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RESEARCH

Cytosine methylation polymorphisms in cotton using TD-MS-RAPD-PCR

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Abstract

Methylation in DNA and chemical modification in histone proteins are the two most studied epigenetic mechanisms in plants. There exist low-throughput and high-throughput DNA methylation detection techniques in epigenetic research. In this study, touch-down polymerase chain reactions methylation sensitive-random amplified polymorphic DNA (TD-MS-RAPD) technique was used to investigate cytosine methylation differences among three cotton varieties; Texas Marker 1 (TM-1), Pima 3-79 (3-79) and Maydos Yerlisi (MY), belonging to *Gossypium hirsutum* L., *G. barbadense* L., and *G. herbaceum* L., respectively. Genomic DNA samples extracted from the mature seeds of these varieties were treated with *Msp*I, a relative methylation-insensitive restriction enzyme and *Hpa*II, a methylation-sensitive restriction enzyme before touch-down polymerase chain reactions. Among 16 oligonucleotide primers used, three primers (AT03, W15, and C08) resulted in methylation polymorphisms among three varieties. TD-MS-RAPD-PCR method was cost-effective, required a simple method and basic instrumentation, and could easily be performed in our laboratory with basic setup using a regular DNA thermal cycler and DNA gel electrophoresis system, however, the level of methylation polymorphisms detected with this method were very low in cotton. We concluded that the low level of polymorphisms among the three cotton species were probably due to low occurrences of CCGG sites within the cotton genome. We also noted that TD-MS-RAPD-PCR method could be used in primary scanning studies in epigenetic research.

Keywords: Cotton species, cytosine methylation, restriction digestion, touch-down PCR

Introduction

Methylation such as methylation at the fifth atom of cytosine base usually associated with the changes in gene expression by altering proteins binding to DNA and by changing the nucleosome, a basic unit of DNA packaging in eukaryotes. DNA methylation, as an important epigenetic phenomenon, involves in tuning gene expression during the tissue and organ development, response to biotic- and abiotic-stimuli, allows evolutional adaptation to new conditions without changing the DNA sequence in plants (Osabe et al. 2014; Karaca et al. 2016; Karaca & Ince 2018). Methylation responses to environmental stresses including cold, drought, salt, metals and developmental changes (seedling, mature, flowering, fruiting) have been studied. Many studies revealed that maturation, salt, drought, and metal stresses caused an increase in methylation (hypermethylation) or demethylation (hypo-methylation) in response to these stresses (Osabe et al. 2014; Wang et al. 2016). However, hypo- methylations or hyper-methylation were not common among organisms or developmental stages (Karaca & Ince 2018).

There are different types of DNA methylations in living organisms. Among the DNA methylations, cytosine methylation in sequence contexts, CG, CHG and CHH (where H stands for any base other than G) is the most occurring types of methylation in plants. Changes in the DNA cytosine methylation or de-methylation of genes may indicate that the gene may be under the epigenetic regulations along with

genetic controls. Epigenetic regulations do not only involve in responding to environmental stimuli but also involve in the expression of other agronomically important characteristics such as fruit ripening, seed size, flowering time, plant size, heterosis, plant stature, sex determination, and pathogen resistance (Osabe et al. 2014; Tiwari et al. 2015; Karaca et al. 2016; Wang et al. 2016).

Methods for DNA sequencing can be broadly divided into three categories such as (i) gene or locus-specific method that produces resolution at base pair at particular nucleotide, (ii) regional or proportional methods including the exome sequencing (about 1% of the genome) or genotyping by sequencing studies (<0.1% of the genome) and (iii) whole genome sequencing methods (Karaca & Ince 2017). Detecting methylation at the DNA level could use these three categories mentioned above. Methylation detection studies could utilize "old" or "modified" methods or new methods (Karaca & Ince 2008; Ince et al. 2010a, Ince & Karaca 2011a). Examples for old or modified methods include methylation-sensitive amplified polymorphism (MSAP), a modification of amplified fragment length polymorphism (AFLP), methylation-sensitive random amplified polymorphic DNA (MS-RAPD), a modification of random amplified polymorphic DNA (RAPD), methylation-sensitive single-strand conformation polymorphism (MS-SSCP), a modification of single-strand conformation polymorphism (SSCP), methylation-sensitive inter-simple sequence repeat (MS-ISSR), a modification of inter-simple sequence repeat and many others (Karaca et al. 2008; Tiwari et al 2015; Wang et al. 2016; Ince & Karaca 2017).

Random or Randomly amplified polymorphic DNA (RAPD) is a technique widely used for studying the DNA polymorphism among species without the requirement of prior knowledge of the genome (Ince et al. 2010b; Welsh & McClelland 1990; Williams et al. 1990; Ince et al. 2015). RAPD usually uses single oligonucleotide primers at low stringency to generate amplified products of polymerase chain reactions (PCR). Amplified products showing size differences between two samples are called polymorphic RAPD markers. In RAPD-PCR technique generally, two types of amplified products; dense and light amplified products called bands are generated. Reproducible RAPD bands are usually those dense bands that are produced two primers at their ends while light bands are those amplified products that are produced one primer and they are not usually reproducible (repeatable). RAPD-PCR markers are being utilized in many genetic studies since this method provides a rapid method for detecting the polymorphism (Welsh & McCelland 1990; Ince et al. 2010c; Ince & Karaca 2011b, Tiwari et al. 2015).

The main aim of present study was to investigate whether a touch down based methylation sensitive random amplified polymorphic DNA marker (TD-MS-RAPD-PCR) method, one of the oldest and simplest methods for detection of DNA polymorphisms, could be reliably utilized in studies of cotton (*Gossypium* L.) DNA cytosine methylation which is one of the most studied epigenetic mechanisms in plants.

Materials and Method

Plant materials

Plant samples consisted of old varieties/accessions of *Gossypium hirsutum* L. Texas Marker-1 (TM-1), *Gossypium barbadense* L. Pima 3-79 (3-79) and *Gossypium herbaceum* L. Maydos Yerlisi (MY) (Karaca & Ince 2011; Karaca et al. 2016).

DNA extraction

Genomic DNA samples were extracted from a single seed of each sample using a DNA extraction protocol described in Aydin et al. (2018). Briefly, each seed sample was crushed without using liquid nitrogen with a pestle. The seed coat was removed during crushing to remove maternal tissue. Extracted genomic DNA samples were analyzed using spectrophotometric analysis and agarose gel electrophoresis studies as described in Ince et al. (2010).

Restriction enzyme digestion

Restriction enzyme digestion experiments were performed in 0.2 mL thin-walled microtubes. Fifteen microliter reaction mixtures contained 750 nanograms of genomic DNA, 20 units of restriction enzyme *Msp*I or *Hpa*II and 1x DNA digestion buffer (Tango buffer, Thermo Scientific) placed in an incubator at 37°C for 16 hours (Karaca et al. 2005). At the end of the incubation 60 µL, PCR grade water was added to each sample, mixed well before TD-MS-RAPD-PCR experiments.

Touch-down polymerase chain reaction (TD-PCR) experiments

DNA amplifications were performed in a model GeneAmp System 9700 thermal cycler machine (Perkin-Elmer Corp., Applied Biosystems). Amplification was performed in a 25 µL reaction mixture containing template DNA, primers, 10x reaction buffer, $MgCl_2$, four different dNTPs and *Taq* DNA polymerase as shown in Tab. 1 (Karaca et al. 2005; Karaca & Ince 2011). Targets were amplified using a touch-down PCR as shown in Tab. 2 for increasing the specificity of RAPD-PCR reactions (Ince & Karaca 2011b). *Msp*I and *Hpa*II (Tab. 3) digested cotton samples were amplified using a total of 16 oligonucleotides primers (Operon Technologies, Tab. 4).

Agarose gel electrophoresis

After TD-MS-RAPD-PCR experiments were performed, 5 µL of 6x DNA loading buffer solutions were added to each 25 µL of PCR products, mixed well and these mixtures were loaded in 1.5-3% high-resolution agarose gels containing 0.05 µg/mL ethidium bromide, electrophoresed at 5 V/cm at constant voltage for 3 to 6 hours in the presence of 1x Tris-

Table 1. PCR reaction mixture for TD-MS-RAPD.

Chemicals		Stock solution	Volume	Final
Genomic DNA			8.5 µL	50 ng
Primer		20 µM	3 µL	2.4 µM
10x reaction buffer	TRIS-HCl (pH 9.1)	12 mM	3 µL	80 mM
	KCl	60 mM		19 mM
	Triton X-100	0.012%		0.009%
	$MgCl_2$	1.8		3 mM
$MgCl_2$		25 mM	0.6 µL	
dNTP		10 mM	0.7 µL	0.28 mM
Taq DNA polymerase		5 unite/µL	0.4 µL	1 unite
Steril-H_2O			8.8 µL	
Total volume			25 µL	

Table 2. TD-MS-RAPD-PCR profile.

PCR Profile		Duration	Cycle	Stage
Hot Start	94°C	5 min	1	Pre-denaturation
	94°C	1 min		Denaturation
Pre- PCR	42°C→37°C	1 min	10	Renaturation
	72°C	2 min		Synthesis
	94°C	1 min		Denaturation
PCR	37°C	1 min	30	Renaturation
	72°C	2 min		Synthesis
Final	72°C	10 min	1	Final Synthesis
	5°C	Until removed		

Table 3. Methylation patterns and cleavage behaviors of *Msp* I and *Hpa* II (Salmon et al. 2008).

Targets	Msp I	Hpa II	Methylation Status
CCGG/GGCC	Does cut	Dose cut	Both DNA strands are not methylated
C^{m}CGG/ GG^{m}CC	Does cut	Doesn't cut	Internal cytosine bases are fully methylated
^{m}C^{m}CGG/ GGCC	Doesn't cut	Dose cut	One DNA strand is fully methylated
^{m}CCGG/GGCC	Doesn't cut	Dose cut	One DNA strand is hemimethylated
^{m}C^{m}CGG/ GG^{m}C^{m}C	Doesn't cut	Doesn't cut	Both DNA strands are fully methylated

Table 4. Oligonucleotide primers and number of TD-MS-RAPD-PCR markers.

Primer ID	Sequences (5'→3')	Number of amplified bands* (*Gossypium* L.)		
		G. hirsutum L. (TM-1)	*G. barbadense* L. (3-79)	*G. herbaceum* L. (MY)
U5	TTGGCGGCCT	4	4	4
U10	ACCTCGGCAC	11	10	11
U15	ACGGGCCAGT	9	8	9
U20	ACAGCCCCCA	10	10	13
AT03	GACTGGGAGG	13 (+1)	11 (+1)	13 (+1)
AT04	TTGCCTCGCC	11	11	11
C5	GATGACCGCC	13	14	14
C8	TGGACCGGTG	8	8	8
C10	TGTCTGGGTG	19	16	19
C11	AAAGCTGCGG	10	10	10
E1	CCCAAGGTCC	12	12	12
E2	GGTGCGGGAA	11	11	10
M04	GGCGGTTGTC	13	13	13
N16	AAGCGACCTG	14	14	14
J09	TGAGCCTCAC	15	12	15
W15	ACACCGGAAC	15 (+1)	17 (+1)	15 (+1)

* Please note that numbers in the parentheses are number of polymorphic/methylation sensitive markers

Borate-EDTA buffer and photographed on an ultraviolet (UV) transilluminator for analysis (Karaca et al. 2013).

Cytosine methylation scoring

TD-MS-RAPD-PCR markers were scored as present (1) or absent (0) within and between genomic DNA sample digested with *Msp*I or *Hpa*II for TM-1, Pima 3-79 and MY. An absence of a TD-MS-RAPD-PCR marker was considered that both strands of targets contained unmethylated tetra-nucleotides (5'-CCGG-3'/3'-GGCC-5') while the presence of a TD-MS-RAPD-PCR marker was considered that both strands of targets contained fully methylated tetra-nucleotides (5'-mCmCGG-3'/3'-GGmCmC-5'). When a TD-MS-RAPD-PCR marker was present in *Msp*I digest but the same marker was absent in *Hpa*II digest, this case was considered that one DNA strand of the target was fully methylated (5'-mCmCGG-3'/3'-GGCC-5') or hemimethylated (5'-mCCGG-3'/3'-GGCC-5'). On the other hands, when a TD-MS-RAPD-PCR marker was absent in *Msp*I digest but the same marker was present in *Hpa*II digest, this case was considered that the internal cytosine bases were fully methylated (5'-CmCGG-3'/3'-GGmCC-5'). In the present study, 1 and 0 scorings were used. Scoring as present (1) was considered that target was not cut by the enzyme while scoring as absent (0) was considered that target was cut by the enzyme (Salmon et al. 2008).

Results and Discussion

In the present study, we utilized TD-MS-RAPD-PCR technique for detection of DNA cytosine methylation in mature cotton seeds. The use of TD-MS-RAPD-PCR technique relies on the application of isoschizomers, which are those restriction enzymes that have the same recognition sites but show differential sensitivity to DNA methylation. Isoschizomers utilized in the present study were *Hpa*II and *Msp*I. These enzymes recognize 5'-CCGG-3' sequences and *Hpa*II cuts the external cytosines when only a single DNA strand is methylated (hemimethylated), whereas *Msp*I cuts the internal cytosines when both DNA strands are fully methylated. However,

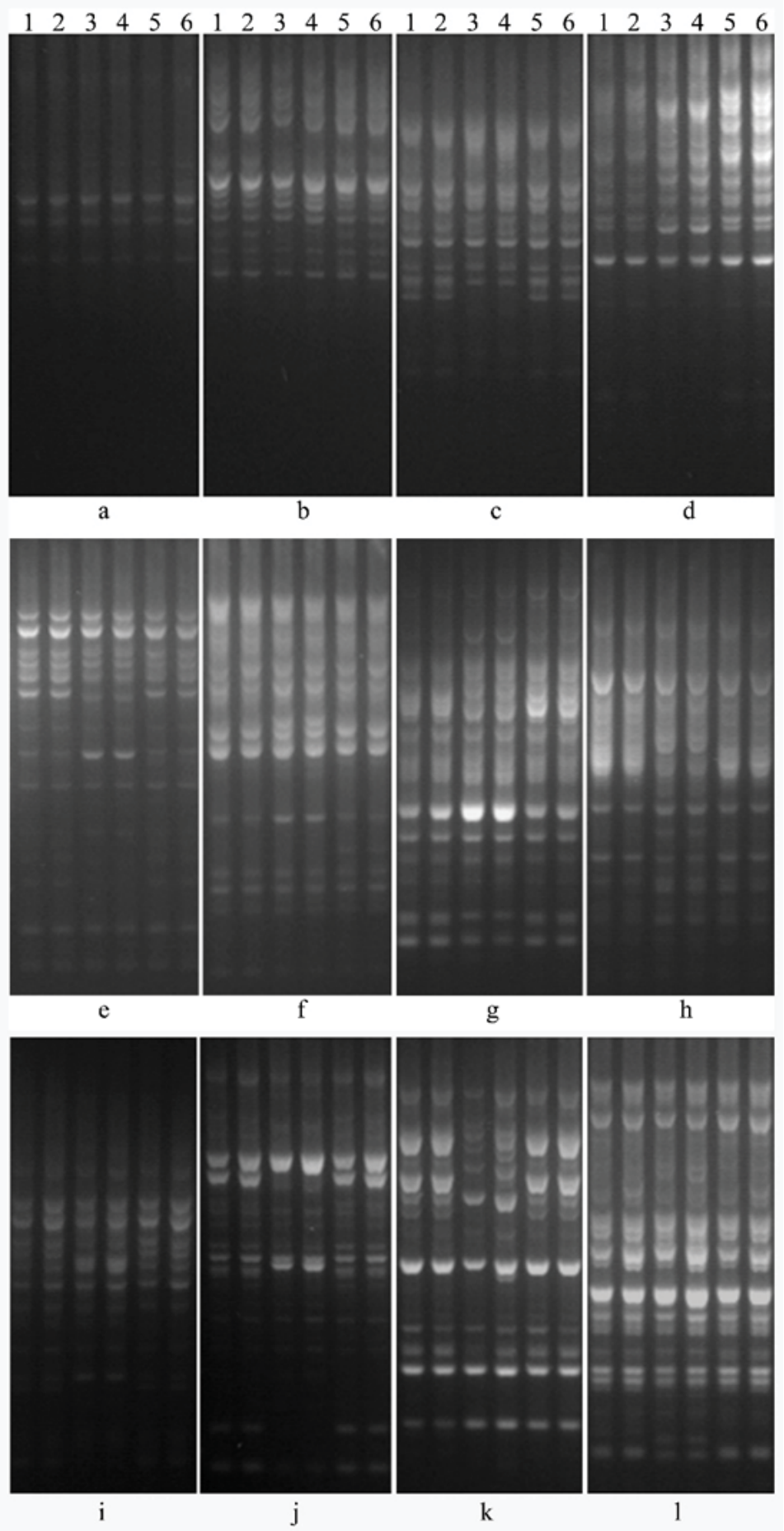

Figure 1. TD-MS-RAPD-PCR markers. Numbers 1-2 in each panel represent cotton variety TM-1, numbers 3-4 represent Pima 3-79, and numbers 5-6 represent MY. In each panel, odd numbers represent templates digested with MspI and even numbers represent templates digested with HpaII restriction enzyme. Letters a to l represent amplified products of primers U05, U10, U15, U20, AT04, C05, C10, C11, E01, M04, N16, and J09.

when both DNA strands are not methylated both enzymes cut the targets. Tab. 3 summarizes cleavage behavior of *Msp*I and *Hpa*II (Salmon et al. 2008). When two different genomic DNA samples representing two different lines or varieties are digested with *Msp*I and *Hpa*II separately, they could be differentiated if they have different methylation patterns at 5'-CCGG-3' target sites. On the other hands, two varieties or lines cannot be distinguished when the target sequences were unmethylated and fully methylated (Tiwari et al. 2015).

In the present study, amplification of MS-RAPD-PCR markers were performed using a touch-down PCR profile (called as TD-MS-RAPD-PCR) to minimize the amplification of nonspecific markers, called artifacts. Production of artificial amplicons in the RAPD technique is one of the main disadvantages of the RAPD method since it reduces the reproducibility of markers (Karaca & Ince 2008).

A number of amplified products that could be reliably scored varied among 16 primers (Tab. 4, Fig. 1 and 2). For instance, oligonucleotide primer U5 produced 4 TD-MS-RAPD-PCR markers while primer C10 produced the highest number of markers (19). The number of TD-MS-RAPD-PCR markers varied among the three cotton species represented with TM-1, Pima 3-79 and MY cultivars in this study. There were a total of 191 TD-MS-RAPD-PCR markers in variety MY (*G. herbaceum* L.) while there were 181 markers in Pima 3-79 (*G. barbadense* L.) and 188 markers in TM-1 (*G. hirsutum* L.). Variety Pima 3-79 and TM-1 have tetraploid genome while variety, MY, has a diploid genome. The number of TD-MS-RAPD-PCR markers was less in tetraploid genomes than that of the diploid genome. Although higher ploidy genomes produce number of RAPD markers than lower ploidy genomes (Karaca et al. 2002), this was not observed for TD-MS-RAPD-PCR markers in the present study (Fig. 1).

Among 16 primers utilized just three (AT03, W15, and C08) as shown in Fig. 2 detected methylation sensitive polymorphisms among cotton species studied. Locus amplified with primer AT03 contained fully methylated internal cytosine bases. However, these methylations were not polymorphic among the cotton varieties (as indicated with an arrow at the bottom of the panel in Fig. 2). On the other hands, there was a TD-MS-RAPD-PCR marker in variety Pima 3-79. However, this methylation polymorphism was not present in other varieties (as indicated with an arrow in the middle of the panel in Fig. 2). Locus amplified with primer W15 contained two kinds of methylation differences, one of them was not polymorphic among the samples, the other band was polymorphic between *Gossypium hirsutum* (TM-1) and *Gossypium barbadense* (3-79), and between *Gossypium barbadense* (3-79) and *Gossypium herbaceum* (MY). Locus amplified with primer C08 contained two methylation differences among the cotton samples.

To date, more than 52 cotton species have been identified. Among these species, *Gossypium hirsutum* L. is the most commercially cultivated cotton species. However, *Gossypium hirsutum* has very limited genetic diversity probably due to relatively recent polyploidization before intense domestication pressures (Osabe et al. 2014). In the present study, based on TD-MS-RAPD-PCR results we reported that genomic DNA samples of mature cotton seeds have a low level of DNA methylation differences at 5'-CCGG-3' sites. However, several previous research groups published papers revealing that DNA methylation polymorphisms were greater compared to the genetic polymorphisms in *G. hirsutum* accessions. For instance, Osabe et al. (2014) reported that DNA methylation diversity was greater than the genetic diversity in the selected cotton genotypes and significantly different levels of DNA methylation were identified between tissues. In the present study we could study CG type methylation while most of the previous studies investigated not only CG site methylation but also CHG and CHH sites were also studied (Correia et al. 2013; Karaca et al. 2016; Wang et al. 2016). Our results clearly indicated that a large number of extensive sequencing of the methylation-sensitive fragments should be used to examine

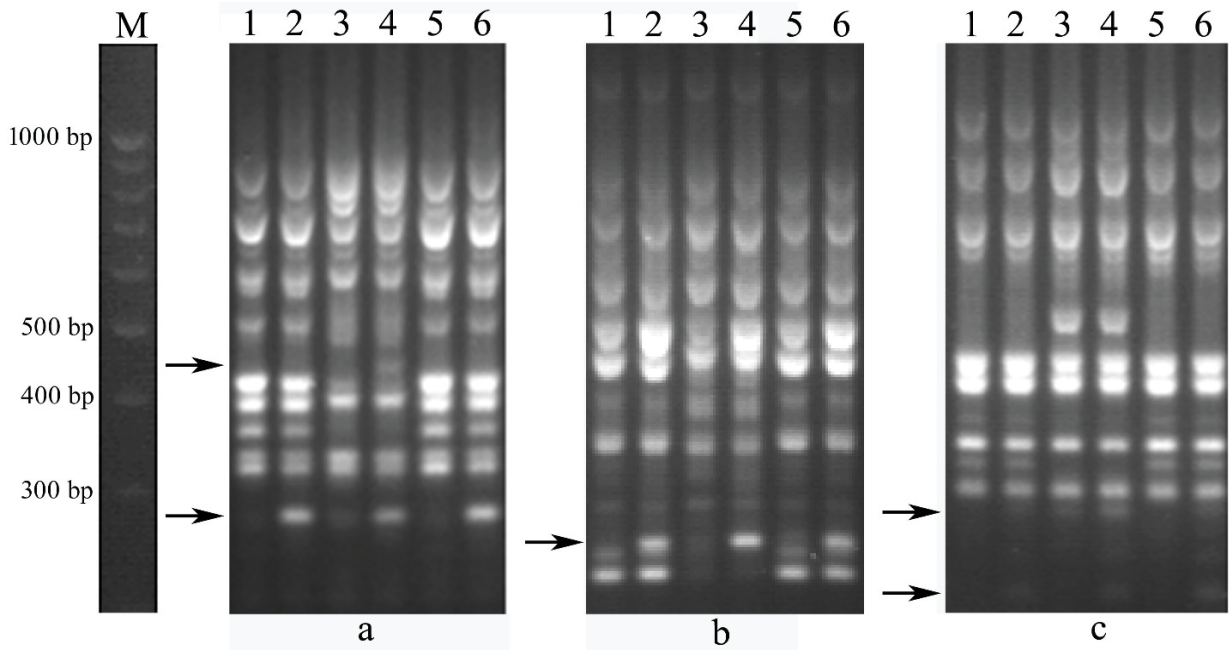

Figure 2. Representation of touch-down TD-MS-RAPD-PCR markers. Panels a to c show TD-MS-RAPD-PCR markers produced using AT03 (a), W15 (b) and C08 (c) oligonucleotide primers. Numbers 1-2 in each panel represent cotton variety TM-1, numbers 3-4 represent Pima 3-79, and numbers 5-6 represent Maydos Yerlisi (MY). Also in each panel, odd numbers represent templates digested with MspI and even numbers represent templates digested with HpaII restriction enzyme. Arrows indicate methylation differences within and between the samples.

genomic regions most affected by genetic and epigenetic changes in cotton.

Based on TD-MS-RAPD-PCR markers obtained from three different cotton varieties belonging to three different cotton species our results indicated that DNA methylation polymorphisms of mature cotton seeds were less than genomic methylation polymorphism in cotton. However, we should mention that TD-MS-RAPD-PCR could only discriminate methylation of cytosine bases present in 5'-CCGG-3' sequence. We should also note that as reported in the literature (Correia et al. 2013) and based on our previous DNA sequencing studies we confirmed that the occurrence of 5'-CCGG-3' sites in cotton genome is low, therefore, some other restriction enzymes specific to CG, CHG and CHH sites could be utilized in TD-MS-RAPD-PCR method in order to enhance the methylation differences in cotton.

Conclusion

In the present study, a touchdown (TD) based methylation sensitive (MS) random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) method (TD-MS-RAPD-PCR) was used to investigate DNA cytosine methylation differences at 5'-CCGG-3' sequences among three cotton varieties, Texas Marker-1, Pima 3-79 and Maydos Yerlisi, belonging to species of *Gossypium hirsutum* L, *G. barbadense* and *G. herbaceum* L., respectively. Target genomic DNA samples were extracted from individual seeds of the varieties. Among 16 oligonucleotide primers utilized, three primers (AT03, W15, and C08) resulted in epigenetic polymorphisms among the varieties. We also confirmed that cotton genomes do not contain high occurrences of 5'-CCGG-3' sites. Many of 5'-CCGG-3' sites studied did not show a high level of DNA cytosine methylations. This indicated that genomic DNA samples extracted from mature seeds were not differentially methylated. We noted that the methylation differences between the species studied in the present study were higher among other tissues of the same species (data not presented). In the present study, our results also revealed that genetic polymorphism among the cotton samples were much greater than the DNA cytosine methylation (epigenetic). Although TD-MS-RAPD-PCR method is cost-effective, requires simple and basic instrumentation, and can easily be performed in any laboratory with basic setup using a regular DNA thermal cycler and DNA gel electrophoresis system, the level of polymorphisms detected were very low in genomic DNA of cotton seeds.

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Authors M. Karaca and A. Aydin contributed equally to this work. This work was partially supported by the Scientific and Technological Research Council of Turkey (TUBITAK, 113O935).

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