RESEARCH ARTICLE

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Novel methylation specific bisulfite primer pairs for epigenetic studies of *Capsicum* spp.

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Abstract
Over the past ten years, interest in epigenetic has rapidly increased. Heritable and stable changes in gene expression without any change in DNA sequence is in the field of epigenetics. Plants have a well-preserved epigenetic signature called DNA methylation. It is an essential epigenetic mark that protects genomic stability, silences harmful transposon insertions, and controls global gene expression in all developmental stages and environmental circumstances. All three sequence contexts, the asymmetric CpHpH context and the symmetric CpG and CpHpG contexts (where H is C, A, or T), are among DNA methylation sites in plants. Particularly, DNA cytosine methylation affects a wide range of biological processes, such as gene expression, chromatin structure, DNA packing, recombination, genomic imprinting, and DNA replication. The choice of primer pairs that flank cytosine methylation contexts is critical when designing for the detection of DNA cytosine methylation using bisulfite sequencing. We have developed and synthesized 26 bisulfite specific primer pairs suitable for DNA cytosine methylation investigations in peppers. These primers are specific to certain promoters, intergenic regions, and gene bodies (exons, introns, and UTRs). DNA samples taken from various tissues and developmental stages of *Capsicum annuum* L. Demre Sivrisi were analyzed by these primer pairs to confirm their utilization.
Keywords: Cytosine methylation, Epigenetics, Exon, Intron, Promoter

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INTRODUCTION

The study of heritable and physiological phenotypic trait variations in gene expression not regulated by alterations in the genetic code sequence of DNA is known as epigenetics. The word "epigenetics" describes the covalent modification of DNA, protein, chromatin or RNA not governed by the rules of central dogma of molecular biology. The location of DNA within the nucleus, adenine deamination at the RNA level, cytosine and adenine methylation of DNA at the sequence level, and remodeling the chromatin, which is impacted by acetylation, deacetylation, methylation, phosphorylation, SUMOylation, ubiquitination, ADPribosylation, proline isomerization, and deimination of histon and non-histon proteins, are some of the major enzyme-related epigenetic modifications (Smulders and Klerk, 2011; Karaca et al., 2016a; Araz et al., 2022; Cai et al., 2022).

DNA cytosine methylation of nucleotides is the most extensively researched epigenetic alteration in plants and is a fundamental mechanism for epigenetics in eukaryotic genomes. Nuclear DNA (nDNA) methylation is a unique characteristic of plant genomes. DNA methylation is a specific property of the plant genome that is known to control all genetic functions, including DNA replication and repair, gene transposition and transcription, cell differentiation and gene

silencing, imprinting and biodefense, and the expression of transgenes and foreign DNA in cells (Karaca et al., 2016a). It is known that methylation in nDNA can be species-specific, tissue-specific, organ-specific, and development stage-specific. Methylation of the nDNA can occur at either the adenine or cytosine nucleotide. Cytosine nucleotide is regarded as the fifth base in plant genomes and is known to be the most often methylated base (Smulders and Klerk, 2011). Numerous organisms have had their cytosine methylation investigated in relation to biotic and abiotic stressors, hormone control, cancer, bacterial host defense, embryonic and postnatal development, heterosis, imprinting and evolution (Peng and Zhang, 2009; Araz et al., 2022; Cai et al., 2022).

There are already several widely used methods for testing and identifying both global and gene-specific cytosine methylation. The gold standard for determining whole genome DNA methylation is bisulfite-mediated deamination since it can be done in large batches using massively parallel sequencing techniques and shows the methylation state of each cytosine nucleotide in a genome. The selective and total conversion of unmethylated cytosine to uracil by sodium bisulfite is exploited by bisulfite sequencing. After being chemically changed, cytosine nucleotides are amplified as thymine nucleotides using the polymerase chain reaction (PCR) (Figure 1) (Jin et al., 2013). Although using PCR has a technical benefit for bisulfite sequencing, this step is frequently the most challenging one overall. Using primer pairs that flank cytosine methylation contexts is essential for accurate bisulfite sequencing investigation of DNA cytosine methylation (Warnecke et al., 1997; Rand et al., 2006; Dhringa et al., 2014; Araz et al., 2022; Cai et al., 2022).

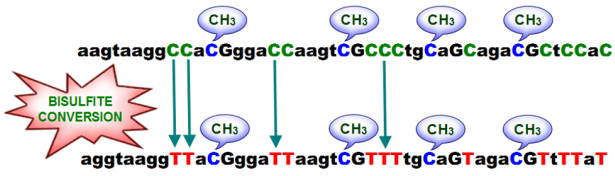

Figure 1. Alteration of Cytosine to Thymine with Bisulfite Conversion and Conseving of 5mC.

Numerous biological processes, such as chromatin structure, DNA packing, gene expression, genomic imprinting, recombination, and DNA replication are influenced by DNA cytosine methylation. In the 5′ regulatory regions of genes, methylation of CpHpG leads to transcriptional silencing. According to some research, transcribed sequences frequently have lower levels of methylations than silent gene promoters and specific coding regions. Significant variations exist in the pattern or amounts of cytosine methylation within the same tissue in different tissues or under different functional conditions. Certain degrees of DNA methylations may influence tissue-specific transcription, according to certain research. Furthermore, understanding the relationship between tissue-specific gene expression and tissue-specific methylation requires the measurement and analysis of levels and patterns of genome-wide and tissue-specific methylation in various tissues (Araz et al., 2022; Cai et al., 2022).

Epigenetic studies in pepper (*Capsicum* spp.) lags behind many other important crop species. The *Capsicum*, closely related to other members of the Solanaceae family such as potato, tomato, and tobacco, that originated in the New World, is a diploid and self-pollinating plant. Pepper is a crop of considerable economic significance that is also used as a raw material in industry (Ince et al., 2010a; Liu et al., 2017). The *Capsicum* genus contains 39 species and only *C. annuum* L., *C. baccatum*, *C. frutescence*, *C. chinense*, *C. pubescens*, and *C. assamicum* species are cultivated. Based on the presence and absence of capsaicinoid compounds, *Capsicum* species are grouped as pungent (hot/spicy) and nonpungent (sweet) pepper. The pepper fruits consist beneficial metabolites such as carotenoids (provitamin A), vitamins C and E, flavonoids, and capsaicinoids. Due to its vast variation in fruit form and the biochemical actions of unique metabolites like capsanthin and capsaicin, pepper is also a valuable model plant for fruit development (Paran and van der Knaap, 2007; Mazourek et al., 2009). Many studies were targeted toward various aspects, including the development of genetic and genomic resources for crop improvement in pepper. Recent studies on pepper genome sequencing have established a crucial basis for understanding the function of pepper genes (Kim et al., 2014, 2017; Qin et al., 2014; Hulse-Kemp et al., 2018). Additionally, the molecular mechanisms behind a number of significant pepper phenotypes have been clarified. A comparative analysis of the genomes of various *Capsicum* species revealed that the large appearance of leucine-rich domain protein (NLR) genes by retroduplication could give rise to functional nucleotide-binding (Kim et al., 2017). Up to date, methylation-sensitive amplified polymorphism (MSAP), gas chromatographic method, high-performance liquid chromatography (HPLC), and bisulfite sequencing have been used in pepper (Potris et al., 2004; Xu et al., 2015; Shams et al., 2020; Ince and Karaca, 2021; Araz et al., 2022; Cai et al., 2022).

The most popular methodology for studying DNA methylation is bisulfite sequencing, which provides details on the methylation profiles of each CpG, CpHpG, and CpHpH. Nevertheless, research on pepper's bisulfite-mediated cytosine methylation is scant. Pepper requires the construction of primer pairs to use bisulfite sequencing to determine epigenetic information, specifically cytosine methylation. In current study, primer pairs for bisulfite sequencing targeting specific gene bodies and promoters have been devised and established for several key pepper genes.

MATERIALS AND METHODS

Target DNA sequences

Pepper (*Capsicum annuum* L.) genomic DNA sequences were downloaded from NCBI GenBank databases (ftp://ftp.ncbi.nih.gov/). EpiOne software (Karaca and Ince, 2016) was utilized for mining the gene body entities such exons, introns, untranslated regions (5'-UTR and 3'-UTR), and promoters. Reversibly Knolle protein kn gene, disease-related protein-1 (PR-1) gene, capsanthin/capsorubin synthase gene, lipid transfer protein gene, pathogenesis-related protein 10 (PR10) gene, bHLH transcription factor Upa20 gene, SAR82A gene, defensin gene, SP gene, snakin (Sn) gene, hydroxycinnamoyl transferase gene, acyltransferase (Pun1) gene, and 3-oxoacyl-(acyl-carrier-protein) synthase gene were among the gene sequences examined in this study. These primer pairs are a set of 40 primer pairs derived from promoters and gene body components. Using the Primer 3 program (Untergasser et al., 2012), degenerate primer pairs were created depending on the following primary parameters: The predicted amplified product size was defined as 400–800 bp, the annealing temperature (Ta) as 58°C–62°C, and the GC content value as 40%–80%. Following design, Y (C/T) was used to replace the cytosine bases (C) in the forward primers and R (A/G) was used to replace the guanine bases (G) in the reverse primers (Ince et al., 2010b; Ince and Karaca, 2021).

Plant materials and genomic DNA extraction

Studies on the extraction of genomic DNA were conducted using mature seeds of *Capsicum annuum* cv. Demre sivrisi. Using a mortar and pestle, mature seeds were ground into a powder in order to extract DNA. The following adjustments were made to a DNA extraction process that was previously published in Karaca et al. (2005) and the modified protocol in Ince et al. (2011). Before incubating for two hours at 65°C, powdered 0.5–1.0 g tissues were mixed vigorously with a vortex using 2.48 mL of the preheated (65°C) 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). The samples were incubated at 65°C in a water bath for 45 minutes with vortex intervals of 15 minutes. After centrifugation steps. DNA was revealed using 0.9 volume isopropanol and 0.1 volume NaCl buffer. Then, ethanol washing was performed. Finally, it was dissolved in 100 µL of TE and stored at 4°C before use. Genomic DNA's quantity, integrity, purity, and accessibility to enzymes were all verified (Ince et al., 2010b). Additionally, primer pairs screened on mature seed genomic DNA were tested from different tissue sources of pepper including pericarps and flowers before bisulfite treatment to confirm the integrity of extracted DNAs (Ince and Karaca, 2017; Ince and Karaca, 2021).

Bisulfite conversion

Using a bisulfite conversion kit from Invitrogen Corp. in Carlsbad, California, USA, genomic DNA samples of mature pepper seeds were bisulfite treated. 900 µL of ddH_2O, 50 µL of M-dissolving buffer, and 300 µL of M-dilution buffer were used to apply the C-T conversion buffer. After one minute vortexing of the C-T conversion buffer and five minutes of room temperature incubation, 130 µL of bisulfite-containing C-T conversion reagent was added to 0.5 µg of genomic DNA in 20 µL, thoroughly mixed, and centrifuged for a short while. The samples were incubated with a cycling type of conversion profile, which consisted of eight cycles of incubation at 53°C for 30 min and 37°C for 6 min after an initial denaturation at 98°C for 10 min. The GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used to incubate the conversion reactions for 10 minutes at 4°C. Samples of transformed DNA were purified and diluted in 22 µL of sterile water as soon as the reactions were completed (Ince and Karaca, 2017; Ince and Karaca, 2021).

Touchdown polymerase chain reactions (Td-PCRs)

A 25 µL reaction volume was used for a touchdown PCR (Td-PCR), which included a template of 3 µL bisulfite converted or control genomic DNA, 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 MgCl2, and 1 unit of Taq DNA polymerase (Invitrogen Corp. Carlsbad, CA, USA). Using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA), the Td-PCR amplification profile was performed as follows: initial denaturation at 94°C for 3 min, ten cycles of 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. The same cycling conditions as previously described were used for an additional 40 PCR cycles, with continuous annealing at 55°C. The conditions for denaturation and extension were the same as previously mentioned. Final extension reactions were conducted after the amplification reactions, completing reactions at 72°C for 10 minutes (Ince and Karaca, 2017; Ince and Karaca, 2021).

Purification of amplified products

PCR products were loaded onto 3% (w/v) high-resolution agarose gels (SERVA Electrophoresis GmbH, Heidelberg, Germany) with the presence of 1x DNA loading buffer. The gels were then electrophoresed at 5 V cm-1 at a constant voltage for 4-6 hours. Following electrophoresis, the PCR products were purified using a PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen Corp. Carlsbad, CA, USA) by cutting slits just ahead and behind with a clean razor blade. Samples of purified DNA were eluted in 13 µL of sterile water.

Ligation, transformation, cloning and sequencing reactions

Two microliters of 10x ligation buffer [400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25 °C)] and two microliters of 50% (w/v) polyethylene glycol 4000 were added to tubes holding 13 µL of purified PCR products. At least two hours were spent at 22°C for the ligation processes after adding 2 µL of pTZ57R/T and 1 µL of T4 DNA ligase enzyme (5 u/µL) and gently mixing them. Next, each PCR product was treated to 2.5–4 µL of ligation mixture. By using a Transform Aid Bacterial Transformation Kit (Thermo Scientific, Waltham, MA USA), vectors containing PCR products were then transformed into *E. coli* bacteria strain JM109. GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used to extract plasmid DNA samples following colony selection and subculturing. The M13R sequencing primers (Macrogen Inc., Amsterdam, The Netherlands) were used to commercially sequence a total of 12 plasmids comprising PCR fragments from bisulfite treated genomic DNA and 4 plasmids containing PCR fragments from untreated DNA (Ince and Karaca, 2021).

Detection and statistical analysis of methylation

Using the software Sequencher, sequences were put together into contigs according to the contig assembly settings, which were set to a minimum overlap of 50 bases and a 90% identity match. From each clone sequence, the forward and reverse primer sequences were identified and cut off together with the vector sequences. The default KisMeth program setting, which used alignment lengths equal to or greater than 50% of the reference sequence length and alignment lengths equal to or greater than 80% positive match in the alignment, was used to analyze all data sets containing bisulfite treated sequences and the reference sequences (Ince and Karaca, 2017).

For every cytosine sequence context (CpG, CpHpG, and CpHpH), the methylation percentage values were computed using the percentage methylation (%), which was determined by dividing 100×C by (C+T). Using the nonparametric Mann-Whitney U test, values the three methylation sets of CpG, CpHpG, or CpHpH were determined and statistical significance was assessed within and between samples. The threshold of 0.05 for two-tailed P values was deemed statistically significant. The methylation percentage was utilized as the response, while the methylation context (CpG, CpHpG, or CpHpH) was employed as the factor (Ince and Karaca, 2017; Ince and Karaca, 2021).

RESULTS AND DISCUSSION

A total of 26 primers, named CA primer pairs, were chosen to amplify genomic DNA samples that had been bisulfite transformed. On genomic DNA samples, the amplified products' sizes varied from 402 bp to 1500 bp. But just 26 of the 48 primer pairs were able to amplify genomic DNA samples that had undergone bisulfite conversion (Figure 2). The amplified products had an average size of 556.7 bp per primer pair and ranged in size from 402 bp to 757 bp. As we observed, the primer pairs that amplified bands longer than 757 bp were unable to amplify genomic DNA samples that had undergone bisulfite conversion.

A total of 21 primer pairs were unsuccessful in amplifying bisulfite-converted DNA samples. This is a result of difficulties in the binding of primers during the amplification of genomic DNA that has been treated with bisulfite. One of the challenges encountered in this study was the presence of repetitive sequences in the target sequence, which is caused by the high frequency of cytosine residues. This leads to the formation of extended regions of uracil, which might potentially cause fragmentation of the DNA during the bisulfite-treatment process. It was postulated that this phenomenon was observed mostly in the aforementioned targets, particularly those exceeding a length of 757 base pairs. Additionally, it was shown that the utilization of degenerate primers, specifically generated as Y (C/T) in the sense strand and R (G/A) in the antisense strand (Teyssier et al., 2008; Gallusci et al., 2016; Xiao et al., 2020; Ince and Karaca, 2021), has the potential to enhance the efficiency of amplification reactions.

The findings of this study clearly indicated that the optimization of bisulfite conversion and PCR, as well as the selection of polymerase enzyme and buffers, exhibited lower efficacy compared to the utilization of primer pairs that specifically target the amplification process. It was observed that primers, namely reverse and forward primers, utilized for bisulfite PCR should consist of a length ranging from 24 to 29 bases. This conclusion was drawn based on our findings, which indicated that employing longer primer pairs at elevated annealing temperatures resulted in successful amplification. While

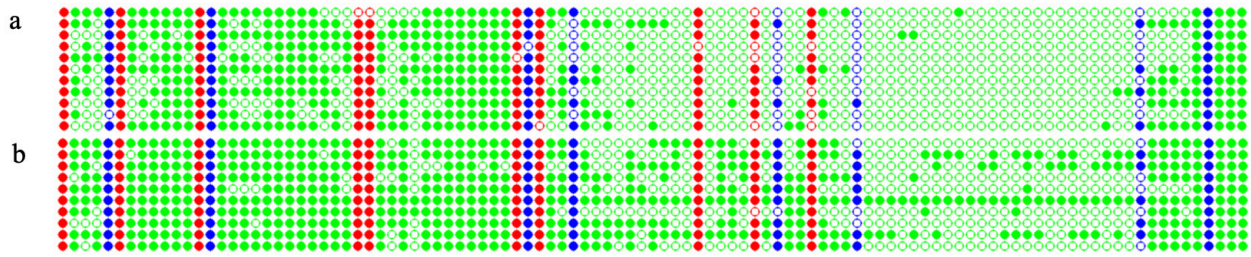

Figure 2. Some representative images for the outcomes of the bisulfite conversion of CA44 primer pairs are from the Kismeth program. Panel a: 15 (Days Post Anthesis) DPA pericarp, panel b: 60 DPA seed samples.

Table 1. Primer Pairs Suitable for DNA Methylation in Pepper.

ID	Acc. #	5′ ➔ 3′ Forward and Reverse sequences*	Genes	Region	Tar.	St./Fi.	TM
CA01	AJ276631	F: TGYTAAAYAATTAAGGGGTAATAATYA	kn gene	Promoter	1	76	60.3
		R: CAAATACATCCAAATACATATRRTACA			800	764	59.2
CA02	AJ276631	F: ATCGATYATAAGAGYAATAAAAATYAT	kn gene	Promoter	800	804	59.7
		R: GATAARCRAGAAAATCAARTAAARAA			1375	1352	58.7
CA05	DQ201633	F: GGCGTYTAGTAGTATTTTYAGTTTTT	PR-1 gene	Promoter	1	159	57.8
		R: CATARACCATAARCATAATCTACCATC			850	820	58.5
CA06	DQ201633	F: GCAGATATYYGTAYAAAAAYTTAAAAT	PR-1 gene	Promoter	850	870	59.3
		R: CATCAACTTTTCCAACTTAACAATTTC			1500	1390	60.4
CA12	DQ907615	F: TTGGYTTTATATAGYAAAAGAAAGTATT	capsanthin/ capsorubin synthase	Promoter	1	130	58.6
		R: ARRAATACTACAARRCCTCCAAC			1000	761	59.9
CA13	DQ907615	F: GGAAATTTTYATYGGGGTTYAAA	capsanthin/ capsorubin synthase	Promoter	1500	1657	60.5
		R: GAATTCTTCCAACARTTCRTTTTT			2309	2309	59.9
CA14	DQ907615	F: GTGAGTAAATAAYTTTGTTGGATGGAT	PR10 gene	Promoter	1	104	59.9
		R: GTTTGACRTTCAAATTTTCTATTTARC			750	607	59.8
CA17	DQ907615	F: CGTTTGAYGTTYAAATTTTYTATTTAG	PR10 gene	Promoter	750	893	60.5
		R: TACTAGRACCTRCTTCTTAAATRTRTT			1500	1486	60.0
CA19	AY804337	F: TAATATGAGTTGTAGTGGGATGATTGA	lipid transfer protein gene	Promoter	1100	1116	60.0
		R: ARAATRATGTTCCCTTTAATTTTTCTT			1700	1603	60.0
CA21	AY804337	F: TATATATGGATGTTTTGGGTYATAYAA	lipid transfer protein gene	Exon 2	2890	2723	59.7
		R: TTRATRATCATTCCATARAACAAATTA			2970	3190	59.9
CA22	AY804337	F: ATTAATTGTATAGYYAAAAGGAAAAYA	lipid transfer protein gene	Exon 3-Intron 3	3585	3406	59.6
		R: AGAAACAAATRRTAAAAARTAACATRC			3694	3856	60.1
CA39	EU046276	F: ATGGAATTGATATYGGAYAYTTTT	Upa20 gene	Exon 7-Intron 7	2609	2609	59.9
		R: GTACTCTTTTRRCTTCCACTRATT			3266	3266	60.0
CA44	X95730	F: TTACAYATAAGGYAYAYAAGTTTTTAG	defensin gene	Intron	2500	2506	59.9
		R: CTTGTTTCRTAAATTCACTTARACCTC			3100	3207	60.0
CA49	AY775331	F: GTGATTAAATTCTYGAGAYAAAGTAYA	SAR82A gene	3 UTR	2245	1831	59.2
		R: TTGCAARTTGARTTTACAATATRAAAR			2498	2377	60.0
CA54	AJ871130	F: CGTACAATGTTTTYGYATGTAATAAT	SP gene	Exon 4	2266	2008	60.4
		R: CTTCTGARAATTTTCRARTACARAATC			2487	2413	60.1
CA56	FJ570809	F: CCACTTATAATGTYYTGTTTYAYTTT	snakin (Sn) gene	Promoter	600	631	59.0
		R: CGGCGTATATAARTTAAATCTTCCTTT			1295	1094	60.4
CA59	EU616565	F: AAGTYYAAAGAAGATGGAAATAYAGT	hydroxycinnamoyl transferase	Exon	750	727	59.8
		R: GTCRRACTTRRCAATATAAAACTTA			1308	1226	59.8
CA60	AY819029	F: GGTCATTAGAAGGTYATAYYGCTY	acyltransferase (Pun1) gene	Promoter	1	1	58.3
		R: ATGATTRTTAAAATARTRARAATTRAAA			650	604	57.9
CA61	AY819029	F: CGTYTGAAAATTGAAATATATYTAGGG	acyltransferase (Pun1) gene	Promoter	650	624	59.7
		R: CCAAAGAARRAACCCTCCAAAATTA			1300	1207	60.7
CA62	AY819029	F: GAAAGAGAATTGGATTTYTAYATTTTT	acyltransferase (Pun1) gene	Promoter Exon 1	1300	1408	59.7
		R: AATRCAAAARCCATAATTAATTTAACA			2000	1912	60.3
CA64	AY819029	F: CTAGGYTATTTAGTYTATTTGTAGAAGYTA	acyltransferase (Pun1) gene	Intron 1	2700	2732	57.3
		R: CTTCTTATARCCATCCATATTTTCA			3400	3349	57.0
CA65	AY819029	F: GTAGTAGAATYAATGAGAGAAGGGAAA	acyltransferase (Pun1) gene	Exon 2 Intron 2	3200	3291	59.6
		R: CAAAARTATTCCTACCTTTRTTTCRTA			3752	3699	60.2
CA66	HQ229922	F: AYTGAAGAAGAAAGAATYAAGAATYAA	3-oxoacyl-synthase gene	Exon 1 Intron 1	1	41	60.1
		R: ATCAAARTATCAAATTCCCACATTTT			500	442	60.2
CA67	HQ229922	F: ACAYAAGGTAAAATTAAGGTTTGTGAG	3-oxoacyl-synthase gene	Intron 1	700	702	60.1
		R: ATAAAATCAAARAACATRRAAACAAAC			1200	1188	59.9
CA68	HQ229922	F: CAGTYTTTGGAAGTGATATYGATAAAT	3-oxoacyl-synthase gene	Exon 2 Intron 2	1300	1335	61.1
		R: ATAATATCARCTTCRCCCCTTCTAAT			2000	1953	62.5
CA70	HQ229922	F: ATTTTAAGCTAGAATGAAAATGTGTYY	3-oxoacyl-synthase gene	Intron 6 Exon 7	2700	2683	59.4
		R: GCTRTATCTTCAARAAATTATRARCTT			3456	3439	59.7

*Acc. #: accession number, Tar.: target, St./Fi.: start and finish sequences of primers, TM: melting temperature of primers

it is worth noting that a few products exceeded the length of 700 base pairs, amplicons within the range of 400 to 600 bases were found to be more suitable for transformation and cloning investigations. Based on empirical observations, it is recommended that researchers employ annealing temperature gradient spanning studies as a means to ascertain the ideal annealing temperature for primer pairs. Additionally, the utilization of a touchdown PCR profile, as proposed by Ince et al. (2010b), may prove beneficial in this regard.

The current investigation involved the selection of 26 primer pairs, as detailed in Table 1. These primer pairs were chosen based on their ability to amplify bisulfite-treated genomic DNA samples, their consistent product size between control DNA (unconverted bisulfite samples) and bisulfite converted samples, and their reliable amplification of various tissue sources of pepper, such as pericarps and flowers. In this study, a set of 26 primer pairs was employed to amplify a 14,473 base pair segment of genomic DNA from pepper plants, which includes 13 distinct genes. These genes include kn gene, pathogenesis related protein-1 (PR-1) gene, capsanthin/capsorubin synthase gene, lipid transfer protein gene, pathogenesis related protein 10 (PR10) gene, bHLH transcription factor Upa20 gene, SAR82A gene, defensin gene, SP gene, snakin (Sn) gene, hydroxycinnamoyl transferase gene, acyltransferase (Pun1) gene, and 3-oxoacyl-(acyl-carrier-protein) synthase gene (Figure 3).

The analysis conducted indicated that the pepper genomic DNA, spanning 14,473 base pairs, included a cumulative count of 2375 cytosine residues across the 13 genes encompassed within it. The dataset consisted of 264 CpGs, 335 CpHpGs, and 1926 CpHpHs, as illustrated in Figure 2. The highest and highly variable CpHpH concentrations were observed among the 26 DNA regions investigated.

Differential amounts and patterns of cytosine methylation were seen in the CpG, CpHpG, and CpHpH contexts, as determined through computational analysis using KisMeth (Gruntman et al., 2008). The CpG sites exhibited the highest levels of methylation, followed by CpHpG sites and CpHpH sites. The findings of the study revealed that genes exhibiting comparable biological functions, such as housekeeping or tissue-specific genes, as well as DNA sequences situated within similar genomic areas, such as chloroplast or mitochondrial DNA, exhibit similar levels of cytosine methylation.

The examination of 14,473 base pair DNA sequences indicated that the CpHpH contents exhibited the highest levels within the promoters, exons, and introns of the 13 genes under investigation. The cytosine methylation contents of CpHpG were found to be the second highest, while CpG contents exhibited the lowest levels among the various cytosine methylation contents that were examined (Figure 4). The disparities in CpG and CpHpG concentrations inside promoters exhibited somewhat smaller magnitudes when contrasted with those observed in introns and exons. Subsequent investigations have provided compelling evidence to support the notion that methylation of cytosine nucleotides within promoters holds greater significance when compared to methylation patterns observed in exons and introns. This finding suggests that methylation processes are significant in the control of gene expressions (Karaca et al., 2016a; Xiao et al., 2020; Cai et al., 2022; Jaiswal et al., 2022).

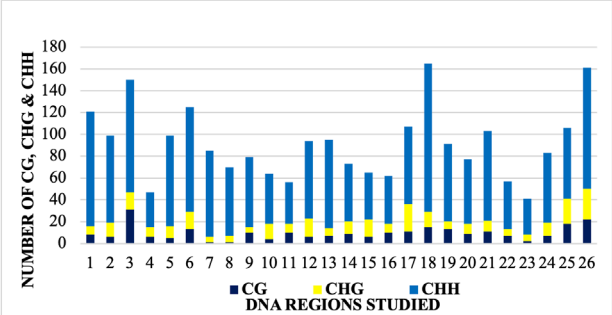

Figure 3. Numbers of CG, CHG and CHH Contents Among 26 Genomic Regions of Pepper

The current work demonstrates that there are variations in DNA cytosine methylation levels among genes, gene locations, promoters, and gene body components such as introns and exons. These findings provide unambiguous evidence of the presence of DNA cytosine methylation. The variations in CpG, CpHpG, and CpHpH contents observed in various genes and gene body entities may be associated with biological processes such as gene expression, genomic imprinting, recombination, and DNA replication. Our results are consistent with prior studies that have shown a lower level of methylation in transcribed regions compared to promoters (Teyssier et al., 2008; Gallusci et al., 2016; Karaca et al., 2016b; Xiao et al., 2020; Cai et al., 2022).

The majority of the genomic areas that were amplified using the proposed primer pairs exhibited variations in the methylation status of cytosine nucleotides. Bisulfite primer pairs have the potential to be employed for the identification and quantification of genomic DNA sequences that are unmethylated, methylated, or differentially methylated. Additionally, they can serve as internal or validation controls in high throughput investigations conducted at the genome-wide level (Xiao et al., 2020; Araz et al., 2022; Cai et al., 2022).

Epigenetic regulations arise as a consequence of differences in the degree of DNA cytosine methylation. The occurrence and variability of methylations have been associated with various genetic phenomena, such as DNA recombination, gene expression, and transposon silencing. While the epigenetic concept was experimentally discovered in pepper as the second crop, the level of DNA methylation in this crop is comparatively lower than that observed in numerous other crop species.

In this study, we presented the amplification efficacy of 26 bisulfite-specific primer pairs for both bisulfite-converted and unconverted genomic DNA samples. The primer pairs utilized in this study consisted of nucleotide sequences ranging from 23 to 30 base pairs in length. These primer pairs were shown to generate amplified products that were both singular and well-defined when subjected to elevated annealing temperatures. While certain amplified products exceeded a length of 700 base pairs, the sizes of the amplicons varied between 400 and 550 bases.

In the genes whose methylation levels were investigated in this study, differences were found in methylation levels according to gene regions. There were a total of 937 cytosine contents in the promoter regions, of which 91 CpG, 97 CpHpG and 749 CpHpH. On the other hand, there were 1270 cytosine contents in the exons, including 141 CpG, 195 CpHpG and 934 CpHpH. Introns contained 245 cytosines, 23 of which are CpG, 32 were CpHpG and 190 are CpHpH. There were only 73 total cytosine contents in the 3UTRs; of these, 9 were CpG, 11 were CpHpG, and 53 were CpHpH. Primer pairs possess the capability to not only facilitate the identification and quantification of unmethylated, methylated, and differentially methylated DNA sequences, but also serve as internal or validation controls in next generation sequencing technologies employed for DNA methylation investigations (Ince and Karaca, 2021).

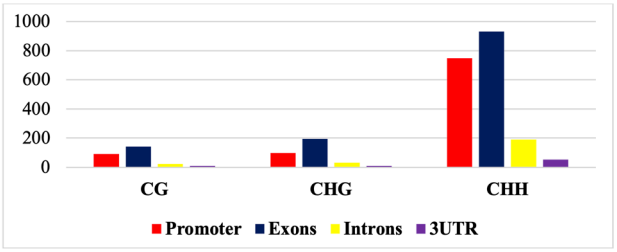

Figure 4. Number of CG, CHG and CHH Contents Presented in Promoters, Exons and Introns

CONCLUSION

The field of epigenetic research in pepper lags behind that of numerous other significant crops. In this study, it was presented 26 bisulfite specific primer pairs that demonstrate successful amplification of both bisulfite converted and unconverted DNA. Based on the empirical evidence and study outcomes, it is recommended that researchers should employ annealing temperature gradient spanning studies as a means to ascertain the best annealing temperature for primer pairs. Additionally, the utilization of a touchdown PCR profile is proposed as a viable approach in this regard. Using these suggestions one can get effective of amplifications, as can be seen by band intensity. The use of these primer pairs target cytosine nucleotides that were differently methylated. The utilization of bisulfite primer pairs may enable the acquisition of data pertaining to allele-specific methylation in pepper. The primer pairs disclosed in this paper have the potential to serve as internal or validation controls in genome-wide high throughput investigations, as they can target unmethylated, completely methylated, and differentially methylated genomic areas.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Ethics committee approval is not required.

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Data availability

Not applicable

Consent for publication

Not applicable

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