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RESEARCH ARTICLE

DNA methylation changes detected by methylation-sensitive amplified polymorphism in the Pacific oyster (*Crassostrea gigas*) in response to salinity stress

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Abstract Salinity is a considerable factor to the survival and distribution for a majority of marine organisms, the drawbacks of which are becoming a serious issue of aquaculture. DNA methylation, an extensively studied epigenetic modification in eukaryotes, plays a significant role in the regulation of gene expression in responding to environmental changes and triggering evolutionary consequences. The Pacific oyster *Crassostrea gigas*, as a eurythermal and euryhaline species, is considered to be tolerant to salinity fluctuation. In this study, fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP) analysis was used to investigate the frequency and variation of DNA methylation in *C. gigas* under different salinity and time. The results showed that total methylation level was generally on a downward trend. At lower salinity, the total methylation level decreased at the earlier process and then increased during experiment process, but continued to shrink at the rest salinity. Fully methylation tended to better reflect the dynamics of total methylation. Recovery treatment showed that the extent and pattern of DNA methylation were difficult to return to the normal level in this research. The sequencing and BLAST analysis indicated that in salt stress most of the selected bands were closely related to the metabolism of nucleic acids and proteins, tropomyosin, and cellular transport, effecting on different biological processes of *C. gigas*. This work provides useful data to further elucidate the molecular mechanisms of salt stress response and tolerance in invertebrates.

Keywords DNA methylation · Salt stress · *Crassostrea gigas* · Fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP)

Introduction

Salinity is a considerable factor influencing the survival states and distribution characteristics for a majority of marine organisms, the drawbacks of which are becoming a serious issue of aquaculture (Gunter 1961). When the salinity changes abruptly, marine invertebrates would suffer the threat of mass mortality (Jorge 1988; Laing 2001), causing huge losses to economic benefits and aquaculture development. The Pacific oyster *Crassostrea gigas* is one of the most important bivalves for their economic importance as an aquaculture species. And as a eurythermal and euryhaline species, *C. gigas* could live at salinity below 10 psu, also could survive in more than 35 psu (Zhao et al. 2012). However, salt stress, as a constraint condition, also influences trophic relationships and interactions with other species so as to upset the balance of ecosystems (Petes et al. 2012). In addition, the changes of osmosis pressure induced by long-term salt stress could cause toxicity to invertebrates which disrupt protein synthesis and interfer with enzyme activity (Kawabe et al. 2010). Due to the effect of osmosis, salinity fluctuation on organism growth and mortality can impact the success of oyster fisheries, causing the loss of valuable ecosystem

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services (Petes et al. 2012). As an osmoconformer, *C. gigas* has adapted to utilize the ionic composition of their external environment, typically seawater, in order to support important biological functions (Bradley 2009). At present, a series of studies have conjoint salinity and *C. gigas* in the terms of pathology, metabolomics, and transcriptomics (Zhao et al. 2012; Segura et al. 2015; Yang et al. 2016). However, the research about mechanism regulation upon epigenetic modifications for salt stress in *C. gigas* is scarce.

Organisms can employ regulatory strategies to adapt quickly to new conditions (Ghalambor et al. 2007). As an extensively studied epigenetic modification in eukaryotes, DNA methylation has showed probable to respond to environmental changes and trigger evolutionary consequences without changing genetic information (Jablonka and Lamb 1998; Morris 2001). Much progress in ecological and evolutionary aspects of DNA methylation has been achieved in various animals and plants, ranging from genomes to specific regions and genes (Zemach et al. 2010; Herrera and Bazaga 2011; Wang et al. 2015a). Furthermore, recent researches upon insects have indicated that DNA methylation played a pivotal role in response to environmental stress and in the regulation of gene expression (Elango et al. 2009; Bonasio et al. 2012; Wang et al. 2013). Environmental stimuli, such as drought, thermal and salt stress as well as heavy metal stress, alter cytosine methylation at specific sites throughout the genome, which would affect the expression of specific genes (Lukens and Zhan 2007; Place et al. 2008). In *C. gigas*, DNA methylation is confirmed crucial for early development (Riviere et al. 2013), and regulation of gene function (Riviere 2014), and its transgenerational inheritance has also been evidenced (Jiang et al. 2013, 2016). Nevertheless, little is known about salinity tolerance between genome-wide DNA methylation and the response to salt stress in *C. gigas*.

Fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP), a modified technique to detect DNA methylation, is in a position to optimize DNA methylation reaction conditions by replacing radiation with fluorescent system in the technique of MSAP (Xu et al. 2005). Compared with other DNA methylation detection methods, F-MSAP is a cheaper and less labor-intensive approach to scan genomic DNA methylation pattern, and its effectivity and reliability in *C. gigas* have been verified in previous study (Jiang et al. 2016).

In this study, we detected and compared the extent and pattern variations of genome-wide DNA methylation among different salt stress with F-MSAP. Polymorphic fragments were amplified and sequenced to explore potential mechanism in vital process. Our work will benefit epigenetics of *C. gigas* in environmental stress.

Materials and methods

Oyster materials and stress treatments

Adult *C. gigas* were collected from Laizhou, Shandong Province, China, in 2016, and were acclimatized for a week in filtered seawater before experiment. These oysters were individually marked and randomly divided into 24 groups and subjected to different salinity treatments (5, 10, 15, 20, 25, 30, 35, and 40 psu) for 8, 24 h, and 5 days, respectively. The individuals cultured in salinity 30 psu were used as control group. Moreover, after 24 h, some of the samples under salinity 5 and 40 psu conditions were transferred to control condition (30 psu) for another 5 days (called RE530 and RE4030, respectively) to assess whether exposure to nature condition enabled genome-wide DNA methylation to recover its normal extent and pattern. After experiment, about ten individuals from each group were randomly selected for sample collection. The gill samples from every group were dissected and saved at DNA guarder (Sangon).

Genomic DNA extraction and F-MSAP analysis

Genomic DNA was extracted using a modified phenol–chloroform protocol (Li et al. 2006). The integrity and concentration of genomic DNA was quantified by 1% agarose gel electrophoresis and Nanodrop-2000. The F-MSAP analysis was performed to detect methylation polymorphism as described by Jiang et al. (2016). Genomic DNA was digested with *EcoR* I+*Hpa* II and *EcoR* I+*Msp* I (Thermo), respectively. Then the ligation reaction was performed at 16 °C, overnight. After an initial denaturation at 72 °C for 2 min, 20 PCR cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C were performed; followed by 60 °C incubation for 30 min. Selective amplification was performed for 2 min of denaturing at 94 °C, then 10 cycles of 20 s at 94 °C, 30 s at 66 °C, and 2 min at 72 °C, with a 1 °C decrease in the annealing temperature of each cycle, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min 72 °C, with 60 °C incubation for 30 min. All adapters and primers are listed in Table 1. Selective amplification was carried out using a total of twelve primer combinations. *EcoR* I selective primers were labeled using a 6-FAM reporter molecule.

After denaturation (94 °C for 5 min), PCR products were loaded simultaneously with an ABI 3130 Genetic Analyzer (Applied Biosystems). Fragment analysis was subsequently obtained using GeneMapper v4.0 software and then the data were rendered in the form of Excel table for further scoring. According to the electrophoresis profiles, the F-MSAP patterns digested with the isoschizomers were divided four types (Additional file: Figure S1). Type I (1, 1) presents, both in *EcoR* I/*Hpa* II and in *EcoR* I/*Msp* I lanes

Table 1 Adapters and primers used for methylation-sensitive amplified polymorphism (MSAP) analysis

Adapter and primer	*EcoR* I	*Hpa* II/*Msp* I
Adapter I	5′-CTCGTAGACTGCGTACC-3′	5′-GACGATGAGTCTAGA A-3′
Adapter II	5′-AATTGGTACGCAGTCTAC-3′	5′-CGTTCTAGACTCATC-3′
Pre-amplification primer	5′-GACTGCGTACCAATTCA-3′(E0)	5′-GATGAGTCTAGAACGGT-3′(HM0)
Selective primer	E0 + CA	HM0 + CA
	E0 + TC	HM0 + GC
	E0 + CG	HM0 + GT
	E0 + CG	HM0 + AC
	E0 + GG	HM0 + AT
	E0 + CT	HM0 + AG
	E0 + AG	HM0 + AT
	E0 + AC	HM0 + AT
	E0 + CA	HM0 + GT
	E0 + CT	HM0 + GT
	E0 + AG	HM0 + AC
	E0 + CA	HM0 + AT

the bands are detected, usually treated as no methylation in analysis. Type II (1, 0) presents, only in *EcoR* I/*Hpa* II (H) lane bands are detected, called hemi-methylation. Type III (0, 1) presents, only in *EcoR* I/*Msp* I (M) lane bands are detected, called fully methylation. Type IV (0, 0) presents absent from both enzyme combinations, regarded as genetic polymorphism or hyper-methylation (Keyte et al. 2006).

Silver staining and DNA sequences of specific fragments

Some samples chosen on the basis of interesting polymorphisms were run on acrylamide gels and silver stained with the aim of isolating (Additional file: Figure S2–S5). DNA was eluted by UNIQ-10 column page DNA kit (Sangon). Eluted DNA was reamplified twice using corresponding primer pairs. The sequences of eluted bands were successfully obtained. Homology searches and sequence analysis were assessed using BLAST searching in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the *C. gigas* genome database (http://www.oysterdb.com) and UniProt (http://www.uniprot.org).

Results

Extent and pattern of genome-wide DNA methylation under different treatments

Twelve primer combinations were used to assay cytosine methylation at 5′-CCGG-3′ sequences in tissue gill for the F-MSAP analysis. Tables 2 and 3 show the general cytosine methylation patterns in tissue gill of each genotype among all these treatments (including recovery groups). A total of 1163 clear and reproducible fragments were amplified in each treatment. Based on the MSAP profiles, the number of DNA methylation sites was determined, a total of 411–554 amplified sites (Type I + II + III), accounting for 35.34–47.64% of the total sites. The extent of DNA methylation ranged from 28.38% (5 days, 40 psu salinity) to 47.84% (RE530) of gill samples.

Overall, a general trend for total methylation was recovery groups > 8 h > 24 h > 5 days. The methylation level of recovery group was significantly higher than that of the experimental group 24 h and 5 days ($P < 0.01$), and there was no significant differentiation between recovery group and 8 h treatment group. Although the overall trends were consistent, there were slight differences in some treatments. In terms of different salinity treatments, the total methylation level of RE530 was significantly higher than the experimental group salinity 5, 10, 15, 20, 25, 30, 35, 40 psu and RE4030 ($P < 0.05$), however, the full methylation level of RE530 was only significantly higher than the experimental group salinity 5, 10, 15, 20, 25, 30, and 35 psu ($P < 0.05$). As for hemi-methylation, no differences occurred in this time. Interestingly, at lower salinity 5, 10, and 15 psu, the total methylation level experienced a downward trend at the earlier 24 h and then increased during experiment process. However, at the rest salinity, the total methylation level continued to shrink. The fully methylation ratios were always higher than those of hemi-methylation ($P < 0.01$). Furthermore, fully methylation tended to better reflect the reality of the total methylation.

Table 2 DNA methylation changes under different salinity conditions

Salinity (psu) and time	Total amplified sites	Total methylation sites	I (11)	II (10)	III (01)	IV (00)	Hemi- methylation state (%)	Fully methylation state (%)	Total methylation state (%)
Number of sites after 8 h									
5	437	149	288	50	99	726	11.44	22.65	34.10
10	411	135	276	53	82	753	12.90	19.95	32.85
15	455	173	283	73	100	708	16.04	21.98	38.02
20	455	171	284	69	102	708	15.16	22.42	37.58
25	473	165	308	79	86	690	16.70	18.18	34.88
30	471	156	315	71	85	692	15.07	18.05	33.12
35	422	158	265	66	92	741	15.64	21.80	37.44
40	469	200	269	98	103	694	20.90	21.96	42.64
Number of sites after 24 h									
5	435	145	291	61	83	728	14.02	19.08	33.33
10	469	149	320	60	89	695	12.79	18.98	31.77
15	492	151	342	49	101	671	9.96	20.53	30.69
20	506	170	336	55	115	657	10.87	22.73	33.60
25	489	163	326	57	106	674	11.66	21.68	33.33
30	479	154	326	50	104	684	10.44	21.71	32.15
35	485	151	333	51	100	679	10.52	20.62	31.13
40	473	171	302	68	103	690	14.38	21.78	36.15
Number of sites after 5 days									
5	512	184	328	64	120	651	12.50	23.44	35.94
10	554	186	368	55	131	608	9.93	23.65	33.57
15	544	193	351	73	121	619	13.42	22.24	35.48
20	499	167	332	78	89	665	15.63	17.84	33.47
25	528	170	358	61	109	636	11.55	20.64	32.20
30	486	146	340	40	106	677	8.23	21.81	30.04
35	541	168	373	70	98	622	12.94	18.11	31.05
40	511	145	366	50	95	652	9.78	18.59	28.38

I nonmethylated sites, *II* hemi-methylated sites, *III* fully methylated sites. IV represents the absence of band in both enzyme combinations. Hemi-methylated state (%) = [(II)/(I + II + III)] × 100%; Fully methylation state (%) = [(III)/(I + II + III)] × 100%; Total methylation state (%) = [(II + III)/(I + II + III)] × 100%

Table 3 DNA methylation changes under different salinity conditions for recovery groups

Salinity (psu) and time	Total Amplified sites	Tottal methylation sites	I (11)	II (10)	III (01)	IV (00)	Hemi- methylation state (%)	Fully methylation state (%)	Total methylation state (%)
RE530	510	244	266	115	129	653	22.55	25.29	47.84
RE4030	522	207	315	80	127	641	15.33	24.33	39.66

Salinity-induced methylation/demethylation changes of recovery samples

According to the approach of Wang et al. (2015a), the patterns of salinity-induced changes of DNA methylation under salt stress and subsequent recovery could be divided into four kinds which include A, B, C, and D. In our study, pattern A (A1, A2, A3) indicates that the methylation status was the same between salt stress and subsequent recovery without changes. Pattern B (B1, B2, B3, B4, B5) indicates that DNA methylation level decreases under subsequent recovery, to be called hypomethylation. Pattern C (C1, C2, C3, C4, C5) indicates that DNA methylation level increased under the salt treatment, to be called hypermethylation. Pattern D (D1, D2) indicates that DNA methylation status changed between the salt stress and subsequent recovery,

but no difference in the statistical level. And 15 DNA methylation banding patterns were identified as methylated/demethylated under salt stress or recovery conditions. The detailed composition of different patterns and their percentages are listed in Table 4. Around 22.66–29.54% of the CCGG sites remained unchanged from salinity induction to recovery. The percentage of demethylation bands (pattern B) under salt stress ranged from 32.47 to 39.21% and the percentage of methylation bands (pattern C) under salt stress from 35.78 to 37.92%, indicating more DNA methylation events in recovery than salt stress for all four treatment.

Analysis of the differentially methylated DNA sequences and gene homology

15 of 32 interesting fragments were sequenced to identify how the DNA sequences involved in methylation/demethylation under salt stress. The BLAST results are summarized in Table 5. The size of polymorphic DNA fragments ranged from 99 to 418 bp. BLAST analysis of sequenced fragments indicated that the homologous sequences were related to many important processes, including tropomyosin, myomegalin, cellular transport (potassium voltage-gated channel subfamily H member 1, sodium-independent sulfate anion transporter and carboxypeptidase B), cellular metabolism (DnaJ homolog subfamily A member 1), synthesis and degradation of protein and nucleic acid (telomere length regulation protein TEL2 homolog, carboxypeptidase B, DNA-directed RNA polymerase II subunit RPB4, E3 ubiquitin-protein ligase MIB2, adenylate cyclase-associated protein 1), protein marking (ubiquitin carboxyl-terminal hydrolase 25), and so on. The sequence results showed that these fragments involved in a wide range of cellular functions were affected mostly by cytosine methylation/demethylation due to salinity stress.

Discussion

DNA methylation is an epigenetic modification that mostly occurs at the fifth carbon position of a cytosine ring. Evidences are increasingly in favor of DNA methylation influencing eukaryotes preparedness for stress adaptation through regulation of gene expression (Zemach et al. 2010; Head 2014). DNA methylation plays a key role of eukaryotes in response to diverse environmental stresses by adjusting various cellular activities and proceeding to regulating

Table 4 Summary of DNA methylation pattern changes under salt stress and subsequent recovery

Pattern	Enzyme digestion types				Number or ratio of loci			
	Control*		Recovery		5D30S*	5D30S*	5D5S*	5D40S*
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	RE530	RE4030	RE530	RE4030
A	No change				177 (24.86%)	167 (22.66%)	197 (28.18%)	213 (29.54%)
A1	1	1	1	1	138	140	150	171
A2	1	0	1	0	10	4	17	11
A3	0	1	0	1	29	23	30	31
B	Hypomethylation				250 (35.11%)	289 (39.21%)	227 (32.47%)	239 (33.15%)
B1	1	0	1	1	8	12	12	12
B2	0	1	1	1	16	26	28	17
B3	0	0	1	1	104	137	76	115
B4	0	0	1	0	60	47	55	39
B5	0	0	0	1	62	67	56	56
C	Hypermethylation				270 (37.92%)	275 (37.31%)	251 (35.91%)	258 (35.78%)
C1	1	1	1	0	34	24	31	23
C2	1	1	0	1	34	36	31	36
C3	1	1	0	0	134	140	116	136
C4	1	0	0	0	18	23	23	23
C5	0	1	0	0	50	52	50	40
D	Methylation pattern changed				15 (2.11%)	6 (0.81%)	24 (3.43%)	11 (1.53%)
D1	0	1	1	0	11	5	12	7
D2	1	0	0	1	4	1	12	4
Total					712 (100%)	737 (100%)	699 (100%)	721 (100%)

5D30S*, 5D5S*, 5D40S* represents the Control* in different 4 comparisons. RE530, RE4030 represents recovery groups. And in the Table 5, we called them R1, R2, R3, R4, respectively

Table 5 BLAST results and methylation patterns of a randomly selected methylated DNA fragments

Primer	Length (bp)	R1	R2	R3	R4	Identity (%)	Accession no.	Gene symbol	Locus tag	Protein homolog/homology description
743	240	A1	A1	A1	A1	99	NW_011934762.1	LOC105335489	CGI_10013163	Tropomyosin
744	166	B5	B5	B5	B5	97	NW_011937848.1	LOC105321055	CGI_10006707	Myomegalin
745	418	C2	C3	C2	/	86	NW_011934847.1	LOC105344012	CGI_10001418	Ubiquitin carboxyl-terminal hydrolase 25
						84	NW_011935252.1	Cap1	CGI_10022483	Adenylyl cyclase-associated protein
747	349	C3	A1	/	A1	98	NW_011935343.1	LOC105329753	CGI_10025113	Protein regulator of cytokinesis 1
748	148	/	B3	/	A1	96	NW_011937782.1	LOC105320372	/	/
573	300	A3	A3	A3	A3	91	NW_011935092.1	LOC105326836	CGI_10011660	Telomere length regulation protein TEL2-like protein
574	126	C3	A1	C3	B3	94	NW_011937756.1	LOC105319990	CGI_10026214	DnaJ-like protein subfamily A member 1
						94	NW_011942123.1	LOC105324970	CGI_10024105	E3 ubiquitin-protein ligase MIB2
577	99	/	/	/	C3	94	NW_011937469.1	LOC105317175	/	/
578	260	A1	A1	A1	A1	96	NW_011935226.1	LOC105328025	CGI_10003384	Potassium voltage-gated channel protein eag
145	157	C3	C3	/	/	97	NW_011937493.2	LOC105317502	CGI_10024549	DNA-directed RNA polymerase II subunit RPB4
146	127	B3	B3	A1	A1	97	NW_011937114.1	LOC105346164	CGI_10022307	/
224	190	C3	C3	C3	C3	98	NW_011936417.1	LOC105339840	CGI_10011152	Sodium-independent sulfate anion transporter
225	251	B3	/	A1	/	88	NW_011935086.1	LOC105326769	CGI_10022593	Carboxypeptidase B
228	171	A1	A1	A1	A1	95	NW_011935294.1	LOC105329134	/	/
2210	134	B3	B3	B1	B3	97	NW_011935478.1	LOC105330625	CGI_10023117	/

genome-wide gene expression (Bird 1992). In both plants and animals, cytosine is primarily methylated in the CG dinucleotide context, and methylation in the 5′portion and the 3′portion may an obstruction to gene expression (Grativol et al. 2012). In eukaryotes, genes with very low level of expression are most likely to be methylated (Zemach et al. 2010). In this case, regulation of gene expression is a particular need for eukaryotes to develop stress tolerance. In this study, we assessed that genome-wide changes in the DNA methylation induced by salinity fluctuation of the tissue gill of *C. gigas*. According to the existing studies, the optimum salinity for *C. gigas* is between 15 and 34 psu (Helm et al. 2004), and oysters will benefit tasty when the growth salinity is between 20 and 25 psu, which is lower than natural seawater salinity. In our current research, individuals in lower salinity (5 and 10 psu) were more susceptible than those in high salinity treatments. However, these results were against previous studies, of which mortality was highest at high salinity (33 psu), intermediate at 25 psu, and lowest in much lower salinity (Chu and Volety 1997; Petes et al. 2012). The different results compared to other existing researches might be attributed to the adoption of different materials and sampling points.

In recent years, much more attentions have been paid to the relationship between DNA methylation and abiotic stress tolerance. Absence of methylation is supposed to facilitate transcriptional opportunities to increase phenotypic plasticity in invertebrates (Roberts and Gavery 2012), and on this very note, there might be a close association between demethylation and gene expression. Similar results have been confirmed in recent studies. When *Aplysia* was in the serotonin environment, the CREB2 gene promoter methylation level increased, however, the gene expression decreased (Rajasethupathy et al. 2012). The methylation level of the 5 ‘UTR and the first exon of hox gene of *C. gigas* has been proved negatively correlated with the gene expression by methyl-DNA immunoprecipitation (Riviere et al. 2013). This phenomenon provides an adaptive potential for invertebrates, especially for the species living in highly fluctuating environment. However, interestingly, a positive association has been also reported between methylation status, in both gene bodies and putative promoter regions, and expression (Olson and Roberts 2014). Therefore, these results imply that the DNA methylation might be related to the abiotic stress response, but the association would obviously differ ranging from species to genes.

The extent and pattern of DNA methylation for *C. gigas* in response to salt stress were investigated by F-MSAP technique. DNA methylation pattern changes occurred under salt stress and subsequent recovery, including hypermethylation, hypomethylation, and other patterns. In general, the overall methylation level and the trend of variation were maintained in the treatment (Table 2), which showed that the stable DNA methylation extent might be vital important to the individuals in stress process. Salinity stress made the overall DNA methylation level reach to a higher value at earlier treatment and then reduced the levels overtime, namely, the extent of DNA methylation of 24 h and 5 days treatment is lower than that of 8 h (Table 2). One possible explanation is that the tissue, gill, is the respiratory and feeding organ of most shellfish, a main regulatory organ in adjusting salt degrees, and the first to experience the salinity changes of sea water; and some epigenetic states are established by transiently expressed or transiently activated factors that respond to environmental stimuli (Bonasio et al. 2010). Furthermore, perhaps because some genes with lower methylation level have greater apparent adaptability and these genes can be regulated by methylation modifications in the available time (Elango et al. 2009). Recently, Li et al. (2017) has reported that total and hemi-methylation of *Cynoglossus semilaevis* prominently went back to its normal levels to obtain equilibrium with continuous exposures to salt stress, which also supports our results in some extent. Given all these factors, it might indicate that biological adaptation might play a part in tolerability in response to salt stress. The total methylation level at lower salinity, 5, 10, and 15 psu, experienced a downward trend at the earlier 24 h and then increased during experiment process (Table 2). The specificity under lower salinity suggested that the response to low and high salinity stress would produce different physiological and biochemical reactions in the tissue gill of *C. gigas*. However, similar results had never been found in previous studies. Meanwhile, the fully methylation bands were always more than hemi-methylation bands, and the change of fully methylation could be better traced the variation of the total methylation of changing (Table 2). The result was consistent with previous research in *C. gigas* (Jiang et al. 2013), and was against those in cotton (Wang et al. 2015a) and *Cynoglossus semilaevis* (Li et al. 2017). It is not known that how full and/or hemimethylation affects organisms, which is vital important for eukaryote survival.

In this study, we left subsequent recovery groups to attempt to answer the question whether returning to natural conditions again to recover the extent and pattern of DNA methylation to normal level. It turned out that there was remarkable epigenetic differentiation between recovery groups and control groups (Table 4). However, it was inconsistent with the condition at the end of last 5 days, namely, DNA methylation and gene expression were unable to recover to the original and control level in this study. Moreover, the extent of CCGG methylation in recovery groups was higher than that in 5 days salinity treatment. On this paradox, we presumed that, during periods of salinity stress, although the DNA methylation had been on the trend as the normal level, these genes had triggered some

disease and irreversible damage. So that the populations of *C. gigas* might be highly susceptible and might therefore improve an adaptive mechanism in this stress. When salinity returned to normal, though the mortality decreased, internal environment may not come back the original in the limited time available. From another perspective, both groups of salt stress and subsequent recovery presented a completely consistent tendency with previous studies. For previous research on the genomic DNA methylation under salinity stress in other species (Wang et al. 2015a, b; Li et al. 2017), and so on, mostly, the first sampling point was ranged from 3 to 7 days, through which the extent of total methylation was lower than that of control group, all were similar to our current research. However, in this study, we paid more attention to the genomic DNA methylation model in 8 and 24 h from the beginning of stress experiment, which showed larger fluctuations in the extent and pattern of DNA methylation.

Related studies have already indicated special regions or gene activities towards stress tolerance. That the invertebrates respond to stress exposure might be strongly linked to the mode of DNA methylation (Gavery and Roberts 2010; Riviere et al. 2013; Saint-Carlier and Riviere 2015). These results imply that the phenotype can be significantly affected by the regulation of gene expression induced by DNA methylation. Therefore, additional information on the dynamics of genomic DNA methylation is useful for revealing the mechanism of epigenetic regulation in the development and stress-adaption processes. In this study, we selected and amplified thirty-two methylation fragments for sequencing, and nearly half of the recycled fragments were homologous to annotated sequences in oyster online databases (summarized in Table 5). The BLAST results showed that salt stress might play a role in the extent and pattern of DNA methylation during vital process, such as cellular transport and metabolism, synthesis and degradation of protein and nucleic acid, protein marking, and so on (Table 5), of or relating to vital process. The sequence Primer 745 was homologous to Adenylyl cyclase-associated protein, and the sequence Primer 578 was homologous to potassium voltage-gated channel protein eag. These above two points have been showed in the BLAST results in previous studies (Wang et al. 2015a, b). In this research, the the sequence Primer 578 maintained the same methylation level in the recovery, which was in accordance with the existing study of rice (Wang et al. 2015b); for all four treatments (R1, R2, R3, R4), their expression should not be changed by DNA methylation in the stress and subsequent recovery, which might be part of the reason that *C. gigas*, as a member of wide-temperature-salinity group, are tolerant and could maintain normal growth for salinity fluctuation. And the the sequence Primer 745 showed a decreased trend in DNA methylation level, except for the treatment R4, in our current research, which is regarded as genetic polymorphism or hyper-methylation (Keyte et al. 2006). However, we couldn't find a correction in the recovery with previous result. Furthermore, some of other genes, for instance, tropomyosin and enzyme activity-related genes, have been proved important for shellfish physiology activity and development (Ishikawa et al. 1998; Xu et al. 2016). Gavery and Roberts (2010) have confirmed DNA methylation has regulatory functions in *C. gigas*, particularly in gene families that have inducible expression, including those involved in stress and environmental responses. The effects of methylation in *C. gigas* against salt stress might need to combine the expression of related genes and specific sites in order to make more accurate judgments in subsequent experiments.

In conclusion, this study examined genome-wide DNA methylation variation in different salinity during limited time with F-MSAP. Our results suggested that there might be an important association between the epigenetic changes and salinity in the *C. gigas* genome. The sequencing and BLAST analysis for specific fragments showed that salt stress might also play a role in regulation of epigenetics to vital process. Our research provided useful information on role of epigenetics in regulating stress response to salt in *C. gigas*. Expression analysis of known sequences is needed for further investigation in environment stress.

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Compliance with ethical standards

Conflict of interest Xin Zhang declares that she does not have conflict of interest. Qi Li declares that he does not have conflict of interest. Lingfeng Kong declares that he does not have conflict of interest. Hong Yu declares that she does not have conflict of interest.

Ethical statement The research was conducted in the absence of any ethical issue on aquatic animal research.

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