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Exonic and Intronic DNA Methylation Differences in a Fiber Specific Gene of Pima Cotton (*Gossypium barbadense* L.)

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Abstract Epigenetic regulations in cotton are poorly understood. In the present study bisulfite sequencing technique was used to assess the level and pattern of cytosine methylation in intronic and exonic regions of a fiber specific gene, GhMADS11, in different tissues (pollen grains, ovules, linter and lint fibers) and development stages of ovules at -1 day before anthesis, 5 and 10 days post anthesis (dpa) and lint fibers (20 and 25 dpa) of Pima 3-79, an extra-long staple cotton. Results revealed that intronic and exonic regions of GhMADS11 gene had different level and pattern of cytosine methylation. Among the tissues studied linter type fibers had the highest level of methylation while pollen grains had the lowest methylation. Exonic region of GhMADS11 gene in elongating lint fibers had the lowest level of DNA methylation while full elongated lint and linter fibers had the highest level of DNA methylation. Differential methylation level and pattern in GhMADS11 gene indicated that level and pattern of cytosine methylation may contribute valuable epigenetic effects on cotton fiber yield and quality.

Keywords development stages, epigenetics, fiber cells, methylation pattern, tissue specific

1. Introduction

Knowledge on epigenetics will be extremely useful to understand cotton ovule and fiber development, yield potential, stress adaptation and bio-defense. Heritable changes in gene function without a change in DNA sequence are commonly defined as epigenetics. Epigenetic variation results from enzyme-mediated chemical modifications at nucleotide and histone levels. Among the enzyme-mediated chemical modifications, DNA methylation is the main mechanism of epigenetics. In plants DNA methylation usually occurs in the three sequence contexts, CpG, CpHpG and CpHpH where H is A, T, or C. De novo methylation in plants is catalyzed by domains rearranged methyltransferase 2 (DRM2) and maintained by different pathways. For instance, methylation of CpG and CpHpG is maintained by DNA methyltransferase 1 (MET1) and chromomethylase 3 (CMT3) while CpHpH methylation is maintained through de novo methylation by DRM2 [1-3].

It is known that the pattern and level of DNA methylation influence the time of flowering, leaf morphology, plant height and fitness, response to heat, salt and light conditions, plant responses to hormones [4-9]. High performance liquid chromatography (HPLC), bisulfite sequencing, methylation specific polymerase chain reaction (MSP), methylation sensitive amplification polymorphism (MSAP), whole genome bisulfite with next generation sequencing are used for assaying and detecting global and gene-specific methylation in plants. Among these methods, bisulfite sequencing is considered the "gold standard" for assessing DNA cytosine methylation since it reveals the methylation status of every cytosine nucleotide in a genome [6, 10].

Up-to-date, a large number of fiber related genes in cotton have been identified. However, methylation levels and patterns of many of these genes are not well characterized. MCM1-AGAMOUS-DEFICIENS-SRF (MADS) box genes have highly conserved 56-amino-acids-long motif known as the MADS box present in animals, fungi, and plants [11-12]. Li et al. [13] isolated and characterized a MADS protein with 151 amino acid residues from cotton fiber cDNA of *G. hirsutum* L. and designated as the GhMADS11. The authors found that the

GhMADS11 was not a transcriptional activator and transcripts of this gene were specifically accumulated in elongating fibers, but low signals of its expressions were detected in other tissues of cotton.

Fiber yield and quality of cotton ovule (seed) may be influenced by the DNA cytosine methylation which is the most studied epigenetic mechanism. The level and pattern of DNA cytosine methylation in the three contexts CpG, CpHpG and CpHpH may have differential effects on gene expression. A large number of studies revealed that methylated genes usually correlate with lower expression level in comparison to unmethylated states. Studies clearly revealed that DNA methylation in promoter regions is known to silence genes while gene body methylation (methylation in intron and exon) is positively or negatively correlated with expression. However, there existed limited number of studies dealing with gene body specific methylation in cotton [6-9, 14].

The main objective of this study was to investigate the level and pattern of CpG, CpHpG and CpHpH methylation in gene bodies of GhMADS11, a cotton MADS box gene. DNA cytosine methylation pattern and level in different tissues (pollen grains, fertilized and unfertilized ovules, and lint and linter fibers) and development stages (ovules at -1 day before anthesis, 5 and 10 days after anthesis, and lint fibers at 20 and 25 days after anthesis) of Pima 3-79, an extra long staple cotton (*Gossypium barbadense* L.) using bisulfite sequencing method.

2. Material and Methods

2.1. Plants tissues

The plant materials used in this study is Pima 3-79 (*Gossypium barbadense* L.) which is a double haploid cotton line. Pima 3-79 produces low yield but it has extraordinary quality fibers. Pima 3-79 is known to have no or very few linter type fibers. We were able to collect linter type fibers from ovules at 25 days post anthesis (dpa). Linter type fibers are short (15 mm or shorter) and coarse fibers while lint fibers are long (25 mm or longer) and thin fibers more desirable in the textile industry [15].

Plants were grown in experiment fields of Akdeniz University, Antalya, Turkey in the growing season of 2012. A total of seven samples consisting of different tissues and development stages were used in this study. Pollen grains from 60-80 flowers were shed on sterile papers at a sterile bench and collected in 2 mL sterile tubes. Samples were stored at -80^{o}C in a deep freezer until DNA extraction studies.

Bracts, stamens, stigmas, styles, sepals and petals of 20-30 unopened flowers at one day before anthesis (-1 dba) were cut off from the ovary base and ovary was removed from these flower parts to collect unfertilized ovules at -1 dba. Bolls at 5 days post anthesis (dpa) and 10 dpa were collected and coats were vertically incised about 3 mm in depth throughout ovary apex to the boll base with a sharp scalpel. Ovules in each of the locule were gently picked up with the help of forceps and collected in 2 mL sterile tubes, stored at -80^{o}C until DNA extraction studies. Bolls at 20 and 25 dpa were collected and bracts were removed. Boll coats were vertically incised about 3 mm in depth and lint and linter type fibers were gently picked up from each ovule with the help of forceps and stored at -80^{o}C until use.

2.2. Target DNA sequence

Genomic DNA sequence of a MADS-box gene (GhMADS11, [13]) was chosen as the target sequence. A pair of bisulfite primers was designed to determine the methylation status of coding (exon) and non-coding (introns) regions of the GhMADS11 gene. Primers were F: 5'-GGATTCGTATTAGATAGGAATATGAG and R: 5'-TTCTACCCTTAAACGCATAAAATC.

2.3. Genomic DNA extraction

Bulked tissue samples were completely frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Approximately 100 mg polyvinyl polypyrrolidone, insoluble (PVPP) were added per gram of the tissues before grinding. A DNA extraction protocol previously described in Karaca *et al*. [16] was used with the following modifications. Preheated (65°C) 2.48 mL extraction solution [0.4 mL 2 M TRIS-HCl, pH 8.0, 0.4 mL 0.5 M EDTA, pH 8.0, 1.6 mL 5 M NaCl, 0.08 mL Triton-X 100], 1.42 mL 5.6% CTAB and 0.1 mL BME were added to powdered 0.5-1.0 g tissues and strongly mixed using a vortex before 2 h incubation at 65°C. During the heat incubation, samples were mixed in every 15 min intervals. Amount, purity, integrity, enzyme accessibility of the genomic DNA samples were verified [17-18].

2.4. Bisulfite conversion

Two independent bisulfite conversion reactions for each DNA sample obtained from bulked samples were performed. Cotton genomic DNA was bisulfite treated using a methyl code bisulfite conversion kit (Invitrogen Corp. Carlsbad, CA, USA). Briefly, 900 µL dd H_2O, 50 µL M-dissolving buffer and 300 µL M-dilution buffer were added per tube of C-T conversion reagent, mixed using a vortex for 1 min, and incubated for 5 min at room temperature. Mixing and incubation steps were repeated one more time. Then 130 µL of bisulfite containing C-T conversion reagent was added into 0.5 µg genomic DNA in 20 µL, thoroughly mixed and briefly centrifuged. Samples were incubated using cycling type of conversion profile as follows: initial denaturation at 98°C for 10 min and incubation at 53°C for 30 min, followed by 8 cycles with incubation at 53°C for 6 min and 37°C for 30 min. Reactions ended with an incubation of 10 min at 4°C using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). After reactions completed, converted DNA samples were immediately purified according to the manufacturer's procedures and dissolved in 22 µL sterile water.

2.5. Touchdown polymerase chain reactions (Td-PCRs)

A touchdown PCR was carried out in 25 µL reaction volume containing 3 µL bisulfite converted or control genomic DNA as the template, 0.5 µM forward and reverse primers (sequences given above), 80 mM TRIS-HCl (pH 8.8), 19 mM $(NH_4)_2SO_4$, 0.009% Tween-20 (w/v), 0.28 mM of each dNTP, 3 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Invitrogen Corp. Carlsbad, CA, USA). The Td-PCR amplification profile was as follows: initial denaturation at 94°C for 3 min, ten cycles with denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec in the first cycle, diminishing by 0.5°C each cycle, and extension reactions at 72°C for 1 min using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA).

Additional 40 more PCR cycles were run using the same cycling parameters mentioned above with constant annealing at 55°C. Denaturation and extension conditions were the same as indicated above. The amplification reactions ended with final extension reactions at 72°C for 10 min [19].

2.6. Electrophoretic purification

A total of 30 µL solutions consisting of 5 µL 6x DNA loading buffer and 25 µL PCR products were mixed and loaded in 3% (w/v) high-resolution agarose gel and electrophoresed at 5 V cm^{-1} at constant voltage for 4-6 hours [18]. After the gel electrophoresis, a plastic wrap between an ultraviolet (UV) transilluminator and the gel was placed. Targeted PCR products were cut a slit just ahead and then a slit just behind using a clean razor blade and purified using a PureLink Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen Corp. Carlsbad, CA, USA). Purified PCR products were eluted in 50 µL sterile water. After adding 5 µL 3 M sodium acetate, pH 5.2 and 125 µL absolute ethanol, samples were stored at -20°C in a freezer until use.

2.7. Cloning and sequencing

Tubes containing the PCR products stored in ethanol were centrifuged at 12000xg for 10 min and the DNA pellet was washed using 80% absolute ethanol. The DNA pellet was dissolved in 20 µL sterile water. After addition of 6 µL 5X ligation buffer, 3 µL pTZ57R/T, and 1 µL T4 DNA ligase enzyme, briefly mixed by vortex and centrifugation 5 second before incubated at 22°C overnight or overnight at 4°C. Using 2.5-4 µL ligation mixture, vectors with PCR products were transformed to *E. coli* bacteria strain JM107 using a Transform Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA USA). After selecting and sub-culturing of colonies, plasmid DNA samples were extracted using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). A total of 16 plasmids containing PCR fragments from bisulfite treated genomic DNA and 8 plasmids containing PCR fragments from untreated DNA (as control) were commercially sequenced using M13R sequencing primers (Macrogen Inc., Amsterdam, The Netherland).

2.8. Detection and statistical analysis of methylation

Sequences were assembled into contigs using the software Sequencher based on the contigs assembling parameters which were set to minimum overlap of 50 bases and 90% identity match. Primer sequences of forward and reverse were identified from each clone sequence and trimmed off along with the vector sequences. Target sequences were formatted into fasta file and analyzed using the KisMeth program [20].

The methylation percentage was calculated for each cytosine sequence context (CpG, CpHpG and CpHpH) using the percentage methylation (%), calculated as 100×C/(C+T). The statistical significance between and within the samples of the entire set of either CpG, CpHpG or CpHpH sites was separately evaluated with the nonparametric Mann-Whitney U test. Two-tailed p values, 0.05 were considered statistically significant. Methylation context (CpG, CpHpG or CpHpH) was used as factor and the methylation percentage was use as response.

3. Results and Discussion

Bisulfite sequencing technique is still considered as a gold standard in DNA cytosine methylation analysis. As judged from reproducibility of methylated and unmethylated CpG, CpHpG and CpHpH contexts between different bisulfite treatments, it was thought that a high molarity, high temperature with increased duration of bisulfite treatment and increased number of thermal denaturation steps [21] followed a touchdown style PCR used in the present study [18] resulted in successful and reproducible C=> T conversion.

Although a total of 16 plasmids from each tissues and development stages were sequenced, 91 sequences had Q20 values. Analysis was performed using these 91 high quality sequences, the number of sequences per sample ranged from 10 to 16 for bisulfite treated samples. A total of 8 control target DNA sequences were compared and a consensus sequence obtained with the help of Sequencher software.

A total of 680 bp DNA sequence was obtained from control DNA samples which were not treated with bisulfite. Searches using basic local alignment search tool (BLAST) revealed that there were 95% identity between sequence of Pima 3-79 (*G. barbadense* L.) obtained in this study and sequence of *G. hirsutum* L. [13] indicating that the sequence studied corresponded to GhMADS11 gene [13]. Further BLAST searches showed that forty-five nucleotides of this sequence were exactly present in the mRNA of GhMADS11, (HM989877) deposited in the National Center for Biotechnology Information (NCBI) database.

Of 680 bp sequence, 1-248 bp and 294-680 bp were intronic sequences while 249-293 bp were exonic sequences [13]. The left intron (intron I) in the target region consisted of 1 CpG, 5 CpHpG and 13 CpHpH cytosine contexts. Exon sequence contained 2 CpHpG and 7 CpHpH while right intron (intron II) consisted of 6 CpG, 5 CpHpG and 27 CpHpH. In total target region consisted of 7 CpG, 12 CpHpG and 47 CpHpH cytosine contexts. Analyses of this study used a total 6005 cytosine containing contexts consisted of 637 CpG, 1091 CpHpG and 4277 CpHpH (Fig. 1).

Pollen grains contained sperm and vegetative cells, unfertilized ovules consisted of haploid egg, synergid and antipodal cells along with the tetraploid outer and inner integuments cells. Lint fiber cells may also contain some linter fibers dissected along. Also there could be some methylated unmethylated cytosine residues treated as methylated or vice versa. For these reasons we used at least 10 replications for each tissue and development stage (Fig. 1).

Among the methylation contexts CpG showed a great level of reproducibility among the replications (sequenced clones). Among 7 CpG sites in the target sequences four were full methylated and 2 were full unmethylated. Among 12 CpHpG, 2 were full unmethylated across the samples while 2 had full methylated cytosine residues. A total of 8 CpHpG showed different level and pattern cytosine methylation and they were very informative in differentiating one tissue from another (Fig. 1). Although CpHpH contexts were the most occurring sites (47) in the target sequence, the level of DNA methylation in this context was very low. Among the 47 CpHpH sites two had full methylation across the samples. Some samples contained tissue specific pattern of CpHpH sites. For instance intron I of unfertilized ovules and linter fibers at 25 dpa contained specific patterns of CpHpH methylation (Fig. 1).

Target DNA regions consisted of 2 introns and 1 exon. Results clearly showed that there were statistically significant methylation level and pattern context differences between exon and intron, and between intron I and II (Table 1). Exon DNA sequence studied did not contain any CpG context (Fig. 1). Among the 7 CpHpH in all the tissues and development stages studied only one, presented between the exon and intron II had full cytosine methylation. Exonic CpHpG contexts in unfertilized ovules (-1 dba) and fertilized ovules (10 dpa) and lint fibers (10 dpa) were not methylated but exonic CpHpG contexts in pollen grains, fertilized ovules (5 dpa), lint and

linter fibers (25 dpa) had varying level and pattern of cytosine methylation. This indicated that CpHpG context methylation involves in tissue and development stages specific expression of GhMADS11 gene in cotton [13].

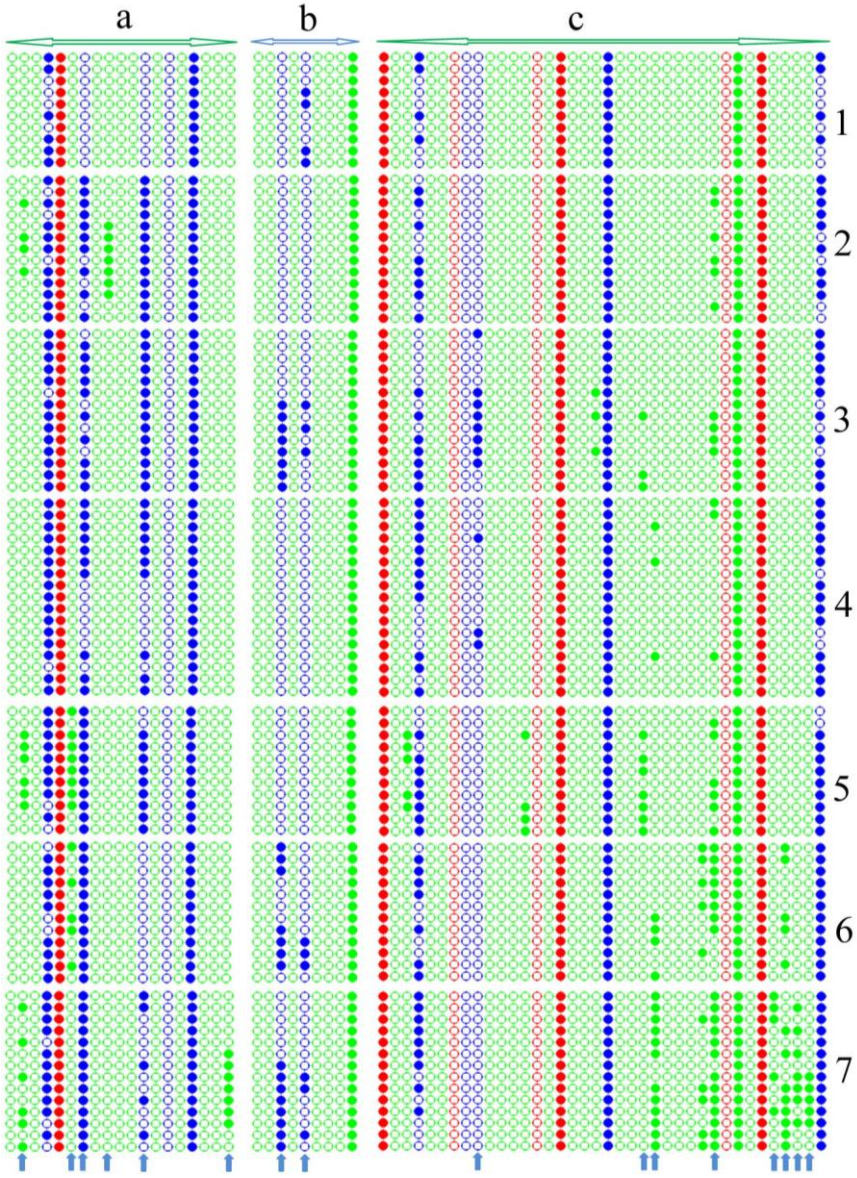

Figure 1: Cytosine methylation patterns of cotton tissues and development stages.
Letters a, b and c are intron I, exon and intron II of GhMADS11 gene. Numbers 1 to 7 are pollen grains, -1 dpa unfertilized ovules, 5 dpa fertilized ovules, 10 dpa fertilized ovules, 20 dpa lint fibers, 25 dpa lint fibers and 25 dpa linter fibers, respectively. Each circle in the panels represents a cytosine residue, methylated cytosine residues are shown with full circles, and non-methylated cytosine residues are shown with empty circles. Cytosine residues are color-coded by their sequence context: red for CpG, blue for CpHpG and green for CpHpH where H is C, T or A. Each row of panels represents a sequence of clone. Box shows one of coding regions of the GhMADS11 gene.

Methylation level and pattern of intron I and II differed. Intron I contained just one CpG which was full methylated while intron II contained 6 CpG contexts, 3 of which were full methylated. Across tissues and development stages the level and pattern of CpG methylation among intron I and among intron II were identical. This indicated that CpG context methylation may not involve in tissue and development stage specific expression of GhMADS11 gene.

Among the tissues and development stages investigated, pollen grains were the only source of haploid cotton genomic DNA resources. In intron I and intron II of GhMADS11 gene in pollen grains, CpHpG methylation was low (32% in intron I and 36% in intron II) in comparison to other samples. Intron I of pollen grains also showed CpHpG pattern methylation differences (Fig. 1). In pollen grains, compared to intron I, intron II had higher level of CpHpG methylation among the samples investigated (Table 1). On the other hand, CpHpG methylation levels in intron I were higher than intron II of GhMADS11 gene in unfertilized and fertilized ovules. CpHpG methylation differences between intron I and II were the highest in unfertilized ovules (-1 dba) indicating that differential gene body methylation may be important in gene regulation.

Table 1: Methylation level and pattern of fiber specific GhMADS11 gene in pima cotton

Sample ID	Gene Body	Size (bp)	Methylation ratio (%)*			
			CpG	CpHpG	CpHpH	C
Pollen grains (Pima 3-79)	Intron I	222	100	32	0	13.68
	Exon	45	na	20	14.28	15.56
	Intron II	363	50	36	3.7	15.26
Unfertilized ovules (-1 dba) (Pima 3-79)	Intron I	222	100	70.76	6.5	28.34
	Exon	45	na	0	14.28	11.11
	Intron II	363	50	46.15	5.41	17.83
Fertilized ovules (5 dpa) (Pima 3-79)	Intron I	222	100	72.85	0	24.44
	Exon	45	na	39.28	14.28	19.84
	Intron II	363	50	57.14	6.34	19.92
Fertilized ovules (10 dpa) (Pima 3-79)	Intron I	222	100	62.35	0	21.67
	Exon	45	na	0	14.28	11.11
	Intron II	363	50	54.11	5.01	18.58
Lint Fibers (20 dpa) (Pima 3-79)	Intron I	222	100	72.72	9.79	31.1
	Exon	45	na	0	14.28	11.11
	Intron II	363	50	50.9	11.11	22.49
Lint Fibers (25 dpa) (Pima 3-79)	Intron I	222	100	55	3.2	21.93
	Exon	45	na	41.66	14.28	20.37
	Intron II	363	50	51.66	10.49	22.15
Linter Fibers (25 dpa) (Pima 3-79)	Intron I	222	100	62.85	7.14	26.69
	Exon	45	na	39.28	14.28	19.84
	Intron II	363	50	52.85	18.25	27.82

*: The methylation levels per sequence motif (CpG, CpHpG, and asymmetric CpHpH), referred as methylation percentage (%) were calculated by dividing the number of non-converted cytosine residues by the total number of cytosine positions within the assay. na: not present.

Compared to intron II, intron I had lower level of CpHpH methylation across samples investigated (Table 1). CpHpH methylation level and pattern in intron I of fibers at 20 dpa were significantly higher from the other samples. Intron I of the GhMADS11 gene in linter fibers at 25 dpa had specific methylation pattern of CpHpH contexts (Fig. 1). In intron II CpHpH methylation levels were higher in lint and linter fibers compared to other samples. Furthermore the level of CpHpH methylation in linter fibers at 25 dpa was even higher than lint fibers at 25 dpa. Although there is no literature about the methylation differences between lint and linter fibers in cotton, Osabe et al. [22] reported methylation differences between cells of inner integument and outer integument of ovules. Since the lint fibers originate from outer integument while linter fibers originate from inner integument observed methylation differences between lint and linter fibers in the present study may indicate that GhMADS11 gene differentially express in lint and linter fibers. Previous study of Li et al. [13] reported that GhMADS11 gene is expressed preferentially during fiber elongation and is either not expressed or lower expressed in other tissues. However further studies on the epigenetic regulations of GhMADS11 gene are needed since this gene has not shown to be associated with a specific phenotype in cotton and is only known via its expression profile and over expression in yeast.

Although the molecular mechanisms controlling cotton fiber initiation, differentiation into linter or lint fibers and elongation remain largely unknown, epigenetic and genetic studies of fiber related genes are expected to produce results that could be used in cotton breeding studies. All the tissues used in this study were dissected from cotton bolls and seeds. Injuries generated during collection and dissection might have affected methylation status of the genomic DNA. Cold, salts, drought, hydrogen peroxide, osmotic stress and light intensity have been reported to affect the status of methylation in plants including cotton [4-6, 22-25]. However, the entire sample used in this study were treated with the same manipulations, therefore, observed variations in the DNA cytosine methylation indicate methylation differences between and within the samples.

There was some certain degree of methylation differences in the GhMADS11 gene across the tissues and development stages used in the present study. Differences in tissue methylation levels have been reported in different crops including cotton using other methods [14, 22, 25-26]. Vining et al. [14] studied seven tissue types consisting of vegetative bud, male and female inflorescence, leaf, root, xylem and phloem in black cottonwood (*Populus trichocarpa*) and found DNA methylation differences among tissues using 5-methyl-cytosine DNA immunoprecipitation followed by Illumina sequencing (MeDIP-seq). Osabe et al. [22] studied DNA methylation level of tissues of cotyledon, stems, roots harvested from 3-week-old plantlets, and mature leaves, stem internodes, mature roots, 0 and 3 dpa ovules, 35 dpa fibers, outer integument and inner integument of 0 dpa ovules collected from 6-month-old mature cotton plants using HPLC. The authors found that plantlet roots had the lowest methylation level and cotyledon, stem internodes, mature leaves, 35 dpa fiber, and inner and outer integument had the highest methylation level. In our study we also found that older tissues contained more methylated DNA in comparison to young ones as reported in Osabe et al. [22]. Tissue level cytosine methylation levels found in our study are in general agreement with previous studies [9, 22, 27-28].

Total DNA cytosine methylation levels compared to individual methylation levels (CpG, CpHpG and CpHpH) across the samples studied were not informative. Total DNA methylation may not show the actual methylation variations within a genome or a sequence target since methylation may increase in a gene body while it may decrease in the other gene body with no gain or loss in total methylation level. In the present study we observed that although total cytosine methylation levels were not statistically significant between some samples such as between intron I of unfertilized ovules at -1 dba (28.34%) and intron I of fertilized ovules at 5 dpa (24.44%), CpHpH in intron I and CpHpG pattern in exon and intron II were quite different. We found that pattern specific methylation contexts were much more informative in terms of tissue identification than the total methylation levels in bisulfite sequencing studies.

In order to utilize epigenetics in cotton breeding, identification of epialleles are very important. We found that there are several CpHpG and CpHpH methylation contexts with single methylation polymorphism in gene bodies of the GhMADS11. Pollen grain, fertilized and unfertilized ovules from TM-1, an upland cotton (*G. hirsutum* L.) and fiber mutant Xu-142fl (*G. hirsutum* L.) cotton lines were included into the study. Analysis revealed that there existed epialleles (alleles with identical DNA sequences but show different level of epigenetic marks such as cytosine methylation) between Pima 3-79, TM-1 and Xu-142fl. Results indicated that since GhMADS11 is fiber elongation specific gene, epialleles of this gene could be used in cotton breeding and epigenetic studies.

There were some minor nucleotide sequence differences in control DNA of GhMADS11 gene among Pima 3-79, TM-1 and Xu-142fl cotton samples studied as noted from the sequence alignment studies. These minor sequence differences were due to insertions or deletions. We noticed that insertion or deletion within the target sequence created new contexts or deleted the existing cytosine contexts. Changes in the level of cytosine methylation due to insertion or deletion have been referred as "effects of genetics on epigenetics" [29]. For instance, Zakrzewski et al. [9] reported that the level of methylation of cytosine was strongly dependent on the sequence contexts. Site specific methylation level differences in cotton species observed in the present study were also previously reported [27].

4. Conclusion

Epigenetic phenomena in cotton are poorly understood, any findings are extremely important for cotton epigenetic studies. Present study used the GhMADS11 gene which is expressed preferentially during fiber

elongation. Our results confirmed that preferential expression of GhMADS11 gene in elongating fibers since its methylation levels were highest in elongated fibers at 25 dpa. Cytosine methylation level and pattern of CpHpG and CpHpH contents between exonic and intronic regions were very different indicating the importance role of DNA methylation in gene expression.

As far as our knowledge this is the first study reporting DNA cytosine methylation differences between lint and linter fibers in cotton. However, GhMADS11 has not been shown to associate with a specific phenotype in cotton and is only known via its expression profile and over-expression in yeast. Further studies are required using other MADS box genes such as GhMYB25-like involved in epigenetic regulation of cotton fiber.

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References

[1]. Vaillant, I. and Paszkowski, J. (2007). Role of histone and DNA methylation in gene regulation, *Current Opinion in Plant Biology*, *10*: 528-533.

[2]. Law, J. A. and Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals, *Nature Reviews Genetics*, *11*: 204-220.

[3]. Shen, H., He, H., Li, J., Chen, W., Wang, X., Guo, L., Peng, Z., He, G., Zhong, S., Qi, Y., Terzaghi, W. and Deng, X. W. (2012). Genome-wide analysis of DNA methylation and gene expression changes in two Arabidopsis ecotypes and their reciprocal hybrids, *Plant Cell 24*: 875-892.

[4]. Karan, R., DeLeon, T., Biradar, H. and Subudhi, P. K. (2012). Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes, *PLoS ONE 7*: e40203.

[5]. Latzel, V., Zhang, Y., Moritz, K. K., Fischer, M. and Bossdorf, O. (2013).Epigenetic variation in plant responses to defence hormones, *Annals of Botany London 110*: 1423-1428.

[6]. Bhardwaj, J., Mahajan, M. and Yadav, S. K. (2013). Comparative analysis of DNA methylation polymorphism in drought sensitive (HPKC2) and tolerant (HPK4) genotypes of horse gram (*Macrotyloma uniflorum*), *Biochemical Genetics*, *51*: 493-502.

[7]. Bian, R., Nie, D., Xing, F., Zhou, X., Gao, Y., Bai, Z. and B. Liu, (2013). Adaptational significance of variations in DNA methylation in clonal plant *Hierochloe glabra* (Poaceae) in heterogeneous habitats, *Australian Journal of Botany*, *61*: 274-282.

[8]. Fan, H. H., Wei, J., Li, T. C., Li, Z. P., Guo, N., Cai, Y. P. and Lin, Y. (2013). DNA methylation alterations of upland cotton (*Gossypium hirsutum*) in response to cold stress, *Acta Physiologiae Plantarum, 35:* 2445-2453.

[9]. Zakrzewski, F., Schubert, V., Viehoever, P., Minoche, A. E., Dohm, J. C., Himmelbauer, H. Weisshaar, B. and Schmidt, T. (2014). The CHH motif in sugar beet satellite DNA: a modulator for cytosine methylation, *Plant Journal*, *78*: 937-95.

[10]. Slotkin, R. K., Vaughn, M., Borges, F., Tanurdzic, M., Becker, J. D., Feijo, J. A. and Martienssen, R. A. (2009). Epigenetic reprogramming and small RNA silencing of transposable elements in pollen, *Cell*, *136*, 2009, 461-472.

[11]. Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saeder, H. and H. Sommer, H. (1990). Genetic control of flower development homeotic genes in *Antirrhinum majus*, *Science*, *250*: 931-936.

[12]. Reichmann, J. L., Wang, M. and Meyerowitz, E. M. (1996). DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. *Nucleic Acids Research, 24:* 3134-3141.

[13]. Li, Y., Ning, N., Zhang, Z., Wu, Y., Jiang, J., Su, S., Tian, F. and Li, X. (2011). A cotton gene encoding novel MADS-box protein is preferentially expressed in fibers and functions in cell elongation, *Acta Biochimica et Biophysica Sinica*, *43*: 607-617.

[14]. Vining, K. J., Pomraning, K. R., Wilhelm, L. J., Priest, H. D., Pellegrini, M., Mockler, T. C., Freitag, M. and Strauss, S. H. (2012). Dynamic DNA cytosine methylation in the *Populus trichocarpa* genome: tissue-level variation and relationship to gene expression, *BMC Genomics, 13*: 27.

[15]. Karaca, M. and Ince, A. G. (2011). New non-redundant microsatellite and CAPS-microsatellite markers for cotton (*Gossypium* L.). *Turkish Journal of Field Crops, 16*:172-178.

[16]. Karaca, M., A.G. Ince, S.Y. Elmasulu, A.N. Onus, and Turgut, K. (2005). Coisolation of genomic and organelle DNAs from 15 genera and 31 species of plants, *Analytical Biochemistry, 343:* 353-355.

[17]. Ince, A. G. and Karaca, M. (2009). The MAGi RNA extraction method: highly efficient and simple procedure for fresh and dry plant tissues, *Journal of the Science of Food and Agriculture, 89:* 168-176.

[18]. Ince, A. G., Karaca, K. and Elmasulu, S. Y. (2014). New microsatellite and CAPS-microsatellite markers for clarifying taxonomic and phylogenetic relationships within *Origanum* L. *Molecular Breeding, 344*, 2014, 643-654.

[19]. Ince, A. G., Yildiz, F. and Karaca, M. (2011). The MAGi DNA extraction method for fresh tissues and dry seeds, *Journal of Medicinal Plants Research, 5*: 5458-5464.

[20]. Gruntman, E., Qi, Y., Slotkin, R. K., Roeder, T., Martienssen, R. A. and Sachidanandam, R. (2008). Kismeth: analyzer of plant methylation states through bisulfite sequencing, *BMC Bioinformatics, 9:* 371.

[21]. Genereux, D. P., Johnson, W. C., Burden, A. F., Stoger, R. and Laird, C. D. (2008). Errors in the bisulfite conversion of DNA: modulating inappropriate and failed conversion frequencies, *Nucleic Acids Research, 36:* 22-150.

[22]. Osabe, K., Clement, J. D., Bedon, F., Pettolino, F. A., Ziolkowski, L., Llewellyn, D. J., Finnegan, E. J. and Wilson, I. W. (2014). Genetic and DNA methylation changes in cotton (*Gossypium*) genotypes and tissues, *PLoS ONE, 9:* e86049.

[23]. Bossdorf, O., Arcuri, D., Richards, C. L. and Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*, *Evolutionary Ecology, 24:* 541-553.

[24]. Zhao, Y., Yu, S., Ye, W., Wang, H., Wang, J. and Fang, B. (2010). Study on DNA cytosine methylation of cotton (*Gossypium hirsutum* L.) genome and its implication for salt tolerance, *Agricultural Sciences in China, 9*: 783-791.

[25]. Jin, X., Pang, Y., Jia, F., Xiao, G., Li, Q. and Zhu, Y. (2013). A potential role for CHH DNA methylation in cotton fiber growth patterns, *PLoS ONE, 8:* e60547.

[26]. Gong, Z., Xue, C., Zhang, M., Guo, R., Zhou, Y. and Shi, G. (2013). Physical localization and DNA methylation of 45S rRNA gene loci in *Jatropha curcas* L., *PLoS ONE, 8*: e84284.

[27]. Keyte, A. L., Ryan, P., Bao, L. and Wendel, J. F. (2006). Intraspecific DNA methylation polymorphism in cotton (*Gossypium hirsutum* L.). *Journal of Heredity, 97:* 444-450.

[28]. Peng, H. and Zhang, J. (2009). Plant genomic DNA methylation in response to stresses: potential applications and challenges in plant breeding, *Progress in Natural Science, 19*: 1037-1045.

[29]. Lieberman-Lazarovich, M., Melamed-Bessudo, C., de Pater, S. and Levy, A. A. (2013). Epigenetic alterations at genomic loci modified by gene targeting in *Arabidopsis thaliana*, *PLoS ONE, 8*: e85383.