Gene 790 (2021) 145692

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Variance in expression and localization of sex-related genes *CgDsx*, *CgBHMG1* and *CgFoxl2* during diploid and triploid Pacific oyster *Crassostrea gigas* gonad differentiation

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ARTICLE INFO

Keywords:
Dmrt1
BHMG1
Foxl2
Crassostrea gigas
Gonadic differentiation

ABSTRACT

Several evolutionarily conserved classes of transcriptional regulators were involved in diverse sex determination and differentiation pathways across taxa, whereas their roles in most mollusks is still limited. The Pacific oyster *Crassostrea gigas*, a dioecious bivalve with sex reversal, could be an ideal model for this issue because of its complex sexuality and potential disruption of sex differentiation in triploid individuals. Here, two mRNA splicing isoforms of a DM domain gene *CgDsx* and two isoforms of a novel sex-related *CgBHMG1* (ortholog of BHMG1 in mammals) were identified in *C. gigas*. Real time PCR showed that two isoforms of *CgDsx* and one isoform of *CgBHMG1* displayed male-specific expression in diploid oysters, opposite with the female-specific *CgFoxl2* (a potential factor of female gonadic differentiation). Interestingly, the four sex-specific transcripts in diploid oyster were expressed in triploid oysters with opposite sex, triploid hermaphrodites and individuals at stage I that sex could not be determined. Subsequent *in situ* hybridization analysis on gonads of diploid oysters revealed predominant expression of *CgDsx* in spermatogonia of testes, *CgBHMG1* in spermatocytes of testes and follicle cells of ovaries, and *CgFoxl2* in follicle cells of ovaries and some male germ cells in testes. And aberrant co-expression of the three genes in triploid oysters was localized in gonadal tubules of gonads at stage I, ovarian follicle cells and undetermined gonial cells in nontypical hermaphroditic gonads with rare female materials. From the above, temporal and spatial expression of sex-related genes in diploid and triploid gonads indicated that *CgDsx* and *CgFoxl2* might mainly function in *C. gigas* sex differentiation, and *CgBHMG1* appeared as a factor involved in meiosis. This work will help to illuminate the gene network of sex differentiation in bivalves and provides new sight on this issue from comparison between diploid and triploid individuals.

1. Introduction

Species propagation of multicellular animals universally relies on sexual reproduction that requires male and female gametes (Gamble and Zarkower, 2012). Before sexual reproduction can take place, sex should be established and after that an undifferentiated gonad differentiates either as a testis or an ovary (Piferrer, 2013; Nagahama et al., 2020). Hence, sex determination and gonadal sex differentiation are considered as the most important physiological processes for the proper reproductive competence and perpetuation of species. And researchers have long been fascinated by molecular mechanisms on how sex is determined and gonads differentiated.

Data so far accumulated in the past decades suggest that sex determination and differentiation is controlled by quite different mechanisms across taxa, but often these involve several conserved classes of transcriptional regulators. Sex determination pathways in diverse organisms, from insects to mammals, converge on conserved downstream regulators in the DM domain gene family that are essential for male development (Gamble and Zarkower, 2012), such as doublesex (*dsx*) in flies (Erdman and Burtis, 1993), mab-3 in nematodes (Shen and Hodgkin, 1988) and doublesex and mab-3 related transcription factor 1 (*Dmrt1*) in human (Raymond et al., 2000). An important member of the

Abbreviations: Dmrt1, doublesex and mab-3 related transcription factor 1; *Foxl2*, forkhead box l2; BHMG1, basic helix-loop-helix and HMG box domain-containing protein 1; *Sox9*, SRY-box containing gene 9; *Wnt4*, Wnt Family Member 4; *Rspo1*, R-Spondin 1.

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https://doi.org/10.1016/j.gene.2021.145692
Received 28 March 2021; Accepted 30 April 2021
Available online 4 May 2021

forkhead domain family, forkhead box l2 (*Foxl2*), is a conserved, early-acting gene in vertebrate ovarian development during female differentiation (Loffler et al., 2003), which functions in a Yin and Yang relationship with *Dmrt1* to maintain the fates of testes or ovaries in adult mammals (Huang et al., 2017). Several invertebrate orthologs of *Foxl2* have also been characterized, such as *NvFoxL2* in the cnidarian *Nematostella vectensis* (Magie et al., 2005) and *SpFoxl2* in the echinid urchin *Strongylocentrotus purpuratus* (Tu et al., 2006), although their roles are not well defined.

There is still much to learn from evolutionally conserved transcriptional regulators employed to determine sexual fate in diverse taxa, especially non-model invertebrates with varying sexuality. Bivalves contributes the major branch of the second largest invertebrate phylum Mollusca and different reproduction strategies (strict dioecy, simultaneous hermaphroditism and alternative sexuality) make it an excellent clade to understand the evolution of sex and sex determination. Gene homologous to sex determination and differentiation pathway genes in vertebrate species have been identified by molecular cloning or gonadal transcriptome analyses in bivalves, including *Dmrt1*, *Sox9*, *Wnt4*, *Rspo1* and *Foxl2* (Naimi et al., 2009a, 2009b; Llera-Herrera et al., 2013; Li et al., 2013, 2014a, 2014b; Santerre et al., 2014; Zhang et al., 2014; Shi et al., 2015; Yang et al., 2015; Li et al, 2016). They are regarded as participants in the sex differentiation cascade of bivalves because of the sexually dimorphic expression patterns in the gonads, which suggests the possible conservation of sex determination and differentiation in animals (Zhang et al., 2014; Shi et al., 2015). Nevertheless, for robust evidence, the exact role of these conserved transcriptional regulators in bivalve should be studied more in detail.

The cosmopolitan Pacific oyster *Crassostrea gigas* is a representative bivalve to understand the sex determination and sex differentiation in this clade because of its complex sexuality, including dioecy, sex change and rare hermaphroditism. Several orthologs of transcriptional regulators known to act in sex determination in vertebrate species were identified earlier than in any other group of bivalves, including *CgFoxl2* (ortholog of *Foxl2*), *Cg-Dmrl* and *CgDsx* (orthologs of *Dmrt1*) (Naimi et al., 2009a, 2009b; Zhang et al., 2014). However, there is some debate on the potential role of *Cg-Dmrl* and *CgDsx* in *C. gigas* and the cellular location of *CgFoxl2* (Naimi et al., 2009a, 2009b; Zhang et al., 2014). From our previous transcriptome analysis, *CgDsx*, *CgFoxl2* and uncharacterized LOC105327228 displayed sexually dimorphic expression since male and female gonads differentiation could be observed by histological analysis (Yue et al., 2018), which means that these three genes may act as upstream components in sex differentiation cascade. Surprisingly, a transcriptomic profiling of gametogenesis in triploid Pacific oyster revealed quite different expression pattern of reported sex-related transcriptional regulators. The female-specific *CgFoxl2* in diploids is upregulated in most triploid males that almost sterile (defined as "3nβ"), and conversely some male-specific genes are upregulated in 3nβ females (Dheilly et al., 2014), not including *Cg-Dmrl* or *CgDsx*. Owing to abnormal expression of numerous sex-related genes and partial sterility, a probable disruption of sex differentiation mechanism in triploid oyster was hypothesized (Dheilly et al., 2014). And this inspires us that triploid oyster will be an interesting model for elucidating the role of potential sex-determining transcriptional regulators.

In the present study, we identified different transcript isoforms of the controversial *CgDsx* and a novel sex-related *CgBHMG1* (uncharacterized LOC105327228) that co-expressed with *CgDsx* in our previous transcriptome data. The expression patterns of the above variant transcripts and the well-characterized *CgFoxl2* transcript (Naimi et al., 2009b) were detected in diploid and triploid Pacific oyster gonads at different gametogenesis stages. Furthermore, cellular localizations of the three genes in different diploid and triploid oyster gonads were also carried out by *in situ* hybridization. This work will enrich the knowledge about the role of evolutionally conserved regulatory elements in bivalves sex determination and differentiation.

2. Materials and methods

2.1. Animal material sampling

Triploid oysters were obtained from a commercial hatchery and then maintained together with diploid oysters from a cultured population generated by 50 sires × 50 dams at Qingdao, Shandong, China (36.2°N, 120.6 °E) and Rushan, Shandong, China (36.4 °N, 121.3 °E). Two-year-old adult triploid and diploid oysters maintained at Qingdao were sampled monthly between April and December 2019. And oysters maintained at Rushan were sampled at April and June 2019. On each sampling, their gills were immediately dissected and prepared for ploidy check by flow cytometry (CytoFlex Beckman Coulter, US). Five tissues including gill, mantle, adductor muscle, gonad and labial palpus were preserved in RNAwait (Solarbio, China) and stored at −80 °C for RNA extraction. Then, partial gonad of each individual was fixed in Bouin's fluid for histological analysis and the remainder was fixed in 4% paraformaldehyde at 4℃ overnight, dehydrated with serial methanol (25, 50, 75, and 100%) and then stored at −20℃ for *in situ* hybridization.

2.2. Histological analysis

Gonad samples were transferred to ethanol, cleared in xylene, embedded in paraffin wax, and cut into 5-μm-thick sections on Leica RM 2016 rotary microtome (Leica, Germany). Serial sections were tiled on glass slides, deparaffinized with xylene, hydrated with graded ethanol to water, and stained with hematoxylin. After that, the glass slides were counterstained with eosin, dehydrated with ethanol, cleared with xylene, mounted with neutral balsam, and covered with coverslips. Finally, the sections were observed under Olympus BX53 microscope (Olympus, Japan). Gametogenesis stage and sex of the diploid samples were determined as described in our previous study (Yue et al., 2018): Stage 0, resting stage with undifferentiated germinal epithelium; stage I, early gametogenesis stage with numerous spermatogonia or oogonia; stage II, advanced gametogenesis stage with primary gametes and some secondary oocytes or spermatozoa; stage III, maturation stage that gonads completely filled with mature gametes; hermaphrodite with both two types of gametes in gonadic area. The sex and gametogenesis stage of triploid oyster were determined based on criteria in previous studies (Jouaux et al., 2010; Matt and Allen, 2021): Stage 0, gonads similar to the appearance in diploid counterparts at the same stage; stage I, gonads with cell proliferations in the gonadal tubules but impossible sex identification based on gonia size and morphological characteristics; stage II, gonads differentiation associated with vitellogenesis in females (α type with numerous proliferating gonia and β type with locked gametogenesis) and presence of follicles containing spermatogenic cells in males; stage III, maturation stage that mature gametes are present in two patterns in female (α type with a considerable number of oocytes and β type with only a few gametes) and one pattern in male (with numerous mature spermatids); hermaphrodite stage II or III with both two types of gametes in gonadic area.

2.3. Total RNA extraction

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was measured on a Nanodrop (Thermo Fisher Scientific, USA). Meanwhile, RNA degradation and contamination were monitored on 1% agarose gels. These RNA samples conformed to the required purity criteria (A260/A230 and A260/ A280 > 1.8) for cDNA library preparation.

2.4. Reverse transcription, cloning and sequencing

A mixture of gonad, gill, mantle, adductor muscle and labial palpus total RNA was reverse transcribed for rapid amplification of cDNA ends using SMARTer® RACE 5′/3′ Kit (Clontech, USA) according to the

manufacturer's recommendations. After this, 5′- and 3′-RACE cDNAs were used as template for PCR amplifications with primer sets in Table 1, designed by Primer 3.0 according to the sequence information of *CgDsx* and uncharacterized LOC105327228 in the *C. gigas* genome (oyster_v9) (Zhang et al., 2012). The PCR thermal cycle was 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min; 72 °C for 10 min. Resulting fragments were purified and cloned into *pEAZY*® -T1 vector using a TA cloning kit (TransGen Biotech, China). At least ten different independent clones were picked for each RACE inserts and sequenced by Sanger sequencing method. The accuracy of complete cDNA sequence was verified by PCR with specific primer pairs in Table 1, then the cDNA was cloned and sequenced as described above.

2.5. Sequence analysis

The complete cDNAs detected by RACE and deduced amino-acid sequences were analyzed and compared with orthologs by using the BLAST algorithm. Motif discovery of amino-acid sequences was conducted by using ScanProsite tool (https://prosite.expasy.org/scanprosite/) (Gattiker et al., 2002).

2.6. Phylogenetic analysis

Full-length protein sequences of orthologs were downloaded from NCBI. Multiple sequence alignments were performed using ClustalW in MEGA7 with default parameters. After manual adjustments, maximum-likelihood trees were constructed using MEGA7 based on the JTT matrix-based model and statistical analysis was performed with the bootstrap method using 1000 repetitions (Felsenstein, 1985; Jones et al, 1992; Kumar et al., 2016).

2.7. Real time quantitative PCR

To analyze gene expression pattern, various samples were selected for cDNA libraries construction. For diploid oyster, gonads at stage 0, ovaries (stage I, II, III) and testes (stage I, II, III) were selected. For triploid oyster, gonads at stage 0, stage I, β type ovaries at stage III, testes at stage III and hermaphroditic gonads at stage III were selected. Moreover, gills, mantles, adductor muscles and labial palpi of diploid oysters were selected for tissue expression pattern analysis by compared with diploid oyster gonads at stage I. The cDNA libraries were prepared with at least six biological replicates. For each cDNA library construction, first-strand cDNA was synthesized from 1 μg total RNA by Prime Script TM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The quantitative real-time PCR was performed with primers in Table 2 on the LightCycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, UK) using QuantiNova SYBR Green PCR Kit (Qiagen, Germany). For *CgDsx* and *CgBHMG1*, the qRT-PCR primers were designed for recognizing each transcript isoform. And for *CgFoxl2*, the primer pair was specific to its only transcript and could not be allowed to measure its natural antisense transcript as demonstrated in *C. gigas* (Santerre et al., 2012). Cycling parameters were 95 °C for 2 min, then 40 cycles of 95 °C for 5 s, 60 °C for 10 s. Each microplate included negative control (total RNA treated with DNase) and blank control (sterile water). Elongation factor I (EF I) gene expression was used to normalize gene expression (Yue et al., 2018). PCR efficiency and proper dilution of cDNA was determined for each primer pair by constructing a five points standard curve from 5-fold serial dilutions of a diploid male or female gonad template. The melting curves of PCR products (60 to 95 °C) were performed to ensure the detection of a single specific product. Relative gene expression levels was calculated by the $2^{-(\Delta\Delta Ct)}$ method (Schmittgen and Livak, 2008). After being examined for homogeneity of variances, data were analyzed by *t* test using software SPSS 18.0 and levels were accepted as significant at P value < 0.05.

Table 1
Primers used for RACE.

Primer name	Primer sequence (5′ to 3′)
CgDsxRACE5′GSP	GGTGGCTGTATAGCATGGCGAGTGT
CgDsxRACE3′GSP	CCTGTGGGTCCTTGCCTTGTCAGAT
CgBHMG1RACE5′GSP	GTCCTCATCAACTGGGCTTCTTCGC
CgBHMG1RACE3′GSP	GCCAGGGAAGAACAAGCGAAAGCAGT
CgDsxRACEdetectF	CAATTTATCTTCAAGTTTTCCCGCA
CgDsxRACEdetectR	TGAGCCAAGAAAAGGCCATATATCT
CgBHMG1RACEdetectF1	CCAGACAAAGTACCCGCTGA
CgBHMG1RACEdetectF2	TTACCACTTCCTGTCGCCATACT
CgBHMG1RACEdetectR	TTTCCACAGGAGTCAGATTTGAATC

Table 2
Primers used for RT-PCR.

Transcript name	Primer sequence (5′ to 3′)	Amplicon lengths (bp)	Amplification efficiencies
CgDsx-1	F: TCGATAGCGAGTTGTCCACG R: AACAGTGGATCAATTTTACTGTCCC	199	1.956
CgDsx-2	F: AGCGAGTTGTCCACGAGACG R: GTCAAACAGTGGATTGTCCCTGGCT	189	1.963
CgBHMG1-1	F: CCAGACAAAGTACCCGCTGA R: TGGGAATGGCTGTAAAGTTGGA	209	2.083
CgBHMG1-2	F: ACCACTTCCTGTCGCCATACT R: GGCTGTAAAGTTGGACTTCATTTGT	186	2.083
CgFoxl2	F: CACAGTGTGGTTACAACGCAATGC R: CCTGTCAGTCCAGTACGAGTAATGC	179	1.996

2.8. In situ hybridization

To synthesize specific RNA probes, specific cDNA fragments were amplified with primers in Table 3 and purified PCR products were inserted in into *pEAZY*® -T1 vector containing T7 promoter (TransGen Biotech, China). After sequence confirmation, choose the plasmid with correct insert as template for subsequent probe generation. Digoxigenin-labeled sense and antisense probes were synthesized using a DIG RNA labelling kit (Roche, Germany). For *CgDsx* and *CgBHMG1*, the corresponding probes bind to both transcript isoforms of each gene. For *CgFoxl2*, the antisense probe binds to its only transcript as demonstrated (Naimi et al., 2009b) and partial sequences of the sense probe (257 nt of 750 nt) are complementary to its natural antisense transcript *Cg-Foxl2os* (Santerre et al., 2012). Various gonad samples were used for *in situ* hybridization, including diploid oyster ovaries and testes at stage II, triploid oyster gonads at stage I, β type ovaries at stage III, testes at stage III and three kinds of hermaphroditic gonads at stage III. *In situ* hybridization was performed with three biological replicates and the details were specified below. Gonad samples for *in situ* hybridization were transferred to methanol, cleared in xylene, embedded in paraffin wax, and cut into 5-μm-thick sections on Leica RM 2016 rotary microtome (Leica, Germany). Serial sections were deparaffinized with xylene, hydrated with graded ethanol to PBS, and digested with 10 μg/ml

Table 3
Primers used for *in situ* hybridization.

Gene name	Primer sequence (5′ to 3′)
CgDsx	F: GTGGGTCCTTGCCTTGTCAGATAC R: AGACTGTGATCCTTGCTCCTGTTG
CgBHMG1	F: GACTCCACCACTGACCACCAACT R: CTCCTCCTCCTCCTCCTCTGATACA
CgFoxl2	F: AATGCGATTAATGCGACCGTCTTCA R: GCTTGTGCCCATCCCTGATATTGAG

proteinase K at 37℃ for 15 min. After pre-hybridization at 60 °C for 4 h, hybridization was performed with 500 ng/μl denatured RNA probe in hybridization buffer at 60℃ for 16 h. Then, the probes were washed away and sections were blocked in 1% blocking reagent at room temperature (RT) for 4 h. And antibody incubation was performed with anti-digoxigenin-AP Fab fragments (Roche, Germany) diluted 1:5000 in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH = 7.5) at 4 °C for 16 h. After extensive washing in maleic acid buffer, sections were incubated with 2% NBT/BCIP solution in darkness at RT. Sections were examined and pictures were taken with an Olympus BX53 microscope coupled with a DP80 camera (Olympus, Japan).

3. Results

3.1. Gene cloning and sequence analysis

The 5′ and 3′ RACE primers were designed to identify all splicing isoforms of the sex-related *CgDsx* as there is divergence on transcripts of this gene among various Pacific oyster genome versions. And two transcript isoforms of *CgDsx* were finally identified and named as *CgDsx-1* (GenBank accession number MW248786) and *CgDsx-2* (GenBank accession number MW248787), respectively. The complete *CgDsx-1* sequence of 1422 bp (without the poly(A) tail) shows a 5′ untranslated region (UTR) of 89 bp, an open reading frame (ORF) of 912 bp with the ATG codon at position 90 bp and a TAA stop codon at position 999 bp and a 3′ UTR of 421 bp including a poly(A) signal (AATAAA) at position 1402 bp, 15 bp upstream of the poly(A) tail (Fig. 1A). Another isoform *CgDsx-2* of 1440 bp shows a longer 5′ UTR of 116 bp, a shorter ORF of 903 bp with the ATG codon at 117 bp and a TAA stop codon at position 1017 bp and the same 3′ UTR with *CgDsx-1* (Fig. 1A). The difference between ORFs of the two isoforms locates at the end of exon 1 and generates two deduced amino-acid sequences (303aa and 300aa long) (Fig. 1A and B). More precisely, an alternative splice donor site nine nucleotides into intron 1 is responsible for the additional amino acid serine-lysine-isoleucine (S-K-I) in deduced amino-acid sequence encoded by *CgDsx-1* (Fig. 1A). When compared with orthologs in model species, a shorter conserved DM domain (44aa long) was identified both in the two deduced amino-acid sequences (Fig. 1B) and shows closest homology to *Dsx* found in *Drosophila melanogaster* (Fig. 1C).

As for the uncharacterized LOC105327228 that co-expressed with *CgDsx*, there were also two transcript isoforms identified by RACE. The two isoforms show different 5′ UTR (491 bp and 131 bp long), the same ORF of 1902 bp and 3′ UTR of 461 bp including a poly(A) signal (AATAAA) at 14 bp upstream of the poly(A) tail. And the significant difference between 5′ UTRs of the two isoforms is due to variant exon 1 generated by alternative splicing donor sites, upstream of the exon 2 (Fig. 2A). Psi-Blast analysis on the deduced amino-acid sequence (633aa long) suggested LOC105327228 gene as an ortholog of basic helix-loop-helix and HMG box domain-containing protein 1 gene (BHMG1). Thus, it was tentatively named as *CgBHMG1* and the two transcripts were named as *CgBHMG1-1* (2853 bp) (GenBank accession number MW252171) and *CgBHMG1-2* (2493 bp) (GenBank accession number MW252172). Subsequent motif analysis identified a basic helix-loop-helix (bHLH) domain and an HMG box A and B DNA binding domain in the shared deduced amino-acid sequence of *CgBHMG1* (Fig. 2B). Interestingly, not all bHLH domain in BHMG1 homologs could be hit by motif profiles in the PROSITE database, such as BHMG1 of *Salmon salar* (Fig. 2B). The bHLH domain in *CgBHMG1* showed low sequence similarity with vertebrate homologs (data not shown), and in contrast the relatively conserved HMG domain was more homologous to BHMG1 found in *Rhinatrema bivittatum* than other vertebrates (Fig. 2C).

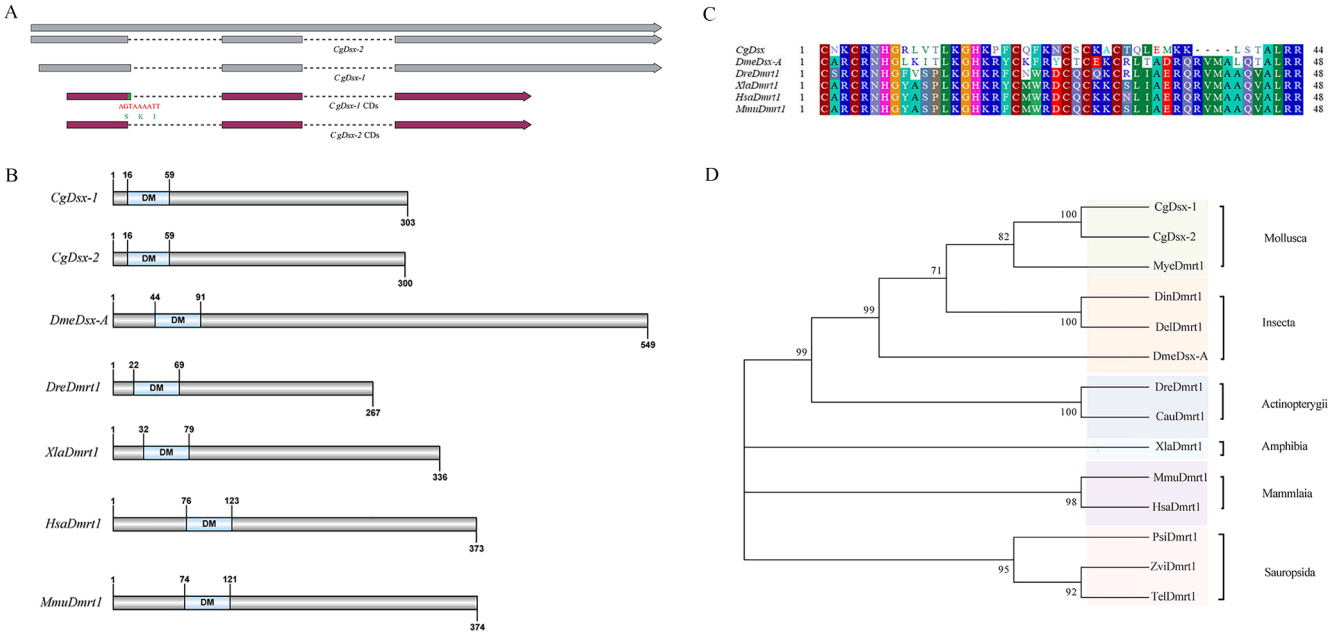

Fig. 1. A DM domain gene in *C. gigas* (*CgDsx*) and phylogenetic tree of the group DMRT1 protein. (A) Schematic presentation of *CgDsx*. Grey boxes, structure of the gene and its alternative transcripts. Darkred boxes, coding regions of two transcripts. Alternative splicing at 3′-end of exon 1 is indicated by green and sequence context is shown below coding region. (B) The structural domains of *CgDsx* and other DM domain containing genes. (C) Alignment of DM domain of *CgDsx* and selected DM domain genes. (D) Phylogenetic tree of protein sequences of *CgDsx* and selected DM domain genes. Species names are abbreviated as Cg for *Crassostrea gigas*, Dme for *Drosophila melanogaster*, Dre for *Danio rerio*, Xla for *Xenopus laevis*, Hsa for *Homo sapiens*, Mmu for *Mus musculus*, Mye for *Mizuhopecten yessoensis*, Din for *Drosophila innubila*, Del for *Drosophila elegans*, Cau for *Carassius auratus*, Psi for *Pelodiscus sinensis*, Zvi for *Zootoca vivipara*, Tel for *Thamnophis elegans*. Accession numbers: CgDsx-1, XP_011441049.2, CgDsx-2, XP_011441050.2, MyeDmrt1, OWF50386.1, DinDmrt1, XP_034479578.1, DelDmrt1, XP_017112743.1, DmeDsx-A, NP_731197.1, DreDmrt1, AAU04562.1, CauDmrt1, XP_026106994.1, XlaDmrt1, NP_001089969.1, MmuDmrt1, NP_056641.2, HsaDmrt1, Q9Y5R6.2, PsiDmrt1, NP_001303976.1, ZviDmrt1, XP_034953368.1, TelDmrt1, XP_032069865.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

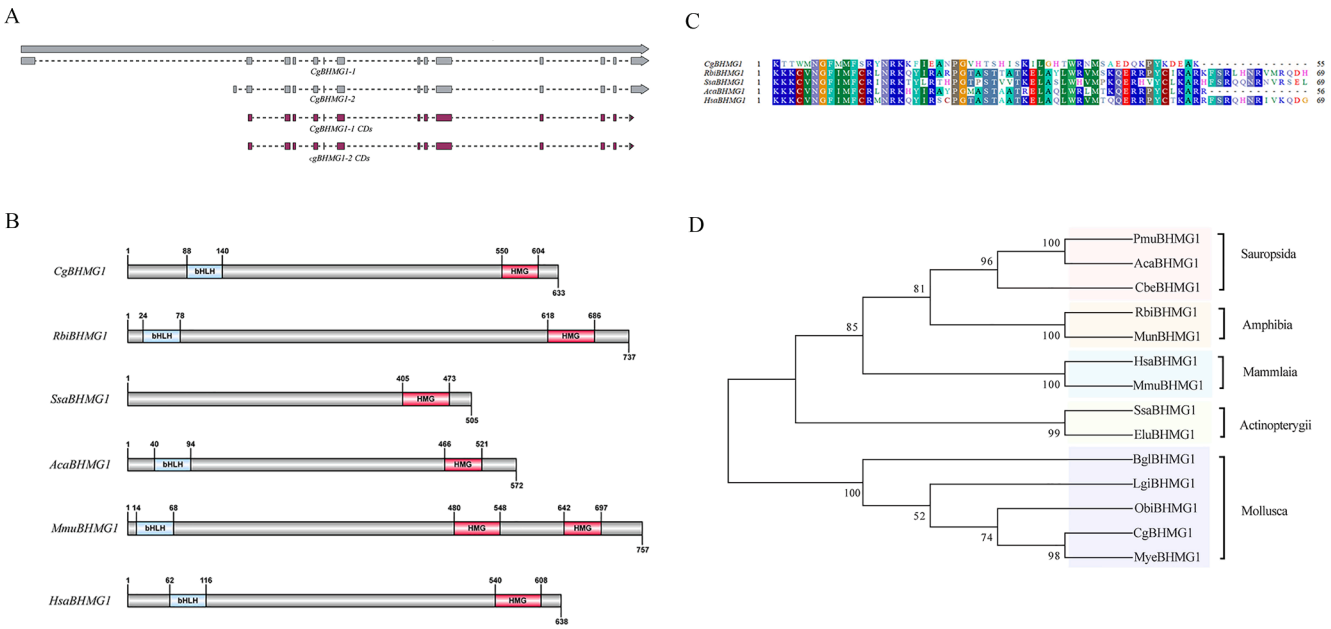

Fig. 2. A BHMG1 gene in *C. gigas* (*CgBHMG1*) and phylogenetic tree of the group BHMG1 protein. (A) Schematic presentation of *CgBHMG1*. Grey boxes, structure of the gene and its alternative transcripts. Darkred boxes, coding regions of two transcripts. (B) The structural domains of *CgBHMG1* and other BHMG1 genes. (C) Alignment of HMG domain of *CgBHMG1* and selected BHMG1 domain genes. (D) Phylogenetic tree of protein sequences of *CgBHMG1* and selected BHMG1 domain genes. Species names are abbreviated as Cg for *Crassostrea gigas*, Hsa for *Homo sapiens*, Mmu for *Mus musculus*, Mye for *Mizuhopecten yessoensis*, Obi for *Octopus bimaculoides*, Lgi for *Lottia gigantea*, Bgl for *Biomphalaria glabrata*, Elu for *Esox lucius*, *Ssa for Salmo salar*, Mun for *Microcaecilia unicolor*, Rbi for *Rhinatrema bivittatum*, Cbe for *Chrysemys picta bellii*, Aca for *Anolis carolinensis*, Pmu for *Protobothrops mucrosquamatus*. Accession numbers: CgBHMG1, XP_011425869.2, MyeBHMG1, XP_021340871.1, ObiBHMG1, XP_014772121.1, LgiBHMG1, XP_009063106.1, BglBHMG1, XP_013072221.1, EluBHMG1, XP_019903667.1, SsaBHMG1, XP_014052367.1, MmuBHMG1, XP_011249053.1, HsaBHMG1, NP_001297053.1, MunBHMG1, XP_030075837.1, RbiBHMG1, XP_029426906.1, CbeBHMG1, XP_029426906.1, AcaBHMG1, XP_008119751.1, PmuBHMG1, XP_029140889.1.

3.2. Phylogenetic analysis of CgDsx and CgBHMG1

Phylogenetic analysis was performed to investigate the relationship between *CgDsx and CgBHMG1* and other family members. Two isoforms of *CgDsx* clustered with Dmrt1 from another bivalve, *Mizuhopecten yessoensis*, then clustered with insect homologs (Fig. 1D). And the Dmrt1 proteins from amphibia, reptilia and mammalia formed three groups separately. In the Fig. 2D, Pacific oyster BHMG1, together with BHMG1 from other mollusca formed a major group distinct from vertebrate homologs.

3.3. Expression patterns of CgDsx, CgBHMG1 and CgFoxl2 in diploid and triploid Pacific oyster

As there are variant transcripts of sex-related *CgDsx and CgBHMG1* in *C. gigas*, one question occurred to us: what are the expression profiles of them? According to RT-PCR using isoform-specific primers, two transcripts of *CgDsx* (*CgDsx-1* and *CgDsx-2*) exhibited similar expression pattern. In diploid oysters, both of them were expressed specially in testis (Fig. 3A–D) and the significant difference could be detected from early gametogenesis stage (stage I) till maturation stage (stage III) (Fig. 3A and C). While, the expression patterns were not sexually dimorphic in triploid oyster. Unexpected high abundance of two *CgDsx* transcripts appeared in gonads of triploid oyster at stage I when sex could not be identified based on gonia size and morphological characteristics (Fig. 3A and C). Though expression abundance of *CgDsx-1* and *CgDsx-2* was found higher in mature testes than β type ovaries and hermaphroditic gonads of triploid oyster, the sex-dependent expression difference was smaller compared to diploid oysters (Fig. 3A and C). Additionally, both two *CgDsx* transcripts were upregulated in triploid oyster β type ovaries and hermaphroditic gonads compared to ovaries of diploid animals (Fig. 3A and C). Unlike the *CgDsx*, only one transcript isoform *CgBHMG1-2* of *BHMG1* ortholog in *C. gigas* showed sex-related expression pattern (Fig. 3E–H). Male-specific expression pattern of *CgBHMG1-2* was detected in diploid oysters from the beginning of germ cell differentiation (stage I) till maturation (stage III) and its expression abundance was also found higher in mature testes than β type ovaries and hermaphroditic gonads of triploid oyster (Fig. 3G). When compared to diploid ovaries, *CgBHMG1-2* expression was upregulated in triploid gonads at stage I, β type ovaries and hermaphroditic gonads at stage III (Fig. 3G), just like *CgDsx-1* and *CgDsx-2*.

Inconsistent expression patterns of the above transcripts between diploid and triploid oysters aroused our interest about the expression profile of *CgFoxl2* that in potential adversarial relationship with *CgDsx*. The female-specific expression of its single transcript was confirmed by RT-PCR in diploid oysters and distinct expression profile was also detected in triploid oysters as the above sex-related transcripts (Fig. 3I and J). As shown in Fig. 3I, expression level of *CgFoxl2* decreased in triploid ovaries, and conversely increased in testes compared to diploid animals. And *CgFoxl2* in triploid hermaphroditic gonads reached an expression level close to that in triploid ovaries (Fig. 3I). Compared to diploid testes, *CgFoxl2* expression was upregulated in triploid gonads at stage I, testes and hermaphroditic gonads at stage III (Fig. 3I).

3.4. Localization of CgDsx, CgBHMG1 and CgFoxl2 in diploid and triploid Pacific oyster gonads

Fascinating expression profiles of the above transcripts urged us to determine the cell localization of their mRNA expression across numerous types of oyster gonads. For *CgDsx*, *in situ* hybridization revealed predominant expression in spermatogonia of testis of diploid oyster at stage II, although it is difficult to exclude the expression in somatic cells and spermatocytes (Fig. 4A). By contrast, no significant signal of *CgDsx* mRNA expression was detected in ovary of diploid oyster (Fig. 4B). In the triploid gonad at stage I, *CgDsx* mRNAs were detected in gonial cells that could not be identified as spermatogonia or oogonia

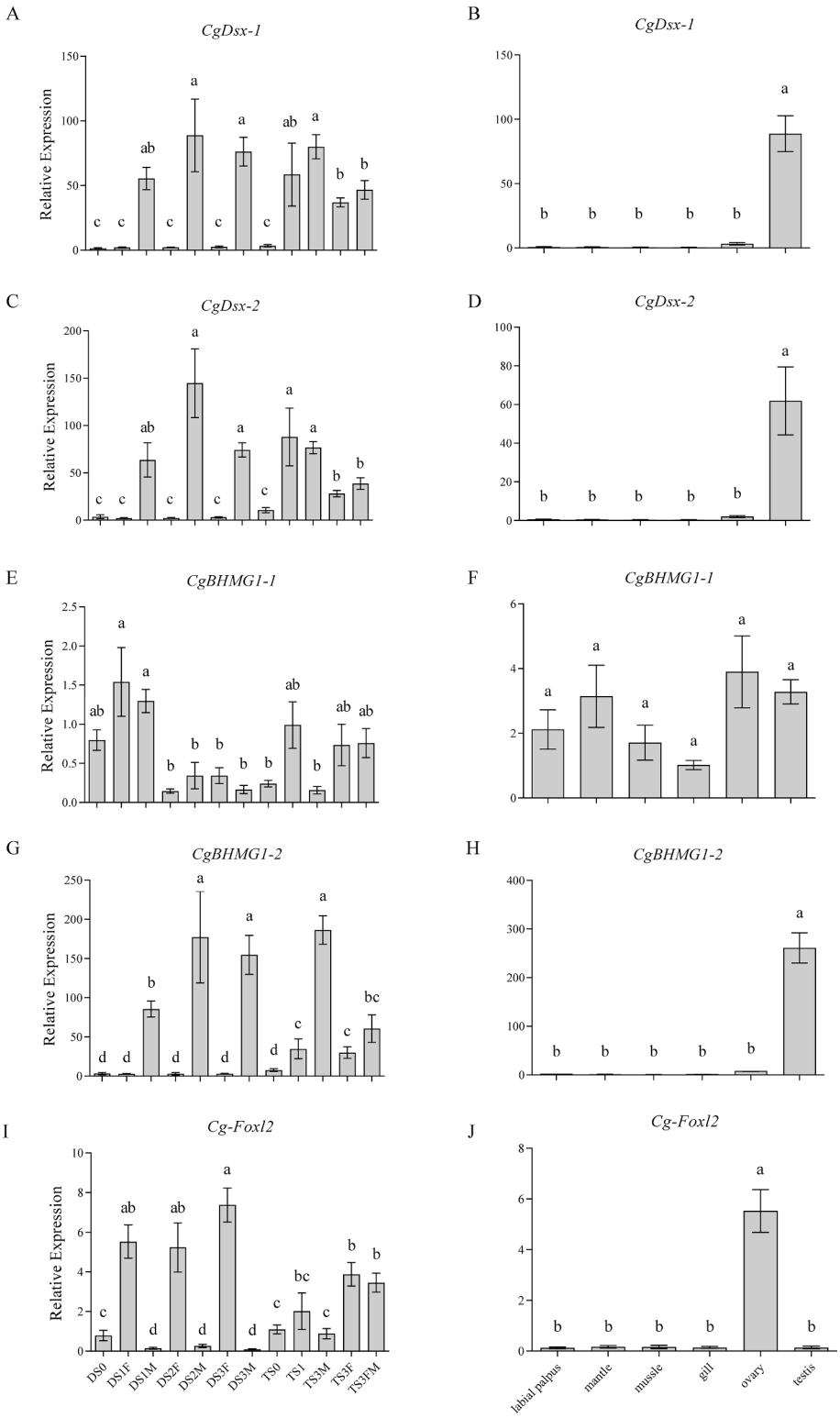

Fig. 3. Expression profiles of five transcripts of potential sex-determining actors. (A, C, E, G and I) Mean transcript expression during different gonadic categories. (B, D, F, H and J) Mean transcript expression in different tissues. Development stage, sex and tissue are indicated at the bottom of corresponding figure. For convenience, DS0, DS1F, DS1M, DS2F and DS3 M stood for diploid oysters assigned to stage 0, stage I, stage II and stage III with suffix by "F" or "M" to distinguish female or male, respectively. TS0, TS1, TS3M, TS3F and TS3FM stood for triploid oysters assigned to stage 0, stage I, male at stage III, female at stage III and hermaphrodite, respectively. Bars represent standard error. Statistical analyses were carried out by *t* test using software SPSS 18.0 and levels were accepted as significant at P value < 0.05. Different letters indicate significant differences between tissues (a–d).

based on cell size and morphological characteristics (Fig. 4C). In mature triploid gonads, predominant expression was observed in spermatogonia of male gonads, and in follicle cells surrounding oocytes in the female gonadal tubules (Fig. 4D and E). Whatever the triploid hermaphrodite that observed with male or female gametogenesis first, *CgDsx* mRNA staining was mainly present in spermatogonia as shown in Fig. 4F and G. In nontypical hermaphrodite with predominantly male and rare female gonad material, *CgDsx* expression was observed in the spermatogonia and undetermined gonial cells surrounding male germ cells (Fig. 4H). For *CgBHMG1*, mRNA expression was essentially found in the spermatocytes and to a lesser extent in spermatogonia from *in situ* hybridization on the diploid and triploid male gonads (Fig. 5A and B). And in the diploid and triploid ovaries, *CgBHMG1* mRNA expression was observed in the follicle cells (Fig. 5C and D). Similar with the expression of *CgDsx* mRNA in the triploid gonad at stage I, *CgBHMG1* mRNAs were also detected in undetermined genial cells in the gonadal tubules

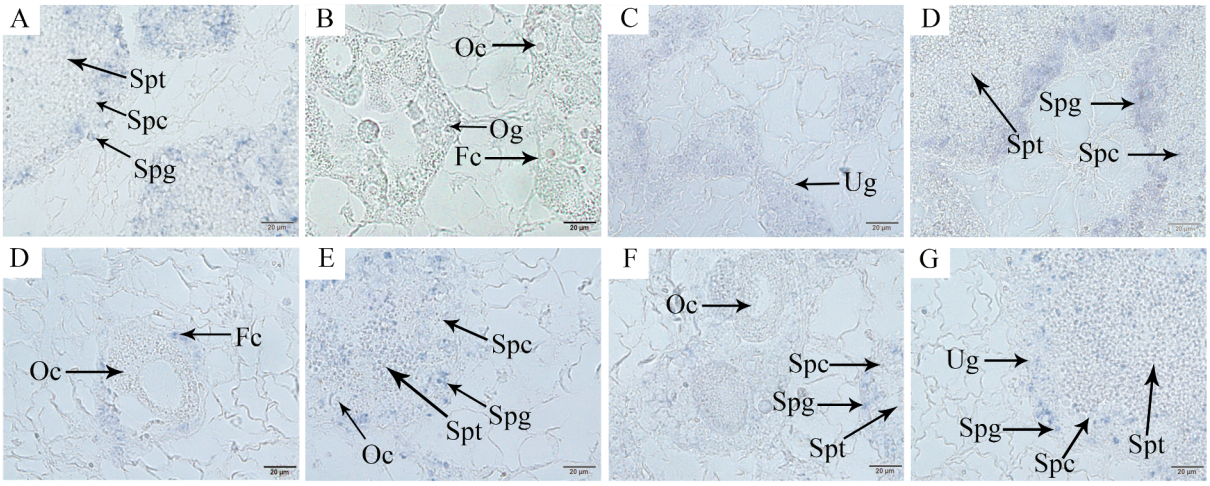

Fig. 4. *CgDsx* mRNA cellular localization in gonadic tubules of diploids (A, B) and triploids (C–H). Localization of *CgDsx* with anti-sense probe in testes of diploids (A), ovaries of diploids (B), gonads of triploid oysters at stage I (C), testes of triploids (D), ovaries of triploids (E), and hermaphroditic gonads of triploids (F–H). Spg: spermatogonia, Spc: spermatocytes, Spt: spermatids, Og: oogonia, Oc: oocyte, Fc: follicle cell, Ug: undetermined germ cell. Bars: 20 μm.

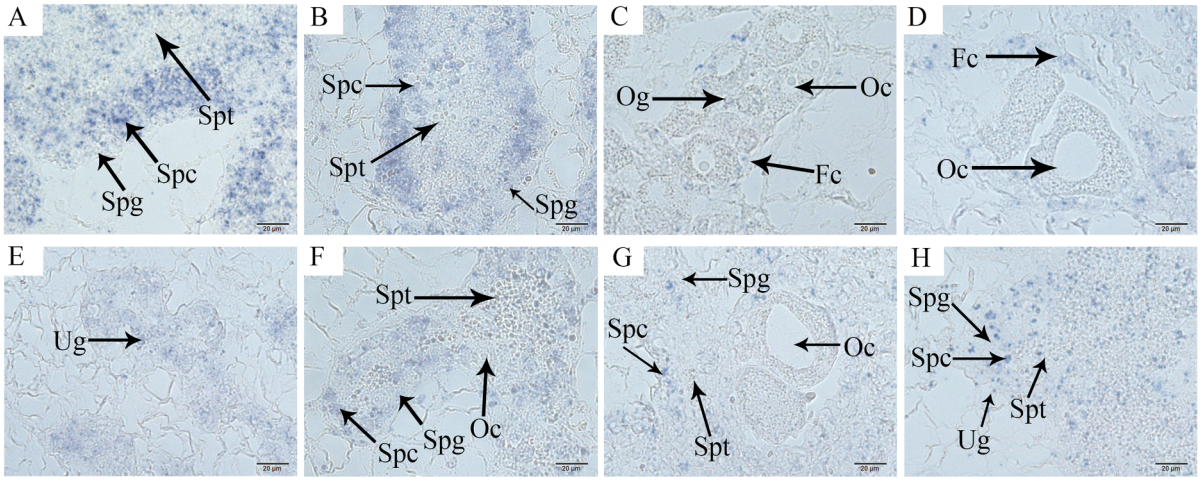

Fig. 5. *CgBHMG1* mRNA cellular localization in gonadic tubules of diploids (A, C) and triploids (B, D, E, F, G and H). Localization of *CgBHMG1* with anti-sense probe in testes of diploids (A), testes of triploids (B), ovaries of diploids (C), ovaries of triploids (D), gonads of triploid oyster at stage I (E), and hermaphroditic gonads of triploids (F-H). Spg: spermatogonia, Spc: spermatocytes, Spt: spermatids, Og: oogonia, Oc: oocyte, Fc: follicle cell, Ug: undetermined germ cell. Bars: 20 μm.

(Fig. 5E). As for triploid hermaphrodite with rare female gonad material, *CgBHMG1* mRNAs were observed in undetermined gonial cells besides male germ cells (Fig. 5H). Moreover, the signal could also be observed in cell nuclei of the connective tissue, as intensely as in the gonads (Fig. 5). Cellular expression of *CgFoxl2* was also observed across diverse diploid and triploid gonads. In ovaries of diploid and triploid oyster, *CgFoxl2* mRNA expression was confirmed exclusively to the follicle cells surrounding oocytes (Fig. 6A and B). Whatever testes of diploid or triploid oyster, specific signal of *CgFoxl2* mRNA expression could be detected in germ cells (Fig. 6C and D). In triploid oyster gonad at stage I, *CgFOXL2* mRNAs were mainly detected in undetermined germ cells that located the center of the tubules (Fig. 6E). In triploid hermaphrodite with male gametogenesis first as shown in Fig. 6F, *CgFOXL2* transcripts were found in some male germinal cells and follicle cells surrounding the gonadal tubules. But *CgFoxl2* mRNA expression was only detected in rare follicle cells in gonadal area of triploid hermaphrodite with female gametogenesis first in comparison (Fig. 6G). It is difficult to know if there is a difference with negative controls as the staining of *CgFoxl2* anti-probes is faint and the sense riboprobe gave the similar faint signal in the testes but not ovaries (Fig. S1E, F). In triploid hermaphrodite with rare female gonad material, *CgFOXL2* mRNAs were also observed in undetermined gonial cells besides follicle cells and male germ cells (Fig. 6H).

4. Discussion

Our previous transcriptome analysis on the mollusc Pacific oyster revealed that several transcriptional regulators displayed sexually dimorphic expression during early gonad differentiation and are regarded as potential participants in the sex differentiation cascade (Yue et al., 2018). And this promoted us to take further investigation on them, including evolutionarily conserved *CgDsx*, *CgFoxl2* and a novel sex-related gene *CgBHMG1* (uncharacterized LOC105327228).

CgDsx, an ortholog of the evolutionarily conserved sex-determining factor *Dmrt1*, was firstly identified as potential testis-promoting transcriptional regulator in *C. gigas* for its specific expression in mature testes (Zhang et al., 2014). Subsequently, we found that the male-specific expression of *CgDsx* could also be detected in gonads at stage I and IV (Yue et al., 2018). And its homologs with male-specific expression were also identified in other bivalve species, such as *Patinopecten yessoensis* (Li et al., 2016). Whereas, there was rare reports about splicing transcripts

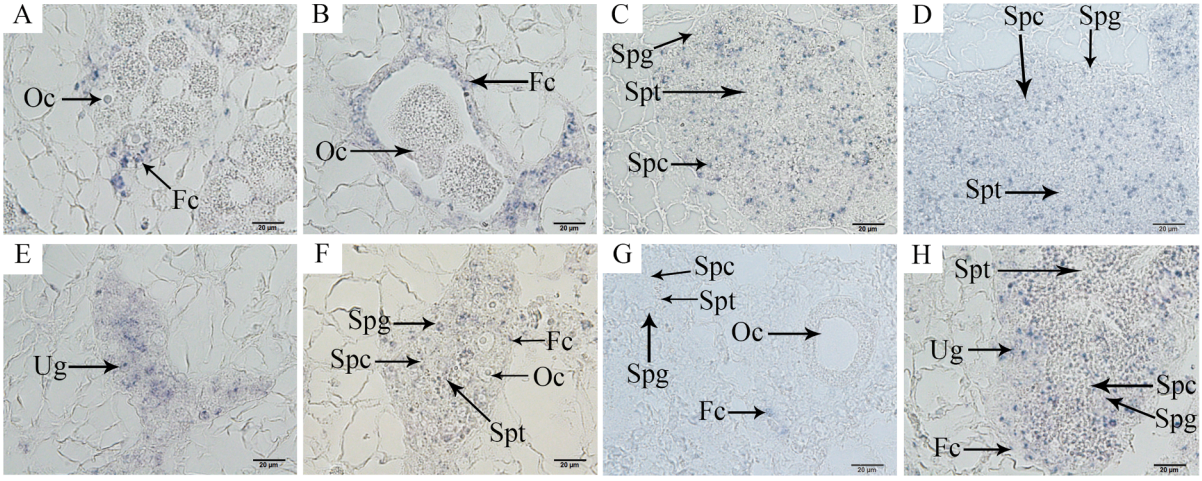

Fig. 6. *CgFoxl2* mRNA cellular localization in gonadic tubules of diploid (A, C) and triploid (B, D, E, F, G and H) gonads. Localization of *CgFoxl2* with anti-sense probe in ovaries of diploids (A), ovaries of triploids (B), testes of diploids (C), testes of triploids (D), gonads of triploid oyster at stage I (E), and hermaphroditic gonads of triploids (F–H). Spg: spermatogonia, Spc: spermatocytes, Spt: spermatids, Og: oogonia, Oc: oocyte, Fc: follicle cell, Ug: undetermined germ cell. Bars: 20 μm.

of *Dmrt1* orthologs in bivalves. In the present study, two splicing isoforms of *CgDsx* were obtained by RACE PCR, which was consistent with the finding that the splicing of *Dmrt1* is common in many animals (Kopp, 2012). Unlike the two *Dsx* isoforms of *Drosophila melanogaster* that encode polypeptides with sex-specific C-terminal domains (Burtis and Baker, 1989), the two *CgDsx isoforms* differ in and 9 bp nucleotides in ORF that encode 3 amino acids outside the conserved DM domain. Whether they have distinct effect on target gene expression is still unknown, although the variance in deduced amino-acid sequence is very low.

As for the uncharacterized LOC105327228 that co-expressed with *CgDsx*, we tentatively identified it as a member of BHMG1 family and named it as *CgBHMG1* according to Psi-Blast result. In mice, the testis-enriched BHMG1 protein (also known as meiosis initiator protein) directly activates the transcription of a subset of critical meiotic genes playing a central role in cell-cycle switching from mitosis to meiosis (Ishiguro et al., 2020; Oatley and Griswold, 2020). And male-specific expression of *CgBHMG1* in our previous transcriptome data is consistent with its expected role as meiosis initiator in oyster germ cells (Yue et al., 2018). But in the transcriptome data from the Pacific oyster genome assembly oyster_v9 (Zhang et al., 2012), no significant abundance difference was observed among oyster tissues except gonads. This indicated the potential multiple roles of *CgBHMG1* besides meiosis initiator in germ cell differentiation. Moreover, there are two isoforms of *CgBHMG1* transcripts that vary in the sequence of partial 5′ UTRs and size of 5′ UTR introns, which contributes to the understanding about the alternative splicing of BHMG1 gene family. From the transcriptome data released on NCBI, there are four and fourteen isoforms of BHMG1 transcripts in human and mice respectively, including variances in 5′ UTRs, CDs and 3′ UTRs (Grimwood et al., 2004; Church et al., 2009). Though Ishiguro et al. (2020) performed a precise study on the function of BHMG1 protein, there is limited knowledge about a great variety of transcript isoforms. Thus, expression pattern and potential role of specific transcript isoform of BHMG1 gene come into question.

Temporal expression analysis during gonad differentiation of diploid and triploid oyster provided us more information for understanding the roles of the sex-related *CgDsx*, *CgBHMG1* and *CgFoxl2*. According to RT-PCR analysis based on transcript-specific primers, both two *CgDsx* isoforms may participate in male differentiation and sex maintenance for their same testis-specific expression in diploid Pacific oyster with fertile gonads, which is quite different with male and female-specific isoforms that have opposite effects on sex differentiation in fruitfly (Jursnich and Burtis, 1993; Waterbury et al., 1999). For *CgBHMG1*, only the transcript variant with shorter 5′ UTR showed testis-specific expression pattern in diploid oyster and was thought to be acting in germ cell differentiation as its ortholog in mammals (Ishiguro et al., 2020). What's more, RT-PCR in this study revealed interesting expression pattern of sex-related transcripts in triploid animals, including the three male-specific transcripts and the female-specific *CgFoxl2* identified in previous study (Naimi et al., 2009b; Santerre et al., 2012). Similar with that found in transcriptomic profiling of gametogenesis in triploid Pacific oyster (Dheilly et al., 2014), the sex-specific transcripts in diploid oysters were expressed in triploid individual with opposite sex. Even more interesting is that the expression of all the four transcripts could also be detected in triploid hermaphrodites and individuals at stage I that sex could not be determined. The abnormal expression of sex-related genes in triploid oysters were logically associated with the reduced gametogenesis, higher proportion of hermaphrodites and undifferentiated individuals compared to diploid oysters (Normand et al., 2009), which corresponded with their expected roles as regulator of sex differentiation and/or gametogenesis in oysters.

Subsequent spatial expression analysis might hold the clue to the roles of the three transcriptional regulators in oyster. In gonads of diploid oysters, *CgDsx* were significantly expressed in the spermatogonia of testis and nearly unexpressed in ovarian tissue and oocytes. Various *Dmrt1* expressed cells have been observed in testis of animals, but often these involve the spermatogonia (Lei et al., 2007; Xia et al., 2007; Wu et al., 2012; Li et al., 2014a, 2014b). Additionally, *Dmrt1* in spermatogonia was demonstrated to determine whether spermatogonia undergo mitosis and spermatogonial differentiation or meiosis in mice (Matson et al., 2010; Griswold, 2016). Therefore, the predominant expression of *CgDsx* in spermatogonia suggested that it might be related to Pacific oyster testis differentiation like its homologs in mice. *In situ* hybridization in gonads of diploid oyster revealed prominent expression of *CgBHMG1* in spermatocytes, mild expression in spermatogonia, follicle cells surrounding oogonia and oocytes and cell nuclei of the connective tissue. In mice, BHMG1 (MEIOSIN) was only observed in spermatocytes during the preleptotene stage and germ cells of embryonic ovary during E13.5–15.5 at the time of meiotic initiation, which is consistent with its role as a meiotic initiator (Ishiguro et al., 2020). From the cellular location of *CgBHMG1* and its homolog in mice, the *CgBHMG1* might play other role besides potential regulator in oyster male germ cell differentiation. In the present study, the well-characterized *CgFoxl2* were mostly localized in the ovarian follicle cells, similar with its vertebrate orthologs (Govoroun et al., 2004; Nakamoto et al., 2006; Uhlenhaut and Treier, 2006), and also in germ cells of male gonadal tubules of diploid

oyster. This suggested *CgFoxl2* may be involved in female sex differentiation and maintaining ovarian function as *Foxl2* in other species (Crisponi et al., 2001; Govoroun et al., 2004; Nakamoto et al., 2006; Uhlenhaut and Treier, 2006). But what the expression of *CgFoxl2* in male germ cell means is still unpredictable for now.

The most interesting finding of the spatial expression analysis on gonads of triploid oysters is about the co-expression of the three genes in gonadal tubules of gonads at stage I, undetermined gonial cells in nontypical hermaphroditic gonads with rare female materials and ovarian follicle cells. We realized that the aberrant expression of these sex-related genes in triploid oyster compared to diploid individual was extremely rare and this led us to the battle between male and female-determining factors for sex control that hypothesized in various animal taxa (Herpin and Schartl, 2011; Matson et al., 2011; Li et al., 2018). In another bivalve *P. yessoensis*, sex differentiation was assumed to co-occur with the formation of follicles, in which relative expression of *FOXL2* vs *DMRT1L* changes, possibly leading to the differentiation of undifferentiated germ cells into spermatogonia or oogonia (Li et al., 2018). Simialar hypothesis could be raised in Pacific oyster, although the co-occureence of sex differentiation and follicles formation needs more evidence. And in triploid oyster sex differentiation that depends on the antagonism of male and female-differentiation factors, such as *CgDsx* and *CgFoxl2*, might be impaired by the presence of the third set of chromosomes that bring about duplication of sex-related genes (Dheilly et al., 2014), which was inspired by more and more observations that sex determination across taxa might be sensitive to dosage, such as the sex revesal caused by duplication of *Sox9* and *Wnt4* in humans (Huang et al., 1999; Mandel et al., 2008; Jordan et al., 2001). In our view, the distrupted sex differentiation of tripolid oysters and the aberrant expression of some sex-related genes also indicated the important roles of these genes in nomal sex differentiation from an indirect perspective.

5. Conclusions

This study identified two mRNA splicing isoforms of the Pacific oyster DM domain gene *CgDsx* and two isoforms of a novel sex-related *CgBHMG1*. Temporal and spatial expression analysis of the two genes and a previously characterized *CgFoxl2* in diploid and triploid oysters provided supporting data for their involvement in sex differentiation and/or germ cell differentiation. Additionally, the aberrant expression of these sex-related genes in triploid oyster leads us to the battle between male and female pathway in *C. gigas*. This work will enrich the knowledge about the role of potential actors in *C. gigas* sex differentiation pathway and provide new sights on this issue from triploid individuals with abnormal gonad differentiation.

Author Contributions. CY carried out the sample collection, histological analysis the data analysis, and drafted the manuscript. QL conceived of the study, participated in experimental design and coordination, and contributed to the manuscript preparation. HY participated in the data analysis. All authors read and approved the final manuscript.

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.

Acknowledgements

This study was supported by grants from National Natural Science Foundation of China (31672649), National Key R&D Program of China (2018YFD0900200), Science and Technology Development Project of Weihai City (2018NS01), and Industrial Development Project of Qingdao City (20-3-4-16-nsh).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.145692.

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