polymerase promoters in opposite orientation was selected to generate dsRNA in an inducible manner in *E. coli*. DNA fragments of the *Foxl2* (GenBank accession no. LOC105319597) transcripts and *Dmrt1* (GenBank accession no. LOC105337844) transcripts were amplified from the cDNA template of *C. gigas* by PCR amplification using specific primers (Table 1). The *eGfp* (GenBank accession no. U55761.1) transcripts as the control group were amplified in the same manner. Appropriate restriction sites were added to the sense and antisense primers respectively (Fig. 1), and the above DNA fragments were used as templates for amplification to obtain fragments with enzymatic cleavage sites. After the fragments and L4440 vectors were digested by double enzyme, the T4 ligase was applied to obtain target vectors. The connected plasmids were transformed into DH5 competent cells, and five monoclones were selected for sequencing verification. Then target plasmids were extracted and transformed into HT115 (DE3) strain without dsRNA endonucleases RNase III. The induction of dsRNA was performed by overnight culture in LB medium with 50 μg/mL ampicillin and 12.5 μg/mL tetracycline, and 0.4 mM IPTG was added for 4 h at 37 °C.

2.3. RNA interference assay

The experiment was divided into a control group (*eGfp*i) and an experimental group (*Foxl2*i and *Dmrt1*i) with 50 oysters in each group and two repetitions were conducted in February 2021. At this time point, oysters were at resting stage to early proliferating stage, which correspond to undifferentiated or developing early active gametogenesis stage according to the reproductive phases of previous studies (Steele and Mulcahy, 1999). Oysters were fed with alga/dsRNA producing bacteria every 24 h and continuously exposed to the co-inoculum for 60 days. The methods of induction and feeding of dsRNA, the bacteria adsorption rate on algae and the concentrations of algae and bacteria were all carried out according to a previously published article (Feng et al., 2019). The gonads of ten oysters in each experiment were sampled on day 20 and 60, preserved with RNAstore (Solarbio, China) for RNA extraction, and fixed with 4% paraformaldehyde (Solarbio, China) for histological analysis of sex identification. The remaining oysters were used to measure the condition of gonadal development on day 60.

2.4. Quantitative real-time PCR (qPCR)

Total RNA from the oyster gonads were isolated by using TRIzol reagent (Ambion, USA), following the manufacturer's instruction. The relative mRNA expression levels of *Foxl2* and *Dmrt1* were assessed by qPCR after RNA interference. Templates cDNA were synthesized from 1 μg RNA applying the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). ChamQ SYBR Color qPCR Master Mix (Vazyme, China) was used for qPCR analysis and the reaction system (20 μl) consisted of 10 μl 2 x ChamQ SYBR Color qPCR Master Mix, 0.4 μM primers and 2 μl cDNA template. The qPCR was carried out in triplicate for each sample on the LightCycler 480 real-time PCR instrument (Roche Diagnostics, UK) with the following program: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and

Table 1
Primer used in this study.

Gene	Sense primer	Anti-sense primer	Purpose
*Foxl2*i	ACGCCTAACGCTTAGTGGAA	GCCGGATAGTCGTTTAGCTG	RNAi
*Dmrt1*i	GATACCAGCCCCATAGAGCA	GATGGAAGCTCGAAGAAACG	RNAi
*eGFP*i	ACGTAAACGGCCACAAGTTC	TGTTCTGCTGGTAGTGGTCG	RNAi
*Foxl2*out	AATCCTTCAAACAGCAAACCAG	CATTTCCGGCAGAGGACTTC	qPCR
*Foxl2*in	CCAGTGCTATGAACCAAATCG	GATATCTGCATGGCCTGCTG	qPCR
*Dmrt1*out	GCACTCAGTTGGAGATGAAGAAA	TGGCTTTGTAGGGCTGGTATC	qPCR
*Dmrt1*in	GAGCCAGTGTTACAGCCCGCCTG	ATTGCTATACCTCCTCATTTCAG	qPCR

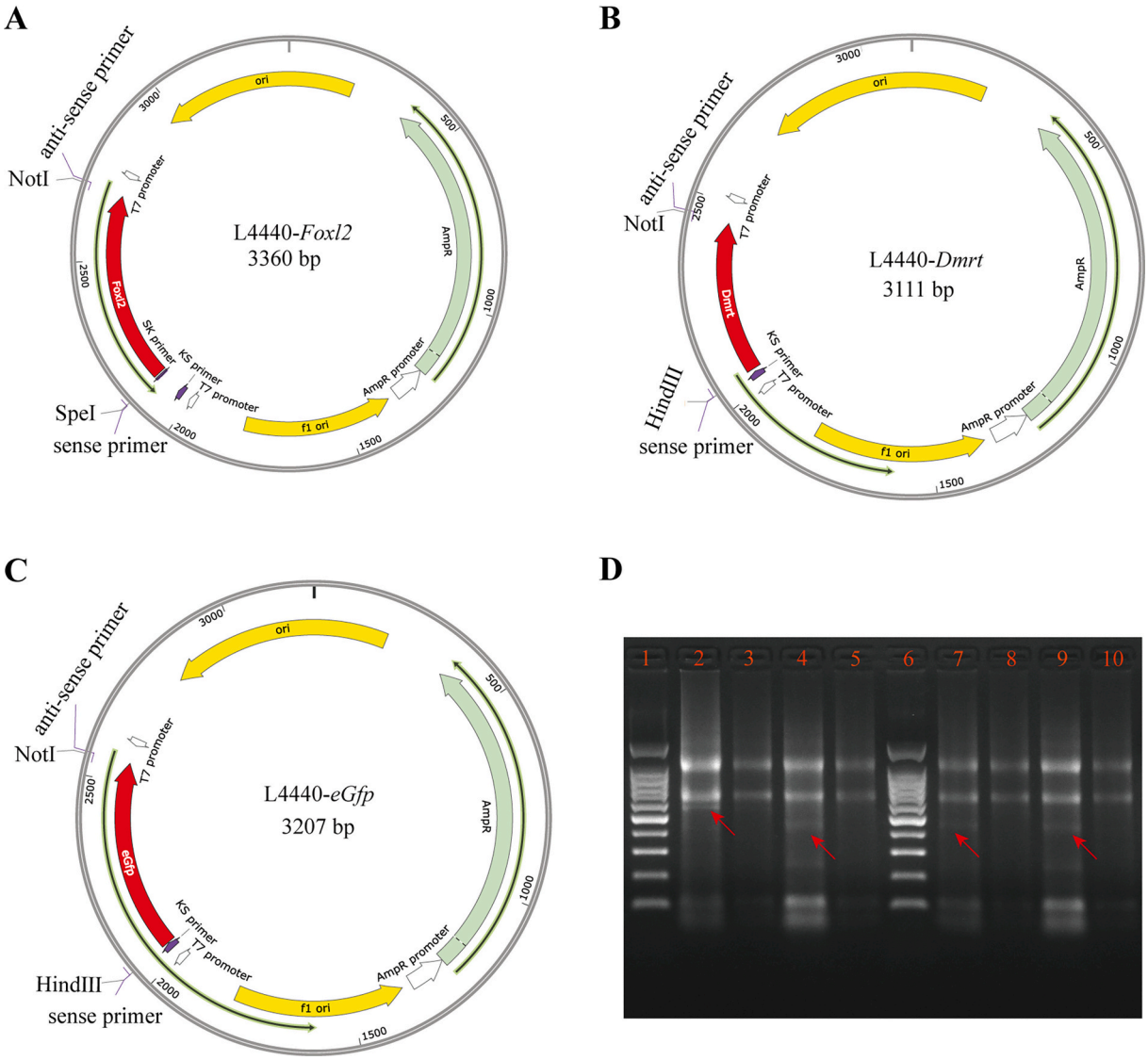

Fig. 1. Construction of plasmid and induction of dsRNA assays. Plasmid construction of *Foxl2*-L4440 (A), *Dmrt1*-L4440 (B) and *eGfp*-L4440 (C) with *Foxl2*, *Dmrt1* and *eGfp* fragment and total plasmid sizes. D Lane 1 corresponded to a 100 bp DNA ladder (Transgene, China). Red arrows indicated bands associated with *Foxl2* (Lane 2), *Dmrt1* (Lane 7) and *eGfp* (Lane 4 and 9) double-stranded RNAs, from RNA extraction of the IPTG-induced *E. coli* transformed with *Foxl2*-L4440, *Dmrt1*-L4440 and *eGfp*-L4440. Lane 3, 5, 8 and 10 represent *Foxl2*, *eGfp*, *Dmrt1* and *eGfp* dsRNAs, from RNA extraction of the non-IPTG-induced *E. coli* transformed with *Foxl2*-L4440, *Dmrt1*-L4440 and *eGfp*-L4440. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

60 °C for 30 s, and a final dissociation curve analysis of 1 cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The elongation factor I (*Ef1*) (Renault et al., 2011) was selected as the internal control and amplified under the same conditions. Relative expression was determined by the $2^{-\Delta\Delta CT}$ method and data expressed as the mean ± SE that analyzed by using the Student's *t*-test with SPSS19.0, and $P < 0.05$ indicated a significant difference.

Detection of RNAi effects in oyster gonads was assessed as the mean ± SE of individuals $2^{-(\triangle Ct(Inside)-\triangle Ct(Outside))}$. i.e., the ratio of the *Foxl2* mRNA level measured with primer sets *Foxl2*in (quantification of both endogenously expressed *Foxl2* mRNAs and *Foxl2* dsRNAs) to the *Foxl2* mRNA level obtained with primer sets *Foxl2*O (quantify the endogenously expressed *Foxl2* mRNAs). Therefore, this ratio is equal to (gene dsRNAs + gene mRNAs)/gene mRNAs = (gene dsRNAs/gene mRNAs) +

1.

2.5. Histological sections of gonads

Gonads were fixed in 4% paraformaldehyde for 24 h, then stored in 70% ethanol. The samples were dehydrated by a series of increasing concentrations of ethanol, removed by xylene, infiltrated with liquid paraffin at 60 °C, and finally embedded in paraffin. The gonads were cut into 5 μm sections and stained with hematoxylin-eosin, then the slides were examined with light microscope. Oyster samples were divided into developed individuals and undeveloped individuals based on whether germ cells could be observed.

2.6. Gonadal coverage area

The methodology standardized by Gómez-Robles and Saucedo (2009) to *Pinctada mazatlanica*, further applied by Rodríguez-Jaramillo et al. (2017) for *C. gigas* and *Crassostrea corteziensis* was adopted here. For each gonadal tissue, finished slides were digitalized and archived. Three different slices were taken from each specimen, and the average area occupied by the gonads was determined by using an image system analyzer (Image Pro Plus v. 6.0) at 4 × scope. Image analysis was based on the intensity of tissue-specific colors and gonadal area was automatically calculated in pixels and represented in μm^2. The gonadal coverage area (GCA) was calculated as: GCA = (gonad occupation area/total area) × 100. The significant differences ($P < 0.05$) of GCA were analyzed by one-way ANOVA.

2.7. Oocyte diameter

The diameter and area of oocytes were determined with digitalized images at 20 × scope by performing the image system analyzer (Image Pro Plus software v. 6.0). Three regions were randomly selected on the slide to detect about 30–40 oocytes with visible nuclei. Since the oocytes deviate markedly from a sphere, the theoretical diameter (TD) is calculated from the total area (A) of each oocyte by the following formula: $TD = \sqrt{(4A/\pi)}$ (Saout et al., 1999). Nested ANOVA was used to test the values of three samples.

3. Results

3.1. dsRNA induction experiment

The expression of target dsRNA was induced in the HT115 strain with 0.4 mM IPTG at 37 °C for 4 h. Total RNA was extracted from the induced and non-induced bacteria by using the Bacteria RNA Extraction Kit (Vazyme, China) and analysis was performed by 1% gel electrophoresis. A band of dsRNA corresponding to the *Foxl2* sequence (576 bp) was observed in the induced bacteria transformed with *Foxl2* constructed plasmid (Fig. 1, Lane 2), while not in the non-induced bacteria (Fig. 1, Lane 3). Similarly, bands of dsRNA corresponding to the *Dmrt1* (399 bp) and *eGfp* (509 bp) sequence were observed in the induced bacteria with *Dmrt1* (Fig. 1, Lane 7) and *eGfp* (Fig. 1, Lane 4 and 9) constructed plasmid, respectively. However, the bands were unobserved in the non-induced bacteria with *Dmrt1* (Fig. 1, Lane 8) and *eGfp* (Fig. 1, Lane 5 and 10) constructed plasmid. Taken together, the target fragments of the genes were successfully induced to express dsRNA in HT115 strain.

3.2. Analysis of RNAi product in gonadal tissues

To determine whether dsRNA was delivered to oyster gonads by feeding bacteria, we assessed the ratio of interfering *Foxl2/Dmrt1* dsRNA RNAi products to endogenous *Foxl2/Dmrt1* mRNA levels in oyster gonads. In the *eGfp*i group, the ratio was closed to 1. While, in the *Foxl2*i group, the ratio of exogenous dsRNA expression to endogenous *Foxl2* gene expression was significantly more than 1, which was 4.1 times and 3.4 times higher than the *eGfp*i group on day 20 ($P = 0.047$) and day 60 ($P = 0.018$), respectively (Fig. 2A). Similarly, the ratio was 2.5 times and 2 times higher than the *eGfp*i group in the *Dmrt1*i group on day 20 ($P = 0.02$) and day 60 ($P = 0.009$), respectively (Fig. 2B). Consequently, dsRNA was successfully delivered into the gonads by feeding the bacteria with the plasmids of target interference fragments.

3.3. Detection of RNAi efficiency of target genes

The plasmids of *Foxl2* and *Dmrt1* were fed to oysters to knockdown their expression, respectively. Since *Foxl2* is specifically expressed in female gonads and *Dmrt1* is specifically expressed in male gonads, the mRNA expressions of *Foxl2* in female gonads of *Foxl2*i and *eGfp*i, and the expression of *Dmrt1* in male gonads of *Dmrt1*i and *eGfp*i were examined by qPCR. The mRNA expression of *Foxl2* and *Dmrt1* in gonads was significantly down-regulated after feeding with plasmids constructed with *Foxl2* and *Dmrt1*. The expression of *Foxl2* mRNA in female gonads was 0.18-fold and 0.38-fold that of the *eGfp*i group on day 20 and day 60, respectively. So, the average knockdown efficiency was 82% and 62% on day 20 and day 60, respectively. In the *Dmrt1*i group, the expression of *Dmrt1* was 0.30-fold and 0.42-fold when compared with the *eGfp*i group in male gonads on day 20 and day 60, respectively. So, the average knockdown efficiency was 70% and 58% on day 20 and day 60, respectively (Fig. 3).

3.4. Impact of RNAi on gonadal development

All oysters were sampled and obvious phenotypic dissimilarities were observed in their developmental status. The developed gonads in *Foxl2*i and *Dmrt1*i group were markedly less plump in gonadal morphology than the *eGfp*i group (Fig. 4A, B and C). In addition, statistical results of histological analysis showed that the proportion of oysters with undeveloped gonads in *Foxl2*i (42%) and *Dmrt1*i (54%) group was higher than that in *eGfp*i (18%) group (Fig. 4H). Subsequently, microscopic examination of the developed individuals reveled that the female gonads with 92.3% (12/13) in the *eGfp*i group were in mature stage, whereas the female gonads in the *Foxl2*i group with 75% (18/24) were in growing stage (Fig. 4D and E). The male gonads in the *eGfp*i group with 88.9% (8/9) and *Dmrt1*i group with 88.2% (15/17) were in mature stage (Fig. 4F). The severity of the knock-down phenotype varied between individuals (Huvet et al., 2012). A hermaphroditic individual was observed in the *Dmrt1*i group (Fig. 4G). Furthermore, The GCA in *Foxl2*i group (52.4%) was less well-developed gonadal tissue than that in *eGfp*i group (82.9%, Fig. 4I), due to the decrease in the number of tubules. For these females, mean oocytes diameter (40.08 ± 4.39) was similar to that of the *eGfp*i group (39.51 ± 5.44, Fig. 4J). However, no remarkable difference was detected in GCA between *Dmrt1*i (78.5%) and e*Gfp*i (72.3%) group (Fig. 4I).

3.5. The expression changes of Esr, Wnt4, β-catenin, Dmrt1 and Foxl2 under RNAi

To explore the effects of RNAi on other genes, we examined the mRNA expression of sex-related genes of females in the *Foxl2*i group and males in the *Dmrt*i group. The mRNA expression of *Wnt4*, *β-catenin* (GenBank accession no. LOC105345286) and *Dmrt1* displayed no striking difference between *Foxl2*i and *eGfp*i group, while the expression of *Esr* was significantly inhibited in *Foxl2*i group (0.55-fold) compared with that in *eGfp*i group. In contrast, the mRNA expression of *Esr* was significantly activated (3-fold) in *Dmrt1*i group compared with that in *eGfp*i group. However, the expressions of *Wnt4* and *Foxl2* were dramatically increased by 4.5-fold and 9-fold as much as that in *eGfp*i group, with no marked variation in the expression of *β-catenin* (Fig. 5).

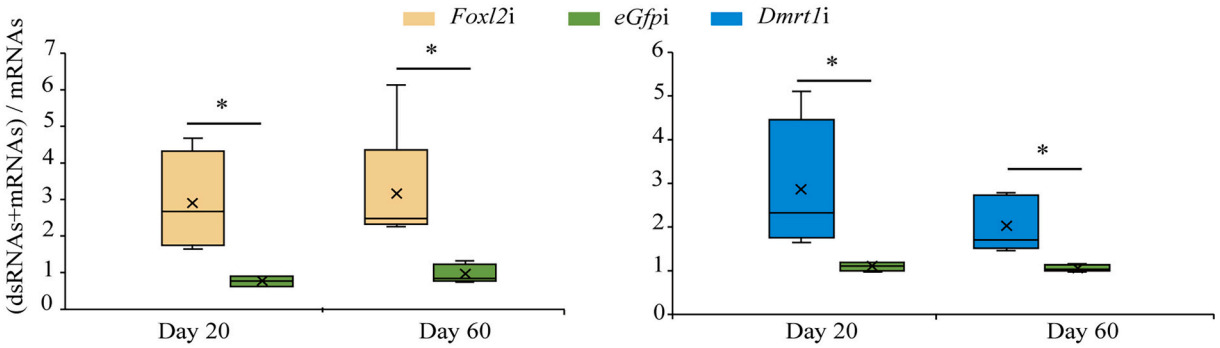

Fig. 2. The ratio of the interfering dsRNA to endogenous mRNA level in females (*Foxl2*i, *eGfp*i) and males (*Dmrt1*i, *eGfp*i) at day 20 and day 60. Only females were detected in *Foxl2*i and males were detected in *Dmrt1*i, and the corresponding genders in *eGfpi* were used as controls. Each group had at least three biological replicates and asterisks indicate significant differences ($P < 0.05$).

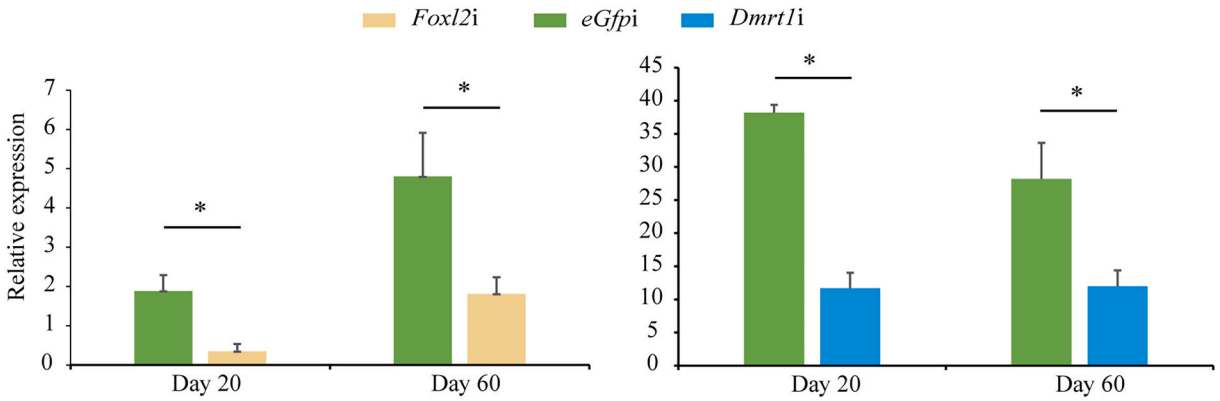

Fig. 3. Relative expression of *Foxl2* and *Dmrt1* in *Foxl2*i and *Dmrt*i after RNAi treatment in gonads, respectively. Only females were detected in *Foxl2*i and males were detected in *Dmrt1*i, and the corresponding genders in *eGfpi* were used as controls. Each group had at least three biological replicates and asterisks indicate significant differences ($P < 0.05$).

4. Discussion

Previous studies have highlighted the important roles of *Foxl2* and *Dmrt1* in sex differentiation and gametogenesis. In oysters, both genes were discovered by genomic homology analysis and the expression of *Dmrt1* presented sexual dimorphism by transcriptome analysis (Zhang et al., 2014). However, the current experiments were only involved in tissues expression distribution, which was limited in verification of gene functions. The method of non-invasive interference was applied to explore the function of genes related to rhythm and shell color in oysters (Feng et al., 2019; Payton et al., 2017), which proved that it was an economical and effective approach to study gene functions. In our present study, we demonstrated that *Foxl2* and *Dmrt1* were involved in sex differentiation and gonadal development in oysters by performing the non-invasive feeding-based RNAi experiment for 60 days. Our experimental results showed that this method successfully induced dsRNA into *C. gigas* cells and led to RNAi effect.

4.1. The roles of Foxl2 in oyster reproduction

As already observed, when targeting genes related to rhythm and shell color (Feng et al., 2019; Payton et al., 2017), we found that RNAi response between individuals was variable. Since no interference of human factor in this non-invasive interference experiment method, individual differences in RNA interference were probably due to the variation in their reproductive status on day 0 (the point at which we feeding of dsRNA) and the amount of dsRNA that actually gets into the gonadal cells (Huvet et al., 2012).

The establishment of gonads during oyster development is still unknown. Gametogenesis is initiated (resting stage) each year with clusters of germ cells scattered in the storage tissue, and then the germ cells proliferate, grow and maturate, during which the *Foxl2* gene expression increases as the female gonadal development (Naimi et al., 2009). *Foxl2* knockdown may inhibit the initiation of gonadal differentiation, as observed in the increased proportion of undifferentiated gonads. The reduction of gonadal area in *Foxl2*i females with mature oocytes was caused by the reduction of gonadal tubules, which was confirmed by the absence of significant differences of oocyte diameter detected between *Foxl2*i and *eGfp*i. Meanwhile, the knockdown of *Foxl2* was involved in the apoptosis of follicular cells (Li et al., 2015), might also cause the decrease of GCA in *Foxl2*i, as *Foxl2* was observed to be predominantly expressed in follicle cells in oysters (Naimi et al., 2009; Yue et al., 2021). In addition, *Foxl2* is involved in the gonadal vitellogenesis associated with follicular cells in *C. gigas* (Matsumoto et al., 2003; Naimi et al., 2009). Therefore, the effect of *Foxl2* on gonadal development may be related to follicular cells and reproductive tubules.

4.2. The roles of Dmrt1 in oyster reproduction and sex determination

Dmrt1 is an important gene that plays an essential role in both sex determination and gonadal development in *C. gigas*. Similar to the results of *Foxl2* interference, undifferentiated oysters were also observed in *Dmrt1*i, indicating that *Dmrt1* participated in the initial differentiation process of gonads. In mammals, *Dmrt* family has a variety of DM structures and sequences, and *Dmrt1* knockout leads to testicular development retardation, germ cell death and sex reverse (Moniot et al., 2000; Raymond et al., 1999). *Dmrt1* is mainly expressed in spermatogonial cells in oysters (Yue et al., 2021). However, no significant differences

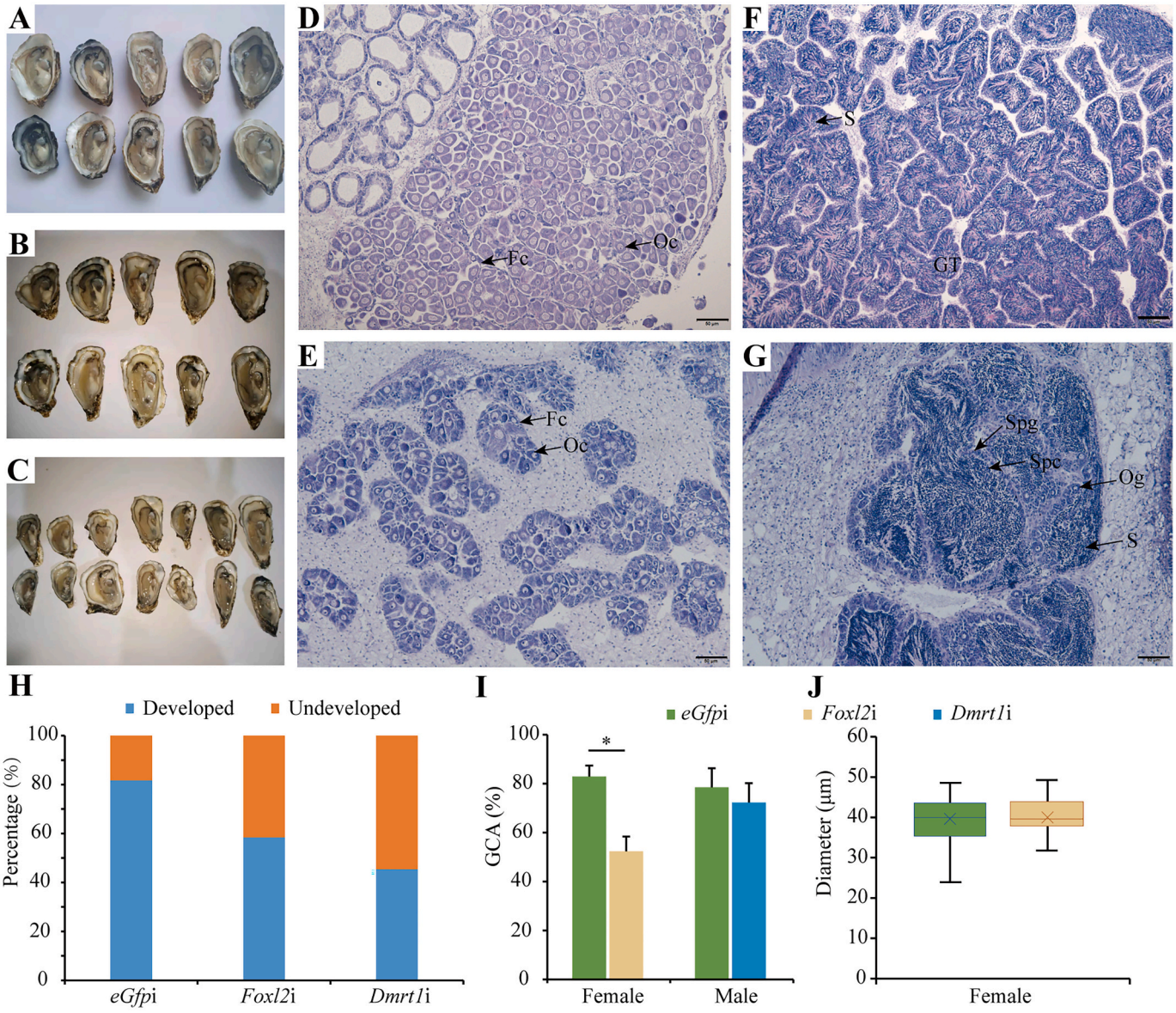

Fig. 4. Analysis of gonadal development after 60 days of RNAi. The phenotype of gonads in *eGfp*i (A), *Foxl2*i (B) and *Dmrt1*i (C). Tissue section analysis of *eGfp*i (females, D), *Foxl2*i (females, E), *eGfp*i (males, F) and *Dmrt1*i (hermaphrodite, G). Since no difference between *eGfp*i males and *Dmrt1*i males, males in *Dmrt1*i were unpresented here. H The percentage of developed and undeveloped gonads in *eGfp*i, *Foxl2*i and *Dmrt1*i. I The gonadal coverage area (GCA) of female and male gonads after RNAi. J The diameter of oocytes after RNAi. Only females were detected in *Foxl2*i and males were detected in *Dmrt1*i, and the corresponding genders in *eGfpi* were used as controls. Asterisks indicate significant differences ($P < 0.05$).

were discovered in GCA, in line with the fact that no substantial alterations in the phenotype of male individuals were observed following injection of *MroDmrt99B*-dsRNA in giant freshwater prawn (Yu et al., 2014), but it was different from the mouse (Kim et al., 2007b) and crab (Ma et al., 2016). Whether the existence of other functionally complementary *Dmrt*s involved in sex determination is unknown; after all, two alternative splicing of *Dmrt* have been detected in oysters (Yue et al., 2021), and multiple *Dmrt*s in other species (Wan et al., 2021). The deletion of *Dmrt1* in medaka resulted in sexual reversal from male to female (Masuyama et al., 2012), and knockdown of *Dmrt1* in Nile tilapia generated abnormal development of male gonads with somatic feminization and loss of germ cells (Li et al., 2013). Oysters without secondary sexual characteristics have sexual reverse phenomenon, so the sex was uncertain at the beginning of the experiment (resting stage). We were therefore uncertain whether the successful application of RNAi mediated gene silencing in oysters resulted in the same sex-reversal phenotype as RNAi in giant freshwater prawn (Lezer et al., 2015), but the emergence of a simultaneous hermaphrodite oyster was more associated with *Dmrt1*i. Oysters with both oocytes and spermatozoa were identified as simultaneous hermaphrodites, which is rare in nature (represented less than 1%) (Broquard et al., 2019), raising the possibility that *Dmrt1* is involved in the sex differentiation.

4.3. The regulation of Foxl2 and Dmrt1 in gonadal development or sex-determining pathway

To date, homologous genes including *Wnt4*, *β-catenin* and *Esr* related to gonadal development and sex differentiation in vertebrates have been identified in *C. gigas* (Matsumoto et al., 2007; Zhang et al., 2014), but they are non-exclusively expressed in female or male gonads (Zhang et al., 2014). The expression of *Wnt4* in both female and male gonads is not surprising. *Wnt4* maintains the correct differentiation of female follicles and is associated with testicular development in males (Review in Bernard and Harley, 2007). The expression levels of *Wnt4* and

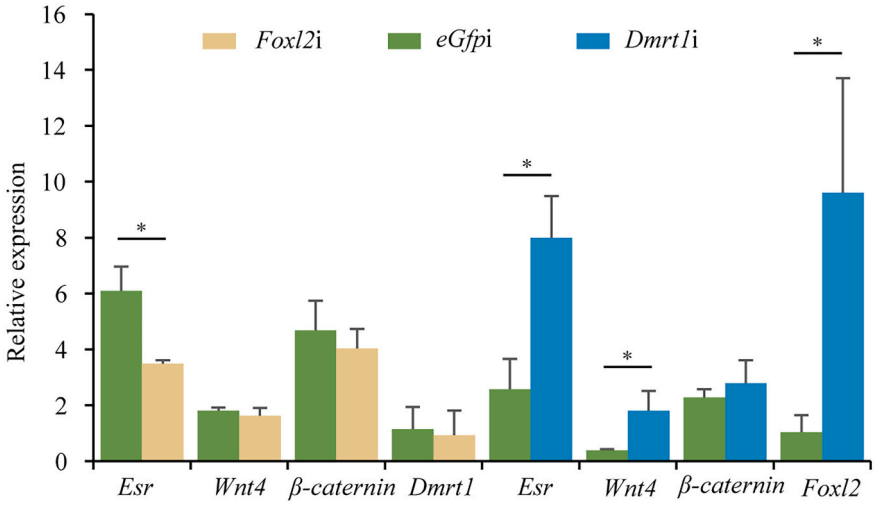

Fig. 5. The relative expression of sex-related genes after RNAi. Only females were detected in *Foxl2*i and males were detected in *Dmrt1*i, and the corresponding genders in *eGfpi* were used as controls. Asterisks indicate significant differences ($P < 0.05$).

β-catenin presented no significantly variation in the *Foxl2*i, in line with studies on goats and freshwater mussel (Kocer et al., 2008; Wang et al., 2020), suggesting that *Foxl2* was uninvolved in the *Wnt/β-catenin* signaling pathway, which is involved in organization development in bivalves (Yang et al., 2015; Xu et al., 2016). In contrast, *Wnt4* was involved in the process of male gonadal development, which could be inferred from the downregulation of *Wnt4* expression by *Dmrt1*. The certain role of *Wnt4* in female gonadal development is unclear, but at least we know that *Wnt4* is involved in male gonadal development or sex differentiation.

It has been proposed that *Foxl2* and *Dmrt1* are involved in the sex determination pathways of female and male in oysters, respectively (Zhang et al., 2014). Estrogen receptors are involved in the vitellogenin production and are mainly located in the nucleus of follicular cells in *C. gigas* (Matsumoto et al., 2007). The expression of *Esr* decreased in *Foxl2*i and increased in *Dmrt1*i, indicating that *Esr* was the downstream of these two sex-determination pathways. This finding is preliminary but consistent with reported interactions between *Fxol2* and *Esr* in vertebrates (Georges et al., 2014). Therefore, *Foxl2* may interact with the *Esr* in oyster gonads to regulate the vitellogenin synthesis and repress the expression of genes related to male development, as observed in the mouse study (Uhlenhaut et al., 2009). Antagonistic effects of *Foxl2* and *Dmrt1* have been demonstrated in vertebrates and are speculated in bivalves (Li et al., 2018; Zhang et al., 2015; Dai et al., 2021; Li et al., 2013), but there is no direct evidence (Dai et al., 2021) that FOXL2 inhibits *Dmrt1* expression or DMRT1 inhibits *Foxl2* expression observed in medaka. Such antagonism was marked on the inhibitory effect of DMRT1 on the expression of *Foxl2* in oysters, as suggested by the altered sex phenotypes observed in *Dmrt1*i, providing supporting data for the hypothesis proposed by Zhang et al. (2014). Although the number of hermaphrodites was low, which may be related to the small sample size, it provided useful information for understanding the function of *Dmrt1*.

5. Conclusions

In order to explore the function of *Foxl2* and *Dmrt1* in sex differentiation and gonadal development, the non-invasive RNAi approach was employed in oysters. This was an efficient approach that carried out for a prolonged period of time to trigger a specific RNAi response. We provided data to support the involvement of *Foxl2* and *Dmrt1* in *C. gigas* gonadal development. These two genes were found in *C. gigas* to regulate the expression of *Esr*, indicating that *Foxl2* and *Dmrt1* are involved in the gonadal development through estrogen signaling pathway. The antagonism between *Dmrt1* and *Foxl2* in vertebrates is widely recognized and the inhibitory effect of DMRT1 on *Foxl2* supports the hypothesis proposed in oysters.

Author statement

Dongfang Sun: Completion of the experiment, data analysis, and manuscript drafting.

Hong Yu: Data analysis and manuscript revision.

Qi Li: Experimental design and coordination and manuscript revision.

Declaration of Competing Interest

There are no conflicts to all authors for this paper.

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