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Identification of differentially expressed proteins involved in the early larval development of the Pacific oyster *Crassostrea gigas*

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ABSTRACT

The trochophore and D-veliger are two pivotal but distinct developmental stages in bivalve larval development. Complex morphological and physiological processes are involved in the transition between these stages, yet the underlying molecular mechanisms remain largely elusive. In this study, a comparative proteomic analysis was conducted to identify the proteins related to early larval development of the Pacific oyster *Crassostrea gigas*. Fifty proteins showing different levels of expression were screened. These spots were further analyzed using LC–MS/MS and twenty of them were identified. Together with information from Western blotting and real-time PCR assay of some annotated genes, our results provide insight into novel aspects of cellular processes and protein function in oyster larvae. For example, it was shown that cell division was unexpectedly slowed down in D-veliger larvae, while substantial gene transcription and biochemical activity were revealed. "Quality control" proteins were identified for the first time to be involved in mollusk larval development. Bivalve larval development provides an elegant system for studying cellular processes and tissue morphogenesis. Our data shed light on the molecular mechanisms underlying these complex processes and regulatory networks.

1. Introduction

Bivalve larval development includes a pelagic (trochophore, D-veliger) phase and a benthonic (pediveliger, postlarva) phase [1]. The morphological and behavioral characteristics of each stage are distinct. These differences in the development of bivalve larvae have been extensively studied by microscopic observation [2–4]. Recently, with the support of newly developed sequencing techniques, available genomic data from bivalve species have been rapidly expanding [5–7]. Transcriptomic analysis has been employed to study bivalve larval development, which revealed the involvement of specific genes in some critical biological processes, such as *insulin*, *calmodulin* and some ribosomal protein genes [8,9]. However, considering the complex cellular processes in bivalve larval development, the current genomic data and functional analyses remain insufficient for revealing the underlying molecular mechanisms. For example how the shell matrix proteins are secreted and organized into larval shell architecture is largely unknown.

Proteomic studies are now becoming powerful tools for the discovery of new proteins involved in developmental processes [10–12]. To date, several proteomic studies have been conducted on the stress resistance of mollusks [13,14]. Proteomic approaches are also feasible ways to identify species-specific [15] and organ-specific [16] peptides in some species of mollusks.

The Pacific oyster, *Crassostrea gigas*, is a widely distributed and economically important species. It occupies a pivotal

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phylogenic position in the Lophotrochozoa and can be an invasive species when introduced into new habitats [5,17]. Because of its economic and ecological importance, its biology, genetics and innate immunity have been extensively studied [8,18,19]. These studies, together with a recent and ongoing whole genome project [20], make *C. gigas* a potential model organism for marine bivalve research. However, a proteomic analysis of *C. gigas* has not yet been undertaken. In the present study, we first screened 50 differentially expressed proteins by comparing the 2-dimensional electrophoresis (2-DE) results of samples from trochophore and D-veliger larvae. Subsequently, 20 proteins were identified using liquid chromatography–mass spectrometry (LC–MS/MS) and mascot searching against a self-constructed *C. gigas* database. Together with Western blotting data and gene expression information, this work enables us to propose novel cellular processes and elucidate the underlying molecular mechanisms during bivalve larval development.

2. Material and methods

2.1. Animals, larvae culture and collection

Sexually mature adults of *C. gigas* were obtained from a hatchery in Qingdao, China. Sperm and oocytes collected from adult oysters (five males and five females) by dissection were mixed and incubated at 25 °C for 10 min with gentle shaking. Unfertilized sperm were eliminated by washing with fresh filtered sea water.

The fertilized eggs were cultured in sea water at 25 °C with constant aeration. Development of the embryos and larvae were observed under a Nikon 80i phase-contrast microscope. The hatched trochophore and D-veliger larvae were collected at 11 and 21 hours post fertilization (hpf), respectively. Three independent samples were collected for each stage; a total of six samples were collected. Larvae were confined to the same developmental stage by controlling water temperature, and the developmental synchronicity was verified by microscopic observation. The collected larvae were frozen in liquid nitrogen immediately and stored at −80 °C until further analysis.

2.2. Protein extraction and 2-DE

Proteins were extracted from each sample of trochophore and D-veliger larvae using the Total Protein Extraction Kit (Bestbio Company, China). Before 2-DE, the protein samples were purified with a 2-D Clean-up kit (GE Healthcare) and quantified using a Bradford assay (QuickStart Bradford Protein Assay kit, Bio-Rad).

In 2-DE experiments, 450 μg of protein was loaded onto the immobilized pH gradient (IPG) strip (Immobiline DryStrip pH 4–7, 18 cm, catalog number 17-1233-01, GE Healthcare) for isoelectric focusing (IEF). IEF was performed at 20 °C using continuously increasing voltage (up to 8000 V) to reach 24,000 Vh. The second-dimension electrophoresis on 12% SDS-polyacrylamide gels, gel fixation and staining with Coomassie brilliant blue (CBB) G250 were performed as previously described [21]. Three replicates were conducted per sample to ensure gel reproducibility.

2.3. Image analysis, in-gel digestion and LC–MS/MS

The 2-DE gels were scanned with a UMAX PowerLook 1120 scanner (Veutron Corporation, Taiwan), and the gel images were analyzed with ImageMaster 2D Platinum 6.0 (GeneBio). The amount of protein in each spot was assessed based on the volume of the spot, which was normalized as the percentage of the total volume of all spots in a whole gel (percentage volume, vol%). Each value for a protein spot was expressed as the mean ± standard deviation (SD), where the SD represents variations among three samples at each stage. Two criteria were used to screen for proteins that were differentially expressed between the two stages. First, all the spots that could only be detected in one stage were considered differentially expressed protein spots. Second, for the spots detected in both stages, only those whose percentage volume varied more than 50% between the two stages (i.e., the ratio between the two stages was less than 0.66 or greater than 1.5), were selected and submitted for Student's *t*-test analysis. The *p* values were further evaluated using Bonferroni correction method [22] and Benjamini and Hochberg's method [23,24] for multiple hypothesis testing.

The differentially expressed protein spots were picked out from the gels and destained in 50% acetonitrile. Proteins in the gel pieces were reduced in 10 mmol/L DTT at 56 °C for 1 h and alkylated in 55 mmol/L iodoacetamide for 45 min in the dark. In-gel digestion was performed by adding 5 μL of 50 ng/μL trypsin to the dry gel pieces followed by an additional 15 μL of 25 mmol/L NH_4HCO_3 after the trypsin solution was absorbed. The gel pieces were then incubated at 37 °C for 16 h to digest the proteins.

LC–MS/MS of the trypsin-digested samples was conducted on a Bruker Daltonics Apex Ultra 70 FTMS Mass Spectrometer System. For liquid chromatography, samples were separated on a PepMap C18 column using a linear gradient from 10% buffer A (0.1% formic acid in acetonitrile) to 40% buffer B (0.1% formic acid in water) in 90 min. The peptides were eluted directly into an ultra apex Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonik). Full-scan spectra over a mass range of 200–2000 Da were obtained. The MS/MS data were automatically acquired on the three multiply charged precursor ions in each full-scan spectrum.

2.4. Protein identification

A protein database was constructed based on the available EST data of *C. gigas*, including 206,388 ESTs in the dbEST of GenBank (as of July 2011) and 1,080,743 ESTs generated by 454 sequencing technology in the SRA database of NCBI (SRA accession SRR074289 and SRR074290). Open reading frames (ORFs) longer than 200 bp were extracted from these 1,287,131 ESTs and translated into protein sequences using the *getorf* program from the EMBOSS package [25]. In this way, 2,225,676 sequences (EST-derived amino acid sequences, designated as tESTs in the following text) were deposited in our local database, designated as CGest.

For protein identification, the mass spectrometry (MS) data of each protein spot was searched against CGest using the *mascot* program (Version 2.3). The search parameters were set

as follows: Type of search, MS/MS Ion Search; Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Mass values, Monoisotopic; Protein Mass, Unrestricted; Peptide Mass Tolerance, ±0.06 Da; Fragment Mass Tolerance, ±0.12 Da; Max Missed Cleavages, 1; Instrument type, ESI-FTICR. Only those hits with $p<0.05$ (mascot score>threshold score) that matched at least two peptides were accepted as significant hits. High redundancy in CGest caused more than one tEST was hit by most of the MS data. To resolve this problem, we employed several steps of blastp searching to evaluate each hit and give an annotation for each MS datum. The hit tESTs were searched against the NCBI nr database for annotation and/or were compared with each other to ensure that the tESTs hit by one MS datum shared sequence similarities (see Supplemental Fig. 1 for detailed procedures).

2.5. Western blotting

Larval proteins were extracted from each of the two developmental stages and loaded on 12% SDS-polyacrylamide gels. After SDS-PAGE, the proteins were transferred to a PVDF membrane. The following procedure was conducted using a One Step Western Kit (CoWin, China). A mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (PC10, 1 mg/mL) (CoWin, China) was used at a 1:1000 dilution. β-Actin was detected by a mouse monoclonal antibody (3E8, 1:1000) as the internal reference. The signals were detected using Pro-light HRP Chemiluminescence Detection reagent (Tiangen, China).

2.6. Real-time PCR

Gene expression profiles were explored using a real-time PCR assay. RNA was extracted and reverse-transcribed to complementary DNA (cDNA) as described previously [26], with minor modifications. Briefly, total RNA was extracted from each sample using an E.Z.N.A. total RNA kit II (Omega BioTek, USA). RNA was then treated with RQ1 RNase-free DNase (0.06 U/μL) (Promega, USA) at 37 °C for 30 min to eliminate potential genomic DNA contamination. The cDNA was synthesized in a 50 μL volume from 2 μg of total RNA by using M-MLV reverse transcriptase (Promega, USA) with a combination of primer oligo-dT primer (5′-GGCCACGCGTCGACTAGTAC(T)16(A/C/G)-3′) and BDA oligo primer (5′-AAGCAGTGGTATCAACGCAGAG-TACGCGGG-3′). The cDNA was diluted eight-fold before PCR amplification. Elongation factor (ELF) of *C. gigas* was employed as a reference [27]. Primers for the target genes were designed according to the ESTs identified during mascot searching. Real-time PCR reactions were conducted in triplicate in a 20 μL volume containing 0.5 μL diluted cDNA, 0.2 mmol/L of each primer and 1× SuperReal Premix (Tiangen China) on an Eppendorf PRISM 7000™ real-time thermocycler. Melting curve analysis was used to confirm the specificity of each primer pair. The relative expression levels of target genes were calculated using a two-standard-curve method as follows. Standard curves of both the target gene and the reference gene were constructed using one cDNA sample from the trochophore stage (reference cDNA). The relative amount of each gene in each sample compared with that in the reference cDNA was calculated based on the Ct value and the standard curve of the gene. The expression of each gene was then normalized to ELF (amount of target gene/amount of ELF). The expression levels of each gene in each of the two developmental stages were compared using Student's *t*-test, and differences were considered statistically significant at $p<0.05$.

3. Results

3.1. Development and morphological characteristics of trochophore and D-veliger larvae

The embryonic, larval and post-larval developmental stages of *C. gigas* are typical morphological processes in bivalves. The trochophore and D-veliger stages appeared at 11 and 21 hpf, respectively, at 25 °C. We observed the morphological and behavioral characteristics of the larvae at each stage. The major characteristics of the trochophore stage were several ciliary structures such as the prototroch and the telotroch (Fig. 1a, black arrows). At this stage, the developing shells could not be observed by phase-contrast microscopy. No other organs were visible in the trochophore stage, and the larvae did not feed. The trochophore developed quickly to D-veliger with double calcified shells and a velum (Fig. 1b, black arrows), the swimming organ which developed from the prototroch of the trochophore. At this stage, the larvae began to feed on small plankton. The D-veliger larvae presented behavioral characteristics distinct from trochophore larvae. The motility of D-veliger larvae improved significantly. They swam in a helical ascent pattern coupled with intermittent stops, while the trochophore larvae moved in a straight-line pattern, suggesting that the D-veliger larva has more robust muscular and nervous systems.

3.2. Identification of differentially expressed proteins at two oyster larval stages

The proteins of trochophore and D-veliger larvae were extracted and used for 2-DE analysis. High resolution 2-DE gels were obtained for trochophore (Fig. 2a) and D-veliger (Fig. 2b) larvae. After CBB staining, 564±19 protein spots were detected on gels from each larval stage. Image analysis revealed 28 protein spots that were only detected in images of one of the two stages. Among the spots detected in images of the both stages, a total of 26 protein spots with percentage volume varied more than 50% between the two stages were submitted for statistical analysis. Student's *t*-test revealed the *p* values of all the 26 protein spots were less than 0.05. Multiple hypothesis testing by Bonferroni correction method and Benjamini and Hochberg's method declared that seven and 26 protein spots were significant, respectively (see Supplemental Table S2 for details). We selected the 26 protein spots for further analysis in order to list more proteins as significant targets for further investigation, as proposed in a recent report [28].

Thus, the total 54 protein spots were picked out for LC–MS/MS analysis and database searching. MS data were successfully

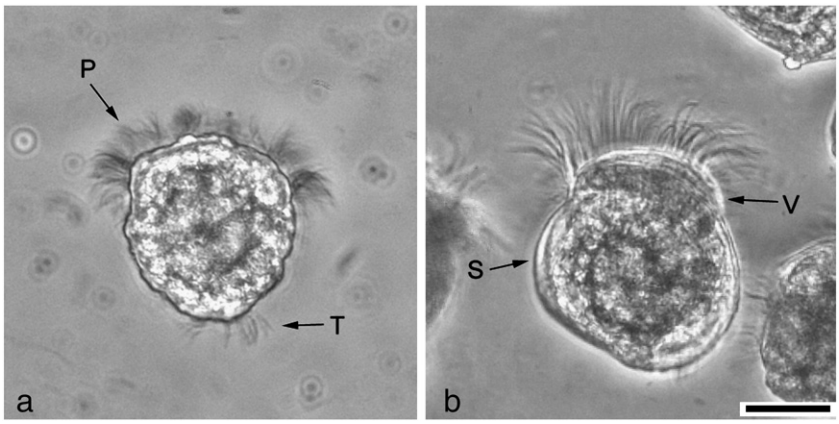

Fig. 1 – Morphological characteristics of *C. gigas* larvae as observed by phase-contrast microscopy. a, the trochophore larva (11 hpf, 25 °C). b, the D-veliger larva (21 hpf, 25 °C). P: prototroch; T: telotroch; V: velum; S: shell. Scale bar: 25 μm.

generated for all the 54 protein spots, and they were used to search against CGest. After searching, 26 protein spots matched tESTs from CGest, including nine spots that only detected in images of one of the two stages, and 17 spots detected in images of both stages. Next, several steps of blastp searching were conducted to annotate. After sequence comparisons, four protein spots were revealed to be mixtures of different proteins, and they were excluded from further analysis. Of the remaining 22 protein spots, which generated significant hits during mascot searching, 20 were annotated (identified) successfully (see Supplemental datasets 1 for detailed annotation and Supplemental datasets 2 for sequences).

As summarized in Table 1, among the 50 differentially expressed proteins spots, 27 were up-regulated and 23 were down-regulated in D-veliger larvae. Sixteen proteins appeared in only D-veliger larvae, and 12 proteins expressed in trochophore larvae could not be detected in D-veliger samples. Based on their functions, the 20 identified proteins were categorized into five groups: cytoskeletal components, cell proliferators, biochemical regulators, biological response modifiers and protein modification factors (Table 1). These proteins are proposed to be involved in various biological processes, including tissue/cell contraction, cell division, cell motility, energy production and protein synthesis and modification.

3.3. *Down-regulation of Cg-PCNA*

The expression levels of proliferating cell nuclear antigen (PCNA) protein were explored using Western blotting, with β-actin as the internal reference. The results showed that the expression of *Cg*-PCNA (PCNA homolog in *C. gigas*) was significantly lower at the D-veliger stage (Fig. 3b). This result was consistent with the result of proteomic analysis by 2-DE (Fig. 3a).

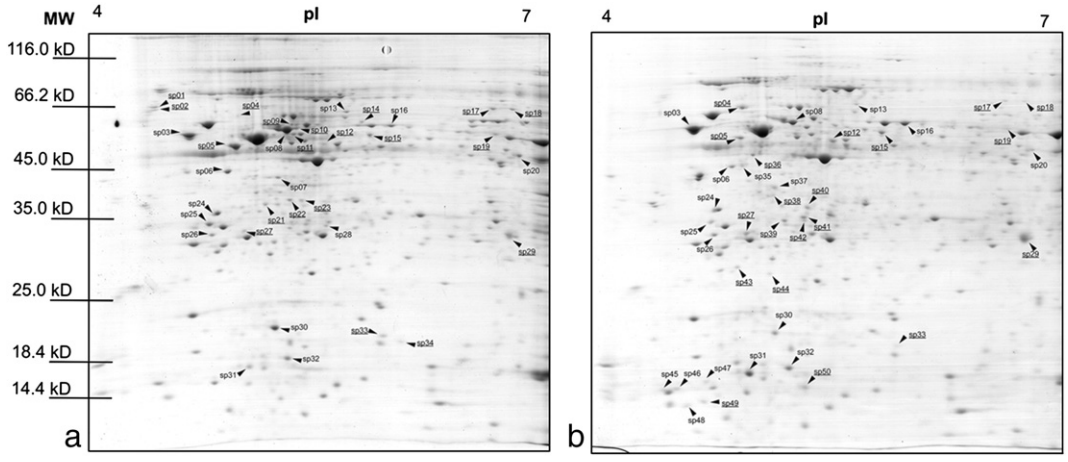

Fig. 2 – Images of 2-DE gels of trochophore (a) and D-veliger (b) larvae of *C. gigas* (pH 4–7 IPG, 12% SDS-PAGE). Fifty differentially expressed protein spots are indicated in the images (black arrow heads). The underlined spots do not display significant hits from mascot or blastp searching.

Table 1 – Differentially expressed proteins between trochophore and D-veliger of C. gigas. (see Supplemental Tables S1 and S2, Supplemental data set ds1 and ds2 for more details).

Code	Protein	Matched EST with highest score [a]						D-veliger/ trochophore (mean ± SD)
		EST [b]	E value (blastp)	Score	Threshold ($p<0.05$) [c]	Matched queries	Matched peptides	
Cytoskeletal components								
sp08	alpha-Tubulin [*Nodipecten subnodosus*]	SRR074290.672961	5.00E−58	96	39	6	2	0.46 ± 0.08
sp24	RecName: Full=Tropomyosin>emb|CAA53028.1| tropomyosin [*Haliotis rufescens*]	SRR074290.283873	2.00E−40	81	36	20	2	3.33 ± 2.09
sp28	Thymosin beta-4 [*Harpegnathos saltator*]	SRR074290.152318	1.00E−15	55	30	4	2	0
sp35	beta-Tubulin [*Haliotis discus discus*]	SRR074290.589100	1.00E−63	124	38	8	2	∞
sp37	beta-Tubulin [*Saccostrea kegaki*]	FP003451	6.00E−166	330	34	39	5	∞
sp46	RecName: Full=Myosin regulatory light chain A, smooth adductor muscle	FP006412	2.00E−33	102	35	8	2	∞
sp48	RecName: Full=Myosin regulatory light chain A, smooth adductor muscle	SRR074289.111347	7.00E−50	59	32	7	2	∞
Biochemical regulators								
sp20	Phosphoribosylaminoimidazole carboxylase [*Bombyx mori*]	SRR074290.476160	1.00E−41	729	39	49	4	0.6 ± 0.05
sp26	RecName: Full=14-3-3 protein homolog 2; Short=14-3-3-2>gb|AAB02100.1| isoform 2 [*Schistosoma mansoni*]	HS119365	9.00E−85	1092	38	53	2	2.16 ± 0.77
sp31	Calmodulin [*Komagataella pastoris* GS115]	AM868565	1.00E−21	1634	42	152	7	3.66 ± 0.65
Cell proliferators								
sp25	Proliferating cell nuclear antigen [*Litopenaeus vannamei*]	FP011289	2.00E−129	855	38	99	4	0.62 ± 0.23
sp30	RecName: Full=Eukaryotic translation initiation factor 5A-1	SRR074290.119633	4.00E−64	60	36	9	3	0.59 ± 0.1
Biological response modifiers								
sp06	Ribosomal protein SA [*Lepidochitona cinerea*] (laminin receptor precursor)	CX069219	4.00E−129	87	35	37	2	0.59 ± 0.06
sp07	Heterogeneous nuclear ribonucleoprotein D0 isoform d [*Mus musculus*]	HS115394	6.00E−58	73	33	5	3	0
sp32	Mitochondrial F1F0 ATP-synthase subunit Cf6 [*Argas monolakensis*]	SRR074290.110536	4.00E−06	231	33	8	3	1.69 ± 0.12
sp47	Mitochondrial H+ATPase a subunit [*Pinctada fucata*]	HS110778	3.00E−170	113	39	10	3	∞
Protein modification factors								
sp03	Calreticulin [*Crassostrea gigas*]	HS111868	5.00E−149	3299	40	124	4	2.16 ± 0.18
sp13	Chaperonin containing tcp1 [*Haliotis discus discus*]	HS110588	2.00E−131	173	32	9	4	0.5 ± 0.09
sp16	Protein disulfide isomerase family A, member 3 [*Danio rerio*]	HS111659	6.00E−103	1216	38	66	5	2.37 ± 0.79
sp45	Putative nucleoplasmin [*Phragmatopoma lapidosa*]	HS118764	1.00E−37	102	39	7	4	∞

(continued on next page)

Table 1 (continued)

Code	Protein	Matched EST with highest score [a]						D-veliger/ trochophore (mean ± SD)
		EST [b]	E value (blastp)	Score	Threshold ($p<0.05$) [c]	Matched queries	Matched peptides	
Unknown proteins								
sp43	Unknown protein	SRR074290.105302	NA	200	38	5	5	∞
sp04	Unknown protein	HS109850	NA	148	41	4	4	3.53 ± 1.56
sp01	NA	NA	NA	NA	NA	NA	NA	0
sp02	NA	NA	NA	NA	NA	NA	NA	0
sp05	NA	NA	NA	NA	NA	NA	NA	0.35 ± 0.08
sp09	NA	NA	NA	NA	NA	NA	NA	0
sp10	NA	NA	NA	NA	NA	NA	NA	0
sp11	NA	NA	NA	NA	NA	NA	NA	0
sp12	NA	NA	NA	NA	NA	NA	NA	2.21 ± 0.18
sp14	NA	NA	NA	NA	NA	NA	NA	0
sp15	NA	NA	NA	NA	NA	NA	NA	1.87 ± 0.08
sp17	NA	NA	NA	NA	NA	NA	NA	0.58 ± 0.33
sp18	NA	NA	NA	NA	NA	NA	NA	0.47 ± 0.18
sp19	NA	NA	NA	NA	NA	NA	NA	0.62 ± 0.22
sp21	NA	NA	NA	NA	NA	NA	NA	0
sp22	NA	NA	NA	NA	NA	NA	NA	0
sp23	NA	NA	NA	NA	NA	NA	NA	0
sp27	NA	NA	NA	NA	NA	NA	NA	3.34 ± 0.28
sp29	NA	NA	NA	NA	NA	NA	NA	2.4 ± 0.29
sp33	NA	NA	NA	NA	NA	NA	NA	0.47 ± 0.09
sp34	NA	NA	NA	NA	NA	NA	NA	0
sp36	NA	NA	NA	NA	NA	NA	NA	∞
sp38	NA	NA	NA	NA	NA	NA	NA	∞
sp39	NA	NA	NA	NA	NA	NA	NA	∞
sp40	NA	NA	NA	NA	NA	NA	NA	∞
sp41	NA	NA	NA	NA	NA	NA	NA	∞
sp42	NA	NA	NA	NA	NA	NA	NA	∞
sp44	NA	NA	NA	NA	NA	NA	NA	∞
sp49	NA	NA	NA	NA	NA	NA	NA	∞
sp50	NA	NA	NA	NA	NA	NA	NA	∞

NA: not available.

[a] More than one EST was hit during mascot searching for some protein spots because of redundancy of EST database.

[b] Actually the sequences hit by MS data were the EST-derived amino sequences (tESTs). Here we listed the corresponding ESTs. The ESTs from the SRA database were noted as the accession no. of SRA run plus the name of spot in the reaction. For example, EST SRR074290. 672961 refers to the sequence of the spot no. 672961 in the SRA run SRR074290. All sequences can be obtained at http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?

[c] Results of mascot searching with scores higher than the thresholds indicated $p<0.05$.

3.4. Comparison of protein expression and mRNA transcription

To investigate the transcriptional levels of identified proteins, six of the ESTs matched by protein spots, namely sp24 (tropomyosin), sp46 (myosin regulatory light chain A, MLC), sp26 (14-3-3 protein homolog 2), sp31 (calmodulin, CaM), sp25 (PCNA) and sp06 (ribosome protein SA, also known as laminin receptor, LR) were chosen for analysis by real-time PCR. The six spot-matched ESTs (designated as "*Cg*" plus the spot name) were used as templates for primer design (Table 2). The melting curve analysis of the PCR products revealed only one melting temperature peak for each amplification reaction, ensuring the specificity of each primer pair. The real-time PCR results showed that *Cgsp24*, *Cgsp26* and *Cgsp31* were up-regulated at the D-veliger stage, while two genes, *Cgsp25* and *Cgsp06*, were down-regulated at this stage. The expression levels of the remaining gene, *Cgsp46*, were not statistically different between the two stages (Student's *t*-test, $p>0.05$) (Fig. 4). Compared with the protein expression patterns revealed by 2-DE analysis, the mRNA expression of five (*Cgsp06*, *Cgsp24*, *Cgsp25*, *Cgsp26*, and *Cgsp31*) of the six genes exhibited similar patterns, while *Cgsp46*, a MLC homolog, showed different variations (Fig. 5).

4. Discussion

The development of bivalve larvae from the trochophore stage to the D-veliger stage involves many developmental events, but the underlying molecular mechanisms remain largely unknown. The present study is, to our knowledge, the first proteomic analysis focusing on bivalve larval development. We observed multiple instances of organogenesis and functional differentiation at the D-veliger stage (Fig. 1), which indicated that D-veliger larvae required more constitutive proteins and a more complex regulatory network than trochophore larvae. Through comparison of 2-DE gels using

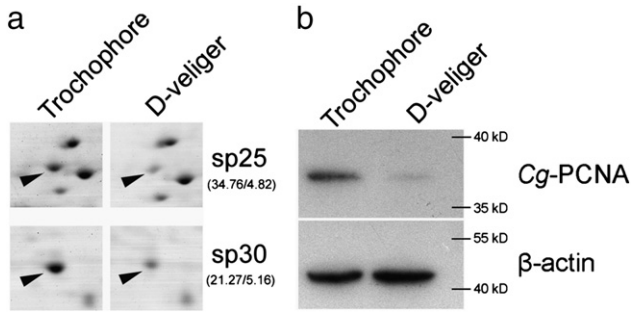

Fig. 3 – Proteins related to cell proliferation are down-regulated in D-veliger larvae. a. Two proteins related to cell proliferation were down-regulated in D-veliger larvae as revealed by 2-DE analysis. The number below each spot name indicates the observed molecular weight (MW) and isoelectric point (pI) of the protein. Protein sp25, proliferating cell nuclear antigen (PCNA); sp30, eukaryotic translation initiation factor 5A (EIF5A). b. Western blotting of the PCNA homolog of *C. gigas* (*Cg*-PCNA) at the two larval stages. β-Actin was used as an internal reference.

samples from these two larval stages, we successfully screen out 50 differentially expressed proteins.

Because the *C. gigas* genome data was not yet published, we collected the 1,287,131 publicly accessible ESTs and constructed one database (CGest) for protein identification. We elected to construct CGest because it had maximal coverage. For instance, it included more ESTs than the publicly accessible database GigasDatabase [5]. However, we also noticed that CGest has a high rate of redundancy because the ESTs we used were not assembled. To resolve this problem, we conducted additional blastp searching for final identification and 40% (20/50) of the differentially expressed proteins were identified. Alternatively, the use of a compiled EST library might lead to a better identification result. The identified proteins were divided into five classes, namely cytoskeletal components, biochemical regulators, cell proliferators, biological response modifiers and protein modification factors. The differential expression of these proteins between the two developmental stages indicates that they might function in complex cellular processes and morphological transitions of tissues during larval development.

A number of cytoskeletal components and cytoskeleton-binding proteins were identified from our proteomic analysis, such as MLC, α-tubulin, β-tubulin and tropomyosin. These proteins play crucial roles in the organization and regulation of actin and microtubule polymerization and dynamics. As an organizer and regulator of cellular architecture and multiple cellular processes, the actin/microtubule network has been implicated in cell shape changes, cell migration and intracellular protein trafficking [29,30]. Changes in cell shape generally result from polymerization of actin filaments and their interaction with the motor protein myosin, which causes the actin filaments to contract. Thymosin β4 is supposed to bind to monomeric (G-) actin and stabilize it in its monomeric form. Therefore, it could remove G-actin from the dynamic assembly and disassembly processes of actin filaments [31]. Tropomyosin could regulate the interaction between actin and myosin, which plays important roles in multiple morphological events, including muscle constriction, tissue invagination, tube closure and cell migration [32]. Up-regulated expression or the emergence of these proteins (Table 1) indicates an enhancement of actin/microtubule based cellular activities. This idea is consistent with the more complex morphological characteristics and enhanced motility observed at the D-veliger stage (Fig. 1). Additionally, dramatic changes occurred in the ciliary organs at the D-veliger stage, the most prominent of which was the transition from prototroch to velum (Fig. 1). The development of the gills and the digestive system, as reported to occur at this stage [33], also involves transitions in ciliary structures [34,35]. As major components of cilia, tubulin proteins have been demonstrated by immunofluorescence to be key molecules in the velum [36]. The variation in tubulin proteins detected in our 2-DE analysis (Table 1) might reflect the development of these ciliary organs. We also noticed a discrepancy between the protein level and the mRNA expression of the MLC gene (Fig. 5). Post-translational modification may be responsible for this discrepancy, as many modifications, such as phosphorylation, glycosylation and protein fragmentation, can change the isoelectric points and/or molecular weights of proteins and thus alter protein mobility on 2-DE gels. For instance, phosphorylation of MLC proteins has been widely reported [37,38]. Among mollusks, phosphorylation of a 19-kDa MLC has been found in *Aplysi* [39]. We speculate that the discrepancy between the protein level and the mRNA expression of the MLC gene might be caused by a post-translational

Table 2 – Primer sequences and reference ESTs for realtime PCR assay.

EST (designated name)	Primers	Referring spot in 2-DE gels
FP006412 (Cgsp46)	F: CCATGATTGACCAGAACAGAGATGG R: GAGTTTGTCGCTGAAGAGGGAGAGG	sp46
SRR074290.283873(Cgsp24)	F: ATCTCCGTCCACATTTTGTTTATATCTC R: GGATATTAATTTTTCCTCTCTCCCTCAC	sp24
HS119365(Cgsp26)	F: GAGGACCACAGGGTGAGGTTGTCT R: GGGCTGGCTCTTAATTTTTCAGTCT	sp26
AM868565 (Cgsp31)	F: AGACGGAGAGAAGGACGGCAGT R: CCAACATGAATTAAAAAAATGAAACAC	sp31
FP011289 (Cgsp25)	F: GTAGCAGTCAACTTACGGAGTGATG R: TTGCACATTTTAGAACTTTGGACAT	sp25
CX069219 (Cgsp06)	F: GTGACGACCAACAAACGGGGCT R: CAGATGGAACAATATGTCTTCAAGAGGA	sp06

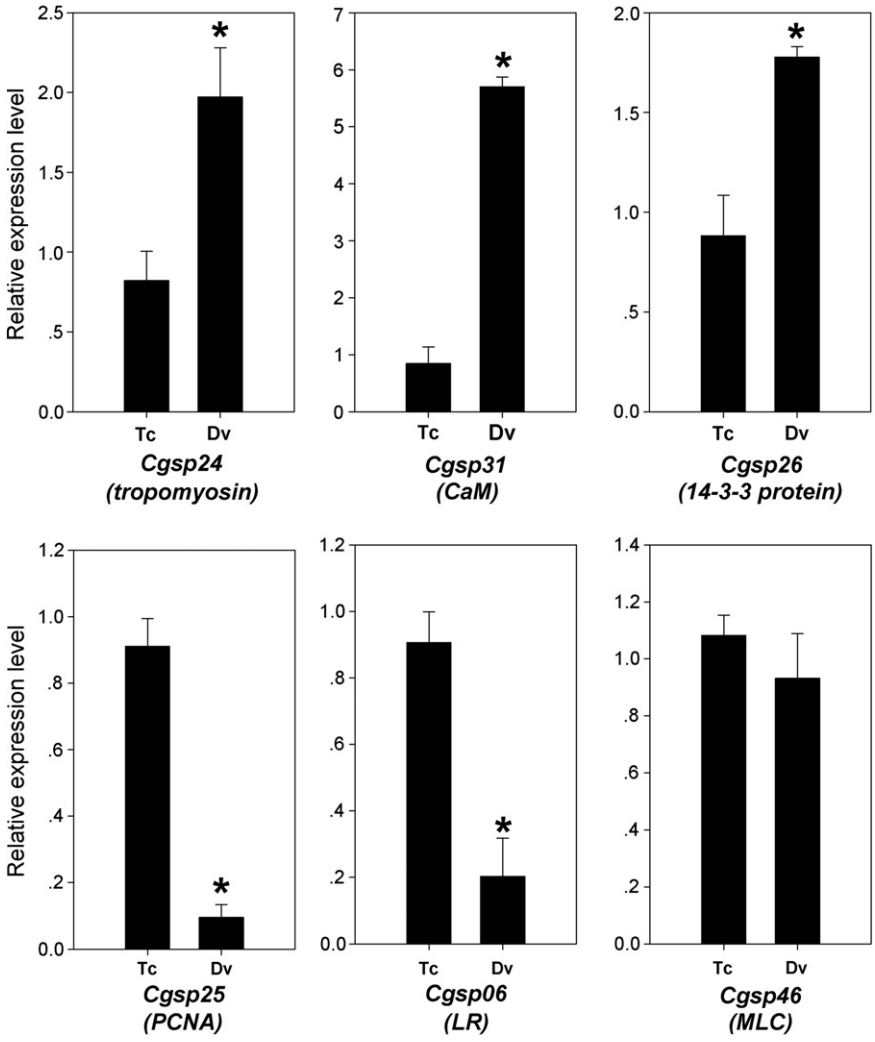

Fig. 4 – The mRNA expression of six differentially expressed genes. Three of the genes were up-regulated in D-veliger larvae and two were down-regulated; the expression levels of the remaining one (*Cgsp46*) were not statistically different between the two stages. Tc, trochophore; Dv, D-veliger. Asterisks indicate that variations were statistically different by Student's t-test analysis ($p < 0.05$).

modification at the larval stage of *C. gigas*. Further studies are required to verify any post-translational modifications of MLC and uncover their roles in oyster larval development.

Calmodulin (CaM) and 14-3-3 protein are two biochemical regulators that can bind to functionally diverse signaling proteins including kinases, phosphatases and transmembrane receptors [40,41]. By interacting with their effectors, they participate in the regulation of diverse biological processes including neuronal development, cell growth control and viral and bacterial pathogenesis [41]. Remarkably, the 14-3-3 gamma protein and CaM play important roles in the nervous system as the activators of tryptophan 5-monooxygenase and tyrosine 3-monooxygenase [42,43]. These two enzymes are responsible for the biosynthesis of serotonin, noradrenalin and adrenaline, which, as neurotransmitters, are crucial in neuronal activities [44,45]. The greater abundance of the 14-3-3 protein and CaM in D-veliger larvae (2.16- and 3.66-fold increase, respectively, over trochophore larvae) might point to their critical roles in neuronal function and suggest enhanced development of the nervous system as *C. gigas* larvae transition from the trochophore stage to the D-veliger stage. Indeed, immunofluorescence techniques recently revealed that the nervous system of the mussel *Mytilus trossulus* are composed of relatively few cells in trochophore larvae but develop into complex systems in D-veliger larvae [36]. In addition, neurotransmitters have been widely used to study the nervous system and metamorphosis of bivalves, but their biosynthesis and regulation are not yet understood [36,46,47]. The identification of 14-3-3 and CaM proteins in our study suggests that the activation mechanism of tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in *C. gigas* might be reminiscent of that in mammals [42,43].

Another group of differentially expressed proteins was represented by two proteins related to cell proliferation, including eukaryotic translation initiation factor 5A (EIF5A, sp30) and proliferating cell nuclear antigen (PCNA, sp25) (Table 1). Both proteins were down-regulated in D-veliger larvae (Fig. 3a). EIF5A, a highly conserved protein found in all eukaryotic organisms, is the only protein containing the unusual amino acid hypusine

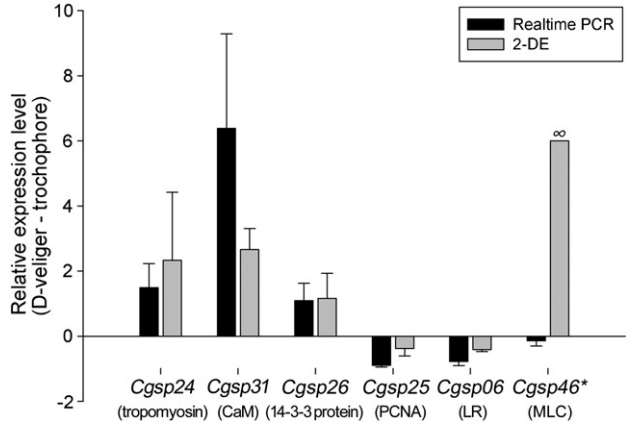

Fig. 5 – Comparison of mRNA and protein expression levels for six genes in trochophore and D-veliger larvae of *C. gigas*. Values in each column represent the relative expression level of the gene (on the mRNA or protein level) at the D-veliger stage minus the expression level at the trochophore stage. Values greater than zero (columns above the horizontal line) indicate up-regulations in D-veliger larvae, and those less than zero (columns below the horizontal line) indicate down-regulations. Five of the genes exhibited the same trend, but a discrepancy was observed between the mRNA expression and the protein level of the MLC gene (*Cgsp46*, *). Variations in both the mRNA and protein levels of the five other genes were statistically significant by Student's t-test analysis ($p < 0.05$).

[48,49]. EIF5A regulates cell division, growth and apoptosis [48–51], and knockdown of EIF5A in a plant model resulted in a dwarf phenotype [49]. PCNA is a cofactor of DNA polymerase delta, which functions in DNA replication. Its synthesis rate correlates directly with the proliferative state of cultured cells and tissues [52] and it can be used as an indicator of cell proliferation [53]. Both proteins are involved in cell proliferation. Unexpectedly, their expression levels were consistently down-regulated in D-veliger larvae (Fig. 3a). Although D-veliger larvae displayed more complex morphological and behavioral characteristics than trochophore larvae, the down-regulation of these two proteins suggested that cell division was slowed at the D-veliger stage. To verify this result, we performed Western blotting using an antibody against PCNA, which has been used to investigate the cell cycle in *C. gigas* [54,55]. The Western blotting result confirmed the significant decrease of PCNA expression in D-veliger larvae (Fig. 3b). Additionally, a real-time PCR assay also revealed down-regulation of PCNA mRNA expression in D-veliger larvae (Fig. 4). These results support our hypothesis that cell proliferation is slowed at the D-veliger stage of larval development in *C. gigas*. A slowing of cell division after the shells fully develop might be conserved among mollusk larvae. In snail larvae, proteomic analysis indicated that cell division became slower at the shell-emerging stage [16]. We speculate that, rather than cell proliferation, other processes including cell shape changes and cellular rearrangement could contribute to the complex morphological changes during organogenesis in D-veliger larvae.

Two protein-modifying factors were identified, including chaperonin-containing tcp1 (sp13) and a putative nucleoplasmin (sp45). In addition, two protein-folding-related proteins, protein disulfide isomerase (PDI) (sp16) and calreticulin (sp03), showed an approximately twofold up-regulation (Table 1). Remarkably, they can bind to misfolded proteins in the endoplasmic reticulum (ER) and either correct the protein folding or direct them toward a degradation pathway [56,57]. These proteins constitute the cellular protein "quality-control" system [58–60]. This system is very important for developmental processes. It ensures the normal development of tissues and organs by correcting or eliminating cells that deviate from the normal route while allowing for their individuality. For example, calreticulin is essential for cardiac development in mouse; a calreticulin knockout was lethal due to impaired cardiac development [61]. Up-regulated "quality-control" protein levels might indicate a high frequency of protein modification processes at the D-veliger stage. In the future, it will be extremely intriguing to elucidate the mechanisms behind these "quality-control" systems in the context of bivalve larval development. Furthermore, regulatory mechanisms used by the "quality-control" system for protein folding, secretion and degradation, might have potential applications in aquaculture. It has been demonstrated that organ size control and immune functions are dependent on specific "quality-control" proteins [62,63]. Regulation of the spatiotemporal expression and trafficking of such "quality-control" proteins might be employed to improve production and disease control in aquaculture.

Besides the complex cellular processes mentioned above, changes in calcium metabolism were also important for D-veliger larvae. Deposition of calcium occurred on the trochophore shells, which made them visible under the light microscope at the D-veliger stage [4]. Two calcium-related proteins involved in shell development, CaM and calreticulin [64–66], were revealed in our proteomic analysis. These two proteins were also detected in the larvae of the snail *Pomacea canaliculata* during shell development [16]. The up-regulation of both proteins suggests that they contribute to the calcification of larval shells. Elucidation of their functions in shell calcification during larval development warrants further study.

5. Conclusion

In summary, the proteomic analysis in this study uncovered important information about the early larval development of *C. gigas*. Biological processes which occurred in the development from the trochophore to the D-veliger stage, including striking morphological changes in organs (such as the development of the velum and shells), transitions in tissues (such as the development of muscles and nervous system) and numerous cellular processes, were reflected or indicated by variations in protein expression. Novel aspects were proposed to be involved in bivalve larval development, such as the "quality-control" system. The annotations of differentially expressed proteins shed light on the molecular mechanisms that underlie these complex cellular processes and regulatory networks at the protein level. Additionally, as proteomic analysis has been efficient for bivalve larval

development research, we believe that more information will be obtained through related technical approaches, such as high-throughput "shotgun" proteomic analysis [67,68].

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