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Analysis of DNA Methylation of *Gracilariopsis lemaneiformis* Under Temperature Stress Using the Methylation Sensitive Amplification Polymorphism (MSAP) Technique

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Abstract *Gracilariopsis lemaneiformis* is an economically important agarophyte, which contains high quality gel and shows a high growth rate. Wild population of *G. lemaneiformis* displayed resident divergence, though with a low genetic diversity as was revealed by amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) analyses. In addition, different strains of *G. lemaneiformis* are diverse in morphology. The highly inconsistence between genetic background and physiological characteristics recommends strongly to the regulation at epigenetic level. In this study, the DNA methylation change in *G. lemaneiformis* among different generation branches and under different temperature stresses was assessed using methylation sensitive amplified polymorphism (MSAP) technique. It was shown that DNA methylation level among different generation branches was diverse. The full and total methylated DNA level was the lowest in the second generation branch and the highest in the third generation. The total methylation level was 61.11%, 60.88% and 64.12% at 15℃, 22℃ and 26℃, respectively. Compared with the control group (22℃), the fully methylated and totally methylated ratios were increased in both experiment groups (15℃ and 26℃). All of the cytosine methylation/demethylation transform (CMDT) was further analyzed. High temperature treatment could induce more CMDT than low temperature treatment did.

Key words DNA methylation/demethylation; epigenetics; *Gracilariopsis lemaneiformis*; MSAP; temperature stress

1 Introduction

DNA methylation, a biochemical process involving the addition of a methyl group to the cytosine or adenine, plays a key role in the epigenetic regulation of eukaryotic gene expression (Iqbal *et al*., 2011) in a tissue-specific or a developmental stage-dependent manner (Angers *et al*., 2010; Zhang *et al*., 2010). DNA hypomethylation can induce altered flower morphology (Ronemus *et al*., 1996; Tanaka *et al*., 1997; Vyskot *et al*., 1995) and early-flowering (Fieldes *et al*., 2005; Iwase *et al*., 2010). The level of cytosine methylation has been observed with significant difference among various tissues in rice (Dhar *et al*., 1990), tomato (Messeguer *et al*., 1991), maize (Lund *et al*., 1995), banana (Kay, 2001) and pepper (Portis *et al*., 2004). Under high salinity conditions (Dyachenko *et al*., 2006), increased methylation level was observed in *Mesembryanthemum crystallinum* genome. Drought stress was found to induce a genome-wide change in DNA methylation level in rice (*Oryza sativa*), and only 70% of methylated DNA can be restored after a return to non-drought conditions (Wang *et al*., 2011). In maize (*Zea mays*), cold stress led to an increase of DNA methylation level (Shan *et al*., 2013). Salt stress was associated with the demethylation of genomic DNA in tobacco (Choi and Sano, 2007). Under metal stresses, specific hypomethylation of DNA was induced in white clover (*Trifolium repens*) and industrial hemp (Aina *et al*., 2004). In *Sorghum bicolor*, DNA methylation alteration has been shown to be associated with aluminum toxicity and low acidity (Kimatu *et al*., 2011).

Gracilariopsis lemaneiformis, an economically important agarophyte, has been cultured on a large scale in China (Ren *et al*., 2006). It is the source of phycoerythrin (Mazumder *et al*., 2003), soluble sulfated polysaccharides (Melo *et al*., 2002), and good material for agar extraction compared to *Gracilaria* sp. (González-Leija *et al*., 2009; Santelices and Doty, 1989; Zou *et al*., 2004). It has become one of the best commercialized macroalgae in China (Zhou *et al*., 2013). *G. lemaneiformis* can complete its life cycle under laboratory conditions, therefore it is also an ideal material for genetic studies (Zhang *et al*.,

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2004). *G. lemaneiformis* can be divided into holdfast, middle segment and tip (Wang *et al*., 2014). Significant difference was found in the growth and agar yield in the three parts of *G. lemaneiformis* (Wang *et al*., 2014). Different strains of *G. lemaneiformis* have diversity on morphology and environmental suitability. For example, compared to wild *G. lemaneiformis*, the cultivar 981 of *G. lemaneiformis* has less tetraspore, more branches and smaller diameter of branch (Zhang and Fei, 2008). Although it is from wild *G. lemaneiformis*, the cultivar 981 could endure high temperature till 26 degrees of centigrade (Zhang and Fei, 2008). Wild population of *G. lemaneiformis* distributed mainly in the low tide and subtidal zone of Yellow Sea and Bohai Sea in China which display relative diverge environments (Sui, 2005). However, samples from different geographic populations showed a low genetic diversity by AFLP and SSR analyses (Ding *et al*., 2012; Pang *et al*., 2010). The highly inconsistence between genetic background and physiological characteristics recommends strongly to the regulation at epigenetic level.

Methylation sensitive amplified polymorphism (MSAP) is a method developed by Reynalópez *et al*. (1997) based on methylation-sensitive restriction enzyme digestion on genomic DNA. Two methylation-sensitive restriction isoschizomers *Hpa* II and *Msp* I were used in the MSAP method, which recognized the tetranucleotide sequence of 5'-CCGG-3', however, displayed different sensitivity to cytosine methylation (Chakrabarty *et al*., 2003; Chen *et al*., 2009; Mcclelland *et al*., 1994). MSAP has been widely employed to detecting cytosine methylation in various plants, such as pepper (Portis *et al*., 2004), apple (Xu *et al*., 2000), Siberian ginseng (Chakrabarty *et al*., 2003), maize (Shan *et al*., 2013), wheat (Meng *et al*., 2012), banana (Baurens *et al*., 2012), *Leymus chinensis* (Yu *et al*., 2013), and *Saccharina japonica* (Qu *et al*., 2013).

In order to investigate the potential role of DNA methylation change in response to temperature stress in *G. lemaneiformis*, we detected and compared the genomic DNA methylation level among different generation branches and temperature-treated samples with MSAP method, which revealed the potential impact of DNA methylation during temperature adaptation process and provided an epigenetic theoretical basis for genetic breeding application of *G. lemaneiformis.*

2 Materials and Methods

2.1 Materials Sampling and Pre-Treatments

Samples were collected from Taiping Corner (36.05°E, 120.37°N), Qingdao, Shandong Province, China. Wild-type *G. lemaneiformis* tetrasporophytes used in this experiment were identified by the existing of tetraspores on the surface of thallus. After the visible fouling organisms were cleaned, the samples were acclimatized in sterilized sea water which was changed every three days, and under the light intensity of 50 μmol photons $m^{-2} s^{-1}$ with a 12:12 (L:D) cycle at 22℃.

2.2 Material Treatment for Testing of Different Branches

The first generation branches (FB), second generation branches (SB) and third generation branches (TB) were distinguished according to Wang *et al*. (2010). The FB grew directly on the holdfast which produced the SB, while TB grew on the SB (Wang *et al*., 2010). Segment, about 3 cm in length, was cut from each FB, SB and TB of three wild-type *G. lemaneiformis* tetrasporophytes, respectively. The segments were cultivated in 300 mL Pro culture medium (Pflugmacher and Steinberg, 1997) for 7 days under the light intensity of 50 μmol photons $m^{-2} s^{-1}$ with a 12:12 (L:D) cycle and at 22℃. The sea water with Pro culture medium was replaced every three days (Wang *et al*., 2016). After that the segments were used for DNA extraction.

2.3 Material Treatment for Testing of Different Temperatures

Three SB with the length of 8 cm of each strain were cut from wild-type *G. lemaneiformis* tetrasporophytes, and each one from the three fragments was treated at 10℃, 22℃ and 26℃, respectively. Totally 9 strains were applied for the analysis. The tips were cultivated in Pro culture medium (Pflugmacher and Steinberg, 1997) under the light intensity of 50 μmol photons $m^{-2} s^{-1}$ with a 12:12 (L:D) cycle and at different temperatures, and used for DNA extraction after a week (Wang *et al*., 2016).

2.4 DNA Extraction

Samples about 0.1g in weight were cut into small segments. Total genomic DNA was extracted using the Plant Genomic DNA Kit (TIANGEN, Beijing, China). The quality and quantity of the extracted DNA were determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific) and agarose gel electrophoresis (1%). The isolated DNA was stored at −20℃.

2.5 Methylation-Sensitive Amplification Polymorphism (MSAP) Analysis

MSAP method was modified according to Xiong *et al*. (1999). Genomic DNA samples were double-digested by *Eco*R I plus *Hpa* II and *Eco*R I plus *Msp* I, respectively, at 37℃ for 5 h, and then treated at 80℃ for 30 min. For each sample, the reaction mixtures contained 600 ng of genomic DNA, 2 μL of 10× T Buffer, 2 μL of 0.1% BSA, 12.5 U of *Eco*R I, and 10 U of *Hpa* II (or *Msp* I) in a final volume of 20 μL. The digested DNA fragments (10 μL) were ligated with the double-stranded *Eco*R I adapter and the *Hpa* II/*Msp* I adapter simultaneously using T4 DNA ligase (Takara, Japan) according to the manufacturer's instructions. Subsequently, the ligation products were used as the template for the following pre-selective amplification with *Eco*R I 1 A and *Hpa* II/*Msp* I 1 T primers. The sequence of adaptors and pre-amplification primers were modified according to Ma *et al*. (2012) as shown in

Table 1. The PCR mixtures with a total volume of 20 μL consisted of 12.1 μL deionized H_2O, 2 μL reaction buffer, 1.2 μL 25 mmol $MgCl_2$, 1.5 μL 2 mmol dNTP (each), 1 μL of 10 mmol forward primer and reverse primer each, 0.2 μL *Taq* DNA polymerase, and 1 μL of template DNA (the ligation product). The thermal cycles for the PCRs were programmed from an initial denaturation at 94℃ for 5 min, followed by 30 cycles of denaturing and annealing at 94℃ for 45 s or at 60℃ for 45 s according to the different primers and extending at 72℃ for 1 min, and a final extension at 72℃ for 10 min. The pre-amplification products were 20-fold diluted (v:v) with deionized H_2O, and used as the template for the selective amplification reactions. In this step, *Eco*R I and *Hpa* II/*Msp* I primers with three additional selective nucleotides were used. The products of selective amplification were separated by electrophoresis on 6% polyacrylamide gel and stained with silver as described by Bassam *et al*. (1991).

Table 1 Primers/adapters used in MSAP analysis

	Primers/adapters	Oligonucleotide sequences (5'-3')	
		F	R
Adapters	*Hpa* II/*Msp* I-adapter	CTCGTAGACTGCGTACC	GATCATGAGTCCTGCT
	*Eco*R I-adapter	AATTGGTACGCAGTC	CGAGCAGGACTCATGA
Pre-amplification primers	E-A	GACTGCGTACCAATTCA	
	H/M-T		ATCATGAGTCCTGCTCGGT
Selective amplification primers†	E-AAG+H/M-AAA	GACTGCGTACCAATTCAAG	ATCATGAGTCCTGCTCGGAAA
	E-AAG+H/M-TAT	GACTGCGTACCAATTCAAG	ATCATGAGTCCTGCTCGGTAT
	E-ATC+H/M-ATT	GACTGCGTACCAATTCATC	ATCATGAGTCCTGCTCGGATT
	E-ATT+H/M-GAA	GACTGCGTACCAATTCATT	ATCATGAGTCCTGCTCGGGAA
	E-AAT+H/M-TCT	GACTGCGTACCAATTCAAT	ATCATGAGTCCTGCTCGGTCT
	E-GAA+H/M-CAA	GACTGCGTACCAATTCGAA	ATCATGAGTCCTGCTCGGCAA
	E-ACA+H/M-AAA	GACTGCGTACCAATTCACA	ATCATGAGTCCTGCTCGGAAA
	E-GAA+H/M-TCT	GACTGCGTACCAATTCGAA	ATCATGAGTCCTGCTCGGTCT
	E-CAG+H/M-ATC	GACTGCGTACCAATTCCAG	ATCATGAGTCCTGCTCGGATC
	E-TAC+H/M-CTC	GACTGCGTACCAATTCTAC	ATCATGAGTCCTGCTCGGCTC
	E-GAG+H/M-AAA	GACTGCGTACCAATTCGAG	ATCATGAGTCCTGCTCGGAAA
	E-CCT+H/M-GAA	GACTGCGTACCAATTCCCT	ATCATGAGTCCTGCTCGGGAA
	E-TCT+H/M-CTC	GACTGCGTACCAATTCTCT	ATCATGAGTCCTGCTCGGCTC
	E-ACT+H/M-GAG	GACTGCGTACCAATTCACT	ATCATGAGTCCTGCTCGGGAG
Selective amplification primers††	E-AAT+H/M-AAA	GACTGCGTACCAATTCAAT	ATCATGAGTCCTGCTCGGAAA
	E-AAG+H/M-AAA	GACTGCGTACCAATTCAAG	ATCATGAGTCCTGCTCGGAAA
	E-AAG+H/M-TAT	GACTGCGTACCAATTCAAG	ATCATGAGTCCTGCTCGGTAT
	E-ATC+H/M-ATT	GACTGCGTACCAATTCATC	ATCATGAGTCCTGCTCGGATT
	E-AGC+H/M-AAT	GACTGCGTACCAATTCAGC	ATCATGAGTCCTGCTCGGAAT
	E-CCT+H/M-TAT	GACTGCGTACCAATTCCCT	ATCATGAGTCCTGCTCGGTAT
	E-AGT+H/M-TAC	GACTGCGTACCAATTCAGT	ATCATGAGTCCTGCTCGGTAC
	E-ATC+H/M-TAC	GACTGCGTACCAATTCATC	ATCATGAGTCCTGCTCGGTAC
	E-ACA+H/M-AAA	GACTGCGTACCAATTCACA	ATCATGAGTCCTGCTCGGAAA
	E-ACT+H/M-AAA	GACTGCGTACCAATTCACT	ATCATGAGTCCTGCTCGGAAA
	E-GAA+H/M-TCT	GACTGCGTACCAATTCGAA	ATCATGAGTCCTGCTCGGTCT
	E-GAA+H/M-ATC	GACTGCGTACCAATTCGAA	ATCATGAGTCCTGCTCGGATC
	E-GAG+H/M-AAA	GACTGCGTACCAATTCGAG	ATCATGAGTCCTGCTCGGAAA
	E-AGC+H/M-TAC	GACTGCGTACCAATTCAGC	ATCATGAGTCCTGCTCGGTAC
	E-CAG+H/M-ATC	GACTGCGTACCAATTCCAG	ATCATGAGTCCTGCTCGGATC
	E-CTC+H/M-GAA	GACTGCGTACCAATTCCTC	ATCATGAGTCCTGCTCGGGAA
	E-CCT+H/M-GAA	GACTGCGTACCAATTCCCT	ATCATGAGTCCTGCTCGGGAA
	E-GAG+H/M-CAA	GACTGCGTACCAATTCGAG	ATCATGAGTCCTGCTCGGCAA
	E-CCA+H/M-CAA	GACTGCGTACCAATTCCCA	ATCATGAGTCCTGCTCGGCAA
	E-ACT+H/M-GAG	GACTGCGTACCAATTCACT	ATCATGAGTCCTGCTCGGGAG

Notes: † used in different generation branches. †† used in different temperature treatment.

3 Results

3.1 Global DNA Methylation Profiles in *G. lemaneiformis*

In the present study, 14 primers combinations (Table 1) were used to assaying cytosine methylation in 5'-CC GG-3' sequences among different generation branches of *G. lemaneiformis*. The PAGE electrophoresis of MSAP were showed in Fig.1.

The presence of bands for both enzyme combinations indicated unmethylated sites. Bands observed only in *Eco*R I/*Hpa* II digestion combination indicated sites with hemi-methylation. Absence of bands in both enzyme combinations digestion together with present band only for *Eco*R I/*Msp* I indicated full methylated sites (Wang

et al., 2011) .

For the same sample, patterns of different generations produced by the same digestion combination were different, suggesting that the methylation types among different branches were different. While patterns of the same branch generation raised by the same digestion combination on different strains were also different, the methylation status of different strains was different.

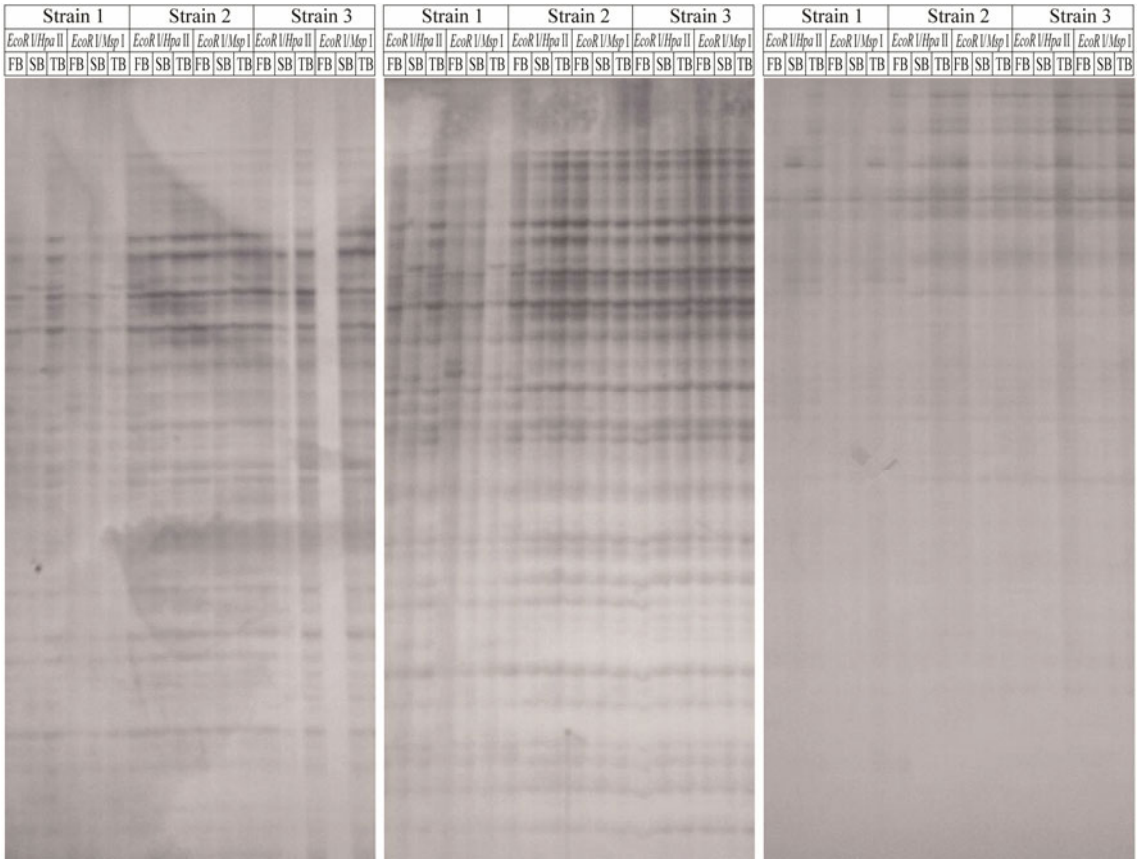

Fig.1 PAGE electrophoresis of MSAP among different generation branches of *G. lemaneiformis* by the number 5, 8 and 29 primers from left to right. FB, the first generation branches; SB, second generation branches; and TB, the third generation branches.

The restriction patterns were artificially categorized into four types, representing different methylation status. The statistic data of amplified bands from different generation branches were shown in Table 2. In total, 63 bands were amplified by 14 pairs of primers. According to the category described above, there were 51 (80.95%), 46 (73.02%) and 53 (84.13%) fully methylated DNA sites in FB, SB and TB, respectively (Table 2), indicating that the fully methylated DNA level was the lowest in the second generation branch but the highest in the third one, and was up-regulated by 11.1% (Table 2). The total methylation ratio, like full methylation ratio, was the highest in the third generation branches (87.30%) but the lowest in the second branches (76.19%) (Table 2). However, the hemi-methylation ratio showed no difference between different generation branches (Table 2).

Table 2 Analysis on the methylation level of *G. lemaneiformis* in different generation branches by 14 pairs of primers as revealed by MSAP

Patterns		MSAP band types	Branch		
*Eco*R I/*Hpa* II	*Eco*R I/*Msp* I		1st	2nd	3rd
1	1	I	10	15	8
1	0	II	2	2	2
0	1	III	1	0	6
0	0	IV	50	46	47
Hemi-methylated sites			2 (3.17%)	2 (3.17%)	2 (3.17%)
Fully methylated sites			51 (80.95%)	46 (73.02%)	53 (84.13%)
Totally methylated sites			53 (84.13%)	48 (76.19%)	55 (87.30%)

Notes: A score of 1 or 0 represents the presence or absence of bands, respectively. Type I represented unmethylated site. Type II represented hemi-methylated site. Types III+IV represented fully methylated sites. Totally methylated sites = II+III+IV. Hemi-methylated percentage = [(II)/(I+II+III+IV)] × 100%. Fully methylated percentage = [(III+IV)/(I+II+III+IV)] × 100%. Totally methylated percentage = [(II+III+IV)/ (I+II+III+IV)] × 100%.

3.2 DNA Methylation Status in *G. lemaneiformis* Under Different Temperature Treatments

Twenty pairs of primers (Table 1) were used to demonstrating cytosine methylation in 5'-CCGG-3' sequences of the strains under different temperature treatments. The PAGE electrophoresis of MSAP was showed in Fig.2. The pattern of the detected bands was categorized into four types by the same typing standards as the experiment of different generation of branch.

For the same sample, pattern from different temperature treatment produced by the same digestion combination were different, suggesting that different temperature treatment affect the methylation degree of the same site. While pattern from the same temperature treatment raised by the same digestion combination on different strain were also different, suggesting that the methylation level was different among different strain and the methylation type at the same site were different.

The detected bands from different temperature treatments were shown in Table 3. The patterns of 432 bands which were amplified by 20 pairs of primers were categorized into four types. The samples cultivated at 22℃, which was considered as the optimal growth temperature of *G. lemaneiformis*, were taken as control. Compared with the control, the full methylation and total methylation ratios were increased in both the two experiment groups (Table 3). The highest full methylation ratio was displayed by 10℃-treated group (49.31%) which was 4.87% higher than that of the control group. Meanwhile, the total methylation ratio reached the highest in the 26℃-treated group (64.12%), 3.24% higher than that of the control group (Table 3). The lowest hemi-methylation ratio was detected in the 10℃-treated group (11.81%), with a 4.63% declining compared to the control group. From 10℃ to 26℃, the hemi-methylation ratio rose from 11.81% to 18.06%, increased by 6.25% (Table 3).

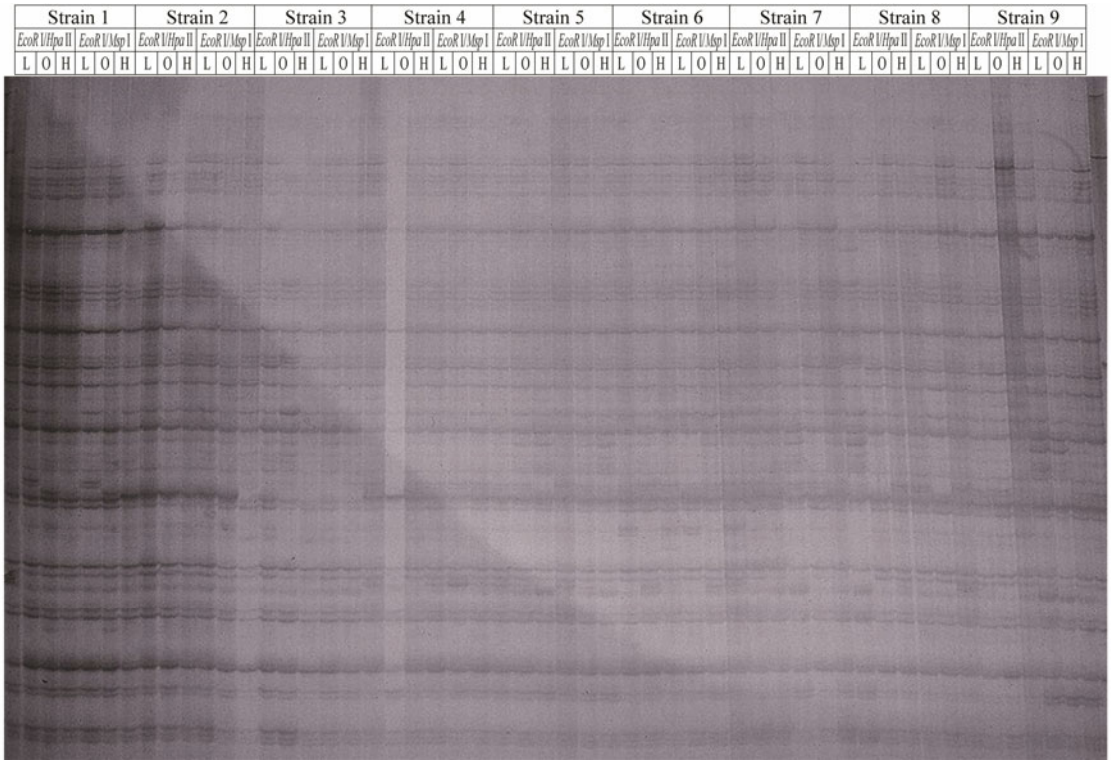

Fig.2 PAGE electrophoresis of MSAP in 9 strains treated at different temperatures by the number 22 primer. The letter L, O and H represent 10℃, 22℃ and 26℃ treatments, respectively.

Table 3 Analysis on the methylation level of *G. lemaneiformis* at different temperature treatments by 20 pairs of primers as revealed by MSAP

Patterns		MSAP band types	Material		
*Eco*R I/*Hpa* II	*Eco*R I/*Msp* I		10℃	22℃	26℃
1	1	Ⅰ	168	169	155
1	0	Ⅱ	51	71	78
0	1	Ⅲ	67	60	87
0	0	Ⅳ	146	132	112
Hemi-methylated sites			51 (11.81%)	71 (16.44%)	78 (18.06%)
Fully methylated sites			213 (49.31%)	192 (44.44%)	199 (46.06%)
Total methylated sites			264 (61.11%)	263 (60.88%)	277 (64.12%)

Notes: Same as those in Table 1.

Based on the results described above, all of the cytosine methylation/demethylation transform (CMDT) were divided into three major groups: without change, demethylation, and methylation, involving 15 classes totally. The information on CMDT in samples treated at different temperatures was shown in the Table 4.

The first type, without any change, was monomorphic with no cytosine methylation alteration and included classes A, B, and C, indicating that the same 5' -CCGG-3' sites were detected both in the control and experiment groups. The second type was demethylation and included classes D through I, including III-II, IV-II, II-I, III-I, IV-I, and IV-III, showing that cytosine was demethylated at different extents under temperature stress compared with the control group. The third type was methylation and included classes J through O, including I-II, II-III, II-IV, I-III, I-IV, and III-IV, representing the cytosine methylation events induced by temperature stress. Among them, the transformation of II-III and III-II suggested one chain was re-methylated while the other was non-methylated, in which the overall level for methylation remained the same.

Table 4 Cytosine methylation/demethylation transform (CMDT) in samples treated at different temperatures as was revealed with MSAP

Cytosine methylation/demethylation transform	Class	Band pattern				Methylation type		Detected sites	
		Control		Experiment		Control	Experiment	10℃	26℃
		Hpa II	*Msp* I	*Hpa* II	*Msp* I				
Without change	A	1	1	1	1	I	I	111	98
	B	1	0	1	0	II	II	23	26
	C	0	1	0	1	III	III	24	24
								158 (45.01%)	148 (40.66%)
Demethylation	D	0	1	1	0	III	II	6	8
	E	0	0	1	0	IV	II	13	19
	F	1	0	1	1	II	I	23	18
	G	0	1	1	1	III	I	15	19
	H	0	0	1	1	IV	I	19	20
	I	0	0	0	1	IV	III	19	25
								95 (27.07%)	109 (29.95%)
Methylation	J	1	1	1	0	I	II	9	25
	K	1	0	0	1	II	III	6	16
	L	1	0	0	0	II	IV	19	11
	M	1	1	0	1	I	III	18	22
	N	1	1	0	0	I	IV	31	24
	O	0	1	0	0	III	IV	15	9
								98 (27.92%)	107 (29.40%)

Notes: A score of 1 or 0 represents the presence or absence of bands, respectively. Type I represented unmethylated site. Type II represented hemi-methylated site. Type III + Type IV represented fully methylated sites. Without change percentage = [(A+B+C)/(A+B+C+D+E+F+G+H+I+J+K+L+M+N+O)] × 100%. Demethylation percentage = [(D+E+F+G+H+I)/(A+B+C+D+E+F+G+H+I+J+K+L+M+N+O)] × 100%. Methylation percentage = [(J+K+L+M+N+O)/ (A+B+C+D+E+F+G+H+I+J+K+L+M+N+O)] × 100%.

Compared with control group, the ratio for the sites without change accounted for 45.01% and 40.66% of the detected bands in the 10℃-treated and 26℃-treated group, respectively. The result suggested that higher temperature treatment induce more CMDT events than lower temperature. Compared to control group, 27.07% and 27.92% of testing sites were demethylated and methylated, respectively in the 10℃-treated group, including class F, transformed from type II to type I, which was the most demethylated group of demethylation type. Meanwhile, class N, transformed from type I to type IV, was the most methylated group of methylation type. The methylation ratio was 0.85% higher than that of demethylation in the 10℃-treated group. Therefore, low temperature stress tended to induce sites methylation. The high temperature stress induced 29.95% and 29.40% of the testing sites demethylated and methylated, respectively, including class I, transformed from type IV to type III, which showed the most demethylation ratio. While class J, transformed from type I to type II, showed the most methylation ratio. Demethylated sites were 0.55% more than the methylated ones under high temperature treatment, suggesting that high temperature stress tended to induce demethylation. Although high temperature stress tended to induce sites demethylation, the genomic DNA methylation level under this condition was raised (Table 3). It may be resulted from the results that the quantity of the sites transformed from unmethylated type to fully methylated ones (including M and N with the number of 46) was more than that transformed from fully methylated type to unmethylated ones (including G and H with the number of 39). These two transform modes had great

influence on the genomic methylation level.

4 Discussion

4.1 MSAP Analysis of Global DNA Methylation Profiles in Different Generation Branches of *G. lemaneiformis*

The DNA methylation levels were different among the first, the second and the third generation branches. The sampling location should be set in the same generation branch of *G. lemaneiformis* in DNA methylation study. Otherwise it will affect the accuracy of the results.

Epigenetic mechanisms like DNA methylation/demethylation are thought to play key roles in the expression regulation process (Iqbal *et al*., 2011). Qu *et al*. (2013) detected the DNA methylation level in different tissues of *S. japonica* by MSAP technology. They found that the methylation level displayed diversity among different *S. japonica* strains. Therefore, they inferred that demethylation may affect the morphological development of *S. japonica* by regulating temporal-spatial expression. In our research, *G. lemaneiformis* showed diverse characteristics among different generation branches and tissues. Wang *et al*. (2010) found that the rate of increase in biomass was the highest in the 3rd generation branches and the lowest in the 1st generation branches. The quantity, survival rates and germination number of tetraspores released from the first generation branches were higher than those from other generation branches (Ye *et al*., 2006). Our study indicated that the methylation levels in different generation branches of *G. lemaneiformis* were different. The hemi-methylation level remained on the same level among different generation branches. The full and total methylation levels were both the highest in the 3rd generation branch and the lowest in the 2nd generation branch. The change in DNA methylation level may be controlled by the regulatory mechanisms of gene expression during development and differentiation (Finnegan *et al*., 1993). The difference of methylation level in different generation branches may influence the expression of some genes, which lead to the change of some characteristics among different generation branches such as the quantity, survival rates and germination number of tetraspores (Ye *et al*., 2006). However, further researches should be exploited to address issues such as which genes are influenced by the changed methylation level, how the changed expression of these genes affects the characters in different generation branches, and what is the biological significance of the changed characters among different generation branches in *G. lemaneiformis*.

4.2 Analysis of DNA Methylation Status in *G. lemaneiformis* at Different Temperatures

DNA methylation is a fundamental mechanism of epigenetic regulation, which often carries on bidirectional adjustment to regular gene expression. Environmental stimulation, such as cold, heat, drought, salt stress and pathogen infection, have been confirmed to cause changes in DNA methylation status in plants (Bender, 1998; Boyko and Kovalchuk, 2011; Lukens and Zhan, 2007). Qu *et al*. (2013) found that chilling treatment could cause the change in DNA methylation level that may protect *Saccharina japonica* from temperature stress by making chromosome structure tight and stable.

In our study, under temperature stress, the increased DNA methylation level in *G. lemaneiformis* may inhibit the expression of some genes to contribute to the temperature adaptation of *G. lemaneiformis*. It was reported that the number of released tetraspores of *G. lemaneiformis* was the highest at 20℃ (Wang *et al*., 2010). We found that the full and total methylation levels were the lowest at 22℃ which is close to the temperature for releasing of the highest number of tetraspores, while lower (10℃) and higher temperatures (26℃) both associated with increased methylation level. Temperature could affect the methylation level in *G. lemaneiformis*. We inferred that low DNA methylation level may relate to the big number of released tetraspores. However, the correlation between physiological traits and methylation level needs to be investigated by further exploiting of more samples and characterization of different biological traits.

High temperature treatment could induce more CMDT in *G. lemaneiformis* genome than that under low temperature treatment. The suitable growth temperature of *G. lemaneiformis* ranges from 15℃ to 22℃, while 10℃ and 26℃ were also growth tolerant temperature, which were too low to induce conformational change of DNA (Zhou *et al*., 2006). Therefore, the change of methylation level in *G. lemaneiformis* under temperature stress probably correlated to its temperature adaptation, while the methylation type of CMDT related to high temperature adaption.

5 Conclusions

From the results of our study, DNA methylation plays an important role during the adaptation of *G. lemaneiformis* to temperature stress. The levels of DNA methylation in genome of *G. lemaneiformis* from different generation branches were diverse.

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