DNA-METHYLATION PATTERNS IN COTTON (*GOSSYPIUM HIRSUTUM* L.) UNDER SALT STRESS

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(Received on Date: 16 December 2017 Date of Acceptance:20 January 2018)

ABSTRACT

Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) technique was employed to assess DNA methylation patterns induced into four cotton varieties under salt stress. Two salt-tolerant [(Niab78 (N78) and Deir-Ezzor22 (DE22)] and two salt-sensitive [Deltapine50 (DP50) and Aleppo118 (A118)] cotton varieties exposed to 0 (control) and 200 mM NaCl (stressed) for 7 weeks. Data presented herein mentioned that, salinity induced variation in DNA methylation profile among the 4 tested cotton varieties. Salt-tolerant varieties exhibited highest methylation level compared to sensitive one. In this respect, the highest methylated bands (MB) (35 and 36 bands) number, hemi-methylation ratio% (17 and 15%), full methylation ratio% (10 and 8%) and methylation ratio% (32 and 31%) were recorded into DE22 followed by N78 salt–tolerant variety for control and treated plants, respectively. However, the two salt-sensitive varieties showed the lowest values regarding the previous parameters. Data presented herein revealed that, MB and methylation ratio% could be served as useful parameters to detect DNA epigenetic changes induced by salt stress in plants breeding program. Therefore, CRED-RA technique could be considered as a potential tool to study epigenetic mechanisms for salinity tolerance screening in cotton.

Keywords: DNA-methylation, Cotton, CRED-RA technique, Salt stress.

No: of Tables: 6 **No: of Figures: 1** **No: of References: 31**

INTRODUCTION

Cotton is one of few economic important fiber crops cultivated worldwide. According to recent reports 2014/15, world cotton cultivated area recorded to be 33.83 million hectares of which Syria occupied 0.06 million hectares; with estimated cotton Syrian production of 65000 metric tons (USDA 2015). Salinity is one of the most serious and biggest problems in Mediterranean region. It has demonstrated that, approximately 50% of irrigated soil in Syria suffers from salinity which caused the departure of vast areas of agricultural lands from the scope of investment (Saleh 2012a).Overall, when plants grow in unfavorable environmental conditions *e.g*. salt stress, they develop different mechanisms to escape their exposure to this constraint. In this respect, they tend to minimize their life cycle or to induce DNA methylation process in order to avoid the stress harmful effects (Dyachenko *et al*., 2006; Yaish 2013). DNA methylation as an epigenetic DNA changes has been discovered too earlier for the first time by Avery *et al*. (1944), and could play an essential role in genes expression regulation.DNA methylation has a potential value in plant breeding program. This importance could be manifested by: agricultural traits improving *e.g*. flowering time, plant height (Fieldes *et al*., 2005), creating of new materials for plants breeding *e.g*. in *Brassica oleracea* (Salmon *et al*., 2008) and in cotton (*Gosspium hirsutum* L.) (Keyte *et al*., 2006). The DNA methylation advantages I) could be inherited for several generations (Kakutani 2002) even for hundreds years; II) It is reversible phenomena *e.g*. DNA methylation variation in white clover and hemp plants are similar before and after metal stress (Aina *et al*., 2004). Yaish *et al*. (2013) reported that abiotic stress caused alteration in few genes expression *e.g*. induction of some modified histones as affected by DNA methylation, leading to stress tolerance enhancement. Labra *et al*. (2002) and Guangyuan *et al*. (2007) reported that 10-1000 mM NaCl may cause not alteration in DNA methylation, but also DNA mutation. Similar findings have also been demonstrated in rape (*Brassica napus* L.) exposed to potassium dichromate (Labra *et al*., 2004).

Previously, Tsaftaris *et al*. (2000), investigated expression of 35 genes between hybrids and their respective parents using HPLC chromatography and CRED-RA techniques. The previous study mentioned DNA methylation differences among the different genotypes may be linked to hybrid vigor. Zhong and Wang (2007) reported that CRED-RA technique could be successfully used for salinity tolerance screening in wheat (*Triticum aestivum* L.), where wheat salt-sensitive variety had a lowest DNA methylation level than the salt-tolerant one.Zhong *et al*. (2009) applied methylation-sensitive amplified polymorphism (MSAP) technique to investigate DNA methylation induced by NaCl in two wheat (*T. aestivum* L.) cultivars differed in their salinity tolerance. The previous study revealed DNA methylation changes in stressed plants compared to their reference control. Where, CCGG

sequences was more methylated in control salt tolerant cultivar compared to other tested sensitive one. It has been indicated also that, these alterations could be served as a useful mechanism in plant salt tolerance. Whereas, Peng and Zhang (2009) reported different DNA methylation patterns induced by different abiotic stresses (chilling, planting density, salt stress and heavy metals….). The previous investigation suggested that plants adaptation via abiotic stress; and this phenomena was heritable that arise in response to stress. This phenomenon plays an important role for salt tolerance improving in plant breeding programs. Indeed, Demirkiran *et al*. (2013) applied the same technique to study barley (*Hordeum vulgare* L. cv. Tokak) genetic variation produced *in vitro* culture via salt stress (0, 50 and 100 mM NaCl) for 20 days. The previous study showed that, salinity induced some genetic variations in root DNA but not in shoot DNA. While, the highest NaCl concentration lead to DNA methylation variation especially in shoot. Moreover, Marconi *et al*. (2013) used MSAP tool to investigate the impact of salt stress on DNA methylation changes in two *B. napus* var. *oleifera* cultivars differ in their salinity tolerance. The previous study revealed that, DNA methylation increased in sensitive-salt cultivar, while it decreases in salt-tolerant one under 100 mmol/l NaCl. Otherwise, 19 amplicons were polymorphic linked to DNA alterations. Recently, Wang *et al*. (2015) applied Methyl-Sensitive Amplification Polymorphism (MSAP) technique to investigate DNA methylation patterns among 3 cotton accessions differed in their salinity tolerance. Indeed, Lu *et al*. (2015) used MSAP technique to detect DNA methylation profiles between 2 cotton accessions differed in their salinity tolerance.

It has been mentioned that CRED-RA technique has been also employed for other purposes *e.g.* Temel *et al*. (2008), used this technique to investigate epigenetic variability in callus culture of barley (*H. vulgare* cv. Zafer-160). The previous study revealed that, 16 RAPD primers out of 20 RAPD tested primers yielded 103 amplicons in mature embryos of barley after 24 weeks. Whereas, Sokolova *et al*. (2014), investigated the impact of UV-C on DNA methylation in corne seeds on their germination rate and seedling resistance to UV-C stress. This study showed that DNA methylation induced by UV-C has different effects on previous tested parameters. More recently, Sun *et al*. (2015) investigated DNA methylation profiles in maize hybrids and their parents. The previous study showed that maize parents exhibited highest total relative methylation levels and higher number of demethylation events compared to their respective hybrids. As yet, few recent researches focused on DNA methylation patterns investigation towards salt stress in cotton using MSAP technique (Wang *et al*., 2015; Lu *et al*., 2015). To our knowledge of the salinity tolerance in cotton cultivated in Syria has been focused on physiological, biochemical and molecular studies (Saleh 2012a; 2016). However, epigenetic effects on cotton salinity tolerance based on

CRED-RA technique have not yet been examined in detail. Thereby, CRED-RA technique has been applied with the hope for screening of different DNA bands which differently methylated among 4 cotton varieties differ in their salt tolerance degree. So the current study focused on DNA methylation patterns evaluation within the same enzyme for each examined variety separately on one hand. On the other hand, to give overview image about DNA methylation profiles among different examined cotton varieties. Genotyping variation among these varieties in their salinity tolerance level was previously assessed at physiological, biochemical and genetic levels.

MATERIALS AND METHODS

Plant materials and growth conditions

Four cultivated cotton (*G. hirsutum* L.) varieties have been previously selected on the basis of their wide range of tolerance towards salinity [(Niab78 (N78) and Deir-Ezzor22 (DE22) as salt-tolerant (Saleh 2012a); whereas; Deltapine50 (DP50) and Aleppo118 (A118) as introduced salt-sensitive varieties]. Seeds of upland cotton (*G. hirsutum* L.) were provided by the General Commission for Scientific Agricultural Research of Syria (GCSAR). Growth conditions and experiment design were carried out in a greenhouse for 7 weeks (five replicates/treatment) as previously described by Saleh (2012a).

Genomic DNA extraction

Plant genomic DNA was extracted from cotton young leaves including the control and stressed plants (200 mM NaCl) using CTAB (cetyltrimethylammonium bromide) protocol as previously described by Doyle and Doyle (1987).

Leaves tissue (150 mg) were ground in liquid nitrogen, the powder was transferred to a 2 ml Eppendorf tube, mixed with 0.9 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.0018 ml β-mercaptoethanol, 2% CTAB), and incubated at 65°C for 20 min. DNA was extracted with one volume of a chloroform:isoamyl alcohol mix (24:1, v/v) and centrifuged at 12,000 g for 10 min at 4°C. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with an equal volume of cold isopropanol and kept at -20°C for 10 min. Then centrifuged at 12,000 g for 10 min at 4°C, the supernatant was discarded; DNA was then spooled out and washed with 1 M ammonium acetate and 100% ethanol. The cleaned DNA pellet was air dried and dissolved in 0.1 ml of 0.1x TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). After addition of 0.005 ml of RNase (10 mg/ml), and incubation for 30 min at 37°C. DNA concentration was quantified by DNA Fluorimeter at 260/280 nm and adjusted to final concentration of 10 pmol. DNA was stored at -80°C until needed.

CRED-RA assay

CRED-RA technique based on potential of *Hpa*II and *Msp*I restriction enzymes to recognize the same cut sequence, but with different restriction efficiency based

on the methylation status of the cytosine residues. *Msp*I and *Hpa*II enzymes are isoschizomers which differ in their CpG sensitivity. Where, *Hpa*II cannot cut, if the inner cytosine is methylated while *Msp*I can. Thereby, *Hpa*II was methylation sensitive, while, *Msp*I was methylation insensitive one.One µg of genomic DNA for each variety including control and stressed plants was separately digested with *Hpa*II (ER0511, Fermentas) and *Msp*I (ER0541, Fermentas) restriction enzymes in final volume of 0.02 ml according to manufacturers' recommendations. The digested DNA was considered as DNA template for PCR amplification with RAPD primers. Where, 20 RAPD (17 primers belonging to Operon Technologies Inc., USA, and 3 primers from the University of British Columbia) (Table 1) were tested for detection of DNA methylation patterns induced into four cotton varieties under salt stress. CRED-RA performed as the same for RAPD marker as previously described by Williams *et al*. (1990). The PCR products were separated on a 1.8% ethidium bromide-stained agarose gel (Bio-Rad) in 0.5xTBE buffer. Electrophoresis was performed for 2-h at 85 V and visualized with a UV transilluminator. Band sizes were determined by comparison with a VC 100bp plus DNA Ladder (Vivantis).

DNA methylation patterns analysis

As previously reported, Hpa*II* and Msp*I* isoschizomers could recognize CCGG sites with differences DNA methylation sensitivity (Yang *et al*., 2011). Thereby, different bands patterns could be detected by CRED-RA technique using digested DNA by above enzyme, as template for PCR amplification.

In this respect, three bands types could be revealed using this technique: Type I: when *Hpa*II and Msp*I* are active in methylation of both strands (band presented in both samples); Type II: when *Hpa*II is sensitive and *Msp*I is inactive in methylation of both strands (band presented in *Hpa*II and absent in *Msp*I in both samples). While the type III: when *Msp*I is active and *Hpa*II is not sensitive to cytosine methylation (band presented in *Msp*I and absent in *Hpa*II in both samples) (Yang *et al*., 2011). Consequently, the proved pattern could be gave an image of DNA methylation level. NaCl stress induced polymorphism pattern included the appearance and disappearance of some types of bands, not only with each enzyme digestion within the same individual; but also between stressed plants compared to their respective control for the same individual.

RESULTS AND DISCUSSION

DNA change patterns induced by NaCl stress, within the same enzyme into the four studied cotton varieties were presented in Tables 2-5. Firstly, DNA epigenetic changes induced by NaCl treatment within each variety have been screened separately (Tables 2-5). That could be allowed somewhat to understand salinity tolerance differences among the 4 tested cotton varieties. Data obtained herein showed DNA methylation alteration induced by NaCl stress compared to their respective

control for each variety separately. One RAPD primer (OPV03) of examined 20 RAPD primers gave (6 bands in total) no polymorphic bands in the four studies cotton varieties. Thereby, 19 RAPD primers were used for screening DNA-methylation patterns under salt stress of NaCl.

DNA epigenetic changes induced by NaCl treatment between *Hpa*II and *Msp*I under control and stress conditions, among the four studied cotton varieties have been investigated (Table 6). DNA methylation change patterns yielded by CRED-RA with UBC159 primer was illustrated in Figure 1.

Genetic modification as a critical indicator could be manifested by DNA methylation. Since, under normal conditions, 20-30% of cytosine may be methylated in the nuclear genome (Lu *et al*., 2015). However, plant exposure to unfavorable conditions such as salinity stress caused increasing in DNA methylation degree (Wang *et al*., 2015).

DNA methylated bands under control and stressed conditions in 4 cotton varieties were evaluated between control and treated plants within the same examined enzyme for each variety and also between *Hpa*II compared to *Msp*I profiles for the same individual. Total bands (TB) yielded by CRED-RA technique were estimated for each variety. In this regards, estimated TB number was in the following order of A118 > DP50 > DE22 > N78. Where, this value was recorded to be 134 and 139 TB for A118 and found to be 99 and 97 for N78 with *Hpa*II and *Msp*I, respectively.

Overall, salt tolerant varieties (N78 and DE22) exhibited highest TB with *Hpa*II compared to *Msp*I (Tables 2-3). Contrary trends was observed in salt-sensitive (DP50 and A118) varieties (Tables 4-5).

As for the polymorphic bands (PB) generated by CRED-RA technique, between treated plants and their respective control, they were also screened. For N78 var. CRED-RA technique produced 70 and 72 PB with *Hpa*II compared to *Msp*I, respectively in treated plants compared to their respective control. While, in A118 var. these values were recorded to be 20 and 19 PB with *Hpa*II compared to *Msp*I, respectively (Tables 2, 5).It worth noting that, the salt-tolerant varieties (N78 and DE22) exhibited highest PB ratio than the salt-sensitive one. In this regards, the polymorphism level (P%) varied according to the tested variety and salt stress conditions. Where, the highest P% was recorded for N78 (70.7 and 74.2%), followed by DE22 (53.2 and 63.6%) with *Hpa*II and *Msp*I, respectively. While, the lowest P% value was recorded for DP50 salt-sensitive var. (10.3 and 13.2%) followed by A118 (14.9 and 13.7%) with *Hpa*II and *Msp*I, respectively. Overall, in salt-tolerant var. *Msp*I treated plants exhibited highest PB compared to *Hpa*II. Indeed, treated plants exhibited highest PB compared to their respective control with both *Hpa*II and *Msp*I enzymes.From data presented in Tables 2-4, it worth noting that, examined OPB12 primer gave the highest polymorphic characteristic bands compared to the other tested primers into three cotton varieties (N78, DE22 and A118) with 17, 16 and 8 PB, respectively.Otherwise, Polymorphism patterns of CRED-RA products after PCR

amplification between *Hpa*II and *Msp*I were presented in different manners according to the RAPD tested primers. All over, in sensitive-salt varieties, *Msp*I and *Hpa*II digestions amplified patterns were mainly monomorphic in most case.

Comparative DNA methylation patterns induced by NaCl treatment among the 4 studied cotton varieties have been carried out. CRED-RA technique revealed different patterns DNA methylation in both salt-tolerant and salt-sensitive cotton varieties. However, methylated bands number was less in salt-sensitive than tolerant once (Table 6). Where, salt-tolerant varieties showed higher polymorphic DNA patterns when the same sample cut with the two examined enzymes *Hpa*II and *Msp*I in the contrary, to salt-sensitive varieties. Thereby, salt-tolerant varieties exhibited highest methylated sits compared to the sensitive once (Table 6). In this respect, the highest methylated bands (MB) were recorded to be 35 and 36 MB in DE22 followed by N78 with 15 and 19 MB for control and treated plants, respectively. While, the lowest MB were presented in salt-sensitive varieties of DP50 and A118 control and treated plants.

Otherwise, methylation ratio% was also higher in salt-tolerant varieties for both control and treated plants than the sensitive once. In this respect, DE22 exhibited the highest methylation ratio% with 31.8 and 31.4%; followed by N78 with 14.9 and 17.6% for control and treated plants, respectively. Overall, DE22 followed by N78 var. exhibited highest total MB, hemi-methylation ratio% and full methylation ratio% than the other tested sensitive one (Table 6). Our data were in accordance with recent findings of Wang *et al*. (2015) and Lu *et al*. (2015) in salt tolerance screening in cotton using MSAP technique. Wang *et al*. (2015) reported that methylated loci were recorded to be 64.3% and 86.2% for CCRI35 salt-tolerant for control and their respective cotton stressed plants, respectively. Whereas, it was 71.4% and 84.8% for Zhong07 salt-tolerant; and 75.8% and 68.1% for CCRI12 salt-sensitive cotton accession for control and their respective cotton stressed plants, respectively. Whereas, Lu *et al*. (2015) used also MSAP technique to detect DNA methylation leaves patterns induced by NaCl into two cotton varieties differ in their salt stress tolerance. The previous investigation revealed that Zhong07, a salt-tolerant cotton variety exhibited more demethylation than methylation loci using MSAP technique. Whereas, ZhongS9612, a salt-sensitive cotton variety showed different tendency. This epigenetic variation could be related to varietal differences. The previous study showed that total bands amplified were recorded to be 341 and 307, total bands methylated 85 and 80, ratio of methylated bands 24.93 and 26.06% and full methylated ratio 20.82 and 20.85% for Zhong07 and ZhongS961, respectively.

Thereby, the highest MB combined with highest methylation ratio% recorded into DE22 and N78 varieties could be explain genotyping variation in their salinity tolerance level compared to the other tested varieties. This finding makes it possible that MB, and methylation ratio%

could be served and considered as an epigenetic marker for plants salinity tolerance screening in plants breeding program. Similar findings were reported in salt-tolerant wheat (*T. aestivum* L.) (Zhong and Wang 2007). Previously, Dyachenko *et al*. (2006) reported that, plant DNA methylation could be considered as one of fewer mechanisms involved in plants salt tolerance.Other recent investigation however showed that, the lowest estimated genomic template stability (GTS%) values recorded for the two salt-tolerant varieties (N78 and DE22) [Where, GTS% was recorded to be 36.7, 26.4, 79.1 and 58.2% for N78, DE22, DP50 and A118 cotton varieties respectively exposed to salt stress (0 and 200 mM NaCl for 7 weeks) using the RAPD marker (Saleh 2016)] could explain their salinity tolerance compared to the other tested varieties.In Saleh (2012b) investigation, AFLP analysis in cotton revealed a level of polymorphism (P%) of 50.569% using twenty-one AFLP PCs primer combinations. Due to the narrow genetic base of cotton germplasm that cotton breeders have been utilizing and low efficiency of traditional selection methods, cultivar improvement in cotton has slowed down in the past 10–15 years (Saleh 2012b).

In the current study, it worth noting that, increased P% within the same variety under salt stress (NaCl) application between control and treated plants in salt tolerant varieties. This finding could be reflected in the lowest estimated GTS% value recorded for N78 and DE22 varieties (Saleh 2016) compared to salt-sensitive once. However, the lowest estimated GTS% value recorded for N78 and DE22 varieties could be related to genetic variability getting new protein induction in relation to salinity tolerance. Similarly, Yaish (2013) reported that, DNA methylation under salt stress conditions lead to individual gene expression diversity of the same plant species. Otherwise, salt-tolerant varieties revealed increased in methylated sits in treated plants compare to their respective control. This could be explain by the fact that, polymorphism in nucleotides occurrence, lead to best recognition sits in treated plants than their respective control. This observation was in accordance of Demirkiran *et al*. (2013) in barley.

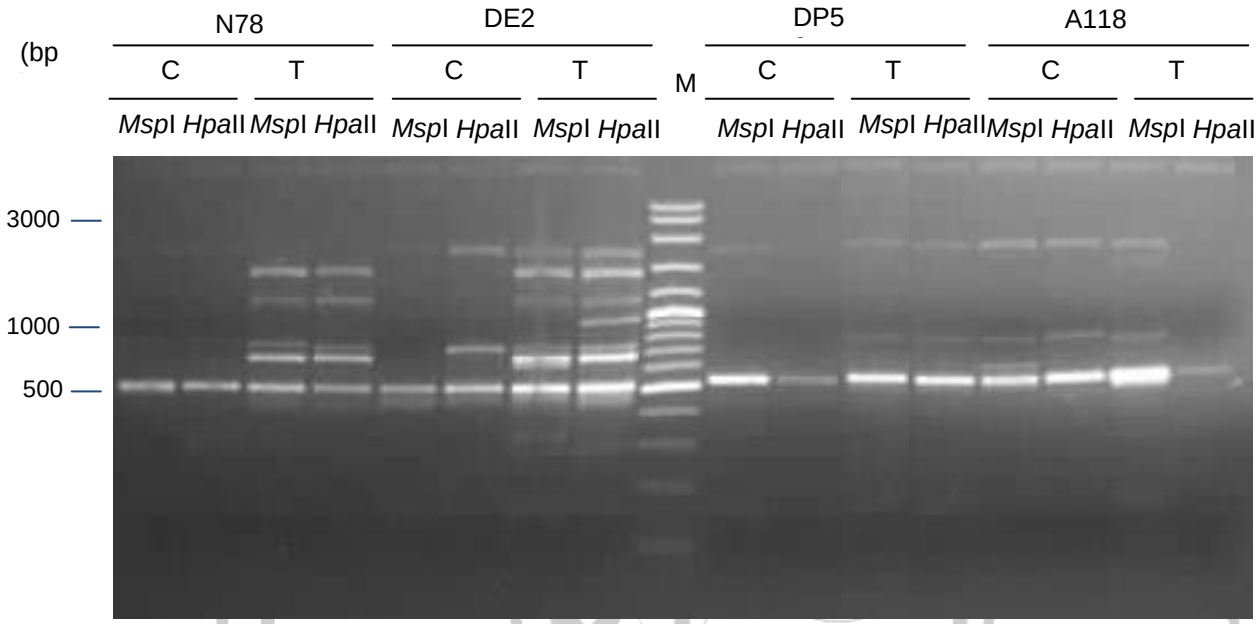

Figure 1. DNA methylation alteration patterns produced by CRED-RA with UBC159 primer in the four cotton tested varieties under stressed conditions (200 mM NaCl) compare to their respective control. M: a VC 100bp plus DNA Ladder (Vivantis).

Table 1. Tested RAPD primers used in the current study in terms of primer sequence and G+C content (%).

Primer N°	Primer name	Primer sequence 5'-3'	G+C content (%)
1	OPA02	TGCCGAGCTG	70
2	OPB12	CCTTGACGCA	60
3	OPB17	AGGGAACGAG	60
4	OPC13	AAGCCTCGTC	60
5	OPD20	GGTCTACACC	60
6	OPE07	AGATGCAGCC	60
7	OPE15	ACGCACAACC	60
8	OPG11	TGCCCGTCGT	70
9	OPJ01	CCCGGCATAA	60
10	OPJ07	CCTCTCGACA	60
11	OPK08	GAACACTGGG	60
12	OPK12	TGGCCCTCAC	70
13	OPQ18	AGGCTGGGTG	70
14	OPR12	ACAGGTGCGT	60
15	OPT18	GATGCCAGAC	60
16	OPV03	CTCCCTGCAA	60
17	OPY10	CAAACGTGGG	60
18	UBC132	AGGGATCTCC	60
19	UBC159	GAGCCCGTAG	70
20	UBC702	GGGAGAAGGG	70

Table 2. Total and polymorphic bands within the same enzyme into N78 variety.

Primer	Event	C	T	C	T
			*Hpa*II		*Msp*I
OPA02	-	11	5 (650, 900, 1100, 1550, 1850)	11	7 (400, 650, 900, 1100, 1350, 1850 & 2400)
	+		3 (600 & 1500)		2 (600 & 1500)
OPB12	-	5	5 (350, 650, 750, 900 & 1000)	5	3 (650, 750 & 1000)
	+		5 (300, 400, 450, 600 & 800)		4 (400, 450, 600 & 850)
OPB17	-	10	3 (300, 350 & 500)	11	4 (150, 300, 350 & 500)
	+		2 (650 & 1200)		3 (650, 1200 & 1500)
OPC13	-	5	1 (550)	4	ND
	+		3 (600, 800 & 900)		3 (600, 800 & 900)
OPD20	-	13	7 (350, 380, 400, 500, 700, 1500 & 2500)	11	5 (400, 500, 700, 1500 & 2500)
	+		1 (550)		2 (350, 550)
OPE07	-	6	1 (250)	6	1 (250)
	+		2 (400 & 800)		2 (400 & 800)
OPE15	-	3	ND	5	2 (300 & 550)
	+		3 (350, 800 & 1000)		3 (350, 800 & 1000)
OPG11	-	4	ND	4	ND
	+		2 (400 & 700)		2 (400 & 700)
OPJ01	-	3	ND	3	ND
	+		2 (800 & 2000)		3 (600, 800 & 2000)
OPJ07	-	2	ND	2	ND
	+		1 (2000)		ND
OPK08	-	5	ND	4	ND
	+		1 (700)		5 (450, 600, 700, 800 & 1500)
OPK12	-	5	ND	3	ND
	+		1 (500)		4 (350, 550, 600 & 700)
OPQ18	-	7	2 (300 & 400)	7	2 (300 & 450)
	+		2 (350 & 550)		2 (350 & 550)
OPR12	-	2	ND	2	ND
	+		3 (550, 700 & 800)		3 (550, 700 & 800)
OPT18	-	4	ND	4	ND
	+		ND		1 (400)
OPY10	-	4	2 (500 & 550)	5	1 (300)
	+		6 (400, 450, 650, 700, 800 & 900)		5 (400, 650, 700, 800 & 900)
UBC132	-	5	2 (200 & 600)	5	2 (200 & 600)
	+		1 (650)		1 (650)
UBC159	-	2	ND	2	ND
	+		3 (700, 800 & 1200)		3 (700, 800 & 1200)
UBC702	-	3	ND	3	1 (800)
	+		6 (350, 500, 600, 800, 900 & 1200)		3 (350, 500 & 600)
Total	-	99	25	97	24
	+		45		48

ND: no differences

Table 3. Total and polymorphic bands within the same enzyme into DE22 variety.

Primer	Event	C	T	C	T
			*Hpa*II		*Msp*I
OPA02	-	15	4 (450, 550, 650 & 850)	15	4 (480, 650, 1000 & 1500)
	+		1 (600)		1 (600)
OPB12	-	7	4 (200, 450, 700 & 900)	5	4 (600, 700, 900 & 1000)
	+		3 (350, 400 & 550)		5 (350, 400, 450, 550 & 800)
OPB17	-	10	ND	8	2 (200 & 280)
	+		4 (450, 480, 900 & 1100)		3 (600, 900 & 1100)
OPC13	-	4	ND	4	ND
	+		2 (600 & 800)		2 (600 & 800)
OPD20	-	12	3 (900, 1500 & 2500)	13	5 (380, 400, 650, 1500 & 2500)
	+		1 (550)		1 (550)
OPE07	-	10	ND	10	1 (350)
	+		ND		ND
OPE15	-	10	3 (750, 1500 & 2000)	9	4 (600, 1500 & 2000)
	+		3 (300, 400 & 500)		3 (350 & 400)
OPG11	-	4	ND	4	ND
	+		2 (400 & 600)		2 (400 & 600)
OPJ01	-	3	ND	2	1 (450)
	+		3 (600, 700 & 2000)		3 (550, 650 & 2000)
OPJ07	-	3	1 (2000)	2	ND
	+		ND		1 (2000)
OPK08	-	4	1 (250)	6	2 (250 & 300)
	+		6 (450, 500, 550, 600, 700 & 800)		3 (450, 650 & 750)
OPK12	-	8	1 (1200)	6	1 (400)
	+		ND		2 (500 &800)
OPQ18	-	8	2 (900 & 1350)	9	1 (400)
	+		2 (400 & 500)		1 (850)
OPR12	-	7	3 (250, 350 & 600)	5	2 (2200 & 3000)
	+		1 (550)		2 (550 & 900)
OPT18	-	4	ND	4	ND
	+		1 (1850)		1 (1850)
OPY10	-	6	1 (250)	5	1 (500)
	+		4 (400, 750, 800 & 900)		7 (300, 350, 400, 650, 750, 800 & 900)
UBC132	-	5	2 (200 &600)	5	2 (200 &600)
	+		1 (1500)		1 (1500)
UBC159	-	3	ND	2	ND
	+		5 (700, 850, 950, 1100 & 1200)		4 (700, 800, 1200 & 1500)
UBC702	-	3	ND	4	ND
	+		3 (350, 500 & 600)		4 (350, 600, 900 & 1100)
Total	-	126	25	118	30
	+		42		45

ND: no differences

Table 4. Total and polymorphic bands within the same enzyme into DP50 variety.

Primer	Event	C	T	C	T
			*Hpa*II		*Msp*I
OPA02	-	13	ND	13	ND
	+		ND		ND
OPB12	-	6	ND	5	1 (350)
	+		2 (400 & 450)		2 (400 & 450)
OPB17	-	12	2 (350 & 400)	13	3 (380, 400 & 800)
	+		1 (1200)		ND
OPC13	-	7	ND	7	ND
	+		ND		ND
OPD20	-	9	ND	9	ND
	+		ND		ND
OPE07	-	11	ND	11	ND
	+		ND		ND
OPE15	-	4	2 (250 & 500)	4	1 (250)
	+		1 (400)		2 (350 & 500)
OPG11	-	4	ND	4	ND
	+		ND		ND
OPJ01	-	4	1 (600)	4	1 (600)
	+		ND		ND
OPJ07	-	2	ND	3	ND
	+		ND		ND
OPK08	-	5	2 (450 & 550)	6	3 (350, 500 & 550)
	+		ND		ND
OPK12	-	9	ND	9	ND
	+		ND		ND
OPQ18	-	15	ND	15	ND
	+		ND		ND
OPR12	-	7	ND	7	ND
	+		ND		ND
OPT18	-	4	ND	4	ND
	+		2 (800 & 1350)		2 (800 & 1350)
OPY10	-	5	1 (480)	6	2 (300 & 650)
	+		ND		ND
UBC132	-	4	ND	4	ND
	+		ND		ND
UBC159	-	2	ND	2	ND
	+		1 (700)		1 (700)
UBC702	-	3	ND	3	ND
	+		ND		ND
Total	-	126	7	129	11
	+		6		6

ND: no differences

Table 5. Total and polymorphic bands within the same enzyme into A118 variety.

Primer	Event	C	T	C	T
		*Hpa*II		*Msp*I	
OPA02	-	13	ND	13	ND
	+		ND		ND
OPB12	-	6	1 (350)	6	1 (350)
	+		4 (300, 500, 750 & 580)		3 (500, 580 & 750)
OPB17	-	13	1 (1200)	11	1 (1200)
	+		2 (250 & 280)		2 (250 & 280)
OPC13	-	7	ND	7	ND
	+		1 (550)		ND
OPD20	-	11	ND	11	ND
	+		ND		ND
OPE07	-	11	ND	11	ND
	+		ND		ND
OPE15	-	6	ND	7	ND
	+		1 (350)		1 (350)
OPG11	-	5	1 (800)	5	1 (800)
	+		ND		ND
OPJ01	-	3	ND	3	ND
	+		1 (600)		1 (600)
OPJ07	-	2	ND	2	ND
	+		ND		ND
OPK08	-	4	ND	6	ND
	+		2 (700 & 800)		2 (700 & 800)
OPK12	-	9	ND	9	ND
	+		ND		ND
OPQ18	-	15	ND	15	ND
	+		ND		ND
OPR12	-	9	ND	9	ND
	+		2 (550 & 800)		2 (550 & 800)
OPT18	-	5	ND	6	ND
	+		ND		ND
OPY10	-	4	ND	5	1 (280)
	+		2 (480 & 600)		2 (450 & 600)
UBC132	-	4	ND	4	ND
	+		ND		ND
UBC159	-	3	1 (700)	4	1 (550)
	+		ND		ND
UBC702	-	4	ND	5	ND
	+		1 (350)		1 (350)
Total	-	134	4	139	5
	+		16		14

ND: no differences

Table 6. DNA methylation changes in the 4 tested cotton varieties under control and stressed conditions.

Type	N78		DE22		DP50		A118	
	C	T	C	T	C	T	C	T
I	86	112	98	118	130	124	132	154
II	4	13	22	23	3	3	8	5
III	11	6	13	13	2	9	1	5
Total amplified bands	101	131	133	154	135	136	141	164
Total methylated bands[1]	15	19	35	36	5	12	9	10
Hemi-methylation ratio[2] (%)	3.960	9.924	16.541	14.935	2.222	2.206	5.674	3.049
Full methylation ratio[3] (%)	10.891	4.580	9.774	8.442	1.481	6.618	0.709	3.049
Methylation ratio[4] (%)	14.891	17.580	31.774	31.442	4.481	9.618	8.709	8.049

Type I: band presented in both samples; Type II: band presented in *Hpa*II and absent in *Msp*I in both samples; Type III: band presented in *Msp*I and absent in *Hpa*II in both samples.
[1]Total methylated bands = II+III; [2]Hemi-methylation ratio = II/I+II+III; [3]Full methylation ratio = III/I +II+III; [4]Methylation ratio = II+III/I+II+III.

Plants developed different protective mechanisms against salt stress. Thereby, DNA methylation could be considered as one of fewer epigenetic mechanisms regulating gene expression in salt stress adaptation. In this regards, Zhong and Wang (2007) reported that methylation level was related to observed difference in wheat salt tolerance. Where, the wheat salt-tolerant exhibited higher DNA methylation level compared to salt-sensitive one after 10 days of salt stress application. Otherwise, Zemach *et al*. (2010) reported that DNA methylation is usually caused inactivated gene expression and vice versa. Indeed, different genes involved in abtiotic stress are methylated in different manners that could suggest that methylation process could play an important role in plants salt stress adaptation.

Whereas, Karan *et al*. (2012) investigated DNA methylation pattern induced by 150 mM NaCl in four rice genotypes differ in their salinity tolerance level. The previous study mentioned that no methylation pattern was specific for salt or sensitive genotypes. While, significant correlation was observed between salt treatment and methylation level in shoots into the four tested genotypes. This finding was also observed in root of only salt-sensitive one. Indeed, Karan *et al*. (2012) reported that the association between methylation pattern variation and salt tolerance has been observed only in some cases suggesting that many methylation modifications seem to be not ''directed''.

Overall, DNA methylation could be appeared as one of fewer mechanisms adopted by plants for enhancing their salinity tolerance. Thus, the observed differences in cotton salt tolerance among the four examined cotton varieties could be attributed to differences methylation levels. These findings were coherent with

previous reports (Zhong and Wang 2007; Peng and Zhang 2009).

CONCLUSION

DNA-methylation pattern was detected in stressed cotton plants and their respective control into 4 cotton varieties subjected to 0 and 200 mM NaCl for 7 weeks using CRED-RA technique. Salt stress application in cotton caused epigenetic variation. In this regards, N78 and DE22 (salt-tolerant varieties) exhibited highest DNA methylation levels compared to sensitive once (DP50 and A118 varieties). Data observed in the current investigation supported previous physiological, biochemical and molecular studies carried out in the same cotton varieties; especially, the lowest estimated GTS% values recorded for the two salt-tolerant varieties (N78 and DE22) compared to the other sensitive once. This observed variation could be exploiting in plant breeding program for improving salinity tolerance in cotton germplasm.

ACKNOWLEDGEMENTS

I thank Professor Ibrahim Othman (director general of AECS) and Professor Nizar MirAli (head of Molecular Biology and Biotechnology Department in AECS) for their support, and also the Plant Biotechnology Group for technical assistance.

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