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Pattern of DNA cytosine methylation in *Aeluropus littoralis* during temperature stress

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Abstract

DNA methylation as an epigenetic mediator plays the important role in spatial and temporal gene regulation and ensures the stability and the plasticity of organism. In this investigation, methylation sensitive amplification polymorphism (MSAP) were assessed in CCGG sites on a halophytic plant, *Aeluropus littoralis* in response to different temperature stresses including freezing, low and high temperatures. A combination of 13 primers were able to produce 500 bands, of which 74%, 20.8% and 5.2% were of type I (non methylated fragments), type II (CpG methylated fragments) and type III (CpCpG methylated fragments), respectively. Among these bands, 130 bands were methylated fragments with the highest occurrence of methylation at CpG internal cytosine. The results showed that up to 2% of all methylated bands were polymorphic, which belonged to types II and III. Highest levels of methylation alternations were detected under high and freezing temperatures. The results suggest that apart from cis regulatory logic plant response to the environmental temperatures may be regulated by methylation of CCGG sites of stress-related loci.

Key words: DNA methylation, MSAP, temperature stresses, halophyte, *Aeluropus littoralis*.

Introduction

The genome carries all information required to build up an organism. Genetic and epigenetic information can be coupled to determine the functional state of cells and tissues during differentiation and developmental stages. The epigenetic information can be introduced by cytosine methylation and by nucleosomal histone modifications (Loidl, 2004). DNA cytosine methylation involves the addition of a methyl group to the C-5 position of the cytosine pyrimidine ring at nuclear DNA. The direct addition of a methyl group to a cytosine can offer docking sites for proteins to revise the chromatin state or affect the covalent modification of resident histones (Alis et al, 2007). Cytosine methylation process is a universal DNA modification and is considered to assume wide different roles among organisms (Hendrich, 2003). Methylation plays a crucial role in

regulation of gene expression, genome plasticity and it is correlated with gene silencing at both transcriptional and post-transcriptional levels (Choi, 2007; Loidl, 2004). Transcriptional gene silencing is associated with hyper-methylation of promoter sequences, while post-transcriptional gene silencing is linked with hyper-methylation of transcribed or coding sequences (Paszkowski and Whitham, 2001). In plants, cytosine methylation has been shared in CpG, CpHpG, and CpHpH contexts, where H is adenine, cytosine, or thymine (Chinnusamy et al, 2009; Sasaki, 2010). Recent studies showed that around 24% of CpG dinucleotides had experienced methylation processing in the Arabidopsis genome (Cokus, 2008). DNA methylation monitoring is divided into global genomic methylation patterns and DNA methylation in specific regions, such as CpG islands (Havlis, 2002). Generally two procedures including sodium bisulfite conversion and methylation-sensitive restriction enzyme digestion have been widely used in this field (Siegmund, 2002). Methylation sensitive amplification polymorphism (MSAP) technique is based on the use of the isoschizomer enzymes, *Hpa* II and *Msp* I, that display differential sensitivity to DNA cytosine methylation at the related recognition sites (Xu et al., 2000; Portis, 2004). Seedling development and subsequently plant stability under environmental restrictions is one of the major topics in the field crop research area. Plants, unlike animals, could not escape from environmental inhibitors such as biotic and abiotic stresses. But re-programming of the gene regulation in response to environmental cues leads to phenological and developmental plasticity, which are important mechanisms to sense and respond to different signals (Chinnusamy et al., 2009). Conditions with low and high temperatures are considered as a restriction factor for plant growth and production. To date, few reports are available on the differential difference DNA methylation patterns under freezing, low and high temperature stresses. Recent studies revealed that DNA methylation plays a major role in regulating plant response against water deficit (Labra et al., 2002), chilling (Peredo et al., 2008), cutting (Choi and Sano, 2007), osmotic (Tan et al., 2010), toxic metal (Filek et al., 2008) and salt stresses (Li et al., 2009). Halophytes commonly grow in regions simultaneously combined with high salinity and temperature stresses (Larcher et al., 1990; Lu and Zhang, 1998). The presented results showed that a sub-lethal dose of a given stress could protect plants against a lethal exposure to another stresses (Lewis et al., 1995). Pretreatment of plants with salinity increased their temperature tolerance (Torzilli, 1997). *Aeluropus littoralis* as a monocot halophyte has originated from arid and semi arid regions. The plant has simultaneously experienced salt and different temperature stresses. The present study employed MSAP technique to investigate relations between different temperature stresses and DNA methylation on a halophytic plant.

Materials and Methods

Plant materials and growth conditions

The seeds of *Aeluropus littoralis* were collected from Isfahan province in Iran. The surface sterilized seeds were transferred on 1/2 MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar in glass plates. The cultures were incubated at 25 ± 1 ^{o}C with 16 h photoperiod at 100 µmol m^{-2} s^{-1} photon flux density using cool-white fluorescent light. One month-old seedlings were subjected simultaneously to different temperature stresses including freezing (-20 ± 1 ^{o}C), low (4 ± 1 ^{o}C) and high (37 ± 1 °C) temperatures and were compared with control temperature (25 ^{o}C). A complete darkness was applied for control plants, freezing and high temperatures. Low temperature stress was conducted by exposing plants to 2 conditions (light and dark). After 2 days, seedlings were sampled, and immediately placed in liquid nitrogen and stored at -80 °C until use.

Methlyation sensitive amplification polymorphism

Genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Germany). Qualification and Quantification of isolated DNA was determinedby

agarose gel electrophoresis and spectrophotometer analysis, respectively. MSAP analysis was carried out according to Vos et al. (1995) with minor modifications. The isoschizomers *Hpa* II and *Msp* I (Roche Applied Science, Germany) as frequent-cutter enzymes were employed instead of *Mse* I. Both *Hpa* II and *Msp* I recognize the same tetra-nucleotide sequence (5'-CCGG-3'), but display different sensitivity to DNA methylation (Xu et al., 2000; Fang et al., 2007; Peng et al., 2009). Genomic DNA (250 ng) was digested overnight using the *Msp* I/*EcoR* I and *Hpa* II/*Eco* RI enzyme combinations and the fragments were Subsequently linked to the specified adapters (Table 1).

Pre-amplification was performed in a 20 µl reaction mixture, containing 50 ng ligated DNA, 250 µM of each dNTPs, 1X buffer PCR (10 mM Tric-HCl; 50 mM KCl, pH 8.8; 0.08 Nonidet P40), 2 mM $MgCl_2$, 1 U of *Taq* polymerase (Fermentas, Lithuania) and 0.25 µM each of non-selective primer pairs (Table 1) (Alpha DNA, Canada) using an MJ Mini thermal cycler (Bio-Rad, USA). The PCR program consisted of 20 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 2 min. The pre-amplification product was diluted 20-fold and used as template for selective amplification. The PCR program consisted of 12 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s with annealing temperature decreasing 0.7°C per cycle; followed by 25 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s and finally elongation performed at 72 °C for 10 min. Selective amplified products were separated on a 6% denaturing PAGE using Sequi-Gen GT Cell system (Bio-Rad, USA) and were visualized by silver staining. The gels were dried at room temperature for 12 h and were scanned with an Imaging Densitometer Model GS-800 (Bio-Rad, USA).

Results

Different primer combinations were tested for MSAP profiling. Primer pair of HM + (0) and *Eco* RI (A) for pre-selective and 13 primer combinations for *Eco* RI (ANN) and HM + (TCAW) as selective primers showed more clear banding patterns and were used for further analysis. Of the thirteen primer combinations, five primer pairs created polymorphic bands at different temperatures while the other combinations detected no variation in the methylation sites. Totally, 500 reproducible amplification products were observed in CCGG sites with an average of 38 bands per each enzymatic combination. The highest and the lowest numbers of bands were 56 and 26 for *Eco* RI (AAG) / HM (TCAA) and *Eco* RI (CTA) / HM (TCAC) primer combinations, respectively.

In this study, differences between profiles of *EcoR* I/*Msp* I and *EcoR* I/*Hpa* II were considered as epigenetic changes. Each pair of isoschizomers was classified in three types for analysis of variation in methylation patterns (Table 2). In type I, *Hpa* II and *Msp* I recognition site were not methylated and showed the same banding patterns. In type II, internal methyation occurred in mCpG site at both strands that could be detected by *Msp* I while in Type III, methylation happened in mCmCpG or mCpG sites at one strands were detectable with *Hpa* II enzyme (Salmon et. al., 2008). In figure 1, a sample of DNA methylation pattern in primer combination of *Eco* RI AAC / HM TCAA is presented.

The number of polymorphic bands (P) produced by each primer combination were ranged from one ((*Eco* RI (AAC) / HM (TCAC)) and (*Eco* RI (AAC) / HM (TCAA))) to four (*Eco* RI (AGC) / HM (TCAA) (Table 3). Percentage of monomorphic bands per type I, II and III were 74, 19.6 and 4.4 while the percent of polymorphic bands in type I, II and III were 0, 1.2 and 0.8, respectively. Based on the data, twenty one percent of total detected methylation (24%) was identical among different treatments. Out of 130 bands, total amount of methylation status in

Table 1. The list of primers and adaptor sequences.

Sequence ID	Sequence	Sequence ID	Sequence
Eco RI linker 1	CTC GTA GAC TGC GTA CC	HM Linker 1	GATCATGAGTCCTGCT
Eco RI linker 2	AAT TGG TAC GCA GTC TAC	HM Linker 2	CGAGCAGGACTCATGA
Eco RI (A)	GAC TGC GTA CCA ATT CA	HM + (0)	ATCATGAGTCCTGCTCGG
Eco RI (N)	GAC TGC GTA CCA ATT CN	HM + (TCAA)	ATCATGAGTCCTGCTCGGAA
HM + (TCAC)	ATCATGAGTCCTGCTCGGTCAC	*Eco* RI (AGG)	GAC TGC GTA CCA ATT CAGG
Eco RI (AAC)	GAC TGC GTA CCA ATT CAAC	*Eco* RI (GGA)	GAC TGC GTA CCA ATT CGGA
Eco RI (AAG)	GAC TGC GTA CCA ATT CAAG	*Eco* RI (AAA)	GAC TGC GTA CCA ATT CAAA
Eco RI (ACA)	GAC TGC GTA CCA ATT CACA	*Eco* RI (AGT)	GAC TGC GTA CCA ATT CAGT
Eco RI (ACC)	GAC TGC GTA CCA ATT CACC	*Eco* RI (ACT)	GAC TGC GTA CCA ATT CACT
Eco RI (ACG)	GAC TGC GTA CCA ATT CACG	*Eco* RI (AGC)	GAC TGC GTA CCA ATT CAGC

Table 2. Schematic presentation of cytosine methylation patterns in MSAP analysis (Salmon et. al., 2008). The shaded boxes correspond to cytosine Methylation (mC).

Description	*Msp* I	*Hpa* II	Methylation status of CCGG site
Non methylated; Type I fragment	+	+	C C G G G G C C
CpG methylated; Type II fragment	+	-	C mC G G G G mC C
CpCpG methylated; Type III fragment	-	+	mC mC G G G G C C mC C G G G G C C
Hyper-methylated fragment	-	-	mC mC G G G G mC mC

+ : Enzyme cut - : Enzyme no cut

Table 3. Primer combinations used in methylation sensitive amplification polymorphism. M: monomorphic bands; P: polymorphic bands

Type of Bands		Type I		Type II		Type III	
Status of Bands		M	P	M	P	M	P
Primer combination	*Eco*RI (AAC) / HM (TCAA)	33	-	10	-	2	1
	*Eco*RI (AAC) / HM (TCAC)	19	-	9	1	4	-
	*Eco*RI (ACG) / HM (TCAA)	21	-	7	-	8	2
	*Eco*RI (ACT) / HM (TCAA)	32	-	11	2	1	-
	*Eco*RI (AGC) / HM (TCAA)	23	-	8	3	1	1
	*Eco*RI (AAA) / HM (TCAA)	27	-	4	-	1	-
	*Eco*RI (AAA) / HM (TCAC)	26	-	2	-	-	-
	*Eco*RI (AGT) / HM (TCAA)	32	-	4	-	-	-
	*Eco*RI (CTA) / HM (TCAC)	20	-	6	-	-	-
	*Eco*RI (AAG) / HM (TCAA)	40	-	14	-	1	-
	*Eco*RI (ACA) / HM (TCAA)	31	-	10	-	2	-
	*Eco*RI (ACC) / HM (TCAA)	27	-	7	-	2	-
	*Eco*RI (AGG) / HM (TCAA)	39	-	6	-	-	-
Total		370	-	98	6	22	4
Percent		74	-	19.6	1.2	4.4	0.8

mCpG and mCmCpG or mCpG sites, 10 bands showed polymorphic pattern among all treatments which out of them 6 and 4 bands were in mCpG and mCmCpG or mCpG positions, respectively. A sample of methylation changes in different temperature stresses has been shown in Figure 2.

Discussion

Plant response to environmental cues is partly resulted from the ability of differential regulation of gene expression and protein function. Epigenetic modifications may be one of the molecular mechanisms by which plants could regulate gene function at the genome level (Habu et al., 2010). Plants with relative high levels of 5-methylcytosine (5mC), ranging from 6 to 25% of total cytosines (Steward et al., 2002), appear to use the epigenetic mechanism to cope with environmental challenges.

Our data showed that all of the observed polymorphisms were the direct consequence of increased cytosine methylation at cytosine residues of the sequence CCGG. At low temperature condition (under both, light and darkness), the methylation patterns showed a high degree of similarity to the control. High temperature and freezing stresses caused the same methylation patterns. The data presented here demonstrate that highest methylation alternations were detectable under high temperature and freezing stresses. The total methylation status that was detected in different temperature stresses was approximately 36 %. The occurrence of methylation in the internal cytosines on both strands (CpG) and hemi-methylation on the external cytosine (CpCpG) were 20.8% and 5.2 %, respectively. Despite the different values for the methylation types, the percentages of polymorphic fragments in each type were found to be similar, ranging between 1.2% and 0.8%, respectively. In contrast, type I fragments displayed any methylation alternation compared to Type II and III fragments. It should be noted that MSAP can only reveal cytosine methylation at CG and partial CCG sites, but can not detect methylation at other sites, such as CAG, and CTG

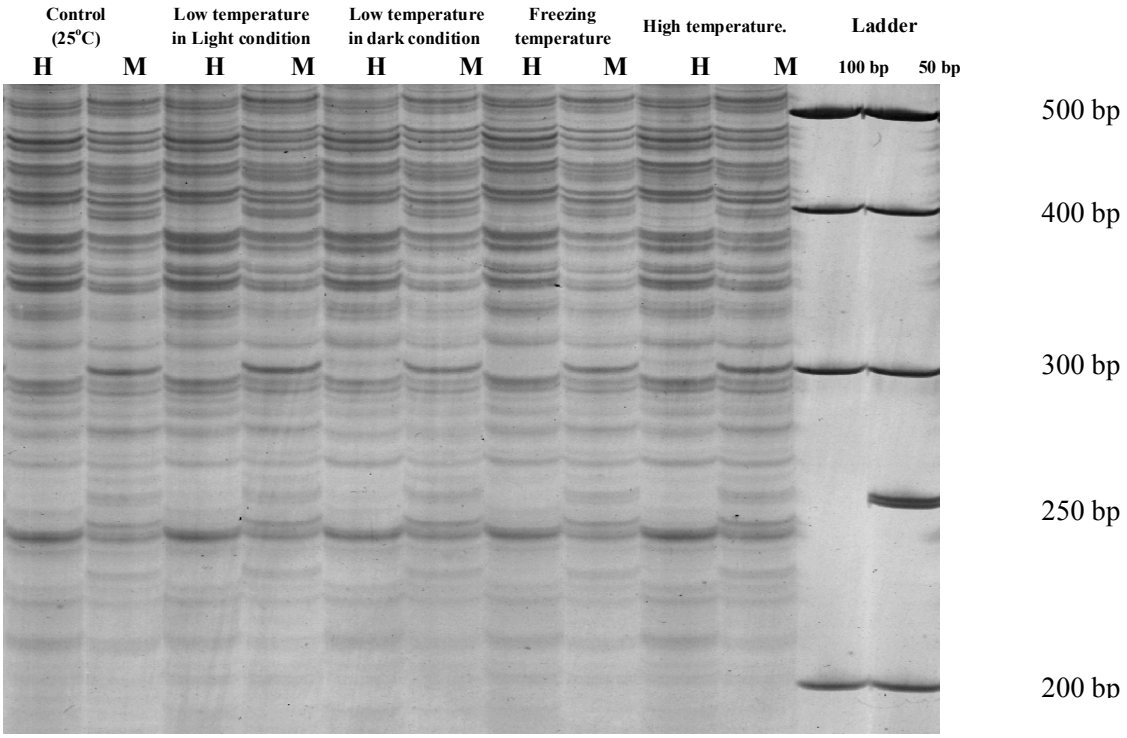

Figure 1. MSAP profile of *Aelouropus littoralis* during different temperature stresses was detected by *Eco* RI (AAC) / HM (TCAA) primer combination. Each sample digested by *Eco* RI+*Hpa* II (represent as H) and *Eco* RI+*Msp* I (represent as M).

sites, which are often methylated in plant genomes (Li et al., 2009). For this reason, analysis of global genome methylation through MSAP possibly underestimates the actual levels of methylation in the genome (Lu et al., 2008).

As reported, the levels of DNA methylation were generally decreased in the experimental conditions such as chilling, planting density, rubbing, cutting, and successive rounds of subculture (Peng, 2009). In plant, DNA cytosine methylation can respond quickly to stresses. For instance, rubbing stress can only reduce the level of methylation within 1 h (Galaud et al., 1993), whereas wounding and chilling stresses can be caused other variations in methylation at longer periods such as 12 h (Wada et al., 2004). The opposite effect was observed for salt stress, and the effects of heavy-metal stress on the methylation pattern are species specific (Peng and Zhang, 2009). Under cold stress, the modification of methylation status in corn seedlings seems to be organ- and site-specific. Methylation was decreased in roots but there was no change in shoots. On the other hand, demethylation only occurred in Ac/Ds transposons, and was not observed in the other genes.

In plants, occurrence of different types of DNA methylation is mostly depending on its own maintenance regulation pathway (Salmon et al., 2008). CG full methylated sites (as recognized by *Msp* I) were maintained during cell divisions by methyltransferase 1 (Finnegan et al., 2000), while CG hypomethylated sites (as recognized by *Hpa* II) were created by chromodomain-containing methyltransferase and domains rearranged methyltransferases (Kankel et al., 2003). It seems

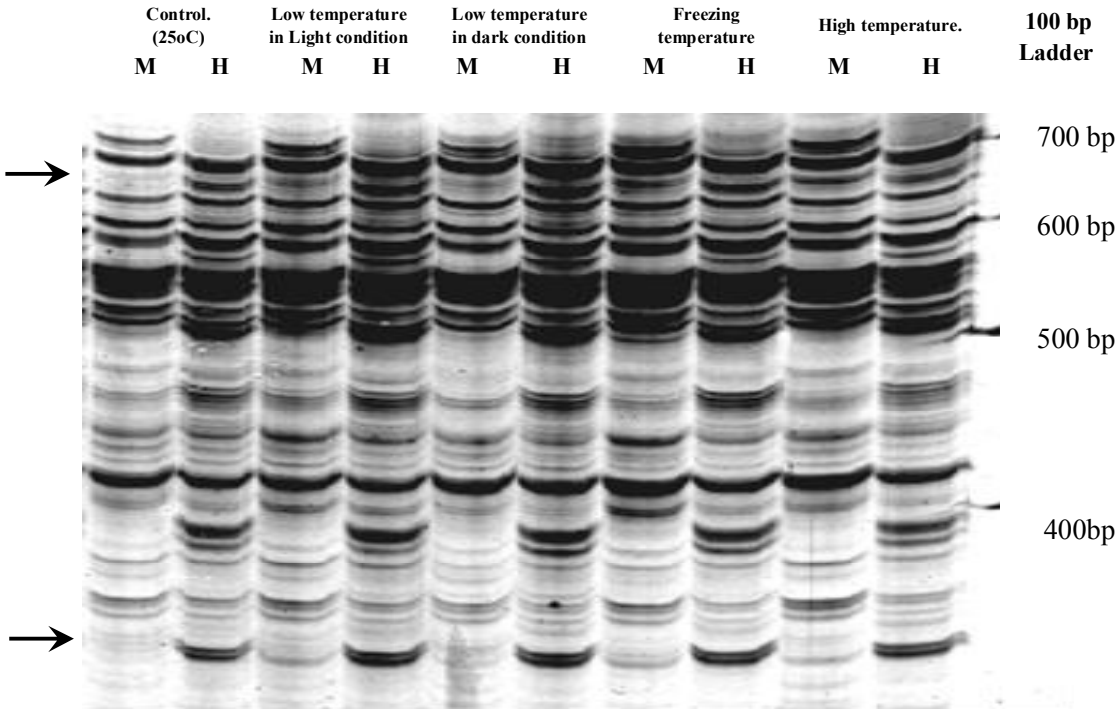

Figure 2. Methylation sensitive amplification polymorphism profile of *Aelouropus littoralis* during different temperature stresses which were detected by *Eco* RI (ACG) / HM (TCAA) primer combination. The arrows indicate methylation alternation.

that the dynamic interaction between DNA methyltransferases and demethylases determines the final genome-wide pattern of DNA methylation (Rival et al., 2008). As Rando and Verstrepen (2007) explained, DNA methylation, unlike genetic modifications, may open a rapid potential way to cope with environmental stresses on very short to long time scales. Our data showed that DNA methylation was predominant feature in response to high temperature and freezing stresses. Since different temperature stresses make different impacts on enzymes involved in methylation pattern, different scales of methylation can be expected.

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