Bisulfite Primer Pairs for Analysis of Cotton (*Gossypium* spp.) DNA Methylation

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Abstract Reliable analysis of DNA cytosine methylation of bisulfite sequencing mainly depends on the selection of target regions and primer pairs that flank differentially methylated cytosine contexts. Bisulfite sequencing method is still the choice of method in cytosine DNA methylation studies of polyploid species. One of the most important aspects of bisulfite sequencing is the development of primer pairs. A total of 82 primer pairs were designed, 18 reliably amplified bisulfite treated and control genomic DNA of *G. hirsutum* L. cv. TM and *G. barbadense* L. cv. Pima 3-79. Target regions of the primer pairs consist of promoters, intergenic and gene body regions. With the exception of two primer pairs specific to mitochondrial and chloroplast regions, nuclear genome specific 16 primer pairs produced varying degree of cytosine methylation polymorphisms between Upland cotton and Pima cotton cultivars. Level and pattern methylation differences within and between the tissues and developmental stages detected with the use of primer pairs reported in this study could provide information on allele specific methylation and their epigenetic effects in cotton.

Keywords differential methylation, epigenetics, *Gossypium*, gene body, promoter, touch-down PCR

1. Introduction

The term epigenetics refers to the study of heritable variations in gene expression, not controlled changes in DNA sequence. DNA cytosine methylation is among the most studied epigenetic modifications in plants since it influences many cellular events including chromatin structure, gene expression, genomic imprinting, and recombination and DNA replication [1]. However, epigenetic studies in cotton (*Gossypium* spp.) lag behind many other important plant species. One of the widely utilized techniques to study DNA methylation is the bisulfite conversion technique because it provides information about the methylation profile of every single cytosine methylation in contexts of CpG, CpHpG and CpHpH, where H refers to A, C or T. This technique has not been widely utilized in cotton due to the difficulties of amplification of bisulfite treated DNA in comparison to methylation-sensitive amplified polymorphism (MSAP) [2-3].

Bisulfite sequencing takes advantage of the selective and complete conversion of unmethylated cytosine nucleotides to uracil nucleotides by the application of sodium bisulfite. Converted cytosine nucleotides are amplified by polymerase chain reactions (PCRs) as thymine nucleotides while methylated cytosine nucleotides are untouched [4-5]. After sub-cloning of the targeted products from different samples, comparison studies between control and bisulfite treated sequences reveal the methylation status of the region of interest. Compared with other approaches, bisulfite methylation analysis has more quantitative accuracy, detection sensitivity, efficiency and simplicity [4-5]. However, there are two main drawbacks of this method. First, methylated and unmethylated DNAs may be amplified with differing efficiencies and second, 5-hydroxymethylcytosine could not be differentiated from 5-methylcytosine nucleotides. Fortunately the use of degenerate bisulfite primer pairs, Tet-assisted and oxidative bisulfite sequencing methods effectively solved these problems [6-7]. A technical advantage of bisulfite sequencing resides in the use of PCR but this step is often the most difficult one in the whole process.

Accurate analysis of DNA cytosine methylation based on bisulfite sequencing mainly depends on the selection of primer pairs that flank differentially methylated cytosine contexts. Here we report 18 primer pairs obtained from scanning of 82 degenerate and non-degenerate bisulfite primer pairs. All the primer pairs reported in this study successfully amplified bisulfite converted and control DNA samples extracted from different tissues and development stages of two cotton species; *Gossypium hirsutum* L. and *G. barbadense* L.

2. Materials and Methods

2.1. Development of bisulfite primer pairs

Exon, intron, 5'-untranslated regions (5'-UTR) and 3'-UTR, promoter sequences of *G. hirsutum* and *G. barbadense* genomic DNA sequences deposited in NCBI GenBank databases were used in primer design studies. Genes included reversibly glycosylated polypeptide (*GhRGP1*), sucrose synthase 3 (*Sus3*), obtusifoliol-14-alpha-demethylase, MADS protein (*MADS11*), *WRKY*, *FSltp4*, ethylene responsive transcription factor *GhERF4*, vacuolar invertase 1 and 2 (*VacInv1* and *VacInv 2*), proline-rich protein *GhHyPRP4*, fiber-specific protein (*SCFP*), *lea* gene for seed protein D-113, cellulose synthase A4 (*CesA4*), alpha-globulin, glucuronosyl transferase, fiber related *E6*, clathrin-associated adaptor *GhAPm*, cellulose synthase A4 (*CesA4*), mitogen-activated protein kinase 7 (*MPK7*), lipid transfer protein (*LTP6*) and (*LTP3*), glutamine synthetase (*GS*), alcohol dehydrogenase (*AdhC*), RNA polymerase C1 (*rpoC1*), 14-3-3-like protein (*14-3-3L*), adenylyl cyclase associated protein (*CAP*), cellulose synthase A1 (*CelA1*) gene, cellulose synthase A3 (*CelA3*), expansin (*Exp*), glycosyl hydrolase (*ManA2*), membrane-anchored endo-1,4-beta-glucanase (*CEL*), sucrose synthase (*Sus1*), fiber-specific protein (*FbL2A*), translation initiation factor eIF-2 subunit alpha (*eIF2A*), 3-ketoacyl-CoA synthase 13 (*KCS13*), 5 pectate lyase (*Pel*), SUR4 membrane family protein (*SUR4*) and alcohol dehydrogenase A (*AdhA*).

A total of 82 non-degenerate and degenerate primer pairs from above gene bodies and promoters were designed and called EPi001-082. Non-degenerate primer pairs were designed using Primer 3 [8] based on the following main parameters: GC content was set between 40% and 80%, annealing temperature (Tm) was set between 58°C and 62°C, and expected amplified product size was defined as 300–800 bp. Degenerate primer pairs were designed from non-degenerate primer pairs. Cytosine bases (C) in the forward primers were replaced with Y (C/T) and the guanine bases (G) in the reverse primers are replace with R (A/G).

Sequences of reference (controls) and bisulfite treated target regions were first aligned to create contigs using Sequencher software. Contigs assembly parameters were set to a minimum overlap of 50 bases, 90% identity match, and the large gap option was implemented. Contigs consisted of the sequenced reads, as well as reverse complements. All data sets containing bisulfite treated sequences and the reference sequence were analyzed using the default setting of Kismeth program which used alignment lengths equal or greater than 50% of the reference sequence length and equal or greater 80% positive match in the alignment [9].

2.2. Plants materials

Plant materials used in this study consisted of Texas Marker-1 (TM-1) and Pima 3-79. TM-1 belongs to *Gossypium hirsutum* L. TM-1 produces high fiber yield but low fiber quality. On the other hand, Pima 3-79, which belongs to *Gossypium barbadense* L., produces low yield but higher quality fibers.

2.3. Genomic DNA extraction

Ovules at the day of anthesis were used as plant materials from TM-1 and Pima 3-79. Ovules were removed from bolls and stored in a -86°C freezer until use. For DNA extraction studies frozen samples were ground to a powder with a mortar and pestle in the presence of liquid nitrogen. Approximately 100 mg polyvinyl polypyrrolidone, insoluble (PVPP) were added per gram of the tissues before grinding. A DNA extraction protocol previously described in [10] was used with the following modifications. Preheated (65°C) 2.48 mL extraction solution [0.4 mL 2 M tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl), pH 8.0, 0.4 mL 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, 1.6 mL 5 M NaCl, 0.08 mL Triton-X 100], 1.42 mL 5.6% cetyltrimethylammoniumbromide (CTAB) and 0.1 mL beta ethidium bromide (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 [11-12].

2.4. Bisulfite conversion

Two independent bisulfite conversion reactions for each cotton lines were prepared. Genomic DNA samples were bisulfite treated using a methyl code bisulfite conversion kit (Invitrogen Corp. Carlsbad, CA, USA). C-T conversion buffer was prepared by adding 900 μL dd H_2O, 50 μL M-dissolving buffer and 300 μL M-dilution buffer. The solution was mixed using a vortex for 1 min, and incubated for 5 min at room temperature. Then 130 μL of bisulfite containing C-T conversion reagent was added into 0.5 μg genomic DNA in 20 μL volume, thoroughly mixed and briefly centrifuged. Samples were then incubated using a 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. Conversion 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 (Table 1), 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 [13].

2.6. Agarose gel purification of amplified products

PCR products in the presence of 1X DNA loading buffer were loaded in 3% (w/v) high-resolution Serva agarose gels (SERVA Electrophoresis GmbH, Heidelberg, Germany) and electrophoresed at 5 V cm^{-1} at constant voltage for 4-6 hours [12]. After the electrophoresis completed, a plastic wrap between an ultraviolet (UV) transilluminator and the gel was placed. 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 DNA samples were eluted in 13 μL sterile water.

2.7. Ligation, transformation, cloning and sequencing

To tubes containing the 13 μL purified PCR products, 2 μL 10X ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25°C), 2 μL PEG (%50 (w/v) polyethylene glycol 4000) 2 μL pTZ57R/T, and 1 μL T4 DNA ligase enzyme (5 u/μL) were added and briefly mixed by vortexing and incubated at 22°C for at least 2 h for ligation reactions. Using 2.5-4 μL ligation mixture, vectors with PCR products were transformed to *E. coli* bacteria strain JM109 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 12-16 plasmids containing PCR fragments from bisulfite treated genomic DNA and 4-8 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 [9].

The methylation percentage was calculated for each cytosine sequence context (CpG, CpHpG and CpHpH) using the percentage methylation (%), calculated as $100 \times C/(C+T)$. 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

Majority of primer pairs designed could amplify genomic DNA samples of Pima 3-79 and TM-1. Sizes of the amplified products of primer pairs ranged from 250 bp to 1000 bp (Figure 1). Among the 82 primer pairs designed in the present study nine pairs failed to amplify both of the template DNAs (Pima 3-79 and TM-1), three primer pairs failed to amplify either Pima 3-79 or TM-1 genomic DNA sample. A total of 8 primer pairs produced amplified products larger than 800 bp (for example lanes 30-31 and 34-35 in Figure 1). Primer pairs producing 800 bp and larger products and those failed ones (for example lanes 32-33 in Figure 1) were excluded from study. Bisulfite treated genomic DNA samples of TM-1 and Pima 3-79 were amplified using selected 44 primer pairs.
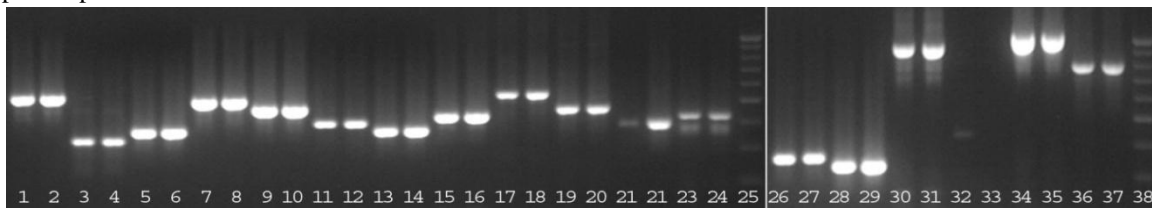

Figure 1: Td-PCR amplification of some primer pairs. TM-1 and Pima 3-79 genomic DNA samples were template DNAs. Lanes 25 and 38 are DNA size markers ranging from 1000 bp (upper band) to 300 bp (lower band).

Bisulfite converted genomic DNA samples of Pima 3-79 and TM-1 were screened using 44 primer pairs. Genomic DNA samples treated with bisulfite produced smear ranging from several kilo bases to 300 bp (Figure 2). A total of 26 primer pairs either failed in amplification of the target regions or produced non-specific amplifications or amplified only one genomic DNA sample of the two cotton species, indicating the existence of the challenge resides in the amplification of the bisulfite-treated genomic DNA [1, 2, 4]. A total of 18 primer pairs listed in Table 1 were selected because they amplified genomic DNAs of both cotton species, produced the same size with the control DNA and produced single amplicons (Figure 2).

Due to the higher ploidy level and allotetraploid nature of the cotton, identification of bisulfite primer pairs in cotton is difficult comparison to small and diploid genomes such as pepper. There are two homologs in tetraploid cotton species, representing descendants from the A-genome and D-genome donors at the time of polyploidy formation. Studies revealed that there existed structural rearrangements, gene loss, disrupted genes and sequence divergence between the A and D subgenomes. This makes sequencing and bisulfite sequencing of cotton more difficult [3, 5, 7, 14]. Bisulfite conversion remains a main method in almost every DNA methylation studies. However bisulfite converted DNA has some disadvantages in PCR studies. Bisulfite conversion results in DNA with reduced sequence complexity because unmethylated cytosines are converted to uracils and this generates homopolymer sequences which is problematic for polymerases to read. Bisulfite conversion denatures double strand and one of the two stands is used in PCR, it differs from conventional PCR studies [7, 15, 16].

Result of the present study clearly showed that optimization studies of bisulfite treatment and PCR, polymerase and buffers were less effective than primer pairs. One of the primer pair (forward) is used in the initial amplification of PCRs and the other primer (reverse) joins to the amplification starting from the second cycle of the PCRs. Our analyses revealed that primers for bisulfite PCR should range from 26-29 bases, with amplicon sizes ranging from 300-750 bases. We observed that higher annealing temperatures with longer than normal primer pairs were key for good amplification. We also noted that, when using a new set of primers, it is always good performing an annealing temperature gradient spanning the calculated temperature to determine where the primers are functioning most efficiently or use a touchdown PCR. We also noted that the use of degenerate primers which are synthesized as Y (C/T) in the sense strand and R (G/A) in the antisense strand [5] could

increase the rate of successful amplifications. Eighteen primer pairs (Table 1) were selected as useful primer pairs for bisulfite conversion in analysis of cotton DNA methylation.

Table 1: Primer pairs for bisulfite conversion studies in analysis of DNA methylation in *Gossypium* species

Primer ID	5' ➔ 3' Forward and Reverse sequences of primers*	Size (bp)	Methylation contexts				Accession	*G. hirsutum* L. Genes	Region
			CG	CHG	CHH	ALL			
EPi002	F: GGATTCGTATTAGATAGGAATATGAG R: GATTTTATGCGTTTAAGGGTAGAA	680	8	12	56	76	HM989877	Fiber-specific MADS11 protein	Gene body
EPi021	F: CAAACCCCATTACTCCTAATCTTCT R: CACAACATAAACTCCAACGTAACTAA	302	13	8	31	52	KP898249	Mitochondrial intergenic region	Intergenic
EPi032	F: GGYTTTAAGTTAAGGGYATCGTGGT R: TGGAAAGAATTAGGAGAAGAAGGYY	648	24	12	76	112	AF031457	Chloroplast RNA polymerase C1 (rpoC1) gene	Gene body
EPi034	F: GTGGGATATAAGAATGTGATTGGAG R: TATTTAAGGTTTATGAGGTGCGTTG	413	7	12	40	59	HQ142993	14-3-3-like protein (14-3-3L)	Gene body
EPi036	F: TAGTTAAATTGGAGGGGTCGTAAAT R:GAGAAGATTAATATTAAGTTATAACGGCC	259	8	3	33	44	GU207869	WRKY15 gene promoter	Promoter
EPi039	F: GGGTATTAGAAGAAGAAAAG R: TAGGTTTAAGTGGTTTGAACGATTC	553	6	20	75	105	HQ143029	Cellulose synthase A3 (CelA3)	Gene body
EPi042	F: ACGAYTGYACAGTYACCAAAGTAYTTG R: TTYAGGGTGAYAGGYAGTGAYAGGC	367	15	17	40	72	HQ143048	Expansin (Exp) gene	Gene body
EPi046	F: GGTTGTTAAAGTTGTTATTTGGATATG R: AGGAGGTTTGATTTAGTTAAATTATGGA	450	3	12	60	75	HQ143018	Membrane-anchored endo-1,4-beta-glucanase (CEL)	Gene body
EPi048	F: TTTGCAGGAGGCCATGCGCAAAGCT R: AATGATAATGAAGAGGTGAGTGGAG	699	9	25	100	134	JQ922565	Translation initiation-factor-eIF-2-subunit-alpha (eIF2A)	Promoter
EPi051	F: CGATGTTACGAAAGTTATAGCGAAG R: GAATAAATTTCGATTTAGAGTAATGGG	475	17	12	60	89	HQ143006	Beta-1,4-glucanase-like gene	Gene body
EPi055	F: AAATGAATAGAATTCGAAATGGTGA R: GGAGTGTATGGATTTTGGAAGTATG	479	13	18	57	88	HQ143089	Sucrose synthase (Sus1)	Gene body
EPi058	F: TGAATTGGTATTTGAATGATTTATGTT R: TTTTTAAGGATAAATTGGAGGAGTTT	432	8	16	46	70	HQ143077	Small GTPase (RacA)	Gene body
EPi062	F:TGAGGATGGTTAYAAYTGGYGTAAGTATGG R:AGTGATTGTGAGGAAGGATGTGATGAAGAG	361	12	15	35	62	FJ966887	WRKY3 transcription factor (WRKY3)	Gene body
EPi063	F:TGAGGAGATAAGYAAAAGAGAYYAGGAGAG R:GGGTGGTYTTGGTGGTAAAGTTATTGTGGG	362	1	16	54	71	FJ966887	WRKY3 transcription factor	Gene body
EPi065	F:TYGGTGYAAGGGGAGTAAGGAAGAGG R:GGTGGGAGTGYAYTTGGGTATGGGATTTG	486	31	28	49	108	AY781120	Ethylene-responsive element binding protein ERF4	Gene body
EPi066	F:TTGGGYTTGGTTAATGATTAATGYYAGGAG R:TAAAAAAGAAAGATGGAGAAYGGGTGGYTG	272	8	7	19	34	FJ864677	Vacuolar invertase 2 (vacInv2)	Gene body
EPi078	F:AAGGGAATTGGTYTATGGTYGAGTYAGTAA R:TTGAGTAAAGAGTAGAYYTTTTTGGGGGGG	474	10	6	47	63	AF031457	Chloroplast RNA polymerase C1 (rpoC1) gene	Gene body
EPi079	F:TGGTTGAGGYYATGAAGAGTGTTGYTAAGT R:TTTGGYAGAATTTAAGGYAGGAGATGATYG	439	6	13	40	59	HQ142993	14-3-3-like protein (14-3-3L)	Gene body

*R: purine (A or G) and Y: pyrimidine (C or T), H: A, T or C. Please note that primer pairs EPi079 and EPi034, EPi078 and EPi032, and EPi062 and EPi063 amplify the same genes but different regions. EPi062 may produce two fragments in some cotton species
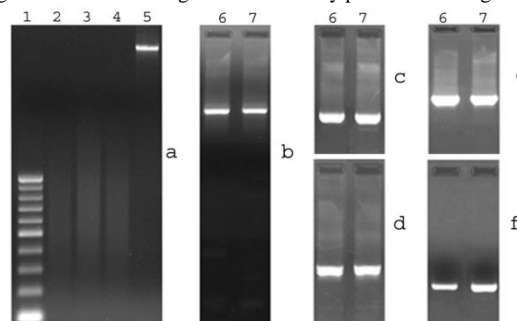

Figure 2: Representative images of bisulfite conversion (panel a) and amplified product of polymerase chain reaction (panel b-f). Panels a: representative bisulfite conversion assays, PCR amplified using primer pair EPi002 (b), EPi034 (c) d: EPi062 (d), EPi046 (e), and EPi021 (f). Numbers 1: DNA size markers ranging from

100-1000 bp, 2-4: different bisulfite reaction profiles, 5: control genomic DNA that is not treated with bisulfite, 6: Genomic DNA from Gossypium hirsutum L. TM-1 and 7: G. barbadense L. Pima 3-79.

Designed and tested primer pairs cover a total of 8151 bp cotton genomic and organelle DNAs. Analyses revealed that there were 1373 cytosine nucleotides consisting of 199 CpGs, 252 CpHpGs and 918 CpHpHs as calculated using Kismeth [9]. Cytosine methylation polymorphisms were greater in CpHpH and followed with CpHpG compared to CpG among gene bodies. Target regions of the primer pairs consisted of promoters, intergenic regions and gene bodies (exons, introns and UTRs) located in the nucleus, chloroplast and mitochondrial genome of cotton (Table 1). Analyses revealed that all the primer pair amplified products produced cytosine methylation polymorphisms within and between the samples of *G. hirsutum* L. and *G. barbadense* L. with the exception of two primer pairs; EPi021 and EPi032. Of 18 primer pairs, 16 could be used to detect differentially methylated genomic region while one (EPi021) could be used as a validation control in bisulfite sequencing studies.

4. Conclusions

Epigenetic studies in cotton lag behind many other important crops. We report 18 primer pairs that successfully amplify bisulfite converted and unconverted DNA with almost similar efficiency as judged from band intensity. Most of the regions amplified with primer pairs contained differentially methylated cytosine nucleotides. The use of bisulfite primer pairs will provide information on allele specific methylation in cotton. Unmethylated, fully methylated, and differentially methylated genomic regions flanking the primer pairs reported in this study could be internal or validation controls in genome-wide high throughput analyses.

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