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Development and Utilization of Bisulfite Specific Primer Pairs for Epigenetic Studies in *Solanum lycopersicum* L.

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Abstract Many agriculturally important traits such as heterosis, inbreeding depression, phenotypic plasticity, and biotic and abiotic stresses are thought to be affected with epigenetic factors. New epigenetic discoveries are likely to have a major impact on strategies for crop improvement in plant breeding. However, assessing the contribution of epigenetics to trait variations in plant species poses major challenges. Methylation of cytosine in DNA is one of the most important and well-studied epigenetic components in plants. DNA methylation not only plays significant roles in the regulation of gene activity, but also it is related with maintenance of genomic integrity. Although several next generation sequencing technologies do not require the use of primer pairs to identify and study DNA cytosine methylation, bisulfite sequencing (BS) requires bisulfite specific primer pairs to selectively amplify and analyze targeted genomic regions. In this study 40 bisulfite specific primer pairs were designed and used in amplification studies of bisulfite converted and unconverted genomic DNA of tomato (*Solanum lycopersicum* L.). Among the 40 primer pairs 28 were found to be useful in epigenetic studies in tomato. Results of the present study revealed the occurrence of CHH contents were the highest in genes while CG content showed higher level of methylation polymorphisms. Results also showed that CG DNA methylation levels were higher in promoters than intron and exons. Analyses indicated that primer pairs reported in this study could be used to detect DNA methylation and other epigenetic mechanisms affecting the development, differentiation, yield or the response to biotic and abiotic stress in tomato.

Keywords differential methylation, epigenetics, gene body, promoter, tomato

1. Introduction

Epigenetics is the study of heritable cellular, physiological and phenotypic variations in gene expression that result from enzyme-mediated chemical modifications of DNA, RNA or nucleosomes [1-3]. Major enzyme related epigenetic modifications include cytosine and adenine methylation of DNA at the sequence level, adenine deamination at the RNA level, spatial location of DNA within the nucleus and nucleosome remodeling which is affected with acetylation, deacetylation, methylation, phosphorylation, SUMOylation, ubiquitination, ADPribosylation, proline isomerization of histone and non-histon proteins [4-5].

In eukaryotic genomes, it is well documented that DNA cytosine methylation of the nucleotides is a major and most studied mechanism for epigenetics in plants [2-4]. Methylation in nuclear DNA is a specific property of plant genomes. Nuclear DNA methylation could be species-specific, tissue, organ, organelle, and development stage specific and it involves in the control of all genetic functions such as DNA replication and repair, gene transposition and transcription, cell differentiation and gene silencing, and imprinting and bio-defense, expression of transgenes and foreign DNA in cell [2-5].

Nuclear DNA can be methylated at both adenine and cytosine nucleotides. It is known that cytosine nucleotide is the most common base methylated and it is widely considered as the fifth base in plant genomes. Cytosine

methylation has been studied in many organisms in embryonic and postnatal development, cancer, bacterial host defense, transgenic silencing, hormone regulation, biotic and abiotic stresses, genome doubling and speciation, heterosis, imprinting, evolution and phylogenetic studies [1-5].

Several common techniques are currently available for assaying and detecting global and gene-specific cytosine methylation. Among these techniques bisulfite-mediated deamination is the gold standard for assessing DNA methylation since it reveals the methylation status of every cytosine nucleotide in a genome. In addition, bisulfite-mediated deamination method is amenable to massively parallel sequencing methods. Bisulfite sequencing (BS) takes the advantage of the selective and complete conversion of unmethylated cytosine to uracil by sodium bisulfite. Chemically converted cytosine nucleotides are then amplified by polymerase chain reaction (PCR) as thymine nucleotides [6]. After sub-cloning of the PCR products, the sequencing reveals the initial methylation profiles of the region of interest. A technical advantage of BS resides in the use of PCR method but this step is often the most difficult one in the whole process. Accurate analysis of DNA cytosine methylation based on BS depends on the selection of primer pairs that flank cytosine methylation contexts [4, 6].

The genus *Solanum* is a member of Solanaceae family, which also contains agronomically important plant species such potatoes, peppers, eggplants, tobacco, deadly nightshade, henbane, datura and petunias, originated in Americas. *Solanum* section Lycopersicon consists of 13 cultivated and wild species. *Solanum lycopersicum* is the only cultivated tomato species [7, 8]. Tomato is the second most consumed vegetable worldwide and a well-studied crop species in terms of genetics, breeding, and genomics. Tomato has been an excellent model plant for both basic and applied plant research including epigenetics in the Solanaceae family [7].

Main breeding objectives of tomato include the development of new candidate cultivars with yield potential equal to or exceeding the current cultivars, improved insects and diseases resistance, widely adapted to several different climates with optimum fruit shape, size, total solids, color, firmness, ripening, nutritional quality and flavor, and longer shelf life [7, 9, 10, 11]. Epigenetic studies in tomato targeted toward fruit development, fruit ripening, growth performance, transgressive phenotypes in hybrids and several epialleles including colorless non-ripening whose expression is silenced by promoter hyper-methylation to inhibit normal fruit ripening have been detected. Epigenetic studies in tomato would identify new epialleles affecting agronomic traits and plant defense systems against biotic and abiotic stress factors [11, 12].

Epigenetic studies utilize several methods including methylation-sensitive amplified polymorphism (MSAP), gas chromatographic method, high-performance liquid chromatography (HPLC), spectroscopy and next generation sequencing technologies. BS methods have been used in tomato for epigenetics studies [9-12]. Due to its high resolution, lower cost and easiness, BS is still the most widely used method to study DNA cytosine methylation for epigenetic variations. BS provides information about the methylation profile of every single CG, CHG and CHH context, where H refers to A, C or T [4-6].

Bisulfite specific primer pairs specifically developed for promoter and gen body entities such as untranslated terminal regions (UTRs), exons and introns are prerequisite for BS application. However, there is a few study of bisulfite mediated cytosine methylation in tomato. Novel bisulfite specific primer pairs could be very useful to study the level of DNA methylation to decipher epigenetic regulations in tomato. The main objective of this study was identify, design and utilize bisulfite specific primer pair specific for promoter and gene body entities.

2. Materials and Methods

2.1. Target DNA sequences

Genomic DNA sequences of tomato (*Solanum lycopersicon* L.) in NCBI GenBank databases (ftp://ftp.ncbi.nih.gov/) were obtained and these DNA sequences were used to obtain gene body entities such as exons, introns, untranslated regions (5'-UTR and 3'-UTR) and promoters using EpiOne software [4]. Gene sequences included in this study were reversibly ethylene-responsive fruit ripening gene E8, ethylene-responsive fruit ripening gene E9, DNA for a fruit specific protein involved in fruit maturation BIP (binding protein)/grp78 (glucose-regulated protein, 78 kD) (BIP/grp78) gene, fruit-specific protein gene, heat shock cognate protein 80 gene, ABA-regulated, and accumulating in developing seeds and drought-stressed leaves LE25 gene

polyubiqutin repeats, RSI-1 protein (RSI-1) gene, Lemmi9 gene, lap17.1a gene, Polygalacturonase 3 (TAPG3) gene, transposon CACTA-like, partial sequence, and cell-wall invertase (Lin5) gene and Hsc70 gene.
A total of 40 primer pairs from gene body entities and promoters were designed and called SL primer pairs. Primer pairs were designed using Primer 3 software [13] based on the following main parameters: GC content value 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 400–800 bp. Cytosine bases (C) in the forward primers were replaced with Y (C/T) and the guanine bases (G) in the reverse primers were replaced with R (A/G) [14, 15] to obtain bisulfite specific primer pairs.

2.2. Plant materials and genomic DNA extraction

Mature seeds of *S. lycopersicon* cv. H2274 were used for genomic DNA extraction studies. Mature seeds were ground to a powder with a mortar and pestle for DNA extraction. A DNA extraction protocol previously described in Karaca et al. [16] was used with the following main modifications [17, 18]. 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-mercapto-ethanol (BME) were added to powdered 0.5-1.0 g tissues and strongly mixed using a vortex before 2 h incubation at 65°C. Samples were mixed in every 15 min intervals during the heat incubation. Remaining of the DNA extraction were as previously described in Karaca et al. [16]. The amount, purity, integrity and enzyme accessibility of genomic DNA were confirmed [18, 19].

2.3. Bisulfite conversion

Genomic DNA samples of mature tomato seeds were bisulfite treated using a bisulfite conversion kit (Invitrogen Corp. Carlsbad, CA, USA). C-T conversion buffer was made using 900 µL ddH_2O, 50 µL M-dissolving buffer and 300 µL M-dilution buffer. The C-T conversion buffer was mixed using a vortex for 1 min, and incubated for 5 min at room temperature and 130 µL 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 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 ^{o}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 and dissolved in 22 µL sterile water [14, 15].

2.4. Touch-down polymerase chain reactions (Td-PCRs)

A touch-down PCR (Td-PCR) was done in 25 µL reaction volume consisting 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 30 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 [14, 15].

2.5. Purification of amplified products

PCR products in the presence of 1X DNA loading buffer were loaded in 3% (w/v) high-resolution agarose gels (SERVA Electrophoresis GmbH, Heidelberg, Germany) and electrophoresed at 5 V cm^{-1} at constant voltage for 4-6 hours [19]. After the electrophoresis completed, 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.

2.6. 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, gently mixed, and incubated at 22°C for at least 2 h for ligation reactions. After then 2.5-4 µL ligation mixture was used for each PCR product, and 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 plasmids containing PCR fragments from bisulfite treated genomic DNA and 4 plasmids containing PCR fragments from untreated DNA (as control or reference) were commercially sequenced using M13R sequencing primers (Macrogen Inc., Amsterdam, The Netherland) [14, 15].

2.7. 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 determined from each clone sequence and trimmed off along with the vector sequences. All data sets containing bisulfite treated sequences and the reference sequences 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 [14, 15, 20].

The methylation percentage was calculated for each cytosine sequence context (CG, CHG and CHH) using the percentage methylation (%), calculated as 100×C/(C+T). The statistical significance between and within the samples of the entire set of either CG, CHG or CHH sites was separately evaluated with the nonparametric Mann-Whitney U test. Two-tailed P values, 0.05 were considered statistically significant. Methylation context (CG, CHG or CHH) was used as factor and the methylation percentage was use as response [14, 15].

3. Results and Discussion

Among 40 primer pairs designed 39 successfully amplified genomic DNA samples of tomato. Sizes of PCR amplified products ranged from 448 bp to 1000 bp and most of the primer pairs produced single amplicon indicating target specific successful primer pairs. However, among 39 primer pairs, only 28 could amplify bisulfite converted genomic DNA samples. Sizes of amplified products ranged from 448 bp to 717 bp with an average of 606.18 bp per primer pair. We noted that those primer pairs amplified products greater than 717 bp could not amplify bisulfite converted genomic DNA samples. A total of eleven primer pairs failed to amplify bisulfite converted DNA samples indicating the existence of the challenge resides in the amplification of the bisulfite-treated genomic DNA. Challenges include the redundancy of the target sequence due to the original C richness creates long stretches of uracil, which might result in DNA fragmentation during the bisulfite-treatment. We assumed that fragmentation of targets were encountered in those targets greater than 700 bp in lengths. In general, results indicated that amplification rate was successful, being 71.79% (28 of 39 primer pairs). We noted that the use of degenerate primers which are synthesized C/T as Y in the sense strand and G/A as R in the antisense strand [9-12] could increase the rate of successful amplifications.

Present study obviously revealed that optimization studies of bisulfite conversion and PCR, polymerase enzyme and buffers were less effective than primer pairs that target the amplifications. We also noted that primer pairs (both reverse and forward) for bisulfite PCR should contain 24-29 bases since our results demonstrated that higher annealing temperatures with longer primer pairs produced reproducible amplification. Although there were some products longer than 700 bp, amplicon sizes ranging from 400 to 600 bases were very effective for transformation and cloning studies. Based on our experience we suggest that researchers who designed new

bisulfite primer pairs could use annealing temperature gradient spanning experiments to determine optimal annealing temperature for bisulfite primer pairs or could use touch-down PCR profiles [14, 15, 19, 21].

In the present study, a total of 28 primer pairs listed in Table 1 were selected because they amplified bisulfite-treated genomic DNA samples, produced the same size between control DNA (unconverted bisulfite samples) and bisulfite converted samples and they produced reliable amplified products from different tissue sources of tomato including pericarps and flowers. Twenty-eight primer pairs could amplify a total of 16.973 bp tomato genomic DNA of 13 genes including ethylene-responsive fruit ripening gene E8, a DNA region for a fruit specific protein, BiP binding protein/grp78 glucose- gene, fruit-specific protein gene, heat shock cognate protein 80 gene, drought-stressed leaves LE25 gene, polyubiqutin repeats, RSI-1 protein RSI-1 gene, Lemmi9 gene, Lap17.1a gene, polygalacturonase 3 TAPG3 gene, cell-wall invertase Lin5 gene and Hsc70 gene (Figure 1).

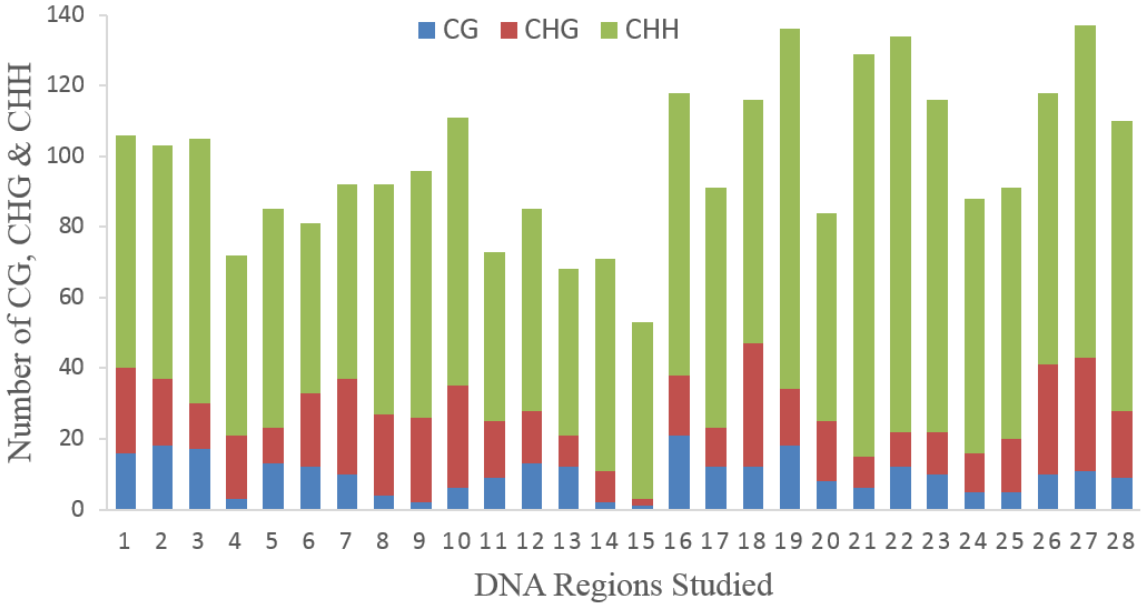

Figure 1: Numbers of CG, CHG and CHH contents among 28 genomic regions of tomato.

Analyses revealed that there were a total of 1,503 cytosine contents in the 16,973 bp tomato genomic DNA of the 13 genes consisting. There were 277 CGs, 494 CHGs and 1990 CHHs (Figure 1). Occurrence of CHH contents were the highest and significantly varied among 28 DNA regions studied.

Table 1: Bisulfite specific degenerate primer pairs suitable for DNA methylation in tomato

ID	5' ➔3' Forward and Reverse sequences of primers*	Accession #	*Solanum lycopersicum* L. Genes	Region	Size
SL01	AAAAAGTGGTTTATAYTAGYAATYTTGA TCTTCACATCCTTRRARAARTCTATTA	X13437	Ethylene-responsive fruit ripening gene E8	Intron	586
SL02	ATATGGYATYYTYATATTGAGATTTT CCAGACTAARAAATRAAAATACTTACCC	X13437	Ethylene-responsive fruit ripening gene E9	Exon	606
SL04	GAGGAGAGAGAYAAAYGATATTAAGAA CGAAARRAACAAAAATACRTATCATAA	X13743	DNA for a fruit specific protein	Exon	658
SL07	ATATTGTAYTTYYAGAGTYTGATGAGG TATCCCTCTRTACTTCCTTATCTTCAA	L08829	BiP binding protein/grp78 glucose- gene	Exon	448
SL09	CAGTTGATATGTYTGGAATTTYG CGAAARRAACAAAAATACRTATCATAA	M87659	Fruit-specific protein gene	Exon	481
SL10	AATGTYGGAYGTAGAGAYGTTTG ACTCACAAAAATACTCARCTRCTCTAA	M87659	Heat shock cognate protein 80 gene	Intron	549
SL11	TTTTAGAGYAGYTGAGTATTTTTGTG TAAACTCTCAAARCRRATCTTRTCTA	M87659	Heat shock cognate protein 80 gene	Intron	630

SL12	TATTTGYAGATYTGGTGAATAAYYT CCAATCATTARTCAARCTCTTRTARA	M87659	Heat shock cognate protein 80 gene	Exon	617
SL13	TATCATGGATAAYTGTGATGAGTTGAT AAACAATRACCTTTTCAACTTTRTCT	M87659	Heat shock cognate protein 80 gene	Exon	659
SL14	CTAGGAGAYAAAGTTGAAAAGGTYAT TAAACTATATCAACCACRAATRTCAAA	M87659	Heat shock cognate protein 80 gene	Exon	630
SL15	AACAGAAAAATGYAGAYAGGAAAG CTACACAAATACRATRRRTTATCAAAAT	M76552	Drought-stressed leaves LE25 gene	Intron	591
SL16	TAAATTTGTAYAAGATYAAATGAGTGG TGATGTRAAACAAAACAATTATARRAA	M76552	Drought-stressed leaves LE25 gene	Exon	624
SL19	CACTAGTTYTTYTTYTYAAGGTAATGG ATAAACATCCCTARRACACAAACAATA	X73156	Polyubiqutin repeats	Intron	643
SL20	TATTGTTTGTGTYYTAGGGATGTTTA TCTGAAATATATTRAARCACAACTRTC	X73156	Polyubiqutin repeats	Intron	533
SL22	TTTTTATTTTGTTTTYGYTAGAGTTTG GCTCARACAACATRATAACACAARTAR	L22189	RSI-1 protein RSI-1 gene	Exon	468
SL24	CATAAYAAGAGYAGAGATATTYGTTTAG AATTGCRATTCTACARTTRRATC	Z69032	Lemmi9 gene	Exon Intron	521
SL25	GTCTTAYTAGGYYTAATGAAAGTGATG ATTCTCATCTCTARCCATRTCATTCT	Y08305	Lap17.1a gene	Intron	593
SL26	ATCCTGTTTYTGTTTTTGTAAYATGA TCTGCATACTTRATTTTCCTCTCTATT	Y08305	Lap17.1a gene	Exon	642
SL27	CCTTCTATTGAYTTYAYYAATAAGGTA CTATTTTRTACRRTTACTCCACTCATT	AF000999	Polygalacturonase 3 TAPG3 gene	Exon	693
SL28	TTTTCAGTATTTGAYATGATGATGAGT TGTTGAAACARAACATTTCTAACAAAR	AF000999	Polygalacturonase 3 TAPG3 gene	Exon	584
SL30	CCAAAAAYAAAGAAGATAATGTATTTGA TAGTGRARTTACCTTTTTARCAACAAT	AY639885	Cell-wall invertase Lin5 gene	Promoter	617
SL31	TCGGTTTGATAYYTAYTAAATGTTTTY AAAGCTTTAACCAATARAARATTRTCA	AY639885	Cell-wall invertase Lin5 gene	Promoter	640
SL32	ATGAGYTATAATTYATYAGYATAATTG GCAAGAACTTTTCTTTRTARAATTTCA	AY639885	Cell-wall invertase Lin5 gene	Promoter	591
SL33	ATCCTATGTTYYATAAGYAAAAYATAA TTCGCTARATARATTCAACTTTTTCAT	AY639885	Cell-wall invertase Lin5 gene	Promoter	589
SL35	GTTAGTTGTGGAAATTYYTTGYTT CTACAATCAARTTAAARAARARTTCARC	L41253	Hsc70 gene	Intron	717
SL36	TTGTAGAYGYTAAGYGATTAATTG AATCAATACCCTCATAAARARAATCAA	L41253	Hsc70 gene	Exon	687
SL37	TGAAATTGATTYTYTTTATGAGGGTAT GAACCATTTTCTCAATCTCATCTTTAR	L41253	Hsc70 gene	Exon	713
SL38	TCTAAAGATGAGATTGAGAAAATGGTT CRAAAARAAAAACAAARAAATTAAARA	L41253	Hsc70 gene	Exon	663

*: R: purine (A or G) and Y: pyrimidine (C or T).

Cytosine methylation levels in CG, CHG and CHH contexts showed different levels and patterns as identified with KisMeth software [20]. The highest methylation levels were observed in CGs, followed with CHGs and CHHs. Results indicated that genes with similar cellular function for example housekeeping or tissue specific genes or DNA sequences located with similar genomic regions such as chloroplast or mitochondrial DNA contain similar frequency of CG, CHG and CHH contexts.

Analyses using 16,975 bp DNA sequences revealed that occurrences of CHH contents were highest in promoters, exon and introns of the 13 genes studied. Occurrences of CHG contents were the second highest while CG contents were the least among the cytosine contents studied (Figure 2). The differences between CG and CHG contents in promoters were lower in comparison to introns and exons. Further studies clearly showed that methylations of cytosine nucleotides in promoters were significant in comparison to exons and introns. This indicated that methylations play important roles in gene regulations [6, 9, 14, 15, 22, 23].

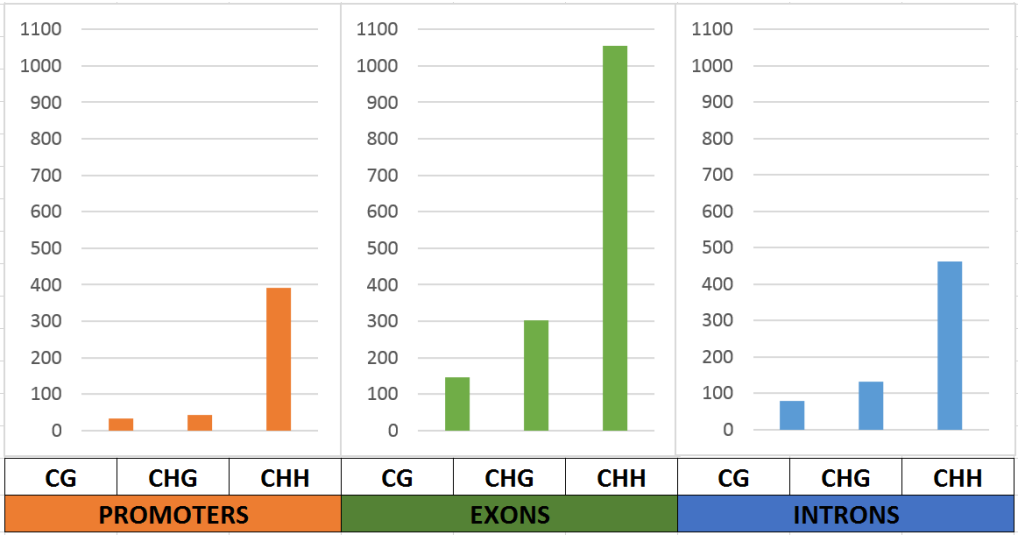

Figure 2: Number of CG, CHG and CHH contents presented in promoters, exons and introns

In the present study, we observed that genes location, promoters and gene body entities such as introns and exons showed different level of DNA cytosine methylations indicating that level and pattern of DNA cytosine methylations play pivotal roles in plant growth and development. Differences between CG, CHG and CHH contents of different genes and gene body entities could be related with cellular events including chromatin structure, DNA packaging, gene expression, genomic imprinting, recombination and DNA replication. Our findings also supported previous research findings indicated that transcribed sequences were often methylated less than promoters [2-5, 9-12, 14, 15, 23, 24].

Most of the genomic regions amplified using designed bisulfite specific primer pairs contained differentially methylated cytosine nucleotides indicating that bisulfite primer pairs could be used to identify and quantify unmethylated, methylated, and differentially methylated genomic DNA sequences. Furthermore, bisulfite specific primer pairs reported in this study could be used for internal or validation controls in genome-wide high throughput analyses of epigenetic research in tomato.

4. Conclusion

Occurrences and variations in the level of DNA cytosine methylation might direct influence phenotypic variations. Occurrences and variations in the methylations have been related with many genetic aspects including DNA recombination, gene expression, or transposon silencing. Although tomato is the second crop in which epigenetic concept was revealed experimentally the level of DNA methylation in this crop lags behind many other crop species. Here we reported 28 bisulfite specific primer pairs that successfully amplify bisulfite converted and unconverted genomic DNA of tomato. Primer pairs contained 24-29 nucleotides and produced single and sharp amplified products in higher annealing temperatures. Although there were some amplified products longer than 700 bp, amplicon sizes ranged from 400 to 600 bases. Based on our experience and findings we suggest that researchers dealing with bisulfite conversion studies could use annealing temperature gradient spanning experiments to determine optimal annealing temperature for primer pairs or could use touch-down PCR profiles. Primer paired amplified products contained a total of 1505 cytosine contexts, consisting of 145 CG, 303 CHG and 1055 CHH in 16,975 bp DNA. Primer pairs reported in the present study not only be useful to identify and quantify unmethylated, methylated, and differentially methylated DNA sequences but they

are very used for internal or validation controls in next generation sequencing technologies dealing with DNA methylation analyses.

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