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RESEARCH PAPER

DNA methylation in differential gene expression during fiber initiation of cotton (*Gossypium* spp.)

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Abstract: DNA methylation is one of the important epigenetic regulatory mechanisms that controls gene expression and contributes to phenotypic diversity. Methylation status was assayed between the *MCU5(wild type, fuzzyless- linted), MCU5 (mutant type, fuzzyless - lintless)* genotypes of *Gossypium hirsutum* and Fuzzy- linted, Fuzzy- lintless lines of *Gossypium arboreum* cotton for ten fiber associated genes which play critical role in fibre initiation and early elongation stages of fiber development by using Methylation Sensitive Restriction Enzyme-Polymerase Chain Reaction (MSRE-PCR) approach. Results revealed that out of 36 In-silico detected restriction sites (5'-CCGG-3'), 4 (12.9 %) sites were methylated in *MCU5* (WT) and 7 (22.5 %) sites in *MCU5 (fl)* of *Gossypium hirsutum* cotton and 8 (25 %) sites in Fuzzy-linted (FL) and 4 (12.5 %) sites in Fuzzy-lintless (Fl) of *Gossypium arboreum* cotton. Methylation polymorphism observed in few sites between the two genotypes of both *Gossypium hirsutum* and *Gossypium arboreum* cotton indicates there is a difference in methylation status of the cytosine at a particular site on the DNA of fibred and fibreless genotypes at +1 Days Post Anthesis (DPA) stage. The difference in the methylation pattern at a particular site hints that, DNA methylation may have role in gene regulation of fiber initiation in cotton.

Key words: Cotton, Fiber Initiation, Methylation polymorphism, Mutants

Introduction

Cotton (*Gossypium* spp.) is an important fibre crop commercially cultivated across the world as a major source of natural textile fibre with 152.31 million bales (1bale =170kg) production and yield of 802 kg/ha during the year 2017-18 (Anon., 2019). The genus *Gossypium* comprises about 45 diploid and 6 tetraploids species (Wendel and Cronn 2003). Among them, only four are being commercially cultivated for natural fibre, which includes, two tetraploids, *G. hirsutum* L. [2n = 4x = 52 (AD1)] and *G. barbadense* L. [2n = 4x = 52 (AD2)] and two diploids, *G. herbaceum* L. (2n =4 x = 26, A1) and *G. arboreum* L. (2n = 4x =26, A2) (Stewart, 1975). Cotton serves as an ideal plant for different biological studies such as genome evolution, polyploidy (Quin and Zhu, 2011) and single-cell system for studying the control of cell differentiation, elongation and carbon partitioning to cellulose synthesis (Haigler *et al.*, 2005; Kim and Triplett, 2011). Fibre development undergoes four distinct but overlapping stages among them fiber initiation and elongation stages have a great effect on number, length and fineness of fibers, which are major factors in determining fibre quality and yield. The molecular processes which trigger or repress the fibre initiation from ovule epidermis is a central issue in understanding fibre biology and one of the major limitations in genetic improvement of cotton fibre is the paucity of information on gene regulation of fibre development at molecular level.

During the cotton breeding several spontaneous mutants defective in fibre development were found and these mutants specific to the fibre trait are serve as excellent system to elucidate molecular mechanism of cotton fibre development and a useful tool for understanding genetics and physiology of fiber development (Salih *et al.*, 2016; Ding *et al.*, 2014 and Wang *et al.*, 2010). Many comparative transcriptome and proteome studies between the normal wild type and their fuzzless- lintless mutants identified key genes and pathways involved in different stages of fiber development and they also revealed down regulation of majority of transcripts, which are involved in an important metabolic processes of fibre initiation and differentiation at the fibre initiation and elongation stages in the fuzzless- lintless mutants (Yu *et al.*, 2000; Li *et al.*, 2002; Liu *et al.*, 2012; Padmalatha *et al.*, 2012 and Hande *et al.*, 2017). Sunil *et al.,* 2019 indicated that fibreless trait is controlled by double recessive genes in cotton.

In the past decade, there has been a remarkable revolution in the field of molecular genetics. DNA methylation is one of the important epigenetic events, which influences many vital processes including gene expression and the most likely method of action is the transcriptional gene repression (Zilberman *et al.*, 2007). Therefore, the information regarding the methylation status of a particular gene provides an important knowledge on transcriptional control (Yaish *et al.*, 2014). In cotton, many functional studies have revealed up-regulation and down-regulation of fibre associated genes at different stages of fibre development. But the reason behind their differential level of expression is still mysterious and as far as the fibre traits are concerned, very less research studies have been focused on DNA methylation. Since fibre development is a very complex process involving a large set of genes, their epigenome data gives a tremendous opportunity to improve the fibre trait by manipulation at DNA level. Therefore, present study we report methylation status of the selected fiber initiation associated genes in cotton.

Material and methods

Plant materials and DNA extraction

The *G. hirsutum* L.cv. *MCU5* (wild-type, fuzzless- linted), its near isogenic fuzzless-lintless mutant, which differed only for fibre trait and other morphological characters like plant height, leaf size, leaf hairiness, stem pigmentation, stem hairiness, boll shape, boll color, petal, pollen color, anther color and anther filament color found similar for both the genotypes (Padmalatha *et al.*, 2012). Fuzzy-linted (FL) and Fuzzy-lintless (Fl) lines of *G. arboreum* diploid cotton were similar for the morphological characters like plant height, leaf size, leaf hairiness, stem pigmentation, stem hairiness, boll shape, boll color, petal, pollen color, anther color and anther filament color except the lint trait (Hande *et al.*, 2017). The Fuzzy-lintless (Fl) line of G. arboreum was derived from limited backcross breeding of a cross between *Gossypium herbaceum* L.cv. Jayadhar (A genome) and *G. anomalum (B genome).* All these four genotypes were grown at the Main Agricultural Research Station (MARS), UAS, Dharwad by following recommended agronomic practices by the University during *kharif* season of 2017. Flowers were tagged on the day of anthesis and considered as '0' DPA. Ovules of +1 DPA stage were collected and frozen in liquid nitrogen immediately and stored at -80ÚC until used for the total genomic DNA extraction and all the molecular experiments were carried out at the Institute of Agricultural Biotechnology (IABT). Genomic DNA was extracted from +1 DPA ovules by following CTAB (Cetyltrimethylammonium bromide) method. DNA quality was determined by Nano-Drop™ 1000 spectrometer at the absorbance of 260/280nm ratio and DNA integrity was assessed by 0.8% TAE (Tris–Acetic Acid EDTA) agarose gel electrophoresis.

In-silico analysis of gene sequences and primer designing

Reference research articles were cited to get the information on cotton genomics and for the present study ten genes having critical role in fiber initiation was selected and listed in Table 1. Gene sequences including 1.5kb upstream regulatory region nucleotide sequenceswere retrieved for each gene from the NCBI Gene Database and *In-silico* custom digestion was performed for the methylation sensitive enzyme (*Hpa*II) to detect 'CCGG' motifs by NEB cutter V2.0 labtool (Vincze *et al.*, 2003). Site specific primers were designed for each restriction site by using NCBI Primer-BLAST software in such a way that primers should not have the restriction site (*i.e.,* CCGG) within the primer sequence.

Methylation sensitive Restriction Enzyme (MSRE)- PCR assay

MSRE-PCR technique followed the principle of, if the cytosine of the CpG is not methylated, then the methylation sensitive enzyme cleaves as expected and cannot be amplified by the further PCR. If the cytosine is methylated, then the enzyme cannot cleave the DNA, therefore, DNA remains intact and can be amplified with suitable primer pairs. This assay was performed by taking 1µl of aliquot (200ng/µl) of genomic DNA of each genotype and digested with 10U of *Hpa*II (New England Biolabs) and *Msp*I (New England Bio labs) separately in a total volume of 20µl with appropriate buffer at 37°C for 12h and reaction was stopped by heating mixture to 65°C for 20 minutes. A parallel reaction was carried out by adding all the components except the enzyme and it served as positive control for the further PCR reaction. Restriction digested DNA samples were diluted in the ratio 1:2 with molecular grade water and used as the template for PCR analysis. PCR amplification was performed by adding 1µl of template DNA, 2 µl of Taq assay buffer (10X), 1.5 µl of dNTPs (2.5mM), 2 µl primers (10 Pico moles), 0.3 µl of Taq DNA polymerase (New England Bio labs) in a total volume of 20 µl and the reaction entailed, denaturation at 94ÚC for 1 min, annealing process by varying the temperature according to the primers followed by 30 cycles of primer extension (72 °C for 2 min) and one cycle for final extension with 72°C for 2 min and finally kept for hold at 4°C. The PCR amplified products were loaded on 2% agarose gel and visualized in Gel documentation unit.

Presence of amplicon at *Hpa*II lane was the indication for the methylation of a particular site. If the amplicon is visualized in the *Hpa*II lane, that site was considered as methylated and scored as "M" and if there is no amplicon, it's considered as "Unmethylated" and scored "UM". A site was considered ''Methylation Polymorphic (MP)" if methylation found in the DNA of the one genotype and not in the other *i.e.*, fibred and fibreless genotypes of *G. arboreum* and *G. hirsutum* cotton.

Table 1. Genes selected for the present study

Sl. No	Gene	Annotation	Reference
1	MYB25	GhMYB25 transcription factor	Lee *et al.*, 2006; Wu *et al.*, 2006; Wu *et al.*, 2007
2	LTP1	Lipid Transfer Protein	Lee *et al.*,2006
3	RDL	Dehydration induced protein RD22 -like protein	Lee *et al.*, 2002; Wu *et al.*, 2006; Taliercio and Boykin, 2007
4	SUS	Sucrose synthase	Notle *et al.*,1995; Ruan and Chourey, 1998; Ruan *et al.*, 2003
5	SPS	Sucrose phosphate synthase	Lee *et al.*, 2006
6	ABP	Auxin binding protein	Ji *et al.*, 2003
7	KCH	Kinesin (KCH1)	Preuss *et al.*, 2004
8	FDH	Fiddlehead like protein	Lee *et al.*, 2002; Wu *et al.*, 2006; Taliercio and Boykin, 2007
9	CESA5	Cellulose synthase catalytic subunit 2	Lee *et al.*, 2002
10	CESA10	Cellulose synthase catalytic subunit 8	Lee *et al.*, 2002

Results and discussion

Cotton fibre is a complex biological system and is a net result of intricate interplay of array of fiber associated genes. The genetic complexity of the cotton fiber transcriptome lies in the involvement of roughly 45,000 genes in allotetraploid cotton genome and accounts for a significant proportion (61%) of all the genes in the cotton genome (Hu *et al.*, 2019). Increasing the number of fibre initials is one of the approaches to obtain maximum fiber yield. A functional genomic study by Padmalatha *et al.* (2012) on the fuzzless- lintless mutant of *G. hirsutum* L. cv. *MCU5* revealed key genes and pathways for the fibre initiation and elongation and also differential expression of genes in fuzzless- lintless mutant compared to wild type. In diploid *G. arboreum* cotton, a study by Hande *et al.* (2017) have also elucidated up-regulation of few genes in fuzzy- linted whereas those transcripts were found down regulated in fuzzy- lintless lines. Unearthing the reason behind differential expression of fibre genes at the molecular level would pave a way to improve the fibre trait by employing advanced biotechnological approaches. In the present study, ten genes were selected based on previous functional genomic studies and listed in Table 1. *In-silico* analysis of these sequences identified 36 restriction sites (CCGG) for methylation sensitive enzyme (*Hpa*II). Out of these, 10 sites were residing on promoter region and 26 are on gene body region and their position on the sequences is as mentioned in Table 2.

In cotton, on the way to explore the significance of DNA methylation, Osabe *et al*. (2014) found DNA methylation diversity was greater than the genetic diversity in selected cotton genotypes of *G. hirsutum* accessions and also studies by Song *et al*. (2015) and Keyte *et al*. (2006) also mentioned the regulatory role of DNA methylation on fiber development. The present study concentrated on detecting the methylation pattern of inner cytosine of CCGG sites within the selected gene sequences in four genotypes. In diploid *G. arboreum* cotton, methylation found at 8 (25%) restriction sites (RDL P2, RDL P4, RDL P6, ABP P3, KCH P1, FDH P4, CesA10 P1 and CesA10 P3) in Fuzzy-linted line and 4 (12.5%) in Fuzzy- lintless line (RDL P5, ABP P2, FDH P4 and CesA10 P3). Only two sites (FDH P4 and CesA10 P3) were commonly methylated in both the genotypes and at 8 sites methylation polymorphism were observed (RDL P2, RDL P4, RDL P5, RDL P6, ABP P2, ABP P3, KCH P1 and CES A10 P1). In tetraploid *G. hirsutum* cotton, methylation observed at 4 sites (12.9%) in normal wild type (RDL P5, SUS P5, ABP P3 and CesA10 P3) and 7 (22.5%) sites in mutant (RDL P5, ABP P2, ABP P3, FDH P4, FDH P5, CesA10 P1 and CesA10 P3). Three sites (RDL P5, ABP P3 and CesA10 P3) were methylated both in wild type and mutant genotypes and at 5 sites polymorphism was observed (SUS P5, ABP P2, FDH P4 and FDH P5 AND CES A10 P1) (Table 3, Fig. 1).

Methylation polymorphism observed between the fibered and fiber less genotypes of *G. hirsutum*, at 6 sites and in *G. arboreum*, at 8 sites. Polymorphism at few sites indicates there is a difference in methylation pattern in the DNA of fibred and fibreless genotypes of *G. hirsutum* and *G. arboreum* cotton at +1 DPA stage of fibre development. When we look at the expression pattern of some genes which were down regulated in fibreless genotypes, we have got few sites like ABP P2, FDH P4, FDH P5 and CES A10 P1 in tetraploid and RDL P5, ABP P2 in diploid cotton, where only fibreless genotype showed methylation. It hints methylation in these sites may have the role in transcriptional repression function. But, to correlate methylation pattern to gene expression with maximum confidence further experiment is needed to find the methylation status of all the cytosines in the sequences because, in plant genome methylation occurs at CHH and CHG along with CG context (Henderson and Jacobsen, 2007). Therefore, almost all the cytosine residues may be prone to methylation and contributes to phenotypic changes and here we examined only a small number of cytosine residues in the gene sequences by

Table 2. Restriction positions on the sequence of selected genes

Gene	Restriction site on sequence	Restriction position (C^CGG)	Region on sequence
MYB transcription factor	MYB site 1	1538 ^ 1539	Gene body
Lipid Transfer	LTP site 1	-417 ^ -146	Regulatory
Protein	LTP site2	268 ^ 269	Gene body
Dehydration	RDL site 1	-471^ -470	Regulatory
induced protein	RDL site 2	-343 ^ -342	Regulatory
RD22-like protein	RDL site 3	478 ^ 479	Gene body
	RDL site 4	919 ^ 920	Gene body
	RDL site 5	1391 ^ 1392	Gene body
	RDL site 6	1644 ^ 1645	Gene body
Sucrose synthase	SUS site 1	-153 ^ -152	Regulatory
	SUS site 2	1855 ^ 1856	Gene body
	SUS site 3	2064 ^ 2065	Gene body
	SUS site 4	2645 ^ 2646	Gene body
	SUS site 5	3379 ^ 3380	Gene body
	SUS site 6	4248 ^ 4249	Gene body
Sucrose Phosphate	SPS site 1	179 ^ 180	Gene body
Synthase	SPS site 2	590 ^ 591	Gene body
	SPS site 3	4732 ^ 4733	Gene body
Auxin Binding	ABP site 1	-173 ^ -172	Regulatory
Protein	ABP site 2	209 ^ 210	Gene body
	ABP site 3	286 ^ 287	Gene body
Kinesin	KCH site 1	-467 ^ -466	Regulatory
	KCH site 2	-264 ^ -263	Regulatory
	KCH site 3	3311 ^ 3312	Gene body
	KCH site 4	5458 ^ 5459	Gene body
	KCH site 5	5855 ^ 5856	Gene body
Fiddlehead like	FDH site 1	-389 ^ -388	Regulatory
protein	FDH site 2	-75 ^ -74	Regulatory
	FDH site 3	127 ^ 128	Gene body
	FDH site 4	1044 ^ 1045	Gene body
	FDH site 5	1178 ^ 1179	Gene body
	FDH site 6	2056 ^ 2957	Gene body
Cellulose synthase A5	CES A5 site 1	912 ^ 913	Gene body
Cellulose synthase A10	CES A10 site 1	-105 ^ -104	Regulatory
	CES A10 site 2	5960 ^ 5961	Gene body
	CES A10 site 3	7431 ^ 7432	Gene body

Table 3. Methylation patterns obtained by MSRE-PCR in *Gossypium hirsutum* and *Gossypium arboreum* cotton

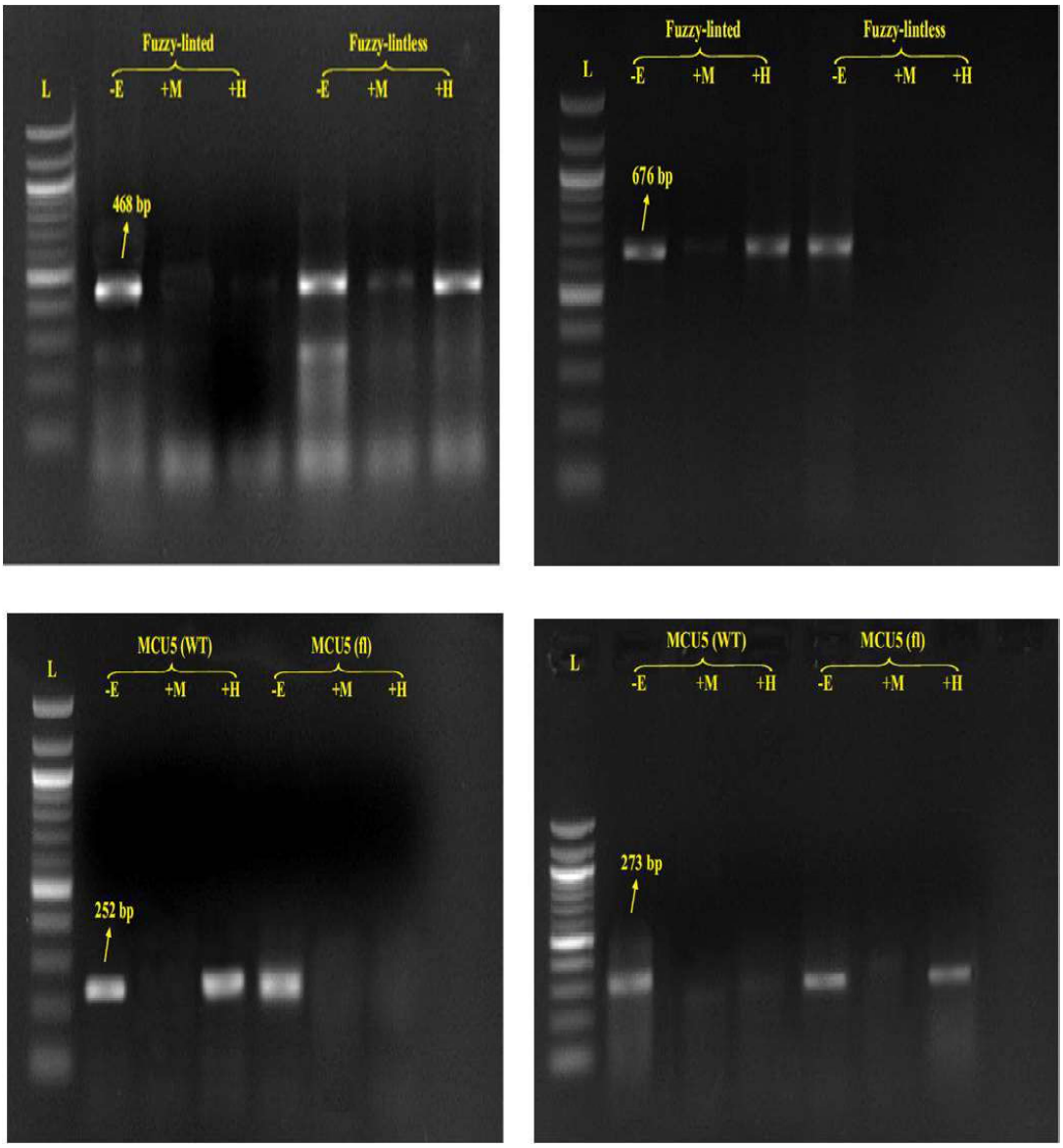
Sl. No.	Gene name	Primer	Fuzzy- linted (FL)				Fuzzy- lintless (fl)				MCU5 (WT)				MCU5 (fl)			
			-E	+M	+H	Score	-E	+M	+H	Score	-E	+M	+H	Score	-E	+M	+H	Score
1	GhMYB25 transcription factor	MYB P1	-	-	-	UM	-	-	-	UM	-	-	-	UM	-	-	-	UM
2	Lipid Transfer Protein	LTP1 P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
3		LTP1 P2	-	-	-	UM	-	-	-	UM	-	-	-	UM	-	-	-	UM
4	Dehydration induced protein RD22-like protein	RDL P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
5		RDL P2	+	+	+	M	+	-	-	UM	+	-	-	UM	+	-	-	UM
6		RDL P3	+	-	-	UM	+	-	-	UM	-	-	-	UM	-	-	-	UM
7		RDL P4	+	+	+	M	+	-	-	UM	+	+	-	UM	+	-	-	UM
8		RDL P5	+	+	-	UM	+	+	+	M	+	-	+	M	+	-	+	M
9		RDL P6	+	-	+	M	+	-	-	UM	+	-	-	UM	+	-	-	UM
10	Sucrose synthase	SUS P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
11		SUS P2	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
12		SUS P3	+	+	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
13		SUS P4	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
14		SUS P5	+	-	-	UM	+	-	-	UM	+	-	+	M	+	-	-	UM
15		SUS P6	-	-	-	UM	-	-	-	UM	-	-	-	UM	-	-	-	UM
16	Sucrose phosphate synthase	SPS P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
17		SPS P2	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
18		SPS P3	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
19	Auxin binding protein	ABP P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
20		ABP P2	+	-	-	UM	+	-	+	M	+	+	-	UM	+	-	+	M
21		ABP P3	+	+	+	M	+	+	-	UM	+	+	+	M	+	+	+	M
22	Kinesin (KCH1)	KCH P1	+	-	+	M	+	-	-	UM	+	-	-	UM	+	-	-	UM
23		KCH P2	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
24		KCH P3	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
25		KCH P4	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
26		KCH P5	+	-	-	UM	+	-	-	UM	+	-	-	UM	-	-	-	UM
27	Fiddlehead like protein	FDH P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
28		FDH P2	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
29		FDH P3	+	-	-	UM	+	-	-	UM	+	+	-	UM	+	-	-	UM
30		FDH P4	+	+	-	M	+	+	+	M	+	+	-	UM	+	+	+	M
31		FDH P5	+	-	+	UM	+	-	-	UM	+	+	-	UM	+	-	+	M
32		FDH P6	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
33	Cellulose synthase catalytic subunit 2	CESA5 P1	+	-	-	UM	+	-	-	UM	+	-	-	UM	+	-	-	UM
34	Cellulose synthase catalytic subunit 8	CESA10 P1	+	-	+	M	+	-	-	UM	+	-	-	UM	+	+	+	M
35		CESA10 P2	-	-	-	UM	-	-	-	UM	-	-	-	UM	-	-	-	UM
36		CESA10 P3	+	+	+	M	+	+	+	M	+	+	+	M	+	+	+	M
		TOTAL	32			8	32			4	31			4	31			7

'+' – presence of amplicon; '-' – absence of amplicon; '-E' – without enzyme; '+M' – with *Mspi;* and '+H' – with *Hpaii*

Figure 1: Morphology of *Gossypium hirsutum* and *Gossypium arboreum* cotton

a) Fuzzy-linted (FL) plant, b) Fuzzy-lintless (Fl) plant, c) MCU5 (wild type, Fuzzless-linted) plant, d) MCU5 (mutant type, Fuzzless-lintless) plant.

e) Fuzzy-linted (FL) seed, f) Fuzzy-lintless (Fl) seed, g) MCU5 (wild type, Fuzzless-linted) seed, h) MCU5 (mutant type, Fuzzless-lintless) seed.



L - 100 bp ladder -E -Without enzyme +M –with MspI +H - with HpaII (methylation sensitive enzyme)
Figure 1: Methylation polymorphism (representative figures)

a) *G.arboreum* diploid cotton Auxin binding protein (ABP P2),
b) *G.arboreum* diploid cotton Cellulose synthse A10 (Ces A10 P1)
c) *G.hirsutum* tetraploid cotton Cellulose synthase A10 (Ces A10 P3),
d) *G.hirsutum* tetraploid cotton Fiddlehead protein (FDH P4)

employing enzymatic method where methylation status of cytosine revealed only within the CCGG motifs and it covered a part of the cytosine residues within the methylome of selected genes. The differences observed in the methylation patterns at a particular site in the DNA of fibred and fibreless genotypes suggests, DNA methylation may have the regulatory role in the gene expression at transcription level and this data gives valuable insight for further research works.

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