Investigation of Both Drought Stress and *In Vitro* Culture Conditions Induced Epigenetic Alteration in Genetically Pure Bread Wheat Line Derived from Pehlivan Cultivar

Kuraklık Stresinin ve *İn Vitro* Kültür Koşullarının Teşvik Ettiği Epigenetik Değişikliğin Pehlivan Çeşidinden Elde Edilen Genetik Olarak Saf Ekmeklik Buğday Hattında Araştırılması

Research Article

Ayşe Şen
İstanbul University, Faculty of Science, Department of Biology, İstanbul, Turkey.

ABSTRACT

In the current study; mature bread wheat (*Triticum aestivum* L.) embryos from doubled haploid wheat line obtained from Pehlivan cultivar were cultured on drought stress induction medium (MS + 0.1 mgL^{-1} 2.4-D + different concentrations PEG 6000 (0, 10, 20, 40 and 60 gL^{-1}) for 28 days. Effects of drought stress on regenerated plants and methylation alterations due to both drought stress treatment and *in vitro* culture conditions were investigated in the combination of Inter-Retrotransposon Amplified Polymorphism (IRAP) with methylation sensitive restriction enzyme (*MspI* and *HpaII*) digestions techniques on 28-day-old control and stress treated regenerated plants. Drought stress decreased the average of plant height and fresh weight of regenerated plants than the controls. A 90% polymorphism rate and 0.6 epigenetic similarity were obtained between controls and drought stress treated experimental groups with the combination of methylation-sensitive restriction enzymes and IRAP. Additionally, the epigenetic similarity ratios between the two controls were detected to 0.733. As a result of these; to investigate methylation alterations due to both *in vitro* culture conditions and drought stress applications under *in vitro* conditions can be obtained more realistically performance with using genetically pure line.

Key Words
DNA methylation, drought stress, *in vitro* screening, doubled haploidy, wheat.

ÖZ

Bu çalışmada; Pehlivan ekmeklik buğday (*Triticum aestivum* L.) çeşidinden elde edilen katlanmış haploid buğday hattının olgun embriyoları, kuraklık stresi teşvik ortamında (MS + 0.1 mgL^{-1} 2.4-D + farklı derişimlerde PEG 6000 (0, 10, 20, 40 ve 60 gL^{-1})) 28 gün boyunca kültüre alınmıştır. Kuraklık stresinin rejenere olan bitkiler üzerine etkileri ve kuraklık stresi uygulaması ve *in vitro* kültür koşulları nedeniyle oluşan metilasyon değişiklikleri metilasyona duyarlı restriksiyon enzimi (*MspI* ve *HpaII*) kesimi ile Inter-retrotransposon Amplified Polimorfizmi (IRAP) tekniklerinin kombinasyonu ile 28 günlük kontrol ve stres uygulanan bitkilerde araştırılmıştır. Kuraklık stresi uygulaması rejenere olan embryoların ortalama bitki yüksekliğini ve taze ağırlığını kontrole göre azaltmıştır. Metilasyona duyarlı restriksiyon enzim kesimi ve IRAP tekniklerinin kombinasyonu ile kontrol ve kuraklık stresi uygulanmış deney grupları arasında %90'lık bir polimorfizm oranı ile 0.6'lık bir epigenetik benzerlik elde edilmiştir. Ayrıca, iki kontrol grubu arasındaki epigenetik benzerlik oranı da 0.733 olarak saptanmıştır. Sonuç olarak; hem *in vitro* kültür ortamının hem de *in vitro* ortamda kuraklık stresi uygulamasının oluşturduğu metilasyon değişikliklerini incelemek için genetik olarak saf hatların kullanımı ile daha doğru sonuçlar elde edilebilmiştir.

Anahtar Kelimeler
DNA metilasyonu, kuraklık stresi, *in vitro* tarama, katlanmış haploidi, buğday.

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Correspondence to: A. Sen, İstanbul University, Faculty of Science, Department of Biology, İstanbul, Turkey.
Tel: +90 212 455570 Fax: +90 212 455570 E-Mail: senayse@istanbul.edu.tr

INTRODUCTION

Wheat is mainly grown on rain-fed land. About 70% of the area of Turkey consists of semiarid environments in which available moisture constitutes a primary constraint in wheat production [1]. Global climate change is the most serious concern for sustainable agriculture production. Major abiotic stresses like drought, salinity, cold, and heat negatively affect wheat survival, biomass production, and yield of staple food crops climate change threatens the food security worldwide [2]. Tissue culture techniques use as tools to study the physiological, biochemical and molecular mechanisms that operate at the cellular level in response to stress conditions in short period of time and in a controlled environment. For example *in vitro* drought stress induction, one of the most popular approaches is to use high molecular weight osmotic substances, like PEG 6000 [3]. It is a non-penetrable and nontoxic osmotic substance, which is used to lower the water potential of the culture medium, and it has been used to simulate drought stress in cultured plant tissues [4].

Instead of *in vitro* screening plant germplasm the response to biotic and abiotic stress conditions, the production of haploids and doubled haploids (DHs), which is one of the plant tissue culture approaches, offers great promise for plant breeding. Haploid plants are of great importance, which are allowed for a single-step development of complete homozygous lines with rapid fixation of genetic variations from heterozygous parents [5]. Thus, to use doubled haploid plants for field screening to abiotic or biotic stress conditions can be made more effective selection or screening criteria, because these plants show more realistic performance than heterozygous plants.

Genetic uniformity of cultured tissues and regenerated plants is expected [6]. However, tissue culture induced variation in regenerated plants [7] and stress-induced variations in cultured explants [8] have been observed in different plant species. These uncontrolled and random spontaneous variations during tissue culture process are mostly unexpected and undesired phenomenon [6]. Temel and Gozukirmizi (2013) reported that due to the highly mutagenic conditions of tissue culture environment, cytogenetic abnormality, sequence changes, DNA methylation variations, transposon and retrotransposon movements occurred in calli, regenerated plants in their progeny. Retrotransposon based markers rely on retrotransposition that results in retrotransposon insertion, which generates polymorphisms. Inter-Retrotransposon Amplified Polymorphism (IRAP), one of the retrotransposon-based molecular markers, amplifies genomic distance between two LTR-retrotransposons. In this technique, polymorphism is detected by the presence or absence of the PCR product for a particular locus [9]. Generally, a high percentage of retrotransposon movements are inactivated by various mechanisms (such as DNA methylation) during plant development [10]. In addition, this activation may be triggered by different abiotic and biotic stress treatments, mutagen applications, *in vitro* cell or tissue culture conditions, etc. [11,12]. Their activation and inactivation can also be linked to methylation levels of DNA. DNA methylation is a major epigenetic modification and one of the important mechanisms involved in plant cell reprograming [13]. In plants, methylcytosine usually occurs in both CpG and CpNpG sequences. Differential DNA cleavage proportion of *MspI* and *HpaII* restriction enzymes are frequently used to identify methylation patterns in genomes. While *HpaII* digests only CCGG unmethylated or hemimethylated sites with one strand, *MspI* digests the CCGG and C^{m}CGG sites but not ^{m}CCGG or ^{m}C^{m}CGG sites [14].

In this context, the purpose of the study was to evaluate the level of epigenetic changes in genetically pure wheat line, induced with both *in vitro* culture conditions and different concentrations PEG 6000 applications in *in vitro* culture conditions. For this purpose, IRAP markers with the combination of methylation-sensitive restriction enzymes digestion were employed.

Table 1. List of primers used in this study and related information.

IRAP Primers	Sequence (5'-3') of IRAP Primers
5'LTR1	TTGCCTCTAGGGCATATTTCCAACA
5'LTR2	ATCATTGCCTCTAGGGCATAATTC
Wis-2-1A	CTAGGGCATAATTCCAACA
Sukkula	GATAGGGTCGCATCTTGGGCGTGAC
Tagermina	AGAGGAGGATATCCCAACAT
Thv19	GCCCAACCGACCAGGTTGTTACAG
Tar1	CTCCCAGTTGACCAACAA

MATERIAL and METHODS

Plant Material

Genetically pure wheat line derived from Pehlivan bread wheat (*Triticum aestivum* L.) cultivar was obtained to utilize anther culture technique utilized by International Center for Agricultural Research in the Dry Area (ICARDA) was used [15].

Drought Stress Treatment of the Mature Embryos of Doubled Haploid Plants Under Tissue Culture Conditions

To screen drought sensitivity of doubled haploid wheat line derived from Pehlivan wheat cultivar, the mature embryo explants were inoculated in an MS (Murashige and Skoog) [16] basal salt mixture of 0.1 mgL^{-1} 2,4-Dichlorophenoxyacetic acid, 20 gL^{-1} sucrose, 0.8% (w/v) agar, and different concentrations of PEG 6000 (0, 10, 20, 40 and 60 gL^{-1}). The pH of the screening media was adjusted to 5.8 before autoclaving. Ten embryo explants with three replications were inoculated in each experimental group under aseptic conditions. Then, the cultures were incubated in a growth chamber for a 16h light/a 8h dark photoperiod, irradiance of 500 $\mu mol\ m^{-2}s^{-1}$ photon flux densities, and at a temperature of 26°C for 28 days.

Molecular Analysis

Genomic DNA Isolation

After randomly collecting leaf samples from each experimental group, fresh leaves (100 mg) frozen with liquid nitrogen were ground with a mortar and pestle. To isolate genomic DNA, 2xCTAB buffers were used [17]. The quality and quantity of purified genomic DNA were estimated with both spectrophotometry and gel electrophoresis and then divided in two parts. Approximately 300 ng of genomic DNA from each part was digested with *MspI* (R0106S, NEB) and *HpaII* (R0171S, NEB) enzymes according to the manufacturers' recommendations. After checking digestion on 2% agarose gel, 100 ng of each digested product was amplified with IRAP primers (Table 1) to screen epigenetic variations between controls and different concentration PEG 6000 applied doubled haploid wheat plants using the combination of methylation sensitive restriction enzyme digestions with IRAP.

IRAP Analysis

Seven LTR-Retrotransposons (Table 1) in forty-nine different combinations were used to determine the epigenetic diversity between control and different concentration PEG 6000 applied doubled haploid wheat plants. The IRAP–PCR mixture (a total of 25 μL) comprised 0.4 μM of each primer, 0.4 mM dNTPs, 2 mM $MgCl_2$, 10X PCR buffer, 1 unit of Taq-DNA polymerase (Promega, USA), and 100 ng of template DNA from each digested genomic DNA. Amplification was carried out in a thermal cycler (Bio-Rad, C1000). Running conditions were initial denaturation for 5 minutes at 94°C, 30 1-min cycles at 94°C, 1 min for annealing at 60°C, 1 min at 72°C adding 3 s per cycle, then a final extinction of 10 min at 72°C and cooling to 4°C. PCR products were separated on 3% agarose gel at 100 V for 3 h, photographed on a UV transilluminator and scored visually.

Data Analysis

One-way analysis of variance (ANOVA) was used to make statistical interpretations, based on plant height and fresh weight. Their means were

compared with the Student–Newman Keuls test [18].

Well-resolved bands were scored as a binary value, (1) for their presence or (0) for their absence to generate a matrix. The binary matrix was used to calculate the genetic similarity by Nei and Li's coefficient [19]. among samples (2 genetically pure control and 12 drought stress applied genetically pure wheat plant samples). All of these computations were performed in Multivariate Statistical Package v3.1 [20]. PIC, MI, and Rp were calculated. PIC was calculated according to Anderson et al. [21], PIC = 1 – Σpi2, where pi is the frequency of the ith allele of the locus in the control set and mutant genotypes. MI was determined according to Powell et al. [22]. as the product of PIC. Rp was calculated using the formula Rp=ΣIb, where Ib is band informativeness and Ib = 1-[2 x (0.5 - p)] where p is the proportion of genotypes containing the band [23].

RESULTS and DISCUSSION

Detection of Sensitivity in Genetically Pure Wheat Plants to Drought Stress

In this study, drought stress induction in *in vitro* culture environment took place from mature embryos obtained genetically pure Pehlivan wheat line on MS medium supplemented with different concentrations PEG 6000 (0 (as a control), 10, 20, 40 and 60 gL^{-1}). The effects of drought stress on 28-old-day doubled haploid Pehlivan wheat plants were given in Table 2. Table 2 shows that increased PEG 6000 concentrations significantly decreased the average plant height (24.11–91.94%) and average plant fresh weight (30.93–91.12%) of doubled haploid wheat plants than control.

It is a well-established fact that shortage of water leads to drought stress and has the primary cause of crop loss worldwide, reducing average yields for most major crop plants adversely affecting the global crop production and the adverse impacts are getting more serious in the past few decades. Simulation of drought stress under *in vitro* conditions during the regeneration process constitutes a convenient way to study the effects of drought on the morphological and genetic responses. Thus, PEG 6000 induced drought stress in *in vitro* tissue culture environment is frequently used technique to screen the capability of drought sensitivity in different plant species [24-26]. These studies reported that PEG-induced drought stress or limited water stress produced substantial dehydration that led to elevated dry matter content, reduced regeneration capacity in callus tissues, and plant growth.

Detection of Epigenetic Instability in Genetically Pure Wheat Line Under Drought Stress Application and *In Vitro* Conditions

Controls and stress-applied regenerated plants on 28-day-old were analyzed with IRAP and methylation sensitive restriction enzyme digestion. Even though forty-nine *HpaII*+IRAP primer combinations did not produce any amplification products, a total of forty-nine *MspI*+IRAP primer combinations were used and five of them were amplified for a total of 20 bands with an average of 4 bands per primer. A total of 18 polymorphic bands (90.00%) with an average of 3.6 bands per primer were produced with all tested primers (Table 3). Most primer combinations resulted in thickness profiles and closely spaced bands, which are difficult to analyze. Figure 1 shows the Msp I+ *Sukkula-Thv*19 (a) and the Msp I+ *Sukkula-Tar1* (b) based epigenetic variations of twelve PEG 6000 applied DH wheat plants and two control DH wheat plants on 3% agarose gel. The highest number of bands (5) was obtained from *MspI+Sukkula-Thv19* and *MspI+Sukkula-Wis-1-2A* primer combinations, respectively, whereas the lowest number of bands (3) was obtained with *MspI+Sukkula-Sukkula* and *MspI*+Sukkula-5'LTR1 primer combinations. The mean PIC, MI, and Rp primer values were 0.95, 2.84, and 3.50, respectively. The highest PIC and MI values were from *MspI+Sukkula-Wis-1-2A*, whereas the highest Rp values were from *MspI*+Sukkula-5'LTR1. According to similarity indices based on Nei and Li's coefficient, control plants were genetically more similar to 40 and 60 gL^{-1} PEG 6000 treated plants than 10 and 20 gL^{-1} PEG 6000 treated plants. The average of *MspI* +IRAP based epigenetic similarity was 0.600 among the samples (two controls and twelve drought stress applied plants). Additionally, the

Table 2. Average fresh plant weights, and plant heights of 28-day-old doubled haploid Pehlivan wheat plants exposed to different concentrations of PEG6000.

Experimental Groups	Average Plant Heights (cm)	Average Plant Fresh Weights (g)
Control	25.18 ± 0.87 a*	0.653 ± 0.509 a*
10 gL^{-1} PEG 6000	19.11 ± 0.69b	0.451 ± 0.094b
20 gL^{-1} PEG 6000	10.87 ± 0.70c	0.298 ± 0.066c
40 gL^{-1} PEG 6000	6.88 ± 1.38c	0.092 ± 0.011d
60 gL^{-1} PEG 6000	2.03 ± 0.18d	0.058 ± 0.015d

Means followed by a different letter are significantly different *p < 0.05 (Student–Newman Keuls test).

Table 3. Used Msp I + IRAP primers, total number of bands, polymorphic bands, polymorphism ratios, polymorphic information content, marker index and resolving power.

No	Msp I with Primer Combinations	Total Band	Polymorphic Band	Polymorphic (%)	PIC	MI	Rp
1	Msp I+ Sukkula-Sukkula	3	2	66.67	0.82	1.64	2.53
2	Msp I+ Sukkula-Wis-1-2A	5	4	80.00	1.32	5.28	3.63
3	Msp I+ Sukkula-5'LTR1	3	3	100.00	0.78	2.34	4.54
4	Msp I+ *Sukkula-Tar1*	4	4	100.00	0.83	3.32	3.87
5	Msp I+ *Sukkula-Thv*19	5	5	100.00	0.99	3.96	2.94
Msp I + IRAP Total		20	18	-	-	-	-
Mean		4.0	3.6	75.00	0.95	2.84	3.50

epigenetic similarity ratios between the two controls were 0.733.

DNA methylation is one of the epigenetic mechanisms that play a role in the transfer of acquired characteristics, creating difference at the regulation stage of gene expression without forming any difference in the DNA sequence line between successive generations. Methylation of the cytosine base has been recognized as the predominant form of epigenetic modification, and this is an important determinant of the information content of eukaryotic genomes. The isoschizomeric pair of restriction enzymes *MspI* and *HpaII* is frequently used to detect methylation status in plant genomes. The recognition sequence of these enzymes are 5'-CCGG-3', but differ in their sensitivities to DNA methylation. *HpaII* does not recognize or cut if either cytosine is fully methylated, whereas *MspI* does not cut if the external cytosine is fully- or hemi-methylated. Variation with respect to patterns of DNA methylation has been widely exploited as having a marked effect on cell differentiation, defense against transposon proliferation, chromatin inactivation, genomic imprinting activated by chromosome doubling, and so on. Additionally, recent studies have shown that epigenetic variations like the DNA methylations are as important as genetic variations in developing salt-tolerant varieties [14]. The cytosine-methylation based on epigenetic variations is independent than genetic variations. So, These bands are needed to confirm which loci changes resulted from IRAP assay with *MspI* digesting. Machczynska et al. [13]. detected that the changes of DNA methylation during

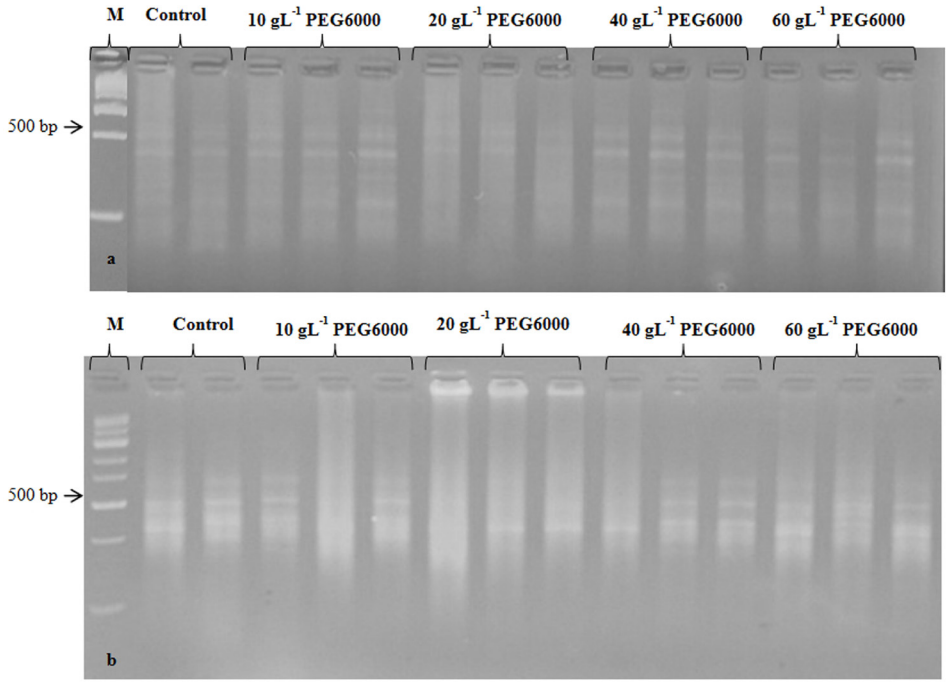

Figure 1. Msp I+ *Sukkula-Thv*19 (a) and Msp I+ *Sukkula-Tar1* (b) based epigenetic variations of twelve PEG 6000 applied DH wheat plants and two control DH wheat plants on 3% agarose gel. M; 100 bp DNA Marker.

doubled haploid triticale productions due to *in vitro* culture conditions, which may lead to a phenotypic variations obtained plants. Genetic and epigenetic changes were studied in barley callus cultures using IRAP and methylation sensitive restriction fingerprinting [6]. The DNA methylation changes to drought stress responses in tissue culture derived banana plants were reported by Msogoya and Grout [8]. All these studies indicated that tissue culture conditions, stress treatments, or chromosome doubling may create genetic shock, epimutations and/or epigenetic modifications, like de-methylation/methylation that altered the restriction site of *MspI*/*HpaII* in genomes, and could be the result of new retrotransposon integrations in the genome.

In conclusion; mature bread wheat (*Triticum aestivum* L.) embryos from doubled haploid wheat line obtained from Pehlivan cultivar were used to investigate methylation alterations due to both *in vitro* culture conditions and drought stress applications under *in vitro* conditions, in this study. As a result of this; drought stress decreased the average of plant height and fresh weight of regenerated plants than the controls and methylation alterations were detected due to *in vitro* culture conditions and *in vitro* drought stress applications in the experimental groups. As far as I know, this is the first report to investigate DNA methylation alterations induced with both *in vitro* drought stress application and *in vitro* culture conditions in genetically pure wheat line.

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