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New insight into evaluation of DNA methylation levels with CRED-RA technique in the genome of *Lycopersicum esculentum* subjected to NaCl and PEG

Ersin ALTUNKAYNAK [1], İlker BÜYÜK [1], Semra SOYDAM-AYDIN [2], E. Sümer ARAS [*1]

[1] Faculty of Science, Department of Biology, Ankara University, Ankara, 06100, Turkey
[2] Medical Biological Products Laboratories, Department of Medicine, Biological and Medical Products Laboratory, Turkish Medicines and Medical Device Agency, Ministry of Health of Turkey, Ankara, 06100, Turkey

Abstract

Levels of methylcytosine in the genome can be detected with multiple approaches from several standpoints, as there are many techniques, such as coupled restriction enzyme digestion-random amplification (CRED-RA), available for determining methylation rates in the genome. CRED-RA, which is a powerful technique for studying the genome methylation status, uses methylation-sensitive enzymes, such as HpaII and MspI. In the present study, we used the CRED-RA technique to determine DNA methylation changes in *Lycopersicum esculentum L.* subjected to 100 mM NaCl and Polyethylene glycol (PEG) for different time intervals (0 h, 6 h, 9 h, 12 h, and 24 h). DNA band variations due to both stressors were revealed by Random Amplified Polymorphic DNA (RAPD) analysis. The DNA band changes obtained by the RAPD analysis at 12 h response to both stressors (PEG and NaCl) indicated that this was the critical point for demethylation/hypomethylation activity. According to the results of the CRED-RA analysis, the PEG treatment led to greater variation in DNA methylation than NaCl in the tomato genome. For both stressors, the maximum decrease in total methylation levels occurred at 9 h compared to the control plants, indicating that this is the critical point for demethylation/hypomethylation activity. In conclusion, the present study showed that DNA methylation changes can be easily observed with a straightforward technique, CRED-RA, in plants subjected to abiotic stress conditions, such as drought and salinity.

Key words: Methylation, CRED-RA, salinity, tomato, drought

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Tuz ve kuraklığa maruz kalmış domates genomunda (*Lycopersicum esculentum* L.) DNA metilasyon seviyesinin CRED-RA tekniği ile araştırılması

Özet

Genomdaki metilsitozin seviyesi veya metilasyon oranı enzimle kesim-rastgele amplifikasyon (CRED-RA) tekniğininde içerisinde yer aldığı bir çok yöntem ile belirlenebilir. CRED-RA HpaII ve MspI gibi metilasyon duyarlı enzimlerin kullanıldığı önemli bir tekniktir. Bu çalışma kapsamında CRED-RA tekniği farklı zaman aralıklarında (0, 6, 9, 12 ve 24 saat) 100 mM NaCl ve Plietilen glikol (PEG) uygulanmış domates (*Lycopersicum esculentum* L) bitkisinde DNA metilasyon değişikliklerinin belirlenmesi amacı ile kullanılmıştır. DNA band değişiklikleri Rastgele Çoğaltılmış Polimorfik DNA (RAPD) analizi aracılı ile ortaya konmuştur. 12 saat süre ile NaCl ve PEG uygulanmış örneklerden RAPD analizi ile elde edilen DNA bant değişikliklerinin de-metilasyon/hipometilasyon açısından kritik olduğu görülmüştür. CRED-RA analizi verilerine göre, PEG uygulamasının NaCl uygulamasına oranla domates genomundaki DNA metilasyon seviyesinde daha fazla değişikliğe sebep olduğu görülmüştür. Her iki stres koşulu içinde toplam metilasyon seviyesindeki maksimum düşüşün 9h uygulamasında olduğu belirlenmiştir. Sonuç olarak; bu çalışma ile CRED-RA tekniğinin kuraklık ve tuz gibi abiotik stres koşullarına maruz kalmış bitkilerde DNA metilasyon seviyesindeki değişikliklerin belirlenmesinde kullanılabileceği görülmüştür.

Anahtar kelimeler: metilasyon**,** CRED-RA, tuzluluk, domates, kuraklık

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +903122126720; Fax.: +903122126720; E-mail: aras@science.ankara.edu.tr
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1. Introduction

Plant epigenetics has attracted unprecedented interest and become a major focus of research activity for both basic and applied research in the last years (Mirouze and Paszkowski, 2011). Epigenetic regulation of plant development could be used to improve crop productivity and to enable plants to adapt to stress because the expression of the genome, including the chromatin structure and DNA methylation, is influenced by epigenetic regulation (Chinnusamy and Zhu, 2009). DNA methylation is a molecular epigenetic heritable enzymatic modification, which results from the addition of a methyl group in the cyclic carbon-5 of cytosine. DNA methylation patterns show differences between animals and plants. In animals, methylation occurs on a cytosine nucleotide in a CpG site, whereas in plants it can take place at CpGCpHpGp and CpHpHp sites in which H can be any nucleotide other than G. It is known that cytosine methylation plays an important role in mediating gene expression, genomic imprinting, and chromatin structure. However, the exact role of methylation, especially in gene expression, remains to be discovered in plants (Suzuki and Bird, 2008).

Abiotic stress, such as drought and soil salinity, is a global issue that adversely affects the yield potential of many crop plants. Such stress has the potential to severely restrict plant growth and development in many parts of the world (Tattersall et al., 2007). Importantly, these two seemingly different types of stress share many commonalities as far as their physiological effects on essential plant cellular processes are concerned. For instance, both water and salt stress negatively influence the ability of plants to take up water from the soil and can therefore induce similar or overlapping metabolic and oxidative stress effects (Kalefetoğlu and Ekmekci, 2010). In plants exposed to such stress, essential cellular constituents, such as proteins/enzymes and nucleic acids, can be severely damaged/inactivated through the accumulation of ions caused by cellular water loss (Campbell et al., 1991; Bray, 1997). At the same time, stress conditions cause changes in DNA methylation and play a key role in the regulation of gene expression (Rassoulzadegan et al., 2006). DNA methylation plays an important role in the development of adaptations, such as genetic and phenotypic variations, tolerance, resistance, and protection (Arnholdt-Schmitt, 2004; Madlung and Comai, 2004).

In one study, DNA methylation led to the repression of gene expression, whereas increased gene expression occurred in response to demethylation (Zilberman and Henikoff, 2007). Cold stress in maize (Steward et al., 2002) and aluminum, salt, and cold stress in tobacco (Choi and Sano, 2007) led to increased DNA demethylation, followed by upregulation of genes related to stress response pathway. In another study, induced DNA hypermethylation occurred in response to osmotic- and salt-stressed tobacco cell-suspension cultures (Kovar et al., 1997). Induced specific cytosine hypermethylation was also seen in the genome of water-deficit stressed pea (Labra et al., 2002). Although the role of methylation in gene expression under abiotic stress conditions is well known, the role of different concentrations of NaCl and Polyethylene glycol (PEG) stress in tomato plants is not fully understood.

Levels of methylcytosine in the genome can be detected with many techniques available for determining methylation rates in the genome (Jost and Saluz, 1993; Grigg and Clark, 1994; Rein et al., 1998). Coupled restriction enzyme digestion and random amplification (CRED-RA) is a powerful technique for studying the genome methylation status (Cai et al., 1996). It is a modification of the Random Amplified Polymorphic DNA (RAPD) technique and uses methylation-sensitive enzymes, HpaII and MspI, which are employed as frequent cutters of DNA (Prakash and Kumar, 1997). The CRED-RA technique has been used successfully in a variety of studies with different plant species to reveal their methylation patterns (Cai et al., 1996; Leljak-Levanić et al., 2004; Tani et al., 2005).

In the present study, the methylation status of the tomato plant (*Lycopersicum esculentum* L. 'Falcon variety') subjected to 100 mM NaCl and 100 mM PEG stress for different time periods (control, 6 h, 9 h, 12 h and 24 h) was analyzed by the CRED-RA technique to shed light on the relation between stress conditions and methylation levels. Possible methylation differences between the various time periods under both stress conditions in comparison to control plants were evaluated.

2. Materials and methods

2.1 Plant material, growth conditions, and stress treatment

Tomato (*Lycopersicum esculentum* L. 'Falcon') seeds were germinated and grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland's solution. Hoagland solution includes macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4.7H_2O$, Ca $(NO_3)_2.4H_2O$, KCl), micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4.5H_2O$, NH_4Mo, $ZnSO_4.7H_2O$) and ions in the following concentrations: 2 mM Ca, 10^{-6} M Mn, 4 mM NO3, 2.10^{-7} M Cu, 1 mM Mg, 10^{-8} M NH4, 2 mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-4} M Fe, and 0^{-6} M B. Five plants were grown in each pot in a controlled environmental growth chamber with light of 250 mmol m^{-2} s^{-1}, photosynthetic photon flux at 25°C, and 70% relative humidity. Twenty-five-day-old plants grown in controlled media were used for the stress treatments. For the salt treatment, NaCl was added to the nutrient solution to a final concentration of 100 mM. The osmotic pressures of the NaCl solutions were determined with a Vapor Pressure Osmometer 5520 (ElitechGroup Wescor Biomedical Systems, UK). The average osmotic pressure of the 100 mM NaCl solution was estimated as 190 mmol/kg. For the drought treatment, PEG was added to the nutrient solution until the osmolality of the solution, as measured using the vapor pressure osmometer, was the same as that of the NaCl solutions (Verslues et al., 2006; Munns, 2011). In this way, an iso-osmotic level of stress was applied to both the NaCl- and PEG-treated plants, with the goal of matching the stem water potentials of the plants exposed to these treatments. Five plants in a single pot were harvested at 0, 6, 9, 12, and 24 h. The harvested plants were frozen in liquid nitrogen and stored at -20°C until DNA isolation.

2.2. Genomic DNA extraction

Genomic DNA was extracted from the tomato samples using the extraction protocol of Lefort et al. (1998). DNA concentrations were measured with the Nanodrop Spectrophotometer (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, USA). DNA purity ranged between 1.87 and 1.94, and the DNA yield was in the range of 745.8–1372.1 ng/µl (Table 1) In addition, agarose gel electrophoresis (1%) was used to analyze the quality of DNA samples.

Table 1. The amount and purity of DNA samples obtained from NaCl and PEG treated tomato plants

Samples	ng/uL	260/280	260/230
Control	806,8	1,91	1,20
100mM NaCl 6h	982,8	1,93	1,22
100mM NaCl 9h	913,8	1,89	1,06
100mM NaCl 12h	745,8	1,88	1,05
100mM NaCl 24h	1346,8	1,92	1,30
100mM PEG 6h	1372,1	1,94	1,43
100mM PEG 9h	1104,4	1,92	1,15
100mM PEG 12h	927,4	1,92	1,21
100mM PEG 24h	830	1,87	1,09

2.3. RAPD-PCR assay

RAPD-PCR analysis was performed using six different primers (OPC04, OPC05, OPA16, OPO07, OPC09 and OPA09) according to conditions previously reported (Soydam Aydin et al., 2012). The RAPD-PCR mix consisted of 1X of enzyme buffer, 4 mM of $MgCl_2$, 0.2 mM each of dNTP, 10 µM of primer, 60 ng of genomic DNA, and 0.5 U of Taq polymerase (Fermentas) in a total volume of 25 µl. Amplification was achieved using a thermocycler program as follows: 1 cycle of 2 min at 94°C; 45 cycles of 40 sec at 94°C, 1 min at 34°C, 2 min at 72°C, and 1 cycle at 72°C for 10 min. The amplification products were electrophoresed in 1.8% agarose gel, stained with 0.5 µg/ml EtBr, and visualized under UV light. Sterile equipment and a negative control were used to prevent any kind of contamination.

2.4. CRED-RA assay

One microgram of genomic DNA from each plant was separately digested with 5U MspI and HpaII enzymes (Fermentas) according to the manufacturer's recommendations. The primers that showed monomorphic band profiles were used in the CRED-RA analysis to ensure that the differences in the band profile resulted from changes in methylation. The amplification and visualization conditions for CRED-RA were the same as those described for RAPD.

2.5. Data analysis

Polymorphisms observed in the RAPD profiles included the disappearance of the control band and the appearance of a new band (Atienzer et al., 1999; Liu et al., 2005). Genomic template stability (GTS) values were calculated according to the results of the RAPD analysis. The GTS% was calculated as GTS%= (1 - a/n)* 100, where "a" indicates the RAPD polymorphic profiles in each sample, and "*n*" is the number of total bands in the control. Changes in the RAPD patterns were expressed as a decrease in GTS, a qualitative measure showing showing the

number of changes in the RAPD profiles generated by the tomato samples exposed to NaCl and PEG stress in relation to the profiles obtained from the control samples (Table 2).

Table 2**.** GTS ratios for all primers in tomato samples

	GTS Ratio (%)	
Samples	**100mM NaCl**	**100mM PEG**
6h	92,95	92,95
9h	95,77	92,95
12h	90,14	90,14
24h	94,36	90,14

HpaII and MspI restriction enzymes have different restriction abilities, depending on the methylation status of cytosines. All the amplified bands obtained from the CRED-RA analysis were divided into four methylation types based on the presence or absence of groups as indicated by Li et al. (2005) and Wang et al. (2011) (Table 3).

Table 3. Methylcytosine sensitivity and restriction patterns of the enzymes (HpaII, MspI).

Type	**Methylationpattern**	**HpaII**	**MspI**	**Band Profile**			
				C	**H**	**M**	
I	CCGG GGCC or GGCC CCGG	Active	Active				No methylatedcytosine on double strands of DNA or inner methylated cytosine on a single strand. It is excepted as non-methylated cytosine according the previous to studies [31,38]
II	CCGG GGCC	Active	Inactive				Outer methylated cytosine on a single DNA strand, (Semi-methylation).
III	CCGG GGCC	Inactive	Active				Inner methylated cytosine on double DNA strands, (Full-methylation).
IV	CCGG GGCC or CCGG GGCC	Inactive	Inactive				Outer methylated cytosine on DNA doublestrands, (Full-metylation).

Presence of a band	Absence of a band

C: PCR product of the DNA which is not digested by the enzyme
H: PCR product of the DNA which is digested by the HpaII enzyme
M: PCR product of the DNA which is digested by the MspI enzyme

According to this classification, the bands indicating each methylation type (I, II, III, and IV) were counted and placed in their location in the table (Table 4 and Table 5). The sum of the band numbers from the four methylation types gives the number of the total amplified bands. The percent ratio of each of the methylation type was calculated by dividing the number of bands of each of the methylation type by the total number of bands, multiplied by 100.

Table 4. Data obtained from CRED-RA analysis for the tomato plants subjected to NaCl stress

CRED-RA Bant Type	**Control**	**6thh**	**9thh**	**12thh**	**24thh**
I	7	8	10	8	7
II	0	0	0	1	1
III	1	1	1	1	0
IV	22	21	19	20	22
Total amplified bands	30	30	30	30	30
I (%)	23,3	26,6	33,3	26,6	23,3
II (%)	0	0	0	3,3	3,3
III (%)	3,3	3,3	3,3	3,3	0
IV (%)	73,3	70	63,3	66,6	73,3
Total methylated bands	23	22	20	22	23
Total methylated bands ratio (%)[a]	76,6	73,3	66,6	73,3	76,6
Fully methylated bands	23	22	20	21	22
Fully methylated bands ratio (%)[b]	76,6	73,3	66,6	70	73,3
Hemi methylated bands	0	0	0	1	1
Hemi methylated bands ratio (%)[c]	0	0	0	3,3	3,3

[a]Total methylated bands ratio (%) = [(II+III+IV)/(I+II+III+IV)]x100
[b]Fully methylated bands ratio (%) = [(III+IV)/(I+II+III+IV)]x100
[c]Hemi methylated bands ratio (%) = [(II)/(I+II+III+IV)]x100

The value showing the methylation level of the samples was obtained by dividing the total number of bands of methylation types II, III, and IV by the total number of bands obtained from all the methylation types multiplied by 100 (II+III+IV/I+II+III+IV×100). To detect full methylation and hemi-methylation, the following two formulas were used: III+IV/I+II+III+IV×100 and II/I+II+III+IV×100, respectively (Karan et al., 2012).

Table 5. Data obtained from CRED-RA analysis for the tomato plants subjected to PEG stress

CRED-RA Bant Type	Control	6th h	9th h	12th h	24th h
I	1	1	2	1	1
II	2	5	3	2	1
III	1	1	1	3	6
IV	18	15	16	16	14
Total amplified bands	22	22	22	22	22
I (%)	4,5	4,5	9	4,5	4,5
II (%)	9	22,7	13,6	9	4,5
III (%)	4,5	4,5	4,5	13,6	27,2
IV (%)	81,8	68,1	72,7	72,7	63,6
Total methylated bands	21	21	20	21	21
Total methylated bands ratio (%)[a]	95,4	95,4	90,9	95,4	95,4
Fully methylated bands	19	16	17	19	20
Fully methylated bands ratio (%)[b]	86,3	72,7	77,2	86,3	90,9
Hemi methylated bands	2	5	3	2	1
Hemi methylated bands ratio (%)[c]	9	22,7	13,6	9	4,5

[a]Total methylated bands ratio (%) = [(II+III+IV)/(I+II+III+IV)]x100
[b]Fully methylated bands ratio (%) = [(III+IV)/(I+II+III+IV)]x100
[c]Hemi methylated bands ratio (%) = [(II)/(I+II+III+IV)]x100

3. Results

Comparison of the band alterations in the NaCl- and PEG-stressed plants with respect to those of the control samples (Table 6) showed that total number of band alterations (a+b) were 19 and 24 for the NaCl- and PEG-stressed tomato plants, respectively. Seven to 15 bands were produced in the control sample, and 71 bands with an average of 11 per primer were obtained in the RAPD analysis.

Table 6. Changes of total bands compared to the control in tomato plants subjected to NaCl and PEG

Lycopersicum esculantum		100mM NaCl								100mM PEG							
Primer	**Control Total Band**	6th h		9th h		12th h		24th h		6th h		9th h		12th h		24th h	
		a	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC04	12	0	2	0	2	0	2	0	2	1	0	0	2	1	2	2	3
OPC05	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
OPA16	14	0	0	0	0	2	2	0	0	1	2	2	1	2	2	0	1
OPO07	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPC09	10	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
OPA09	15	0	2	0	1	0	1	1	1	0	0	0	0	0	0	0	0
	71	1	4	0	3	2	5	1	3	2	3	2	3	3	4	3	4
	a+b	**5**		**3**		**7**		**4**		**5**		**5**		**7**		**7**	
Total		**19**								**24**							

a: Appearance of new bands, b: Disapperance of control bands, a+b: Indicates polymorphic bands.

The highest rate of polymorphisms was obtained with the OPA16 (28.6%) and OPC04 (58.3%) primers in the NaCl- and PEG-stressed tomato plants, respectively (Table 7, Figure 1).

Table 7. The polymorphism ratios of the primers

100 mM NaCl				
Primer	**TB**	**PB**	**Ratio (%)**	**Avarage Ratio (%)**
OPC04	12	2	16,6	11,98
OPC05	13	0	0	
OPA16	14	4	28,6	
OPO07	7	0	0	
OPC09	10	0	0	
OPA09	15	4	26,7	
100 mM PEG				

Primer	TB	PB	Ratio (%)	Avarage Ratio (%)
OPC04	12	7	58,3	19,78
OPC05	13	1	7,6	
OPA16	14	6	42,8	
OPO07	7	0	0	
OPC09	10	1	10	
OPA09	15	0	0	

The highest rate of DNA variations were observed in the tomato plants exposed to NaCl stress for 12 h and PEG stress for 12 and 24 h (Table 6). According to the average rates of polymorphisms, a higher rate was observed in the plants exposed to PEG stress than to NaCl stress (Table 6).

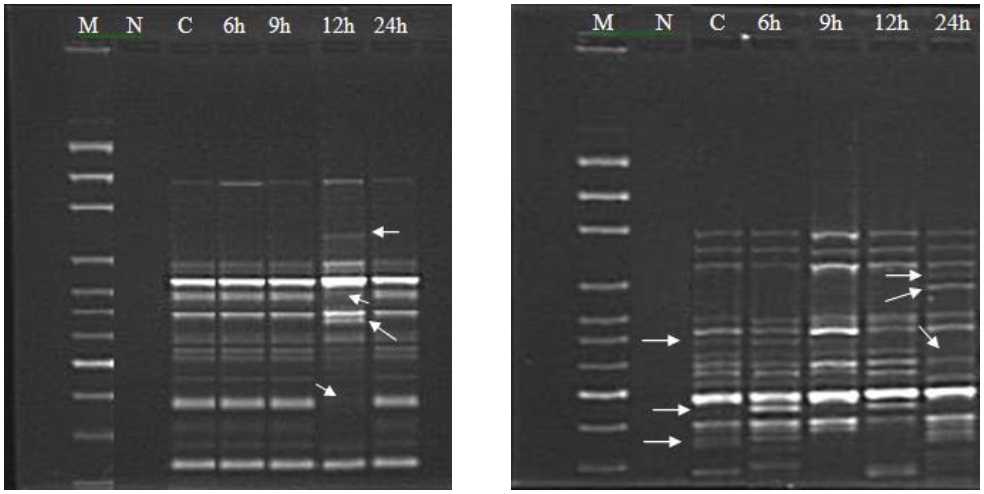

Figure 1. RAPD profile of genomic DNA from tomato plants exposed to NaCl generated by the primer OPA16 and OPC04

The GTS%, a qualitative measure reflecting changes in RAPD profiles, were calculated for each of the six primers tested and presented in Table 2. The highest GTS levels were observed for the NaCl-stressed tomato plants at 9 h and for the PEG-stressed tomato plants at 6 and 9 h. The lowest levels of GTS were detected at 12 h in the NaCl-stressed tomatoes (90%, *n*=14) and at 12 and 24 h in the PEG-stressed tomato plants (90%, *n*=14) (Table 2).

The analysis of the RAPD band profiles showed that the OPC05, OPO07, OPC09, and OPO07, OPA09 primers produced monomorphic band patterns in both the NaCl- and PEG-stressed tomato plants (Table 7).

When the results of the CRED-RA analysis were evaluated based on the methylation types in the NaCl-stressed samples, the maximum level of methylation (33.3%) was observed in type I, and the lowest level of methylation (63.3%) was seen in type IV (Table 4). Type II methylation was not observed for the first 9 h and then occurred at a rate of 3.3% at 12 and 24 h. The rate of type III methylation (3.3%) remained the same until the end of 12 h and completely disappeared at 24 h (Table 4 and Figure 1).

In the tomato plants exposed to 100 mM NaCl stress, the highest rate of total methylation was observed in the not stressed control (76.6%) and the stressed plants at 24 h (76.6%). The lowest rate (66.6%) of total methylation was observed at 9 h (Table 4). As indicated in Figure 3, the methylation rate decreased until 9 h and started to increase again after this hour. Full methylation was observed in the control and in the 6 and 9 h stressed tomato plants, and hemi-methylation was observed in the 12 and 24 h plants at a rate of 3.3%. According to the CRED-RA data obtained from the PEG-stressed tomato plants, type I methylation reached a maximum level (9%) at 9 h. The minimum rate (63.6%) of type IV methylation was observed at 24 h, whereas the maximum level (27.2%) of type III methylation was observed at this time (Table 5 and Figure 2).

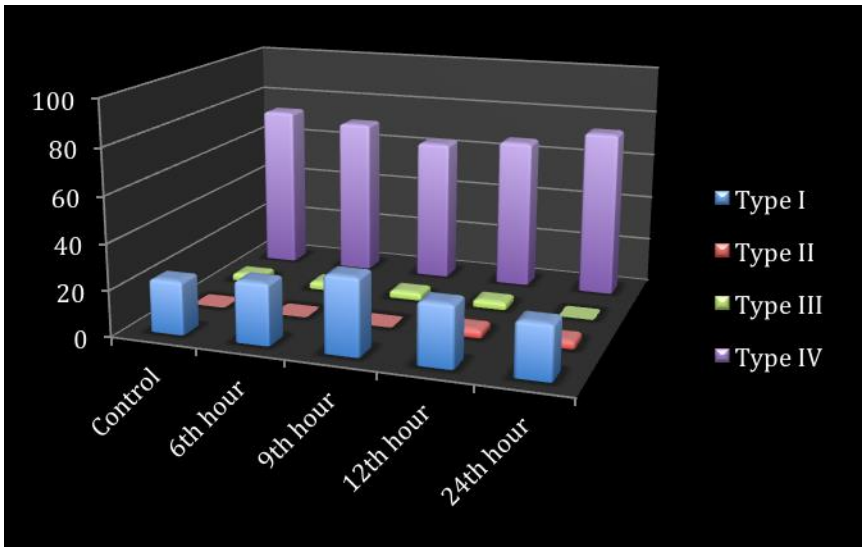

Figure 2. Ratios of the conversion among the cyctosine methylation types in NaCl stressed tomato plants

In the tomato plants subjected to 100 mM PEG, the methylation rate remained as high as 95.4%, and this rate decreased only at 9 h to 90.9%. At 9 h, full and hemi-methylation accounted for 7.2% and 13.6% of methylation, respectively (Table 5). As shown in Figure 4, hemi-methylation reached a maximum level at 6 h, and the minimum level was observed at 24 h..

4. Conclusions and discussion

The tomato plant is sensitive to environmental stress, such as extreme temperature, drought, salinity, and insufficient moisture (Kalloo, 1993). A total of 26% of the world's arable lands suffer from natural drought stress, which is the most severe stress among all abiotic stress factors. Drought stress is followed by mineral stress of 20% part (Blum, 1988). A significant increase in drought and salinity is expected in more than 50% of arable lands due to climatic changes in the next 40 years (Bray et al., 2000). Therefore, salt and drought will be the main stress factors threatening agricultural productivity in the coming years.

In this study, the effects of NaCl and PEG stress for different periods on tomato plants were evaluated in terms of genetic and epigenetic parameters. Genetic and epigenetic changes were determined by RAPD-PCR and CRED-RA analysis, respectively.

The RAPD-PCR results (Table 2) showed that the GTS values were close to each other in the NaCl- and PEG-stressed tomato plants at 9 and 24 h and that they had the same values at 6 and 12 h. The results confirm the idea that osmotic agents, such as PEG, produce similar effects as NaCl when they have the same osmotic pressure (Yeo and Flowers, 1986). Kulkarni and Deshpande (Kulkarni and Deshpande, 2006) reported anatomical changes and RAPD-based DNA polymorphisms in mutant and natural tomato varieties subjected to drought stress using PEG-6000. In another study, Nasiruddin et al. (2004) observed variations in DNA band profiles in 10 *Solanum tuberosum* varieties of different geographic origin exposed to NaCl and PEG stress (Nasuriddin et al., 2004). The current study also shows that both types of stress produce variations/polymorphisms in DNA band patterns as indicated in previous research (Soydam et al., 2012) (Table 6, Table 7).

When the CRED-RA results of the NaCl-stressed tomato plants were evaluated based on their methylation types, the maximum level of type I methylation (33.3%) compared to the control sample occurred at 9 h, whereas the minimum level of type IV methylation (63.3%) occurred at the same time point. In addition, type III methylation completely disappeared at 24 h (Table 4 and Figure 2).

The data derived from the PEG-stressed tomato showed that type I methylation reached a maximum level at 9 h, similar to the salt-stressed tomato plants (Figure 2 and Figure 3). At the same time point, the rate of type II methylation decreased compared to the methylation rate at 6 h.

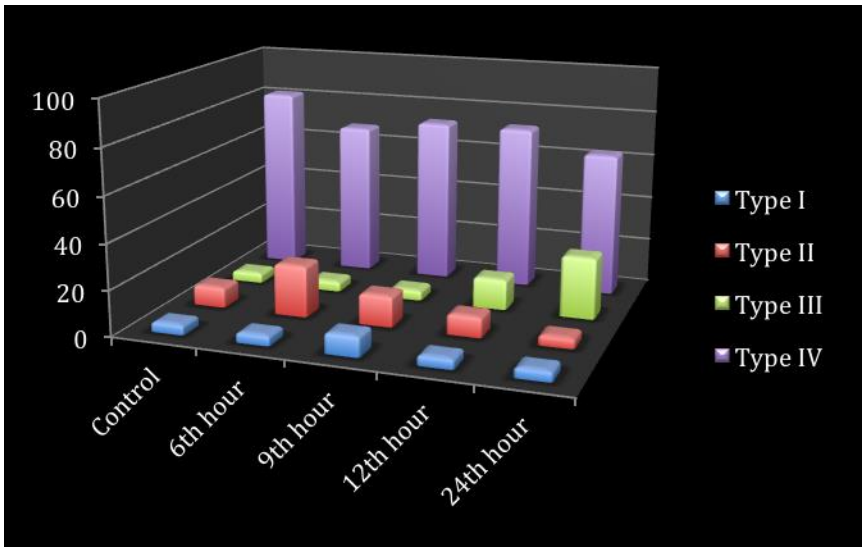

Figure 5. Ratios of the conversion among the cyctosine methylation types in PEG stressed tomato plants

Type I methylation is assumed to represent nonmethylation as indicated in previous studies (Pan et al., 2011; Karan et al., 2012). According to this assumption, demethylation seemed to be present in both the salt- and PEG-stressed tomato plants at 9 h.

Consistent with the results of the present study, Choi and Sano (2007) indicated that some functional active genes of tobacco plants could be demethylated due to exposure to abiotic stress (Choi and Sano, 2007). Although the underlying process of demethylation has yet to be clarified in detail, reactive oxygen species (ROS) have been proposed as possible triggers of demethylation of genomic DNA (Galaud, 1993; Cerda and Weitzman, 1997; Lízal and Relichová, 2001; Steward et al., 2002; Labra et al., 2002). The highest demethylation rate (lowest methylation rate) observed at 9 h for both stressors could be caused by ROS accumulation due to NaCl and PEG stress as suggested by Cerda and Weitzman (Cerda and Weitzman, 1997).

The rate of full methylation in the NaCl-stressed tomato plants was reduced at 6 and 9 h but increased after 12 h. Similarly, the rate of full methylation in the PEG-stressed tomato plants was reduced until 6 h and started to increase at 9, 12, and 24 h. This increment in the full methylation rate may be the result of the addition of a methyl group to the N5 carbon of the primidine ring in cyctosine by the enzyme DNA methyltransferase. Three DNA methyltransferases have been identified in plants: MET1, CMT3, and DRM2 (Ronemus, 1996; Cao and Jacobsen, 2002). In mammals, DNA methylation occurs in the cyctosine of CpG sites, but it also occurs in CpNpG and CpNpN sites (N: A, C or T) in plants (Oakeley and Jost, 1996). As the restriction site of the HpaII and MspI enzymes used in the CRED-RA technique is CCGG, we could not obtain any information about the methylation levels at CpNpN sequences. DRM2 is the only enzyme known to be a *de novo* class of DNA methyltransferase in plants. CMT3, together with MET1, is known to maintain methylation patterns during DNA replication (Cao and Jacobsen, 2002). In this regard, the increments in the rate of full methylation that started at 12 and 9 h in the NaCl- and PEG-stressed plants, respectively, might possibly be due to the activity of the MET1, CMT3, and DRM2 enzymes. Particularly, the higher rate of full methylation at 24 h in the PEG-stressed tomato plants compared to the control plants might be the indication of a *de novo* effect, in other words, the action of DRM2 DNA methyltransferase activity.

In the 100 mM NaCl-stressed tomato plants, the rate of full methylation was lower than in the control sample at 24 h, but the rate of hemi-methylation was higher (Table 4). In a previous study, Karan et al. (2012) used the methylation-sensitive amplification polymorphism technique to evaluate methylation patterns in four rice varieties subjected to 150 mM NaCl for 24 h using DNA extracted from the roots (Karan et al., 2012). They indicated that the rate of full methylation was reduced at the end of 24 h compared to control plants. On the contrary, in the current study, the rate of hemi-methylation increased at 24 h.

In the current study, the increase in the hemi-methylation rate in the plants subjected to 100 mM NaCl for 24 h in association with the decrease in the rate of full methylation might indicate the conversion of double-stranded DNA methylation to single-stranded DNA methylation. Thus, it can be concluded that a demethylation process took place.

Although several studies have been performed in several plant varieties, more efforts are required to understand the complex process of methylation. According to the results of the present study, we can conclude that PEG (drought stress) produces greater variations in methylation in the genome compared to NaCl. In addition, the higher polymorphism ratio obtained at 12 h by both stressors (PEG and NaCl) may indicate that this period is a critical point for demethylation/hypomethylation activity in tomato plants. The maximum decrease in total methylation levels at 9 h compared to the control plants for both stressors suggests that exposure for 9 h is important for demethylation/hypomethylation activity.

In conclusion, DNA methylation polymorphisms in response to very important abiotic stress conditions, drought and salinity, were studied at different time intervals using a straightforward technique, CRED-RA, in an agronomically invaluable plant species, *Lycopersicum esculentum*. Further studies should be conducted to clarify the epigenetic mechanism underlying these polymorphisms and to improve future plant breeding programs.

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