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RESEARCH PAPER

Transcriptional regulation analysis reveals the complexity of metamorphosis in the Pacific oyster (*Crassostrea gigas*)

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Abstract

Many marine invertebrate phyla are characterized by indirect development. These animals transit from planktonic larvae to benthic spats via settlement and metamorphosis, which contributes to their adaption to the marine environment. Studying the biological process of metamorphosis is, thus, key to understanding the origin and evolution of indirect development. Although numerous studies have been conducted on the relationship between metamorphosis and the marine environment, microorganisms, and neurohormones, little is known about gene regulation network (GRN) dynamics during metamorphosis. Metamorphosis-competent pediveligers of the Pacific oyster *Crassostrea gigas* were assayed in this study. By assaying gene expression patterns and open chromatin region changes of different samples of larvae and spats, the dynamics of molecular regulation during metamorphosis were examined. The results indicated significantly different gene regulation networks before, during and post-metamorphosis. Genes encoding membrane-integrated receptors and those related to the remodeling of the nervous system were upregulated before the initiation of metamorphosis. Massive biogenesis, e.g., of various enzymes and structural proteins, occurred during metamorphosis as inferred from the comprehensive upregulation of the protein synthesis system post epinephrine stimulation. Hierarchical downstream gene networks were then stimulated. Some transcription factors, including homeobox, basic helix–loop–helix and nuclear receptors, showed different temporal response patterns, suggesting a complex GRN during the transition stage. Nuclear receptors, as well as their retinoid X receptor partner, may participate in the GRN controlling oyster metamorphosis, indicating an ancient role of the nuclear receptor regulation system in animal metamorphosis.

Keywords Gene regulation network · Indirect development · Lophotrochozoa · Nuclear receptors · Neurohormones · Protein synthesis · Transcription factors

Introduction

Knowledge of vertebrate and arthropod model animals, which are primarily direct developers (Davidson et al. 1995; Sly et al. 2003), is far from sufficient to fully understand the evolution of metazoan development (Xu et al. 2016). Studies on the gene regulation network (GRN) of the transition stage

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of indirect developers (Davidson et al. 1995), a large group of marine organisms forming larvae during development, can provide insights into the evolutionary mechanism of animal ontogeny (Raff 2008). Indirect developers commonly have a transition stage from planktonic larvae to benthic spat, where some fundamental bioprocesses occur, such as settlement and metamorphosis. Furthermore, this transition is usually rapid in marine animals (Hadfield 2000). As the adult body plan is distinct from that of larvae, understanding how the GRN is re-organized is the key to gaining insights into the complexity of the transition (Heyland and Moroz 2006).

While the metamorphosis GRN of model animals, e.g., frogs and fruit flies, has been widely studied, marine indirect developers have not received sufficient attention. The initiation of metamorphosis of frogs (thyroid hormone, TH) (Brown and Cai 2007) and fruit flies (ecdysone) is usually initiated by one or several key hormones, and induces diverse phenotypic transformation and intracellular responses. In tadpoles, thyroid hormone induces GRN through activating transcription factor thyroid receptors (TRs), together with their retinoic acid receptor (RXR) partners (Shi et al. 2012). In the fruit fly *Drosophila,* injection of ecdysone induces puffing of salivary gland chromosomes, resulting in transcription activation of many genes, and the initiation of molting (Hill et al. 2013). Ecdysone receptor (EcR) and its partner USP (ultraspiracle, an ortholog of vertebrate RXR) play a central role during *Drosophila* metamorphosis.

Recent studies suggested that lophotrochozoan species may contain integrated hormone systems (Bauknecht and Jékely 2017; Goulty et al. 2023). Main hormones such as steroids (e.g., ecdysone), tyrosine derivatives (e.g., TH, octopamine, epinephrine), and proteins (e.g., insulin-like peptides) have all been reported in lophotrochozoans (Bauknecht and Jékely 2017; Heyland et al. 2006; Hiripi et al. 1998; Whitehead and Sellheyer 1982; Xu et al. 2021). Furthermore, lophotrochozoans may maintain a more comprehensive hormone regulatory system than vertebrates or insects. For example, molluscs use both epinephrine and octopamine, while vertebrates make extensive use of epinephrine but little use of octopamine, and insects use octopamine but not epinephrine (Adamo 2008; Ji et al. 2016). Genes related to both thyroid hormones and the ecdysone system (synthesis enzymes, TR, EcR, and RXR) have been identified in molluscs (Huang et al. 2015; Laguerre and Veenstra 2010; Zhang et al. 2012). Furthermore, the hormone receptors in molluscs show more diversity than those in common model animals (Jiao et al. 2019). Indeed, vertebrates and insects are thought to be highly specialized in view of their developmental regulatory mechanisms (Peterson et al. 2000). In contrast, lophotrochozoans may represent the most general mode of bilaterian development (Davidson et al. 1995), although their GRNs are not well characterized.

The Pacific oyster *Crassostrea gigas* is a typical indirect developer, and one of the best-studied lophotrochozoans because of its biology and its importance to the aquaculture industry (Hedgecock et al. 2005). The planktonic larval stage usually lasts two weeks and then forms the metamorphosis-competent pediveliger at sizes larger than 300 µm. Pediveligers detect substrata with their foot. Settlement and metamorphosis are induced by a suitable environment, chemical cues or biofilms. Some neurotransmitters, such as epinephrine, can induce direct metamorphosis of pediveligers and has, thus, been successfully used to produce cultchless oysters in hatcheries (Coon et al. 1986). As oyster settlement and metamorphosis are quick and usually nonsynchronous, it is difficult to collect transitioning individuals. Epinephrine-induced pediveligers provide a good model to study the GRN during metamorphosis. At the same time, epinephrine signaling is one of the best-studied pathways in oysters (Coon and Bonar 1987; Vogeler et al. 2021), which comprises a complex transmitter system together with other monoamine signaling pathways (Bauknecht and Jékely 2017). We, thus, focused on the downstream GRN of epinephrine signaling, and collected both epinephrine-treated pediveligers and naturally attached spats (indicating successful settlement and metamorphosis) to conduct multi-omics studies. In this way, the GRN complexity of the transition stage was revealed by comparing gene expression patterns before and after metamorphosis.

Results

Pediveliger-enriched genes

By comparing the gene expression pattern throughout 38 development stages from egg to juvenile, a total of 1016 genes were identified to be enriched in pediveligers (Supplementary Table S1). GO enrichment analysis (Supplementary Table S1) indicated that terms related to receptors and transcription factors are enriched. Some G protein-coupled receptors (GPCRs) that are known to be key regulators of oyster attachment and metamorphosis, including adrenergic receptor, octopamine receptor, gamma-aminobutyric acid receptor, dopamine receptor, and some neuropeptide receptors, were identified in this gene set. Other types of receptors, including acetylcholine receptors, glutamate receptors, and glycine receptors, were enriched in terms related to larval sensory function, nerve activity, or behavior. Homeobox, bHLH, and other transcription factors in the gene set were annotated in terms related to tissue development, neuron differentiation, and morphogenesis.

Enriched genes in early spats

By comparing RNAseq results of early spats to those of swimming larvae (mixed umbo and pediveliger larvae) from study 1 (Fig. 1), a total of 945 spat upregulated genes (URGs) and 260 spat downregulated genes (DRGs) were identified (Supplementary Table S2). URGs were mostly enriched in terms of regulation of protein processing, chaperone-mediated protein folding and tissue (epidermis) development (Supplementary Table S2), and also included numerous structural protein genes (collagens and chitins) and enzymes for their synthesis. Study 3 identified more DEGs by further distinguishing umbo and pediveliger larvae and early spats of the same age. The 1551 spat URGs (spat vs. pediveliger) were enriched with terms for DNA replication, cell cycling, tissue development (digestive system, kidney), as well as collagen and chitin metabolism (Supplementary Table S2). There were 314 overlapped genes between the two URG sets from studies 1 and 3.

Morphological and transcriptomic responses of pediveligers to epinephrine

To investigate the metamorphosis on a fine temporal scale, epinephrine treatment was applied to stimulate quick and concerted metamorphosis. Some pediveligers showed quick morphological responses to epinephrine. Calcified shells were observed as early as 6 hpt (Fig. 2A), although the metamorphosis rate varied (30–80%) between different experiments. Principal component analysis (PCA) of gene expression patterns indicated that EPI treatment groups and control groups were distinctly separated, while the three experiments were also distinguishable, suggesting a significant genomic response of pediveligers to epinephrine stimulation (Fig. 2B). However, 20 mpt treatment groups were clustered together with control groups, indicating small transcriptional changes within a short time post-treatment. We, thus, selected samples (excluding 20 mpt groups) from different time points as biological replicates to calculate DEGs for experiments 2 (Exp2) and 3, respectively. No DEGs were calculated for Exp1, as its EPI treatment group (X1EPI.6h) did not have biological replicates.

To cover as many differential genes as possible, the united set of DEGs from Exp2 and Exp3 was calculated, identifying a total of 780 DEGs (including 610 URGs and 170 DRGs) (Supplementary Table S3). GO enrichment analysis of URGs indicated that the top 10 terms were mainly related to gene transcription, protein biogenesis and protein transport (Supplementary Table S3). Almost all genes of the core translation initiation factors were upregulated

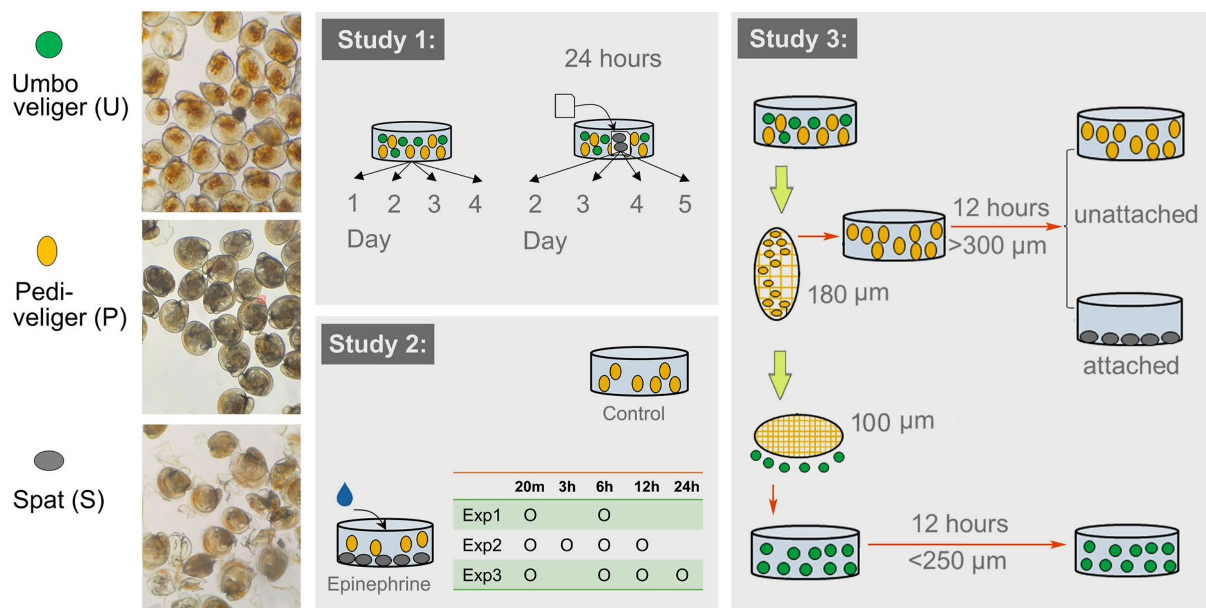

Fig. 1 Experimental design and sampling strategies. The studies focus on competent pediveligers (P) and newly attached spats (S) while using late umbo veligers (U) as the control. In study 1 (conducted in 2011), a mixture of larvae (mainly by U and P) were collected on successive four days (treated as four biological replicates) from a 25 m^3 hatchery tank at ~ 14 days post-fertilization. Spats were then collected in the next four days by immersing plastic plates in the tank for 24 h. In study 2, large-size larvae (almost all Ps) were treated with epinephrine hydrochloride at a final concentration of 1×10^{-4} mol/L. Samples were collected at 20-min and 6-h post-treatment (hpt, three experiments were conducted in 2010 and 2013). Samples at 3, 12, or 24 hpt were also collected from some of the replicates. In study 3 (conducted in 2022), U and P were selected by mesh screening. All larvae were cultured in fresh seawater for ~ 12 h surrounded by nylon screens. S were sampled by collecting spats attached to the screens (four biological replicates)

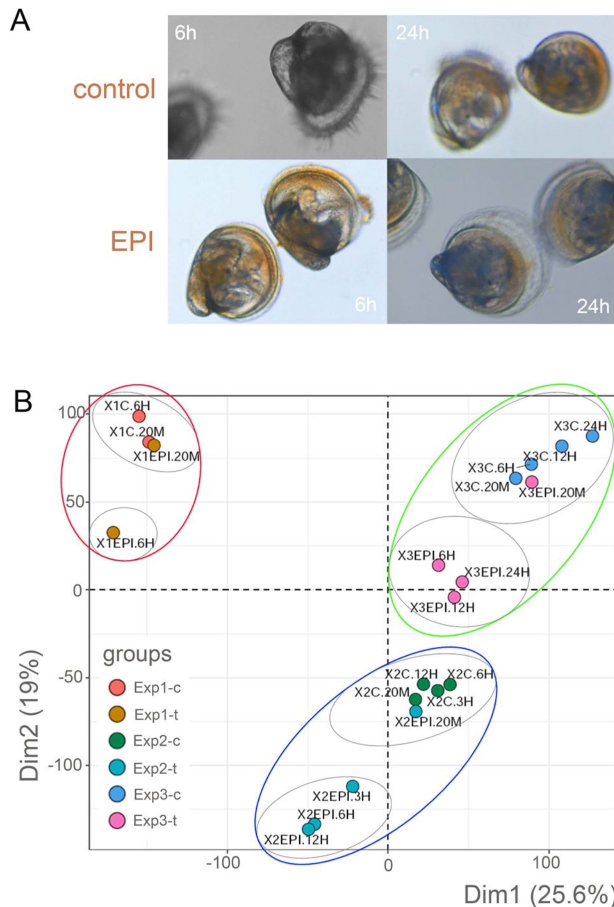

Fig. 2 Morphological changes of competent pediveligers after epinephrine stimulation (**A**) and principal component analysis of the RNAseq data (**B**). The calcified dissoconch shell formed soon after epinephrine stimulation. The red ellipse encompasses samples from the first experiment (controls at 20 min and 6 h: X1C.20M, X1C.6H, treated groups: X1EPI.20M, X1EPI.6h). Samples collected during the second and third experiments are encompassed by the blue and green ellipses. With the exception of the EPI 20-min groups, the EPI-treated groups are separated from the controls in all three experiments

after EPI stimulation, where genes of eIF2S1, 2S3, 3B, 3C, 3D, 3J, 3K, 3M, and 4E were significantly upregulated (Fig. 3; Supplementary Table S4). These genes were also upregulated in spats obtained in study 3 (Supplementary Table S4). At the same time, genes encoding many molecular chaperones including subunits of group I (GroEL, LOC105348623 and GroES, LOC105348624) and group II (subunit beta, LOC105334954; delta, LOC105334189; epsilon, LOC105318294; eta, LOC105332252) chaperonins were also upregulated. Genes related to intracellular receptor signaling pathways, e.g., nuclear *retinoic acid receptor* (RAR, LOC105339440), were enriched. Although the key partner of nuclear receptors, *retinoid X receptor* (RXR, LOC105333288), was not in the URG set, it showed significant upregulation at the early time points (3 and 6 hpt, Fig. 4; Supplementary Table S5). Furthermore, genes functioning in chitin metabolic process and shell formation, such as *PIF-like* (LOC105345525), were also enriched.

Transcriptional regulation assays

An assay for transposase-accessible chromatin was conducted with high throughput sequencing to survey the genomic-wide changes of transcriptional regulation during oyster metamorphosis. Clustering analysis showed that the four early spat samples were grouped together, although the correlation levels were low among samples (Fig. 5A). Mapping results indicated that reads mainly enriched around the transcription start site (TSS), with some enrichment within the gene body (Fig. 5B, C), suggesting that the sample preparation protocol needs to be improved. However, differential peak analysis (Fig. 5D–F) provided much useful information on gene transcriptional regulation. There were 154 genes identified as having both peak variation and gene expression upregulation during development from pediveliger to spat (Supplementary Table S6). This accounted for about 10% of the spat URGs (1551) compared to pediveliger. A total of 300 of the 3327 genes were also detected to be peak-differentiated URGs of spat compared to umbo. In contrast, only 83 out of 1809 genes were identified in pediveliger and umbo comparison groups. The spat-enriched peak-differentiated URGs contained many members involved in collagen biosynthesis and neuronal activities (e.g., *acetylcholine receptor*, LOC105317244). Transcription factors accounted for a large part of the peak-differentiated URGs, which were mainly related to tissue development and cell proliferation, including many nuclear receptors (e.g., *RAR*; *neuronal acetylcholine receptor subunit alpha-3*, LOC105348353; *peroxisome proliferator-activated receptor*, LOC105317849), identified in the transcriptomic studies above. Correspondingly, the nuclear receptor binding motif (RGGTCA) was also within the 20 motifs matched by the spat-enriched peaks (Supplementary Table S7).

Discussion

The oyster life cycle includes the transformation from pelagic to benthic through complex metamorphosis, when larvae experience dramatic tissue transition (Coon and Bonar 1987). Some larval tissues, e.g., the ciliated velum, anterior adductor, and ventral retractors, degenerate during metamorphosis (Li et al. 2019). Histogenesis of some adult tissues (e.g., gills and calcified shells) are initiated during metamorphosis (Cannuel and Beninger 2006). Furthermore, the formation of certain pediveliger-specific tissues (e.g., foot and pigmented eyespot) occurs ahead of metamorphosis. Two apoptosis-related genes, *Caspase3*

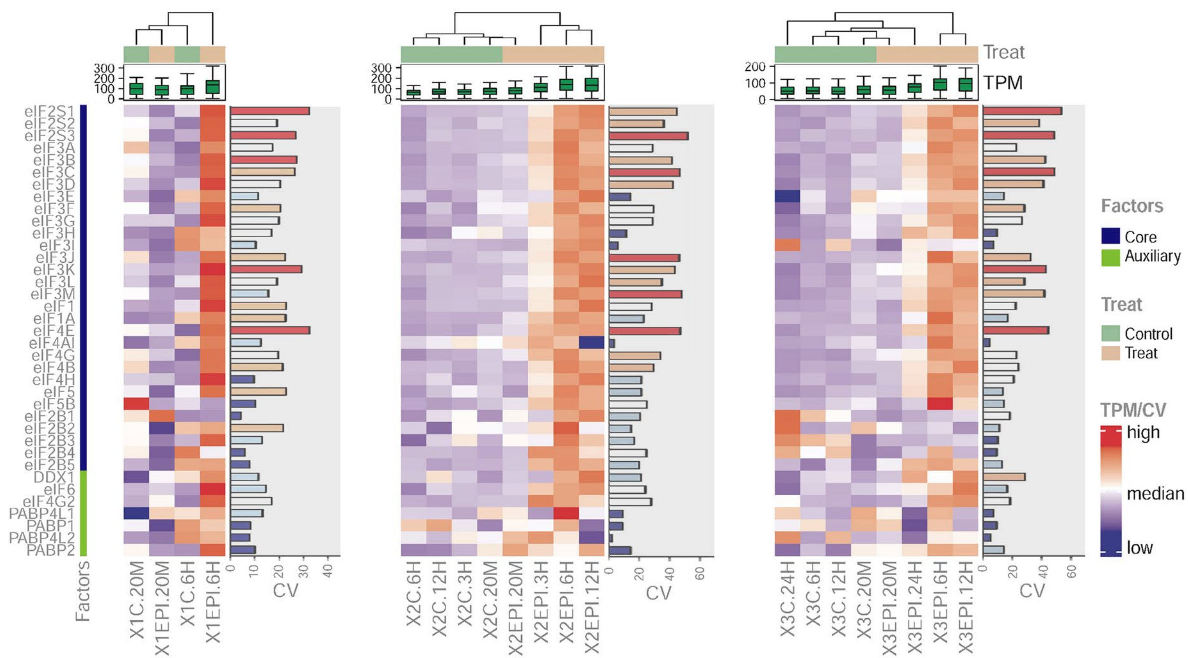

Fig. 3 Expression pattern of translation initiation factors. Factors were classified as core factors (cyan bar on the left) and auxiliary factors (green bar on the left). The box plot at the top indicates the distribution of transcripts per million mapped reads (TPM) of all investigated factors. The colored bars on the right side of each heatmap indicate the coefficient of variation (CV) of the TPM for each gene in the corresponding row. The red bar suggests a high CV, indicating high expression variation of the gene among different samples. Most factors present in the URGs had high CVs. Expression data of the genes are shown in Supplementary Table S4

(LOC105340742) and *Bcl2-like* (LOC105338289), showed upregulation at pediveliger and spat stages (Supplementary Table S2), indicating their possible involvement in larval tissue degeneration. The results from studies 1–3 indicated that pediveliger enriched gene set was distinct to spat URGs. As marine animals usually conduct rapid metamorphosis (Hadfield 2000), the pediveliger should be fully prepared for the quick metamorphosis. So, the sampling time point is key to studying the metamorphosis GRN. Pediveliger and spat samples collected in these studies represented only two static stages reflecting the profiles of two time points of a complex GRN cascade. Epinephrine stimulation on competent pediveligers provides a way to produce larvae, the metamorphosis of which is artificially controlled, for assaying gene expression dynamics during metamorphosis.

Nervous system remodeling before metamorphosis

As a sign of metamorphosis competence, the appearance of eyespots indicated the possible remodeling of the larval nervous system. The development of the foot as a temporary organ to sense a suitable substrate for settlement suggests the biogenesis of novel nerve connections. Correspondingly, genes involved in nervous system development, synapse formation, or neuronal regeneration (e.g., *neuroligins*, *neurotrimin*, *neuroglobin*, *neurocalcin,* and many genes coding neuronal acetylcholine receptor subunits) were highly expressed during pediveliger stage (Supplementary Table S2). At the same time, expression levels of many receptors for sensing environmental stimuli were also upregulated, together with some neuropeptide receptor genes. Many possible receptors of epinephrine, gamma-aminobutyric acid (GABA), dopamine, octopamine and acetylcholine, which have been proven to be effective neuroendocrine compounds to induce oyster metamorphosis (Joyce and Vogeler 2018), were upregulated during the pediveliger stage. However, hormone receptors show high diversity in molluscs (Jiao et al. 2019), and the exact physiology functions of these upregulated receptors are poorly studied and little is known about their functional pathways. The *octopamine receptor* gene (OAR, LOC105328982) for example, which was present in both pediveliger enriched gene set and pediveliger URGs of study 3, is homologous to *Lymnaea* OAR2, and might belong to a poorly studied subfamily of invertebrate OA/TA receptors. The OAR protein is present on the surface of the pediveliger foot, indicating the possible involvement of this protein to sense environmental stimuli. However, direct treatment of competent pediveligers with octopamine resulted in a weak effect on the induction of metamorphosis. Octopamine also showed no effect on the cAMP or Ca^{2+} signal pathway in HEK293 cells stably expressed this gene (Ji et al. 2016). Functional assays to

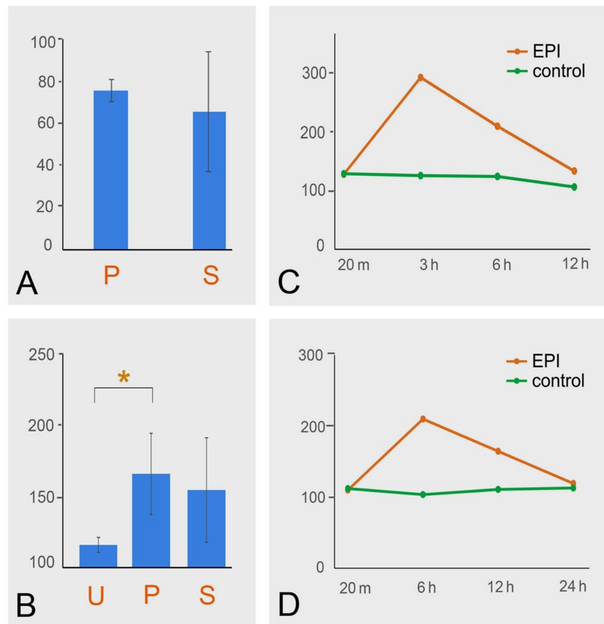

Fig. 4 Expression pattern of retinoid X receptor. **A** Comparison of transcripts per million mapped reads (TPM) values between pediveligers and spat samples of study 1 ($n=4$). **B** Comparison of TPM values between umbo, pediveligers and spat samples of study 3 ($n=4$). **C**, **D**. Temporal pattern of TPM values of samples from experiment 2 (**C**) and experiment 3 (**D**) of study 2 ($n=1$). *U* umbo, *P* pediveliger, *S* spat, *EPI* epinephrine treatment. * indicates a significant difference at $P<0.05$. Expression data of the gene are shown in Supplementary Table S5

these key receptors will provide further insights into their molecular evolution, as well as helping to develop new strategies to control the biofouling caused by marine molluscs.

Shell formation

Oyster shells experience transformation during metamorphosis (Fig. 1A), changing from the aragonite larval shell to the calcite adult shell (Haley et al. 2018; Stenzel 1964). This was well reflected by the 112 intersection genes (Supplementary Table S8) of URGs determined in the studies 1–3. These genes were upregulated during the early spat stage. This gene set differed from previously identified bioadhesive proteinaceous genes in the pediveliger stage (Foulon et al. 2019). Many genes identified in this study were potentially shell related. For example, *perlwapin*, which has been reported to function as an inhibitor of certain crystallographic planes in abalone (Treccani et al. 2006), showed massive upregulation during oyster spat transformation. *Zonadhesin*, *keratin-associated protein*, *spidroin*, *fibrocystin*, and *temptin*-like genes are seldom studied in oysters (Zeng and Guo 2022), but may perform bioadhesion and biomineralization functions during metamorphosis. Many uncharacterized proteins genes in the intersection gene list are also potential novel key molecules in biomineraliztion. Intersection selection is a relatively strict screening method. The URG set of each of studies 1–3 provided additional candidate genes for biomineraliztion studies. Adult oyster shells still have small areas of aragonite (Stenzel 1963), which is usually stacked with chitin sheets and Pif complexes (Pif 97, Pif 80, N16, and other proteins) to form nacreous layers (Suzuki et al. 2009). Some *Pif-like* and *chitin synthase* genes, which were found to be highly expressed in the adult oyster mantle (Zhang et al. 2012), also showed upregulation in attached spats of this study, suggesting that *Pif-like* genes' function might not be limited in aragonite layer.

Global activation of the protein translation initiation system

Oyster attachment or spat transformation requires massive protein synthesis. This was reflected by the global activation of the translation initiation system, a primary regulation stage of protein synthesis (Jackson et al. 2010). EPI stimulation of oyster pediveligers upregulated the expression of core eukaryotic translation initiation factors. Correspondingly, the expression of genes coding for chaperones and chaperonin proteins was also increased, which facilitates protein folding or activate proteolysis of misfolded proteins (Slavotinek and Biesecker 2001). Besides the massive production of structural proteins, there was also biosynthesis of transcription regulators. Upregulation of nuclear receptors (NRs), as well as their interacting partner RXR, indicated the possible involvement of nonpolar regulatory molecules in downstream gene transcription post epinephrine stimulation. These NRs included possible homologs, e.g., ecdysone receptor (LOC105320474) and nuclear hormone receptor HR3, which play important roles in the metamorphosis of insects (Zhao et al. 2018). The widespread presence of ecdysteroids has been reported in many molluscs (Mukai et al. 2001; Romer 1979; Whitehead and Sellheyer 1982) and other lophotrochozoans (Lafont and Koolman 2009). However, their physiological functions remain to be studied. At the same time, the upregulation of the RXR gene in the early stage of metamorphosis and the dynamic opening of the binding motif in the newly attached spat further indicated the possible existence of a hierarchical GRN induced by nuclear receptors (Fig. 6). The metamorphosis GRN becomes more complex when considering the involvement of other transcription factors. For example, the homeobox and bHLH genes *Cranky* (LOC105340685) and *SREBP* (LOC105345607) had a quick response and increased expression within 20-min post EPI stimulation,

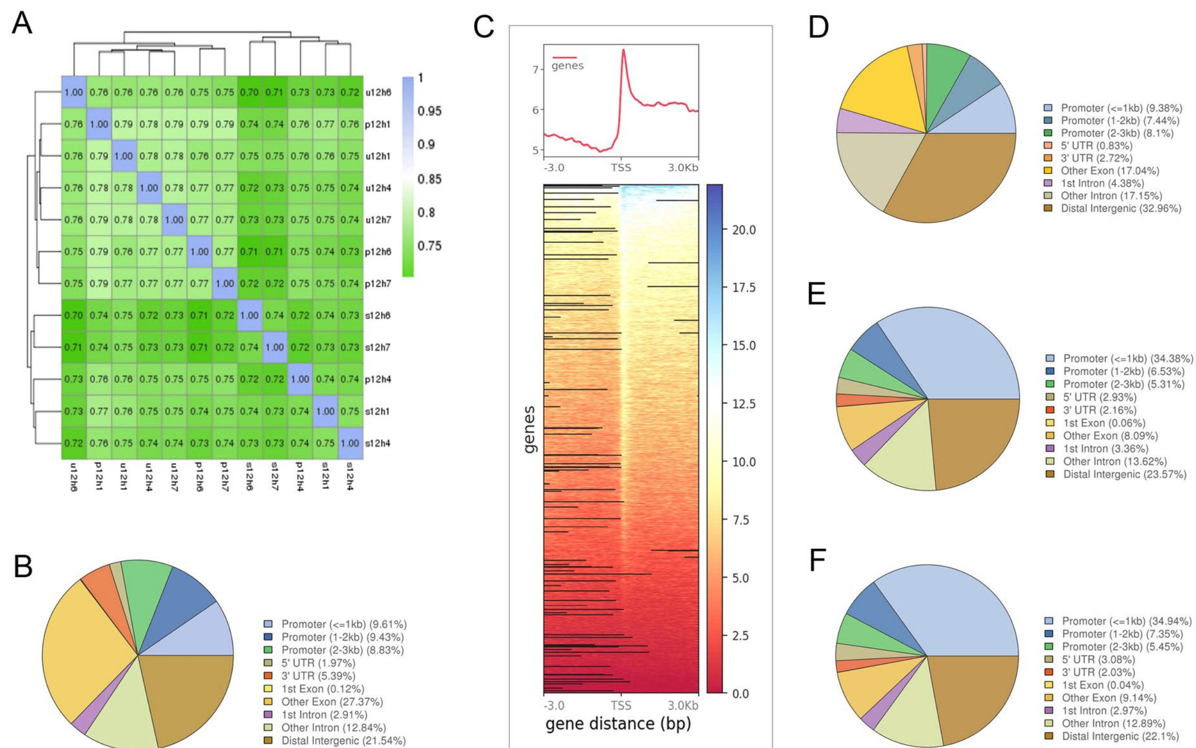

Fig. 5 General analysis of ATACseq data of 12 samples ($n = 4$ biological replicates for three groups). **A** Sample clustering and Pearson correlation coefficients of normalized read abundance. Read abundance in the whole genome was calculated by the sliding window method with a 10 kb interval. **B** Functional annotation on peaks of sample s12h4 as a representative. Other samples showed a similar ratio for different functional regions. **C** Profile plot and heatmap of read density around transcription start site (TSS) of sample s12h4 as a representative. **D**–**F** Functional annotation of differential peaks ($n = 4$ biological replicates) between umbo and pediveliger (**D**), umbo and spat (**E**), pediveliger and spat (**F**). The main differential peak distribution of **D** showed significant difference with **E** and **F**

whereas the homeobox genes *Mkx* (LOC105319734) and *Barh1* (LOC105322672) and the bHLH gene *Mlx2* (LOC105343043) responded later. Although 20 min of EPI treatment was sufficient to initiate metamorphosis and should have triggered the genic response in competent pediveligers, the genomic-scale gene expression response was not fully activated within this short time. PCA analysis indicated that the comprehensive gene expression response occurred between 20 min and 3 h.

Certain transcription factors were downregulated during metamorphosis, among which the turning off of gene *FoxAB* (LOC105338027) can be a biomarker for the start of oyster metamorphosis transformation. *FoxAB* was first identified in the sea urchin genome (Tu et al. 2006). It belongs to an ancient gene family that has been lost several times during the evolution of the Bilateria (Tu et al. 2006). No member from this gene family has been identified among vertebrates or ecdysozoans (Tu et al. 2006; Yang et al. 2014; Yu et al. 2008). However, the *FoxAB* gene has been reported in many other groups, including the sea urchin *Strongylocentrotus purpuratus* (Tu et al. 2006), the cephalochordate amphioxus *Branchiostoma floridae* (Yu et al. 2008), the enteropneust hemichordate *Saccoglossus kowalevskii* (Fritzenwanker et al. 2014), the hydrozoan cnidarian *Clytia hemisphaerica* (Chevalier et al. 2006), the Pacific oyster *C. gigas*, the limpet *Lottia gigantean*, the annelid *Capitella teleta* (Boyle et al. 2014; Yang et al. 2014), and the bryozoan *Bugula neritina* (Fuchs et al. 2011). The developmental expression pattern of *FoxAB* has been well studied in the last species, where *BnFoxAB* was also exclusively expressed in larval tissues, and silenced during metamorphosis (Fuchs et al. 2011). Knowledge of the exact function of *FoxAB* is still limited, but it is possibly recruited to participate in the formation of ectoderm-originated larval tissues, such as the oral ectoderm (Boyle et al. 2014). Although URGs and DRGs may both play important roles in development regulation, we focused mainly on upregulated genes as their function mode should be more direct. While only a few downregulated genes were discussed here, the DRG set should be further investigated in future studies to gain a more comprehensive understanding of metamorphosis GRNs.

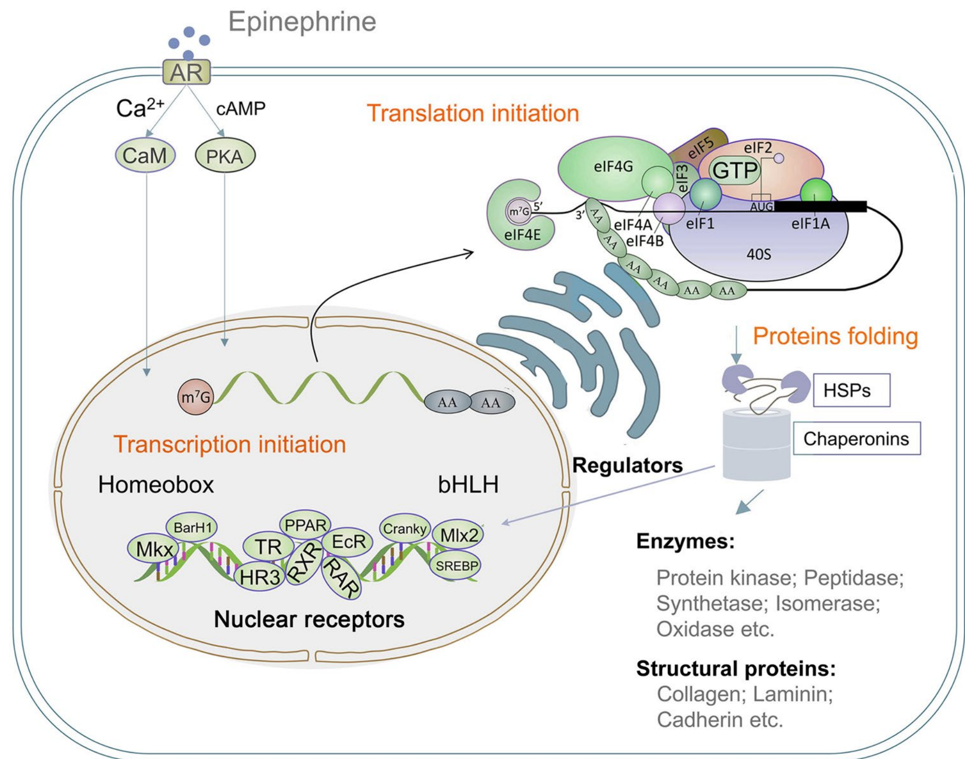

Fig. 6 Overview of gene regulation network during oyster metamorphosis. Shapes with a violet edge denote upregulated genes (URGs) either in pediveliger or early spat stages. Abbreviations: *AR* adrenergic receptor, *CaM* calmodulin, *PKA* cAMP-dependent protein kinase, *Mkx* mohawk homeobox, *BarH1* BarH-like homeobox 1, *PPAR* peroxisome proliferator-activated receptor, *TR* thyroid hormone receptor, *HR3* nuclear hormone receptor 3, *EcR* ecdysone receptor, *RXR* retinoid X receptor, *RAR* retinoic acid receptor, *Mlx2* MAX dimerization protein Mlx2, *SREBP* sterol regulatory element binding transcription factor, *eIF* eukaryotic translation initiation factor, *HSP* heat shock protein. Expression data of the genes are shown in Supplementary Tables S3, S4 and S5

Materials and methods

Oyster treatment and sampling

Three studies and their biological replicates were conducted in 2010, 2011, 2013, and 2022 (Fig. 1). Pacific oyster larvae were cultured in a hatchery as described previously (Zhang et al. 2012). In brief, gametes were collected by dissection from mature oysters. Eggs were washed before artificial insemination. Zygote incubation and subsequent larval culture were conducted in 25 m^3 sand-filtered seawater at 25 °C, with salinity around 30. Larvae were fed with the algae *Isochrysis galbana* and *Nitzschia closterium*. After ~ 14 days, most larvae grew into metamorphosis-competent larvae (pediveliger, P) with some still at the umbo veliger stage (U) without eyespots. For study 1, ~ 5000 larvae, i.e., a mixture of mostly U and P, were collected with a 100 μm nylon screen, while plastic plates were placed into the larval tank as settlement substrata. The plates were then washed to remove unattached larvae and ~ 5000 attached spats (S) were collected the next day (~ 20 h later). This operation was conducted on four successive days (treated as four biological replicates). For study 2, three experiments were conducted independently, and the larvae used for each experiment were from different mass spawnings. Metamorphosis-competent pediveligers were harvested by rinsing the larvae on a 180 μm nylon screen. Pediveligers retained on the screen were resuspended at ten larvae per mL. Epinephrine hydrochloride (EPI, Solarbio, China) solution was added to the EPI treatment groups at a final concentration of 1×10^{-4} mol/L (Bonar et al. 1990). To distinguish the short time effect of epinephrine, the treatment duration was set at 20 min. Pediveligers were then washed out with fresh seawater and transferred into 5 L beakers at ten larvae per mL for culturing. Pediveligers without EPI treatment were cultured in parallel at the same concentration as the blank control. No food was provided during the experiment. All individuals (mixed by P and S) were collected at 20-min post-treatment (mpt) and 6-h post-treatment (hpt) for both blank control and EPI treatment groups. To further investigate the temporal expression pattern, additional time points (3, 12, or 24 hpt) were set in the last two experiments. Study 3 further separated larvae at the same age into U, P, and S groups to study genes expressed exclusively in pediveligers. Metamorphosis-competent pediveligers were harvested by rinsing the larvae on a 180 μm nylon screen. Passed larvae were then filtered through a 100 μm screen to collect the umbo veligers. Both pediveligers and umbo veligers were cultured at ten larvae per mL surrounded by nylon screens. Spats attached to the screens were collected after ~ 12 h, while unattached pediveligers and umbo veligers were collected separately. The samples of all four replicates for study 3 were from

one mass spawning. All samples were immediately frozen in liquid nitrogen before being stored at − 80 °C.

RNA extraction, sequencing, and data analysis

RNA was extracted using TRIzol according to the manufacturer's protocol. Single-end RNA sequencing (RNAseq) was performed for studies 1 and 2 on Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA) as described previously (Zhang et al. 2012). Paired-end RNAseq was performed for study 3 on Illumina NovaSeq 6000 platform. Cleaned sequencing reads were mapped to the oyster genome assembly version "cgigas_uk_roslin_v1" (Peñaloza et al. 2021) with HISAT2 (Kim et al. 2019). Gene expression levels were quantified as transcript per million mapped reads (TPM) with stringtie2 (Kovaka et al. 2019). Functional annotations of the proteins were conducted using the eggNOG-mapper v2 program (Cantalapiedra et al. 2021). Genes enriched during the pediveliger stage were identified by analyzing the published developmental transcriptome data (Zhang et al. 2012). Pediveliger specifically enriched genes were defined as the maximum TPM values at pediveliger stages, while the values of samples before the umbo veliger stage were less than half of the maximum value.

R software (Ihaka and Gentleman 1996) was used for statistical analysis and plotting, and the factoextra package (Kassambara and Mundt 2020) was used to draw the principal component analysis (PCA) plots. The DESeq2 package (Love et al. 2014) was used to identify differentially expressed genes (DEGs). The adaptive Student's t prior shrinkage estimator from the 'apeglm' package (Zhu et al. 2018) was used to estimate the logarithmic fold change (LFC). The systemPipeR package (Backman and Girke 2016) was used to conduct Venn analysis, and the clusterProfiler package (Yu et al. 2012) was used for gene ontology (GO) and KEGG enrichment. During DEG identification for study 2, only data from experiments 2 and 3 were analyzed, as they satisfied the requirement for at least three biological replicates (samples collected at different time points from the same treatment group were treated as biological replicates).

Assay for transposase-accessible chromatin

The ATACseq analysis of samples from study 3 mainly followed previous reports (Xu et al. 2021). In brief, larvae or spats were incubated in 0.8 mg/mL collagenase P (Sigma, 11213857001) at room temperature and gently pipetted to release single cells. About 50,000 cells were centrifuged at 4 °C for 5 min at 500 g. The cells were washed with cold PBS once and suspended with cold lysis buffer. The transposition reaction, purification, library construction and quantitation followed established methods (Buenrostro et al. 2015). The final DNA libraries were run on Illumina platform after purification.

Low-quality reads were removed and then mapped to the *C. gigas* genome assembly version "cgigas_uk_roslin_v1" using Bowtie2 software (Langmead and Salzberg 2012). DeepTools v2.07 (Ramirez et al. 2016) was used to map the density distribution of sequencing reads in a 3 kb interval upstream and downstream of the TSS of each gene. MACS2 v2.1.1 software (Zhang et al. 2008) was used to perform peak extraction. The ChIPseeker package (Yu et al. 2015) was used for the functional annotation of genome-wide peaks. Genes around the peaks were identified according to their distance relationship and were annotated as to whether the peak region was in the TSS, Exon, 5′ UTR, 3′ UTR, Intronic, or Intergenic regions. To analyze peak differences between different experimental groups, "bedtools merge" was used to merge overlapped accessible regions of all samples as candidate differentially accessible regions. The counts of reads supporting these candidate peaks were calculated and normalized. Differentially accessible region analysis of two groups was performed using the DESeq R package (Love et al. 2014). The resulting P values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. MEME-ChIP 4.11.2 (Machanick and Bailey 2011) was used to identify and annotate motifs.

Conclusion

The transcriptional profile of the Pacific oyster during metamorphosis was assayed by RNAseq and ATACseq. Different gene sets were obtained for pediveligers, spats, and epinephrine-stimulated pediveligers, revealing rapid changes in gene regulation dynamics. During the pediveliger stage, genes related to integral components of membrane (receptors) and nervous system formation were massively upregulated, indicating structural preparation for the initiation of metamorphosis. Metamorphosis quickly ensued post epinephrine treatment on pediveligers. Almost entire complexes for protein translation initiation and protein folding control systems were upregulated, indicating massive biogenesis. Genes encoding structural proteins related to shell formation were upregulated during metamorphosis, resulting in rapid growth of the calcified dissoconch shell (sometimes doubling the pediveliger size within 24 h). Different transcription factors, mainly composed of nuclear receptors, showed a significant response during oyster metamorphosis. Lophotrochozoans represent a large clade of Bilateria, and are the key to understanding animal evolution. In light of the key function of nuclear receptors in ecdysozoans and deuterostomes, the results of this study indicate an ancient role of the nuclear receptor regulation system in animal metamorphosis.

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Author contributions FX conceived and designed the study. FX and SD conducted experiments. FX and DG conducted data analysis. GZ contributed to oyster experiments and analysis. FX wrote the paper. All authors approved the final manuscript.

Data availability RNAseq data have been deposited with GenBank under BioProject PRJNA553079. The datasets used and analyzed during the current study are also available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Animal and human rights statement This article does not contain any studies performed with human and vertebrate animals.

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