Molecular Plant • Volume 4 • Number 6 • Pages 996–1013 • November 2011 RESEARCH ARTICLE

Divergent DNA Methylation Patterns Associated with Abiotic Stress in *Hevea brasiliensis*

Thomas K. Uthup[1], Minimol Ravindran, K. Bini and **Saha Thakurdas**

Genome Analysis Lab, Rubber Research Institute of India, Rubber Board PO, Kottayam, Kerala, 686009, India

ABSTRACT Cytosine methylation is a fundamental epigenetic mechanism for gene-expression regulation and development in plants. Here, we report for the first time the identification of DNA methylation patterns and their putative relationship with abiotic stress in the tree crop *Hevea brasiliensis* (source of 99% of natural rubber in the world). Regulatory sequences of four major genes involved in the mevalonate pathway (rubber biosynthesis pathway) and one general defense-related gene of three high-yielding popular rubber clones grown at two different agroclimatic conditions were analyzed for the presence of methylation. We found several significant variations in the methylation pattern at core DNA binding motifs within all the five genes. Several consistent clone-specific and location-specific methylation patterns were identified. The differences in methylation pattern observed at certain pivotal *cis*-regulatory sites indicate the direct impact of stress on the genome and support the hypothesis of site-specific stress-induced DNA methylation. It is assumed that some of the methylation patterns observed may be involved in the stress-responsive mechanism in plants by which they adapt to extreme conditions. The study also provide clues towards the existence of highly divergent phenotypic characters among *Hevea* clones despite their very similar genetic make-up. Altogether, the observations from this study prove beyond doubt that there exist epigenetic variations in *Hevea* and environmental factors play a significant role in the induction of site-specific epigenetic mutations in its genome.

Key words: DNA methylation; epigenetics; abiotic stress; rubber biosynthesis; *Hevea brasiliensis*.

INTRODUCTION

Hevea brasiliensis is a perennial tropical tree species native to the Amazonian forests of Brazil, South America. It is the major source of natural rubber (*cis*-1,4-polyisoprene), a high-priced commodity of great demand for the world rubber industry (Figure 1). Natural rubber is present in colloidal form in the latex (the cytoplasm of laticiferous cells present in the bark of rubber trees). Latex is extracted by wounding the bark of rubber trees where laticifers are present abundantly by a process known as tapping. Since the traditional clones cultivated extensively for rubber production in Asia were derived from a few original seeds collected from the Amazonian forest, they represent only a very small part of the extensive gene pool that exists there. Thus, the genetic base of the presently cultivated clones is insufficient to carry on breeding for achieving substantial genetic improvements in rubber production or in disease resistance. In addition, several years of directional selection for yield and the vegetative method of propagation has also narrowed downed its genetic base (Besse et al., 1994). This low rate of genetic polymorphism among the present popular clones commercially cultivated was demonstrated by several groups using molecular marker techniques (Seguin et al., 1995; Lekawipat et al., 2003; Hernandez et al., 2006).

A puzzling incongruity to the above assumption is the remarkable divergence in quantitative and qualitative characters amongst the popular clones commercially cultivated in Asia pacific region despite their analogous genetic make-up. Wide disparity in traits like yield, disease resistance, cold, and drought tolerance is shown by individuals of the same clone when planted at diverse geographic locations. So far, no satisfactory scientific evidences are available to explain this phenomenon; instead, the changes observed were merely attributed to environmental interactions. This lack of information underlying the behavioral pattern variation of plants under diverse environmental conditions is a serious constraint in the development of high-performing location-specific *Hevea* clones with consistent yield pattern.

[1] To whom correspondence should be addressed. E-mail thomasku79@gmail.com, tel. 919400562247, fax 914812353311.

doi: 10.1093/mp/ssr039, Advance Access publication 24 June 2011
Received 15 November 2010; accepted 20 March 2011

Figure 1. *Hevea* brasiliensis: - The source of natural rubber

Main photo: Rubber planted at low lying areas of southern India without disturbing the natural habitat.
Inset- "From plant to processing centre".
Top: Latex oozing out from the bark of a tapped rubber tree to the collection cup attached to the stem.
Bottom: Natives transporting collected latex to the processing centre over a swinging bridge in the forests of southern India constructed by the British in 1937.

Plants acclimatize to unfavorable conditions primarily through strategies that involve the manipulation of their complex regulatory network of molecular interactions, by controlling the expression of hundreds of genes (Farrel, 2007; Vyroubalová et al., 2009). This is true in the case of rubber also, since it quickly adapts to the non-optimal growth conditions prevalent in the majority of the present rubber-cultivating areas. Since the agroclimatic conditions in these locations are highly flexible, any permanent irreversible changes in the genome will prove to be a hitch once the situation is restored. To cop up with such situations, plants have adopted a strategy by which the genome is reversibly modified through the interaction of external molecules; thereby, the functionality of the concerned gene is altered, keeping the original genetic make-up intact. Since the dynamics involved in such modifications are due to aspects above genetics, they are called epigenetic factors. Recent studies in molecular genetics have revealed that novel gene expressions, and therefore novel phenotypes, can be achieved through a suite of epigenetic mechanisms, even in the complete absence of genetic variation (Peredo et al., 2006). DNA methylation is one such epigenetic modification profusely seen in most of the higher plants and animals that plays an important role in regulating development and developmental processes (Feng et al., 2010; Meijón et al., 2010). DNA methylation in higher eukaryotes is merely the presence of 5-methylcytosine (m5C) nucleotides in the DNA instead of a normal cytosine. By and large, this epigenetic event has been observed abundantly in repetitive sequences, transposons, and in GC-rich regions of promoters and end regions of transcribed portions called CpG islands ('p' designates the phosphodiester bond that joins two nucleotides) (Lippman et al., 2004). While CpG methylation is the more common form in animals, in plants, it has been observed in CpNpG sites ('N' any nucleotide) and CpNpN asymmetric sites (Pradhan and Adams, 1995). Methylation of cytosine has received more attention in the last two decades, given the possibility that they may be associated with the alteration in gene transcription leading to morphological changes without changing the sequence (Lee et al., 2010). This could be either due to hypermethylation, which possibly will alter the chromatin structure, preventing normal interaction of DNA strand with the transcriptional machinery, or due to hypomethylation resulting in the activation of some previously silenced gene (Baylin et al., 2001). So, under particular circumstances, genes that had been previously silenced can be expressed again if the methyl group is removed or can be suppressed if the methyl group is attached.

DNA methylation is considered to be an important mechanism not only for regulating the gene expression, but also in the silencing of transposons and other destructive repetitive sequences (Okamoto and Hirochika, 2001; Villar et al., 2009). More insights into the biology of transposable element methylation and its activity were provided by Palmer et al. (2003), Whitelaw et al. (2003), and Bedell et al. (2005). Besides, it is also established that, under biotic and abiotic-stress conditions, plants employ a great deal of methylation to deactivate the elevated transposomal activity that would otherwise mediate the destabilization of the genome (Lukens and Zhan, 2007). In addition to the above aspects, methylation also stabilizes the genome through the suppression of homologous recombination between dispersed sequences and by restricting recombination events to the hypomethylated gene-rich regions (Maloisel and Rossignol, 1998; Khrustaleva et al., 2005).

When heritable DNA methylation variations occur among individuals of a species that are otherwise genetically identical, they can be considered as epigenetic mutations. Such epigenetic mutations may result in genetic point mutations like 'C' to 'T' transitions in higher organisms but, very often, they may directly generate favorable or unfavorable phenotypic characters in plants. The methylation variations responsible for such changes could be stable and inherited over generations or a transient mechanism to cope with a temporarily stressed condition. While methylation resulting from exposure to prolonged stressed conditions (biotic and abiotic) was often found to be meiotically heritable, those induced by brief adversities may generally be inherited mitotically, with minor exceptions (Saze, 2008). Like genetic mutations, such epigenetic mutations also may have a deep, and hitherto unsuspected,influence on the ecology and evolution of populations. Heritable epigenetic silencing associated with locus-specific DNA methylation changes has been documented for numerous genes involved in plant development, including superman (Jacobsen and Meyerowitz, 1997), agamous (Jacobsen et al., 2000), and flowering locus C (FLC) (Soppe et al., 2000). Alternatively, methylation is also considered as an active factor involved in the flexible short-term stress response memory in plants to counteract hostile conditions in the immediate future (Bruce et al., 2007).

While methylation induced by biotic stress is generally associated with the silencing of parasitic DNA and expression of resistant genes, abiotic stress-induced methylation is supposed to be linked with the numerous biochemical pathways involved in acclimatization and stress response in plants. Finnegan et al. (1998) illustrated this by demonstrating a transient DNA methylation decline in *Arabidopsis* FLC and its two flanking genes during vernalization response. Furthermore, the link between cold stress and hypomethylation was explained by Chinnusamy et al. (2008) and Chinnusamy and Zhu (2009), where they have shown that cold treatment promotes tissue-specific hypomethylation of defined areas of the genome including areas specific to retrotransposon sequences. Similarly, in tobacco stress-induced-DNA demethylation in the coding sequence of the NtGPDL (a glycerophosphodiesterase-like protein) gene by aluminum, paraquat, salt, and cold correlated with its expression (Choi and Sano, 2007). Reports are also there for drought stress-induced DNA hypermethylation in pea (Labra et al., 2002). All the above studies indicate that methylation is a well-synchronized strategy of the plants to regulate gene expression that enables them to adapt to the changing external environment.

It is well understood that the coordinated regulation of hundreds of genes responding in a chronological, spatial, and provisional expression is partially mediated by the transient binding of transcription factors to their specific DNA motifs. DNA methylation has been shown to repress this transcriptional machinery by interfering with the binding of transcriptional activators resulting in transcriptional gene silencing (TGS) in plants and animals (Kinoshita et al., 2007; Zhang and Ogas, 2009). Methylation of CG dinucleotides in the promoter region has been shown to influence the expression of genes downstream significantly due to their direct modification of transcription factor binding target sites resulting in TGS (Turker, 2002; Zemojtel et al., 2008; Cox and Nathanielsz, 2009). Since modifications within these regions may result in remarkable changes in gene expression, screening for methylation target sites at key *cis*-regulatory elements is imperative to fully understand the processes involved in development, stress response, and molecular adaptation machinery in plants. It is assumed that these sites may be the initial action point of environmental or other external factors on the genome having a direct impact on the expression of concerned genes and subsequently on phenotypic characters.

The present study was prompted by the need to identify the direct impact of the environment on rubber genome and the modifications triggered by external stress on the regulatory sequences of genes associated with traits of importance in rubber. Rubber yield is considered to be significantly affected by environmental variations, due to which many high-yielding clones developed are not showing consistent productivity when planted at climatically different locations. The major reason for this discrepancy is attributed to abiotic stress, which may trigger epigenetic changes within the genome (Priyadarshan et al., 2005). Epigenetic modifications like DNA methylation may regulate gene expression at various levels to assist diversification of available nutrients for essential functions compromising production of lower-priority secondary metabolites like isoprenes, which may have a negative impact on latex production (Funk et al., 2004; Wahid et al., 2007). Moreover, the involvement of rubber biosynthesis genes in general stress tolerance renders them more prone to such regulatory processes than other genes. Inappropriate methylation may also result in unfavorable genomic rearrangements often triggered by mobile elements like retrotransposons affecting the expression of essential genes required for the basic cellular functions. In the case of *Hevea brasiliensis*, chances of such genetic rearrangement by retroelements are high due to the abundance of retroelements as reported by Saha et al. (2006). Furthermore, a huge haploid genome size of around 4×10^9 bp (Roy et al., 2004) with large chunks of repeat sequences and the modest digestion of this bulky genome with methylation-sensitive enzymes as reported earlier also point towards the probability of finding a heavily methylated genome in *Hevea*.

Here, we analyzed the genome of three popular clones of rubber planted at two ecologically different locations to find out the impact of environmental stress on their epigenome keeping latex biosynthesis genes in focus. In order to test the hypothesis that abiotic stress have direct impact on the epigenome of plants by inducing site specific methylation/demethylation in the rubber genome, methylation status of the promoter region of rubber biosynthesis genes in the mevolanate pathway (MVA), namely HMG-CoA reductase 1 (HMGR1), HMG-CoA synthase 1 (HMGS1), Farnasyl diphosphate synthase (FDP), Rubber elongation factor (REF), and the disease resistance gene - Coronatine-insensitive1 (COI1), were analyzed by

bisulfite sequencing. CG-rich promoter regions of these genes were selected, assuming that methylation or demethylation of these sites will have a direct impact on the expression of genes downstream. The study aims to identify the influence of stress on the epigenome of plants grown at two different geographic locations as well as the DNA methylation changes it triggers on regulatory motifs of five agronomically important genes in *Hevea*. It is anticipated that the results from this study will help to better understand the fundamental aspects involved in the genome-level molecular modifications by plants under stressed conditions. In this report, we provide support for the conclusion that abiotic stress has a direct impact on the genome and it may result in gene-expression variations even among genetically identical plants. Early epigenome-based screening of juveniles to predict their predisposition to different environmental conditions and identification of stress-specific and location-specific epigenetic markers are the expected outcome of a detailed study in this direction. We speculate that, after validation, these techniques will prove to be effective molecular breeding tools for the release of new high-yielding environmentally better-adaptable *Hevea* clones in the future.

RESULTS

Marker Analysis

RAPD, Microsatellite, and Sequence Analysis

RAPD, SSR, and sequence analysis were carried out to prove the genetic uniqueness of the three clones under study. Alternatively, these techniques were also used to show the genome stability of *Hevea* clones under external stress experienced by the plants at their respective planting locations. OPF-10, OPAI-6, and OPC-5 RAPD profiles showed that there were clear variations in the banding patterns of the three clones studied, whereas individual plants of the same clone showed identical patterns, irrespective of their location (Figure 2). SSR analysis also exhibited identical allele distribution for individuals of the same clones from both locations, while interclonal allele variations were very distinct (Figure 3). On the contrary, the aligned master sequences of the respective gene promoters were found to be highly conserved across all the samples (not shown). Thus, RAPD and SSR results show that the three clones are genetically unique and the external environment does not have much influence on the DNA sequence, at least on the selected loci. The name of each plant, their clonal identity, year of sampling, and location is given in Table 1.

McrBC RAPD

McrBC RAPD was performed to demonstrate the presence of methylation at several sites within the *Hevea* genome. The differences in RAPD banding pattern observed when McrBC-digested and undigested genomic DNA from the same plants were used highlights the presence of methylated bases in Hevea genome (Figure 4). RAPD profiles of *McrBC*-digested genomic DNA showed significant variation from the undigested DNA for all the three primer combinations. OPAI-6 primer exhibited unique profiles for all the three clones. In McrBC RAPD, RRIM 600 pattern stood apart from the other two despite the absence of such a variation in the normal RAPD with the same markers. Even in McrBC RAPD, no polymorphic bands were observed among individual plants of the same clone from two locations. The differences in *McrBC*-digested and undigested RAPD profiles show that there exist several methylated sites within *Hevea* genome and these patterns vary from clone to clone.

Restriction Digestion Using Isoschizomers

Simple restriction digestion was used to identify the existence as well as the type of methylation especially at the recognition sites of three restriction endonucleases within the *Hevea* genome (Figure 5). Polymorphic bands were detected when Ra1 (RRII 105) DNA was digested using *Ava*I/*Bso*BI combination. Digestion using *Bso*BI showed several unique fragments when compared to that of methylation-sensitive *Ava*I. Rb1 (RRIM 600) DNA also showed same trend as that of Ra1, while Rc1 (PB 260) digestion yielded no visible dissimilarity between the two enzymes; instead, it appeared distinct from the other two clones. With *Hpa*II/*Msp*I combination, Ra1 exhibited poor digestion for the methylation-sensitive *Hpa*II, while *Msp*I digestion provided a smear. Rb1 and Rc1 also showed similar results to that of Ra1. With *PspG*I/*BstN*I combination, digestion was partial or 'nil' in most cases. Minor differences were observed in the case of Rc1, whereas no significant variation in the banding pattern was observed for the other two genotypes. Comparative digestion of Ra1 and Ea1, Rb1 and Eb1, Rc1 and Ec1 using all the three methylation-sensitive enzyme combinations did not reveal any clear difference in their patterns (results not shown). Restriction digestion analysis using the isochizomeric pairs proved the existence of DNA methylation variations among the three *Hevea* clones, as well as the type of methylation they harbor.

Bisulfite Sequence Analysis

Bisulfite sequencing of the promoter region of selected genes was performed to find out the exact location, class, and extent of methylation in the samples analyzed. The combined sequence analysis results of all the five gene promoters using CyMATE software was highlighted and is shown in Figure 6. In order to better understand the significance of methylation/demethylation on the functionality of the concerned genes, the location, putative function and the methylation status of the *cis*-regulatory elements present in the analyzed genomic region of all the five genes were given. (Supplemental Table 1).

HMGR1 Promoter

The analyzed 288-bp region during 2007 and 2008 consisted of *cis*-regulatory elements like ARE, CAAT-box, CATT-motif, CCAAT-box, CGTCA-motif, LTR, TATA-box, TC-rich repeats, TCCC-motif, and several unnamed motifs. CGN, CHG, and CHH patterns of methylation were observed in the sequences. In the master sequence, probable maximum CGN frequency was found to be 16.87%, CHG 13.25%, and CHH 69.88%. Of

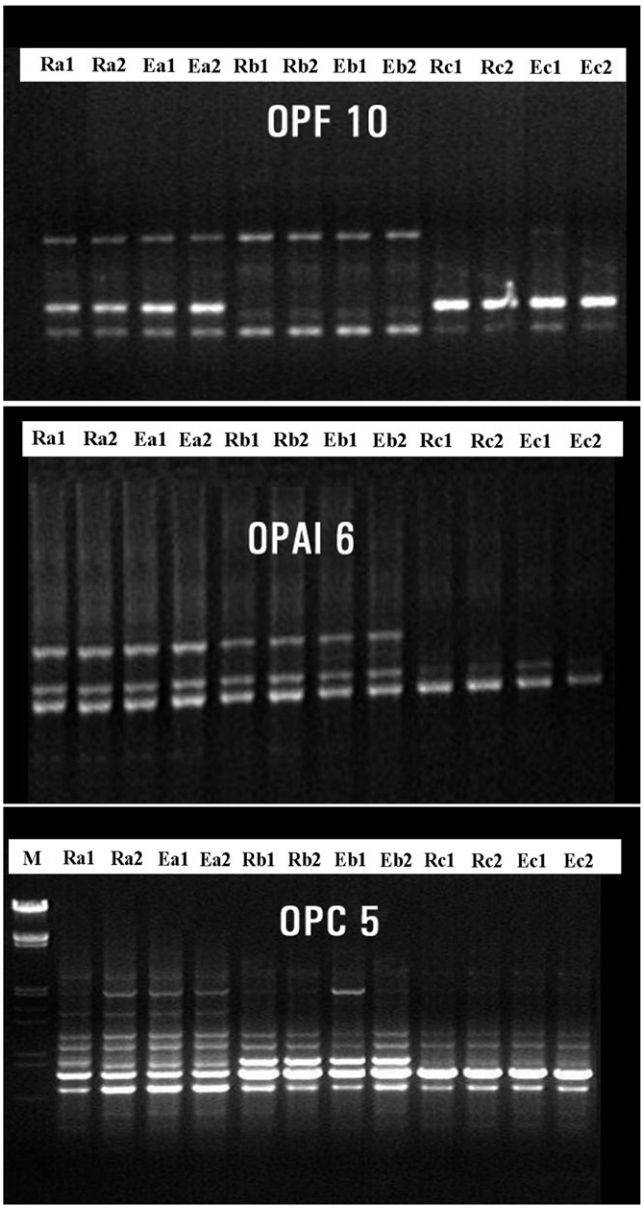

Figure 2. RAPD Profiles Showing Genetic Variation among the Three Clones.

RAPD profiles of all the 12 plants using three random decamers: OPF-10, OPAI-6, and OPC-5.
RRIM 600 pattern is different from the other two for OPF-10 and PB 260 pattern is different from the other two for OPAI-6 and OPC-5.

the 1992 possible sites for different classes of methylation in the alignment file containing representative clone sequences, 113 sites (5.67%) were methylated and 1 879 (94.33%) were found to be unmethylated. Average methylation density for CGN sites was found to be 3.27%, whereas it was 15.53 and 4.38% for CHG and CHH sites, respectively (Figure 7). Contradictory to the earlier reports in other plant species, CHG type was found to be the dominant type of pattern in rubber rather than CGN as far as the analyzed genomic region is concerned.

An interesting observation was made regarding the methylation pattern of RRII 105 clones at their CAAT-box, which is directly involved in controlling the rate of transcription. The CAAT-boxes of the two RRII 105 plants from Elappara seems to be methylated in 2007 as well as in 2008, whereas this site was not methylated in any other samples including control RRII 105 plants. Chromatogram of bisulfite converted HMGR1 promoter region, highlighting the site-specific demethylation and methylation of CAAT-box in RRII 105 control and test clones during 2008 is shown in Figure 8. A reverse pattern was observed at CATT and CGTCA motifs in RRII 105, where the sites were demethylated in plants from Elappara and methylated in those at RRII campus. The clone PB 260 also exhibited a similar trend. Another alteration in methylation pattern was observed in the LTR element where Ra1 and Ra2 were methylated throughout the study period, whereas their counterparts at Elappara (Ea1 and Ea2) remained demethylated during 2007. Surprisingly, Ea2 seems to take up a methyl group in 2008, while Ea1 remained as such. A noticeable phenomenon was the uniform methylation of this site exhibited by all the four plants of the clone RRIM 600 during the two-year study period. PB 260 clones showed an entirely different episode in the LTR motif, where both plants from RRII campus during 2007 and 2008 showed methylation whereas the Elappara plants displayed demethylation of this site in the year 2007 and methylation in the subsequent year. RRII 105 plants also showed methylation at a TC-rich repeat involved in defense and stress responsiveness, whereas no other clones exhibited methylation in this particular site. In general, RRII 105 plants were found to be more prone to methylation changes in the genomic region studied.

HMGS Gene Promoter

The 229-bp HMGS promoter region analyzed from the 2008 samples consisted of *cis*-regulatory elements like CAAT-box, AT-rich element (ATBP1), GARE motif, TATA-box, HSE motif, and several unnamed motifs. Only CHG and CHH pattern of methylation was observed in the analyzed sequence. In the master sequence, probable maximum CGN-type methylation was found to be 6.52%, CHG 26.09%, and CHH 67.39%, respectively. Of the 552 possible sites for different classes of methylation, 35 (6.34%) were methylated and 515 (93.30%) were unmethylated. In the analyzed segment, CHG was the dominant type, with an average methylation density of 18.75%, while CHH was 2.15% (Figure 7). In HMGS gene promoter, CAAT-box showed uniform methylation in Elappara plants and demethylation in RRII plants similar to that in HMGR1. The GARE motif also showed a similar trend in RRII 105. As far as HSE motif was concerned, a specific methylation pattern was observed just two bases before the regulatory site. Except for the two RRII 105 plants from Elappara, this site was methylated in all the samples.

FDP Synthase Gene Promoter

The 166-bp fragment from samples analyzed during 2008 consisted of *cis*-regulatory elements like CAAT-box, CGTCA motif,

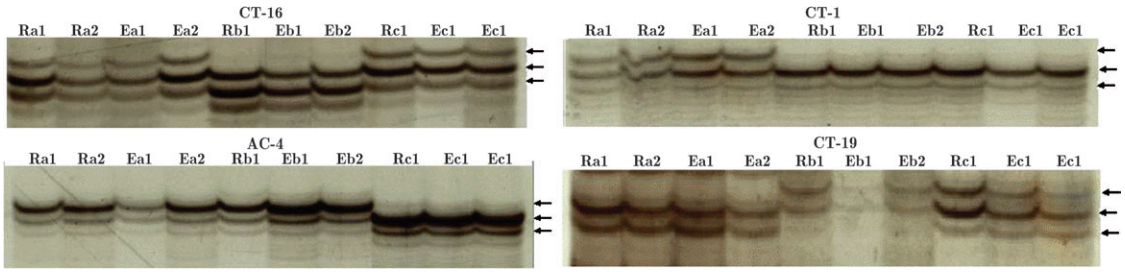

Figure 3. Microsatellite Profiles Showing Genetic Variation among the Three Clones.

Microsatellite profiles of plants using the dinucleotide repeat markers: CT-16, CT-1, AC-4, and CT-19. From the CT-16 marker profile, RRIM 600 can be differentiated from RRII 105 and PB 260. In the CT-1 marker profile, RRII 105 is showing a unique allele that is not present in the other two. In the AC-4 profile, allelic variation was observed for PB 260 whereas the other two displayed similar patterns. In the CT-19 profile, RRII 105 can easily be distinguished from the other two cloes: RRIM 600 and PB 260. (Rb2 and Rc2 plants are not shown.)

Table 1. Sample Details.

Clone	Location	Plants	Year
RRII 105	RRII Campus	Ra1, Ra2	2007 and 2008
	Elappara	Ea1, Ea2	2007 and 2008
RRIM 600	RRII Campus	Rb1, Rb2	2007 and 2008
	Elappara	Eb1, Eb2	2007 and 2008
PB 260	RRII Campus	Rc1, Rc2	2007 and 2008
	Elappara	Ec1, Ec2	2007 and 2008

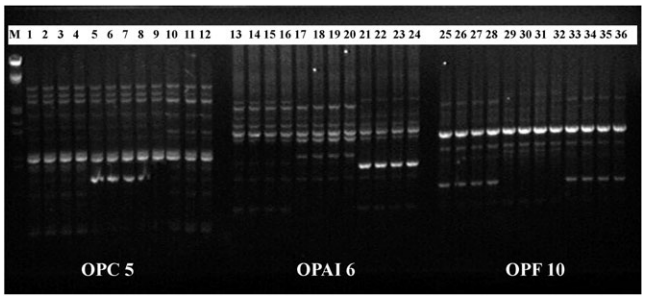

Figure 4. Methylation-Sensitive RAPD Profiling.

Methylation-sensitive RAPD to prove the existence of methyl groups in *Hevea* genome. Reactions were carried out using the same random decamers as used for normal RAPD after digesting genomic DNA with methylation-dependent McrBC enzyme. All the 12 plants belonging to the three clones during 2008 are shown. Loading order for each marker. First four lanes: RRII 105 plants (Ra1, Ra2, Ea1, and Ea2). Second four lanes: RRIM 600 plants (Rb1, Rb2, Eb1, and Eb2). Last four lanes: PB 260 plants (Rc1, Rc2, Ec1, and Ec2). Due to the methylation variation within the decamer-amplified region, a different profile from that of normal RAPD was obtained for all the three markers. The presence of several additional bands to that in normal RAPD indicates methylation at several sites. The single decamer OPAI-6 could successfully differentiate all the three clones as seen in the picture.

and a few unnamed motifs. Only CGN and CHH patterns of methylation were observed in the sequences analyzed. Out of the 35 methylation patterns observed in the master sequence, probable maximum frequency for CGN-type methylation was 11.43%, CHG 28.57%, and CHH 57.14%. Of the possible 420 sites for different classes of methylation, 15 (3.57%) were methylated and 393 (93.57%) were unmethylated. Average methylation density for CGN sites was found to be 10.42% and 3.75% for CHH type (Figure 7). In the examined fragment, CGN type was found to be the dominant type. Patterns specific for RRII 105 and PB 260 clones from Elappara samples were also observed. Moreover, location-specific methylation at CAAT and CGTCA sites of RRII105 clones at Elappara was clearly visible in FDP promoter region. As in the case of HMGR1 and HMGS1, the CAAT-box appears to be methylated in RRII 105 plants from Elappara only, whereas the CGTCA motif was methylated only in the control RRII 105 plants.

REF Gene Promoter

The 348-bp fragment of Rubber elongation factor gene promoter from the 2008 samples consisted of *cis*-regulatory elements like ACE, ARE, BoxI, CAAT-box, G-box, P-box (Gibberellin-responsive element), TATA-box, TCA element, and a few unnamed motifs. CGN, CHG, and CHH patterns of methylation were observed in the analyzed sequences. Out of the 79 bisulfite patterns observed in the master sequence, probable maximum frequency for CGN-type methylation was found to be 20.25%, CHG 13.92%, and CHH 64.56%. Of the possible 948 sites for different classes of methylation, 53 (5.59%) were methylated and 881 (92.93%) were unmethylated. In the analyzed fragment, CGN type was dominant, with an average methylation density of 8.85%, whereas it was 0.76% for CHG and 5.72% for CHH type, respectively (Figure 7). The CAAT motif remained under-methylated in all the samples. A 10-bp *cis*-acting TCA element (CAGAAAAGGA) putatively involved in Methyl Jasmonate responsiveness seems to be methylated in all clones from both locations except RRII 105. Another clone-specific pattern was observed for PB 260 at their ACE motif.

COI1 Gene Promoter

The 167-bp COI1 gene promoter region from 2007 and 2008 samples consisted of *cis*-regulatory elements like ACE, CAAT-box,

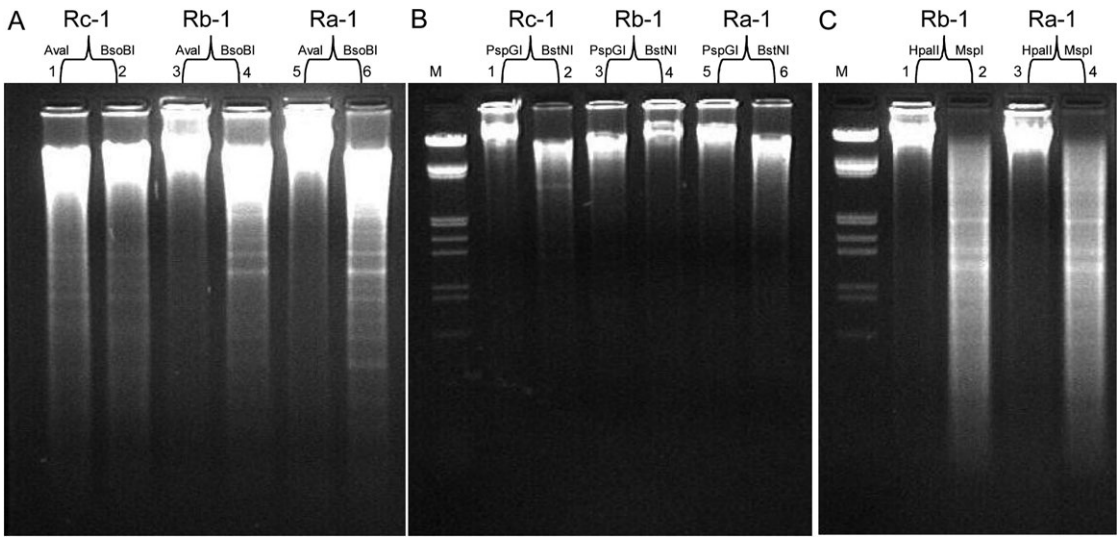

Figure 5. Restriction Digestion of Genomic DNA Using Isochizomeric Enzyme Pairs.

Restriction digestion of genomic DNA using methylation-sensitive/insensitive isochizomeric pairs to examine global methylation variation among the three clones.

(A) *Aval*/*Bso*BI (Lane Nos 1 and 2: PB 260, Lane Nos 3 and 4: RRIM 600, Lane Nos 5 and 6: RRII 105).

(B) *Psp*GI/*Bst*NI (Lane Nos 1 and 2: PB 260, Lane Nos 3 and 4: RRIM 600, Lane Nos 5 and 6: RRII 105).

(C) *Hpa*II/*Msp*I (Lane Nos 1 and 2: RRIM 600, Lane Nos 3 and 4: RRII 105). (PB 260 not included in this combination.)

Sample designation: Rc-1 (PB260), Rb-1 (RRIM 600), Ra-1 (RRII 105).

E2Fb, Skn-1_motif, TATA-box, TATCCAT/C motif, and several unnamed motifs. CGN, CHG, and CHH patterns of methylation were observed in all the sequences. In the master sequence, probable maximum frequency for CGN-type methylation was found to be 32.50%, CHG type 27.50%, and CHH type 37.50%. Of the 960 possible sites for different classes of methylation, 85 (8.85%) were methylated and 851 (88.65%) were under-methylated. Average methylation density for CGN sites was found to be 20.83%, whereas it was 2.27 and 3.89% for CHG and CHH, respectively (Figure 7). In the analyzed fragment, CGN type seems to be the dominant type. Methylation in the ACE motif was observed only for the RRII 105 plants from Elappara. CAAT-box of all RRII105 samples showed methylation irrespective of time and location, while the other two clones appeared completely unmethylated. The E2Fb transcription factor binding site was also blocked in RRII105 planted in Elappara. Site 138, which was not associated with any known regulatory sites, was found to be uniformly methylated in all the samples.

Methylation-Specific–PCR

MS–PCR was carried out to verify the result obtained from the bisulfite sequencing of gene promoters. For the sake of convenience, a single representative locus from the HMGR1 gene was selected for the analysis. When methylation-sensitive primers were used, amplification was observed only for RRII 105 plant from Elappara (Ea1). No amplification was obtained from this DNA when the sensitive primers were replaced by insensitive ones. Contrarily, successful amplification with methylation-insensitive primer was observed for all the other samples. A control reaction using normal primer also yielded an amplicon of similar size (Figure 9).

DISCUSSION

Marker Analysis

RAPD, Microsatellite, and Sequence Analysis

Phenotypic differences in plants may occur due to genetic variation as well as epigenetic variation or both (Wong et al., 2005; Chen, 2007). Genetic variations can be established very clearly using molecular genetic markers like RAPDs, RFLPs, SSRs, SNPs, etc. RAPD profiles of the plants under investigation clearly distinguished the genetic variation of the three clones, irrespective of their planting location. The genotype of selected clones were well apparent from the analysis using the three decamer primers OPF-10, OPAI-6, and OPC-5. However, it should be noted that individual plants of the same clone grown under different environmental conditions showed no obvious genetic variation in spite of the extremely divergent agro-climatic condition that prevailed in those locations. Microsatellite analysis also exhibited identical allele distribution for individual plants of the same clone, irrespective of their planting location, while inter-clonal allelic variations were very clear, which confirmed that the three clones: RRII 105, RRIM 600, and PB 260 are genetically distinct from each other. Thus, RAPD and microsatellite analysis established the genetic variability as well as the genome stability of the three clones grown under different environmental conditions. The highly conserved nucleotide sequences of the

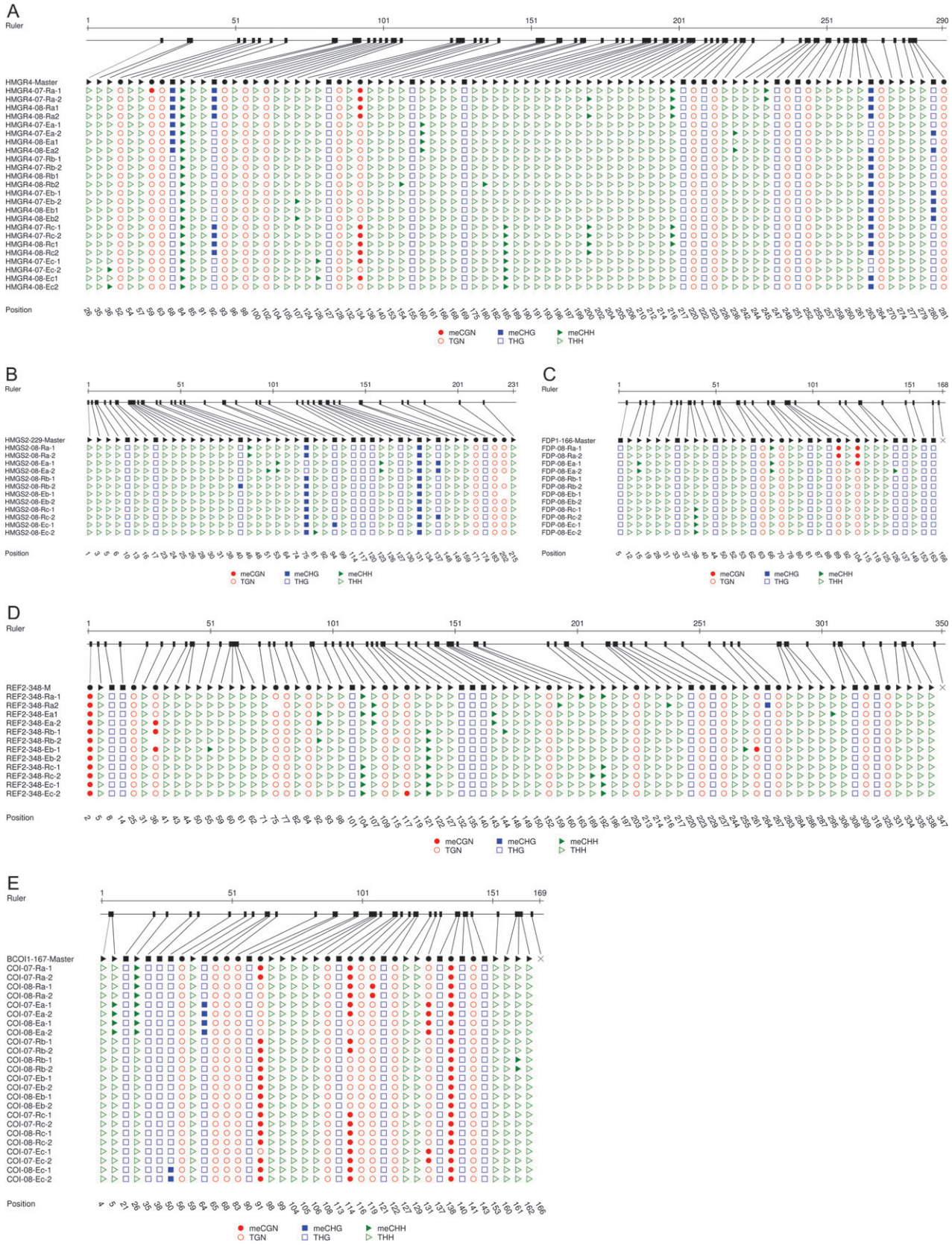

Figure 6. Cytosine Methylation Analysis Results from CyMATE.

respective promoter region of all the plants provided additional support to the latter argument. Therefore, from the above experiments, it was concluded that the three clones under study have distinct genetic make-up and environmental factors are not directly involved in the induction of any significant alteration in the DNA sequence as such, at least in the selected genomic regions.

McrBC RAPD

Since conventional genome analysis tools are incompetent to explain epigenetic variations like DNA methylation, epigenetic tools that could differentiate methylated and non-methylated states of DNA were employed. *McrBC* is an endonuclease that cleaves DNA-containing methylcytosine in one or both strands. Since it will not act upon unmethylated DNA, *McrBC* is used as a tool for determining the methylation status of nucleotides. The smear observed on the gel when *Hevea* genomic DNA was digested using *McrBC* is a good indication of the abundance of methylated cytosines in its genome (Figure 10). However, the genome-wide distribution of methylation in *Hevea* was further established by the differences observed in RAPD profiles between *McrBC*-digested and undigested DNA of the corresponding samples. The abundance of methylated sites within the *Hevea* genome is clearly apparent from the additional number of bands observed in the *McrBC* RAPD profiles compared to normal RAPD. In addition, the profile of each clone also explains their unique epigenetic make-up. The polymorphism that exists between RRIM 600 and the other two clones in the case of OPAC-5 and OPAI-6 despite the absence of any variation in normal RAPD ascertains this postulation. Absence of variation in the banding pattern between individuals of the same clones from two locations is attributed merely to the lack of any methylation variation within the randomly amplified regions and it need not be a global representation of the *Hevea* epigenome.

Restriction Digestion Using Isoschizomers

As methylation is a multifunctional phenomenon that is present globally throughout the genome, there is every possibility that it may modify the recognition site of several endonucleases by binding to cytosines within those sequences and the frequency of this may vary depending on the size as well as the constitution of the genome (Detlev and Reuter, 2005). It is estimated that around 25% of cytosines in plants are methylated whereas, in animals, it is only around 5% (Vaughn et al., 2007). Thus, in plants, the probability of finding a large number of polymorphic DNA fragments subsequent to the methylation sensitive/insensitive isoschizomeric digestion of genomic DNA is high. Since there were no previous records of any kind regarding the presence of methyl groups in *Hevea* genome, a simple digestion of genomic DNA with selected isoschizomers was expected to confirm the existence of methyl groups in the DNA. The isochizomers *Ava*I and *Bso*BI recognize the sequence 'CYCGRG'; however, *Ava*I digestion is blocked if the internal cytosine is methylated, whereas *Bso*BI will digest the fragment irrespective of its methylation status. The difference in digestion pattern when Ra1 DNA was digested with this pair is a clear indication of the presence of methylation in rubber genome and also at several positions. The differentially digested fragments observed reiterate the findings of McClelland et al. (1994) that endonucleases sensitive to m5CpG or m5CpNG methylation, as well as isoschizomers that recognize identical sequences but show differential sensitivity to methylation, provide information about the level and distribution of methylation in eukaryotic DNA. The *Ava*I/*Bso*BI digestion polymorphism is a very good indication of CG type of methylation, which appears more frequently than CHG and CHH type in plants and mammals (Cokus et al., 2008). A noticeable point is the distinct digestion pattern of Rc1, which shows its epigenetic uniqueness. Unlike Ra1 and Rb1, not much variation in the isochizomeric digestion pattern in Rc1 exists, which points out the comparatively lower genome methylation rate in PB 260 clones. The genetic lineage of Rc1 (PB260) may be the reason for this uniqueness, since it is evolved by crossing the Malaysian PB clones, PB49 X PB5/51, while the similar patterns in Ra1 (RRII 105) and Rb1 (RRIM 600) may be attributed to their common maternal parent Tjir1, which is genetically distinct from PB clones (Saraswathyamma et al., 2000). These assumptions are based on the fact that epigenetic factors are inherited by the next generation, as in the case of genetic characters (Kakutani, 2002). Similarly, *Hpa*II and *Ms*PI recognize

Graphical representation of methylation analysis results of all five gene promoters by CyMATE. The master sequence (unconverted) in first position aligned with bisulfite sequences of respective samples.
Probable sites for the three classes of methylation (CGN, CHG, and CHH) as well as actually methylated sites in all the samples were identified by the software and projected symbolically. Blocked symbols represent actual methylation, whereas unblocked ones represent potential sites.
(A) A 288-bp region of the HMGR1 gene promoter in all the 12 samples in 2007 and 2008. Clone-wise or location-wise methylation groupings can be seen at the positions 68, 92, 134, 160, 185, and 200.
(B) A 229-bp region of the HMGS1 gene promoter in all the 12 samples in 2008. Location-wise methylation groupings can be seen at the positions 46, 33, 75, 123, and 137.
(C) A 166-bp fragment from the FDP Synthase gene promoter of all the 12 samples in 2008. Clone-wise or location-wise methylation groupings can be seen at the positions15, 36, and 89.
(D) A 348-bp region of the REF gene promoter of all the 12 samples in 2008. Clone-wise or location-wise methylation groupings can be seen at the positions 84, 104, 121, 143, and 192.
(E) A 167-bp region of the COI1 gene promoter of all the 12 samples in 2007 and 2008. Clone-wise or location -wise methylation groupings can be seen at the positions 5, 26, 64, and 131.

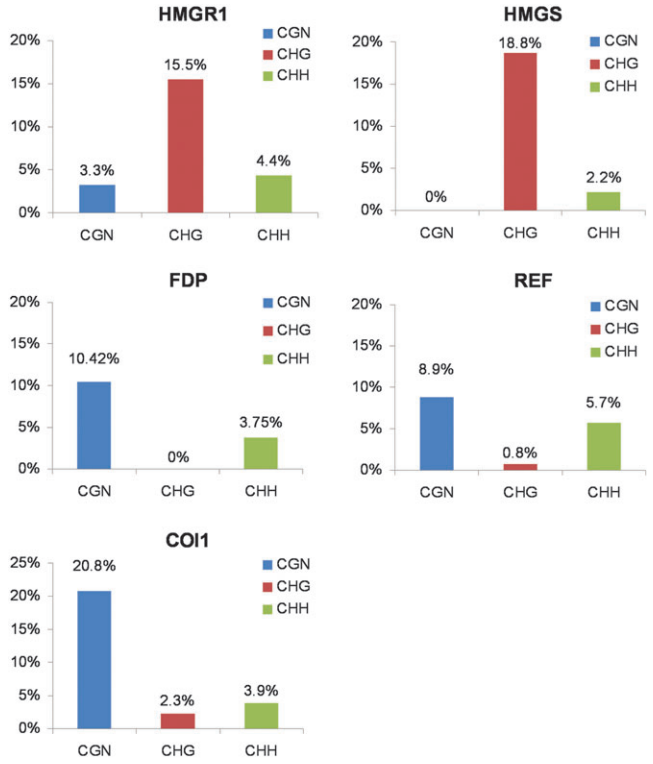

Figure 7. Average Methylation Density of the Three Types of Methylation.

Graph showing the average methylation density for CGN, CHG, and CHH types of methylation within the promoter region of HMGR, HMGS, FDP, REF, and COI1 genes.

the sequence 'CCGG'; however, *Hpa*II is inactive if any of the cytosines is fully methylated, whereas *Msp*I will digest even if the second cytosine is methylated. The modest digestion of DNA by the methylation-sensitive *Hpa*II again points out the-abundance of CGN type of methylation in *Hevea*, which might have blocked the recognition sites of this enzyme. A clean smear on the gel for the methylation-insensitive isochizomer *Msp*I digest further corroborates the above statement. Due to high fragmentation of genomic DNA, as evident from the heavy smearing, Rb1 and Ra1 digestion patters could not be differentiated visually in this case. Similarly, not many differences were noted in the *Psp*GI/*Bst*NI *partial* digestion, except in the case of PB 260, where few faint bands were observed with the methylation-insensitive *Bst*NI, which again signifies its epigenetic uniqueness. All the above results suggest that methylation is present throughout *Hevea* genome and plants can be differentiated based on their methylation pattern.

Promoter Analysis

Rubber Biosynthesis Pathway Genes

Methylation is generally found to be lower in promoter regions and 5′ ends of genes when compared to other coding and intronic regions (Suzuki and Bird, 2008; Lister et al., 2009; Feng et al., 2010). The type of promoter methylation varies significantly from organism to organism and location to location, but, due to their abundance in CG-rich regions, CGN type is considered to be the dominant pattern both in mammalian and plant promoters, whereas CHG seems to prevail mostly in the repetitive and internal exonic regions of plants (Vaughn et al., 2007; Cokus et al., 2008). Though FDP, REF, and COI1 promoter regions showed more CGN-type patterns, as expected, HMGR and HMGS were surprisingly dominated by CHG and CHH types, with CGN totally missing in HMGS. However, the above results may not be a true representation of the methylation status of the entire *Hevea* genome due to the smaller size of analyzed genomic regions. However, it is very likely that CHG type may be a prominent methylation pattern in *Hevea*, which is very unique to the species.

Since regulatory regions are known to contribute to the complex mechanisms governing eukaryotic gene expression, variant methylation patterns at specific transcription-factor binding sites within regulatory regions may result in temporal and tissue-specific patterns of gene expression (Tierney et al., 2000; Choy et al., 2010; Hervouet et al., 2010). Here, in this study, site-specific methylations were identified in the promoter region of rubber biosynthesis genes like HMGR1, HMGS1, FDP, and REF by bisulfite sequencing. Methylation of the CAAT-box within the latex biosynthesis genes is of special interest because genes that have this element seem to require it to be transcribed in sufficient quantities and any mutation in this site may result in a substantial decrease in promoter activity (Weber et al., 2007). As per Deng et al. (2001), DNA methylation within or near sequences of a positive *cis*-element (enhancer) interferes with the binding of a cognate transcription factor to this *cis*-element, which, in turn, causes Transcriptional Gene Silencing (TGS). Since CAAT-box sequences in promoters are enhancers known for their target site regulation and influence on the transcriptional initiation frequency, methylation of such elements upstream of rubber biosynthesis genes may severely affect their transcription. As HMGR1 is considered as one of the rate-limiting enzymes in the latex biosynthesis process, site-specific methylation in the CAAT-box within HMGRI gene promoter of Ea1 and Ea2 may down-regulate its expression, which could result in reduced latex production. The similar trend observed in the other two important genes (HMGS and FDP) in the pathway strongly support the above argument. Since CAAT-box methylation of these genes was not present in the control plants of the same clones at RRII campus (Ra1 and Ra2), this epigenetic mutation may be endorsed essentially to the cold stress encountered by the plants in Elappara region. The visible lag in the growth rate of RRII 105 plants in Elappara compared to its control, combined with the absence of CAAT methylation in RRIM 600 and PB 260 clones, further supports this finding. The critical role of CAAT-box in stress-induced regulatory process was well described in *Arabidopsis*, in which a low-temperature regulatory pathway called the CBF cold response pathway was found to be the key participant in the cold acclimation process (Kumar et al., 2009; Lindlöf et al., 2009). Studies by Guy (1999) and Yamaguchi-Shinozaki and Shinozaki (2005) also exemplified

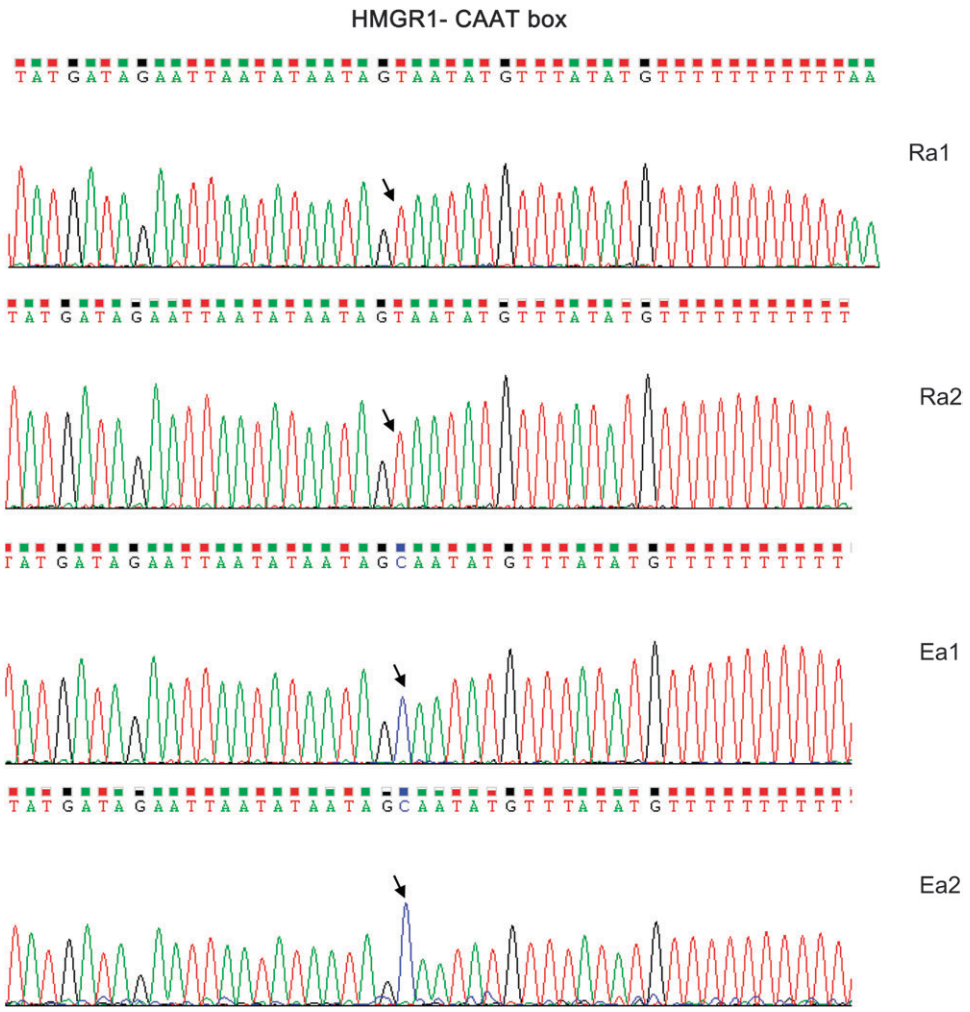

Figure 8. Chromatogram Showing Site-Specific Methylation.

Bisulfite-converted portion of the HMGR gene promoter region highlighting the CAAT-box. The arrow shows the methylation status of cytosine within the *cis*-acting CAAT-box in RRII 105 control and test plants during 2008.

In 2008, RRII 105 plants at RRII campus (Ra1 and Ra2) were not methylated whereas those at Elappara (Ea1 and Ea2) were methylated. [Presence of thymine instead of cytosine in the same site indicate demethylated status. After bisulfite treatment, all cytosines were converted to thymines, except the methylated one, as shown in the figure.]

that the promoter regions of cold-induced and cold-affected genes include the DNA regulatory element, CAAT binding box, which a class of DNA binding proteins called CBF proteins use to bind when the plants are under cold stress. According to their observations, these proteins were not expressed when plants were grown at normal temperature but, under lower temperatures, they were expressed and seen bound to their respective motifs controlling the expression of concerned genes, thereby imparting stress tolerance on the plant. If DNA-binding sites are specifically blocked by methyl groups, access to these sites by CBF proteins will be limited, resulting in a disrupted CBF cold-responsive pathway, making the plant more vulnerable to low temperatures. This phenomenon is expected in the case of *Hevea* also, since HMGR, HMGS, and FDP are among the cascade of genes involved in imparting stress tolerance on plants (Munné-Bosche et al., 2009). As the exact way by which CBF protein regulates the gene expression downstream is not fully known, the precise outcome of methylation on CBF protein-mediated gene regulation could not be predicted at this stage. Given that no methylation changes were observed for the other two clones, it is assumed that their genome is more stable under the conditions studied, which further emphasizes the sensitivity of RRII 105 to environmental variations. The paradoxical methylation of REF 'CAAT-box' in all the samples may be attributed to its isoprene polymerization function rather than biosynthesis or stress-tolerant properties of other genes.

Jasmonates act as signaling compounds for the production of phytoalexins like terpenoids, glycosteroids, and alkaloids, which are all plant defense-related compounds (Seong-Ryong et al., 1993). The Jasmonate signal often spreads systemically throughout the plant and is a major component of systemic acquired resistance (Chinnusamy and Zhu, 2009). This process is

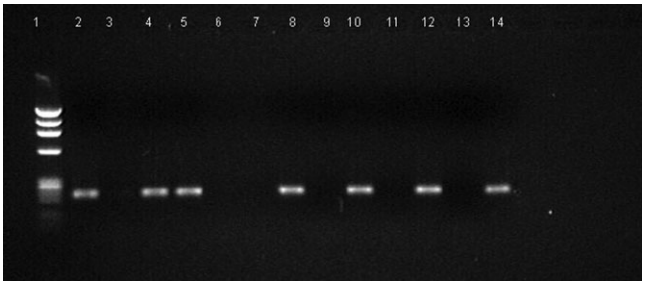

Figure 9. MS–PCR of HMGR Gene Promoter.

Methylation-specific PCR results of a representative CG-rich region within the HMGR genepromoter.

Lane No. 1: Marker, 2: Control (normal Ra1 DNA, amplified using normal HMGR1 promoter specific primer), Lane Nos 3, 5, 7, 9, 11, and 13 (template DNA–bisulfite-treated DNA of Ra1, Ea1, Rb1, Eb1, Rc1, and Ec1; primer-methylation-sensitive). Lane Nos 4, 6, 8, 10, 12, and 14 (template DNA–bisulfite-treated DNA of Ra1, Ea1, Rb1, Eb1, Rc1, and Ec1; primer-methylation-insensitive).

Among the selected Elappara samples, only Ea1 is showing amplification with methylation-sensitive primers, indicating methylation in RRII 105 plants at Elappara.

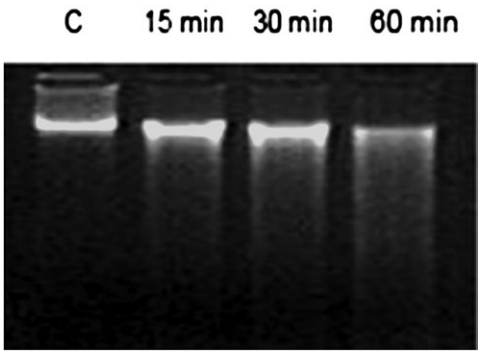

Figure 10. McrBC Digestion of *Hevea* Genomic DNA.

One μg of genomic DNA digested with 10 units of McrBC enzyme in 30-μl reaction volume. Reaction mix incubated at 37°C for 15 min, 30 min, 60 min, and loaded on 1% agarose gel along with undigested sample to check the extent of digestion.

Lane 1: Undigested RRII 105 genomic DNA, Lane 2: 15-min incubation, Lane 3: 30-min incubation, Lane 4: 60-min incubation.

mediated by the co-regulation of several genes involved using their methyl jasmonate-responsive *cis*-regulatory elements like CGTCA (Rouster et al., 1997; He and Gan, 2001). Therefore, it is assumed that the methylation/demethylation of this major regulatory motif may have some role in the methyl jasmonate response system of the plant. The selective demethylation of HMGR1 'CGTCA motif' of all RRII 105 and PB 260 plants at Elappara is a good indication of the influence of environment on this response system. RRII 105 exhibited similar trends in FDP promoter also, but the discrepancy shown by PB 260 clones remains puzzling. It should be noted that CGTCA motif within HMGR1 as well as FDP of all RRIM 600 plants appeared consistently demethylated, which shows its epigenetic uniqueness and stability. The demethylated status of the CATT motif within HMGR1 promoter of Ea1 and Ea2 at Elappara also emphasizes the sensitivity of RRII 105 towards environmental variations. Similar rendering of PB 260 is obvious from the changing methylation status of this light-responsive motif in Ec1 and Ec2 plants, even though Ec1 was methylated in the second year only. Another interesting observation that again highlights the temperature sensitivity of RRII 105 clone was its methylation trend at LTR within the HMGR1 promoter. The demethylated status of Ea1 and Ea2 is a very good indication of the sensitivity of RRII 105 to external temperature variations. In the case of Ea2, subsequent methylation in the second year may be due to its faster acclimatization than Ea1. The same reason can be attributed to the similar trend shown by both Ec1 and Ec2 at this site in the second year. It may be noted that, during 2007, the plants were only 1.5 years old in the field and the PB clones might have adapted to the conditions within a period of 1 year, after which the temperature variations may not be having much impact on them. The consistent methylation at LTR motif by the clone RRIM 600 for the entire period of study asserts the robustness of its epigenomes as well as their steady rate of rubber biosynthesis. Another RRII 105-specific pattern was observed in the TC-rich repeats involved in defense and stress responsiveness. It is inferred that this unique clone-specific methylation pattern is making RRII 105 clones susceptible to environmental fluctuations at the same time, contributing towards higher yield by blocking the transcription factor-mediated down-regulation of the genes downstream.

Like Jasmonates, Gibberellins (GA) were also known to play important roles in mediating the effects of environmental stimuli on plant development. The regulation of biosynthetic pathway-related gene transcription by active GA levels and the influence of environmental factors such as light and temperature on altering these levels has been well explained by Yamaguchi and Kamiya (2000). Moreover, the *cis*-acting Gibberellin response elements (GARE) and their role in escalating transcription rate in the presence of Gibberellins are also well known (Rogers et al., 1994). Since isoprenoids are the precursors for Gibberlin biosynthesis, low levels of their expression will ultimately result in fewer Gibberellins allowing the accumulation of DELLAs (a family of nuclear growth-repressing proteins whose degradation is stimulated by Gibberellins), which may finally result in reduced plant growth. The presence of a methylated GARE motif in the HMGS gene promoter region of RRII 105 is supposed to have the same effect on plant growth as mentioned above.

Salicylic acid (SA) is another important endogenous regulatory signal molecule in plants known to play a major role in abiotic stress responses for low and high temperature, UV-B irradiation, ozone, and heavy metals apart from biotic stress (Hayat et al., 2007). The clone-specific demethylation of the *cis*-acting TCA element (CAGAAAAGGA) involved in salicylic acid responsiveness within the REF promoter region is assumed to be an epigenetic mutation unique to RRII 105. The exclusive demethylation of this element in RRII 105 plants suggests an increased possibility of a unique way of salicylic acid response in RRII 105. Since the site seems to be methylated in the other

two clones, salicylic acid response is believed to have no influence on their REF gene.

Retrospectively, the selective demethylation of the canonical HSE motif within the HMGS gene promoter in Ea1 and Ea2 is supposed to have some direct or indirect role in heat stress response-mediated gene regulation in RRII 105 planted at Elappara. As mentioned earlier, the presence of methyl groups in all other samples underline the uniqueness and sensitivity of RRII 105 to temperature variations, whereas such responses appear to be non-obligatory for the survival of the other two clones. The complete absence of methylation near the proximity of the TATA-box of all the four rubber biosynthesis genes is a clear indication of the incidence of their active transcription, even though at varied levels.

COI1 Promoter Analysis

Cronatine insensitive1 gene is a common defense-related gene that encodes an F-box protein to assemble SCFCOI1 complexes essential for a response to Jasmonate-mediated defense and reproduction in plants (Xie et al., 1998). A methylated cytosine proximal to the COI1 TATA-box of RRII 105 and RRIM 600 plants suggests that transcription of this gene may be severely affected in these clones. Methylation of CAAT-box further supports this argument, even though it was present only in RRII 105 plants. Since COI1 gene is a major defense-related gene, methylation of key motifs like TATA and CAAT may result in reduced expression rate of the gene, which will naturally make the plant more susceptible to pathogen attack. Alternatively, the presence of E2Fb transcription factor binding site in this gene promoter suggests its involvement in the regulation of cell division also. Therefore, an obstruction in this transcription factor binding site is presumed to affect the growth rate of the plant, as evidenced by the growth characteristics of RRII 105 plant at Elappara. The noticeable methylation at base position 138 in all the samples appears to be a permanent one that may be unique to *Hevea* species. Such patterns have the potential to be developed as a species-specific epigenetic marker for epigenetic lineage analysis studies in *H. brasiliensis*.

Other than the regulatory sites mentioned above, a proper explanation for the random methylation/demethylation observed at several sites and identified motifs of five genes analyzed requires extensive investigation. Some of the random patterns across the samples may be due to either temporary methylation resulting from minor encounter with different stressed conditions or bisulfite sequencing errors. Since gene-expression studies and estimation of the exact outcome of each and every site-specific methylation/demethylation on phenotype are beyond the scope of this study, the following results can only be treated as part of propable stress control mechanism in plant systems.

Methylation-Specific–PCR

MS–PCR is a simple, sensitive, and specific method for determining the methylation status of virtually any CpG-rich region (Herman et al., 1996). Primers were designed that can distinguish methylated from unmethylated DNA in bisulfite-modified DNA, taking advantage of the bisulfite-induced sequence differences. The present study employed methylation sensitive/insensitive primer combinations targeted at a single locus at base position 160 of the HMGR1 gene, which appeared methylated in the RRII 105 plant from Elappara (Ea1) and unmethylated in all the other plants tested. The positive amplification obtained using methylation-sensitive primer on Ea1 is well in agreement with the bisulfite sequencing result. The absence of amplicon when insensitive primers were used on Ea1 DNA further corroborates the above results. Failure of methylation-sensitive primers and success of methylation- insensitive primers in rest of the samples emphasize the accuracy of bisulfite sequencing result.

Based on the general trend observed, it is inferred that the methylation pattern is more or less the same for plants of the same clone grown at the same location. A clear location-wise difference in methylation pattern was observed in the case of RRII 105 plants at several key regulatory sites, whereas RRIM 600 and PB 260 plants were found to be epigenetically more stable. Preliminary methylation analysis of a few rubber biosynthesis gene promoter regions showed that certain key regulatory sites had been blocked, especially in the case of RRII 105 clone planted in Elappara, which might negatively affect their gene-expression rate, while their counterparts in RRII campus remained unmethylated. Alternatively, PB 260 clones demonstrated trivial methylation flux whereas RRIM 600 was found to have significant epigenetic stability in terms of methylation in the selected regions. The explicit site-specific methylation patterns in the promoter regions of selected genes and the variation in these patterns from clone to clone and location to location is a clear indication of the environmental interaction on the genome of *Hevea*. In general, it is concluded that stress is having a direct impact on rubber genome and is responsible for the methylation and demethylation of specific sites. This is the first report of its kind regarding the methylation status of gene promoters in any tree species and the observations from this study are supposed to aid in the development of clone-specific and location-specific markers for the evolvement of new location-specific high-yielding *Hevea* clones. Above all, these results furnish certain hints to the understanding of the fundamental molecular mechanisms by which plants interact with their external environment.

METHODS

Sampling and Genomic DNA Isolation

Three popular rubber clones (RRII 105, RRIM 600, and PB 260) were selected for the study. RRII 105 is a high-yielding Indian clone that is widely cultivated in southern India. It is found to be sensitive to cold and drought conditions. RRIM 600 is a high-yielding Malaysian clone widely cultivated in all rubber-growing countries and is one of the highest-yielding rubber clone available. PB 260 is yet another high-yielding Malaysian clone. Both RRIM 600 and PB 260 are supposed to be more

cold-tolerant than RRII 105 as per field trial information. Two plants each of the above clones from two climatically different regions, namely Elappara and Rubber Research Institute of India (RRII) campus were selected for the study. Elappara range forms part of the Western Ghats, a mountain range along the western side of India where the temperature varies from 5°C at night to 25°C at midday—far lower than the surrounding low-lying areas. RRII campus has a typical tropical climate, with a temperature in the range of 20–35°C throughout the year, with high humidity and precipitation, suitable for rubber cultivation. Therefore, plants grown at RRII campus were taken as control and those at Elappara, which are under cold stress, as test plants. Sample details are as given in Table 1.

DNA isolation: tender sprouts were collected from the selected plants during December 2007 and 2008. Genomic DNA was isolated by CTAB protocol as described (Doyle and Doyle, 1990). The isolated DNA was quantified using Nanodrop (Thermo Scientific Inc.) and stored at 4°C until further analysis.

Marker Analysis

RAPD and Microsatellites (SSR)

A set of polymorphic RAPD markers well established in rubber were tested on genomic DNA isolated from Ra1, Ra2, Ea1, Ea2, Rb1, Rb2, Eb1, Eb2, Rc1, Rc2, Ec1, and Ec2 plants. The decamer primers, OPF-10, OPAI-6, and OPC-5 from the Operon primer kit (Operon Technologies, USA) were selected for the final PCR analysis. PCR amplification was performed as per standard procedure and products analyzed on 1.5% agarose gel and documented. SSR markers like CT-16, CT-19, and CT-1 were also used to establish the intraclonal similarity and interclonal variability of the above set of plants. Reactions were performed as per standard conditions given elsewhere and products analyzed by running them on a 6% denaturing polyacrylamide gel containing 7 M urea followed by detection using the silver-staining method (Saha et al., unpublished).

McrBC RAPD

One microgram genomic DNA of all the 12 samples collected during 2007 and 2008 were digested using 10 U of *McrBC* enzyme (New England Biolabs) and incubated at 37°C overnight as per the manufacturer's recommendations. The overnight-digested products were uniformly diluted to 10 ng μl^{-1} and used as a template for RAPD. Primer combinations were the same as used earlier.

Restriction Digestion Analysis Using Methylation-Sensitive/Insensitive Isoschizomers

The following combinations of methylation-sensitive and insensitive isoschizomers were used for the restriction digestion analysis of genomic DNA of the three clones: *Hpa*II/*Msp*I, *Ava*I/*Bso*BI, and *Psp*GI/*Bst*NI (all enzymes from NEB, USA). Ra1, Rb1, and Rc1 DNA were digested using all the three combinations. Five micrograms of genomic DNA was digested with 20 U of respective enzyme in a 50-μl reaction mix. The reaction mix was incubated as per the manufacturer's recommendations. The digested products were analyzed on 1% agarose gel and profile documented. In order to compare the digestion pattern of the three clones from RRII campus as well as Elappara, digestion using methylation-sensitive enzymes (*Hpa*II, *Ava*I, and *Psp*GI) were also performed, on DNA of Ra1 and Ea1, Rb1 and Eb1, and Rc1 and Ec1 respectively (not shown).

Regulatory Motif Search and Bisulfite PCR Primer Designing

Fourteen promoter sequences of REF gene, nine of HMGR, five of HMGS, six of FDP, and two of COI1 were obtained via the ENTREZ search tool of the nucleotide database at NCBI. Multiple short sequences of each gene were aligned using DNASIS® MAX v3.0 (Hitachi Software Engineering, USA) to obtain a contig. The contigs obtained were subjected to regulatory sequence search analysis using the online database at plantCARE (Lescot et al., 2002). The *cis*-regulatory elements present in each sequence were located and their putative functions and base position identified. The contigs were then used to design bisulfite sequencing primes using the online tool, MethPrimer (Li and Dahiya, 2002). The following parameters like optimum product size (~200 bp), T_m (~55°C), primer size (~25 bases) were taken into consideration for primer designing. Primers were designed so that they flanked the CG-rich regions of the promoter sequence. All primers for bisulfite sequencing were designed avoiding CpGs in the sequence. Simultaneously, conventional primers were also designed using the Primer3 software for the amplification of respective promoter regions from the untreated DNA, which serves as the master sequence for bisulfite sequence analysis. All the primers were synthesized by Ocimum Biosolutions, Hyderabad.

Bisulfite Sequencing

Bisulfite conversion of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen GmbH). One microgram of each sample was converted as per the manufacturer's recommendation. Purified converted DNA was stored at –20°C for bisulfite PCR.

Bisulfite PCR using Several primer combinations were tried for each gene promoter to obtain a single clear band and the best pair that gave a unique band of expected size was selected for the final analysis. Details of the normal as well as selected bisulfite PCR primer pairs used for each gene are as given in Table 2. PCR amplification was performed in a total volume of 50 μl containing 100 ng of bisulfite-treated template DNA with 0.5 μM of each primer, 0.2 mM of each dNTP, 2 U of Taq DNA polymerase (GE Health Care Life Sciences), and 5 μl of DNA polymerase buffer (100 mM Tris-HCL, pH 9, 500 mM KCL, 20 mM $MgCl_2$). PCR conditions were as follows. An initial denaturation of 94°C for 10 min was followed by 94°C for 30 s, *°C for 30 s (annealing temperature for each primer given in Table 2), 72°C for 30 s for a total of 40 cycles, 10 min at 72°C, hold at 4°C. PCR products were run on 2% agarose gels along with DNA ladder (Lamda DNA *Eco*RI+*Hind*III digest, Promega Inc.) to estimate the size of the product. Amplified products were eluted from gel using illustra GFX gel extraction kit (GE Healthcare). The eluted products were cloned into the pGEMT easy

Table 2. Primer Details.

Promoter Name	Primer Name and Sequence	Primer Type	Expected Product Size (bp)	*Annealing Temp. (°C)	Expected No. of CpGs
HMG-CoA reductase	HMGP-F 5′-TGCTATTTTTCTATCATCAATTCAGC-3′ HMGP-R 5′- AGAGGCAATGAGGGAGACAA-3′	Normal	706	58	NA
	BHMG4- F 5′-GTAAAAAAAAAAAATAATAAAGTAG-3′ BHMG4- R 5′-TCCTCAACRAATATAACATACTTTC-3′	Bisulfite	288	56.2	14
HMG-CoA synthase	HMGSP-F 5′-AGTCTCTCTTTTTCTCTCCTTGC-3′ HMGSP-R 5′-GTGTATTTCCCTTTGCTTGC-3′	Normal	330	56	NA
	BHMGS2- F 5′-TTTTTTTTGTTGTTTTTAGGGA-3′ BHMGS2- R 5′-ATTCCCACATTCTTTACCATTT-3′	Bisulfite	229	55.8	3
Rubber elongation factor	REFP-F 5′-CCCCATTCTAAATCGACTTCTG-3′ REFP-R 5′-CCTCCCCCTGCTGTTAATTT-3′	Normal	663	59	NA
	BREF2-F 5′-AATYGATTTTTGGAATTGGGATG-3′ BREF2-R 5′-RTACCCCTTATTAATTATCT-3′	Bisulfite	348	53	16
Farnasyl diphosphate synthase	FDPP-F 5′-GTAGCCACAACGCCAAGAAC-3′ FDPP-R 5′-TCAATTACAGAAAGCCCCCTA-3′	Normal	325	55	NA
	BFDP1-F 5′-GGATTTGAAGTTAATTTTTTTGAAGG-3′ BFDP1-R 5′-ACTATCAATTACAAAAAACCCCCTATT-3′	Bisulfite	166	57	4
Coronatine-insensitive 1	COIP-F 5′-CCCCCTCCATAAATCCCAGA-3′ COIP-R 5′-CGTGTAAGGGCATCAAGCTCA-3′	Normal	812	60	NA
	BCOIR1-F 5′-AAGTTATGGAAGAGGAGAATTAGAGTAA-3′ BCOIR1-R 5′-TATAAAAACATCAAACTCATACCAAC-3′	Bisulfite	167	54.5	13

vector system (Promega, USA) followed by transformation in *E-coli* cells (DH5α). Eight to twelve white colonies were selected from the X-Gal/IPTG plates and colony PCR was performed using vector-directed primers to confirm the presence of inserts based on their expected fragment size. Eight to 10 positive colonies from each plate were inoculated into LB medium with ampicillin for plasmid isolation. Plasmids were isolated and sequenced at Macrogen Inc., Korea. Simultaneously, the master sequences for the study were obtained by amplifying, cloning, and sequencing the selected regions of all the five genes from unconverted genomic DNA samples using standard PCR conditions. For HMGR1 and COI1 gene promoter regions, data were collected for two consecutive years (2007–2008) and, for the other three, bisulfite analysis was carried out on 2008 samples only.

Sequence Analysis

The master sequences and the bisulfite-converted sequences were trimmed and aligned using CLUSTAL-W software. 'txt' files with master sequence and clone sequences were created for all the gene promoters separately. The input files were then saved in interleaved format (indicated by 'aln' file extension). The aligned sequence files with the master sequences in the first position were fed to the online methylation sequence analysis software CyMATE (Hetzl et al., 2007) and the results were retrieved from the e-mail ID given. The resulting e-mail had output files such as a converted alignment file for repeated analysis, a summary of the analysis (txt file), graphical representation of results (PDF), and a message log file (txt file). Since the 10 bacterial clone sequences of respective gene promoters of each sample were exact replicates, one representative sequence per sample for each gene promoter was taken for the final analysis for the sake of obtaining a compact graphical output.

Methylation-Specific–PCR

In order to counter-check the bisulfite sequencing results, methylation-specific PCR (MS–PCR) was carried out targeting a representative CpG block within the HMGR1 promoter (base position 160) of Ra1, Ea1, Rb1, Eb1, Rc1, and Ec1 plants. Methylation-sensitive/insensitive primer pairs were designed using the Methyl express software (ABI) to give a pcr amplicon of 260-bp size. PCR amplification was carried out using the methylation-sensitive primers 5′-AGGGTGGTTTAGAACGTTATTC-3′ (forward) and 5′-AAATAATCGAAAAACGATCCTC-3′ (reverse) as well as methylation-insensitive primers 5′-TTAAGGGTGGTTTAGAATGTTATTT-3′ (forward) and 5′- AAATAATCAAAAAACAATCCTCAAC-3′ (reverse) on bisulfite-treated DNA of all samples as per the reaction conditions specified by Herman et al. (1996). PCR products (20 μl) were loaded on 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by the research funds of the Rubber Research Institute of India, Rubber Board (Ministry of Commerce and Industry, Government of India).

ACKNOWLEDGMENTS

We are very grateful to Dr James Jacob, Director of Research and Dr M.A. Nazeer, Joint Director (Project Monitoring), Rubber Research Institute of India for their constant encouragement. We also thank Dr R. Krishnakumar and Dr D.B. Nair for providing plant materials grown at Elappara. We deeply appreciate Mr P.R. Satheesh for the art work and Dr Vinoth Thomas for providing the photograph. No conflict of interest declared.

REFERENCES

Baylin, S.B., Esteller, M., Rountree, M.R., Bachman, K.E., Schuebel, K., and Herman, J.G. (2001). Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Human Mol. Genet. **10,** 687–692.

Bedell, J.A., et al. (2005). Sorghum genome sequencing by methylation filtration. PLoS Biol. **3,** e13.

Besse, P., Seguin, M., Lebrun, P., Chevallier, M.H., Nicolas, D., and Lanaud, C. (1994). Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by nuclear RFLP analysis. Theoretical Applied Genet. **88,** 199–207.

Bruce, T.J.A., Matthesa, M.C., Napiera, J.A., and Picketta, J.A. (2007). Stressful 'memories' of plants: evidence and possible mechanisms. Plant Sci. **173,** 603–608.

Chen, Z.J. (2007). Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Ann. Rev. Plant Biol. **58,** 377–406.

Chinnusamy, V., and Zhu, J.K. (2009). Epigenetic regulation of stress responses in plants. Curr. Opin. Plant Biol. **12,** 133–139.

Chinnusamy, V., Gong, Z., and Zhu, J.K. (2008). Abscisic acid-mediated epigenetic processes in plant development and stress responses. J. Integ. Plant Biol. **50,** 1187–1195.

Choi, C.S., and Sano, H. (2007). Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. Mol. Genet. Genome. **277,** 589–600.

Choy, M.K., Movassagh, M., Goh, H.G., Bennett, M.R., Down, T.A., and Foo, R.S. (2010). Genome-wide conserved consensus transcription factor binding motifs are hyper-methylated. BMC Genomics. **11,** 519.

Cokus, S.J., Feng, S., Zhang, X., Chen, Z., Merriman, B., Haudenschild, C.D., Pradhan, S., Nelson, S.F., Pellegrini, M., and Jacobsen, S.E. (2008). Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. Nature. **452,** 215–219.

Cox, L.A., and Nathanielsz, P.W. (2009). The importance of altered gene promoter methylation and transcription factor binding in developmental programming of central appetitive drive. J. Physiol. **587,** 4763–4764.

Deng, G., Chen, A., Pong, E., and Kim, Y.S. (2001). Methylation in *hMLH1* promoter interferes with its binding to transcription factor CBF and inhibits gene expression. Oncogene. **20,** 7120–7127.

Detlev, H., and Reuter, K.M. (2005). Reliable detection of DNA cytosine methylation at CpNpG sites using the engineered restriction enzyme EcoRII-C. BioTechniques. **38,** 855–856.

Doyle, J.J., and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. Focus. **12,** 13–15.

Farrel, R.E. (2007). The regulation of gene expression in plants and animals. In Regulation of Gene Expression in Plants: The Role of Transcript Structure and Processing, Bassett, C.L., ed. (New york, NY: Springer), pp. 1–34.

Feng, S., et al. (2010). Conservation and divergence of methylation patterning in plants and animals. Proc. Natl Acad. Sci. U S A. **107,** 8689–8694.

Finnegan, E.J., Genger, R.K., Kovac, K., Peacock, W.J., and Dennis, E.S. (1998). DNA methylation and the promotion of flowering by vernalization. Proc. Natl Acad. Sci. U S A. **95,** 5824–5829.

Funk, J.L., Mak, J.E., and Lerdau, M.T. (2004). Stress-induced changes in carbon sources for isoprene production in *Populus deltoides*. Plant Cell Environ. **27,** 747–755.

Guy, C. (1999). Molecular responses of plants to cold shock and cold acclimation. J. Mol. Microbiol. Biotechnol. **1,** 231–242.

Hayat, S., Ali, B., and Ahmad, A. (2007). Salicylic acid: biosynthesis, metabolism and physiological role in plants. In Salicylic Acid: A Plant Hormone, Hayat S. and Ahamed A., eds (The Netherlands: Springer), pp. 1–14.

He, Y., and Gan, S. (2001). Identical promoter elements are involved in regulation of the OPR1 gene by senescence and jasmonic acid in *Arabidopsis*. Plant Mol. Biol. **47,** 595–605.

Herman, J.G., Graff, J.R., Myöhänen, S., Nelkin, B.D., and Baylin, S.B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl Acad. Sci. U S A. **93,** 9821–9826.

Hernandez, R.A., Afanador, K.L., Arango, I.R., and Lobo, A.M. (2006). Analysis of genetic variation in clones of rubber (*Hevea brasiliensis*) from Asian, South and Central American origin using RAPDs markers. Revista Colombiana Biotechnología. **8,** 29–34.

Hervouet, E., Vallette, F.M., and Cartron, P.F. (2010). Dnmt1/transcription factor interactions: an alternative mechanism of DNA methylation inheritance. Genes Cancer. **1,** 434–443.

Hetzl, J., Foerster, A.M., Raidl, G., and Mittelsten Scheid, O. (2007). CyMATE: a new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. Plant J. **51,** 526–536.

Jacobsen, S.E., and Meyerowitz, E.M. (1997). Hypermethylated SUPERMAN epigenetic alleles in *Arabidopsis*. Science. **277,** 1100–1103.

Jacobsen, S.E., Sakai, H., Finnegan, E.J., Cao, X., and Meyerowitz, E.M. (2000). Ectopic hypermethylation of flower-specific genes in *Arabidopsis*. Curr. Biol. **10,** 179–186.

Kakutani, T. (2002). Epi-alleles in plants: inheritance of epigenetic information over generations. Plant Cell Physiol. **43,** 1106–1111.

Khrustaleva, L.I., de Melo, P.E., van Heusden, A.W., and Kik, C. (2005). The integration of recombination and physical maps in a large-genome monocot using haploid genome analysis in a trihybrid *Allium* population. Genetics. **169,** 1673–1685.

Kinoshita, Y., Saze, H., Kinoshita, T., Miura, A., Soppe, W.J., Koornneef, M., and Kakutani, T. (2007). Control of *FWA* gene silencing in *Arabidopsis thaliana* by SINE-related direct repeats. Plant J. **49,** 38–45.

Kumar, G.M., Mamidala, P., and Podile, A.R. (2009). Regulation of Polygalacturonase-inhibitory proteins in plants is highly dependent on stress and light responsive elements. Plant Omics J. **2,** 238–249.

Labra, M., Ghiani, A., Citterio, S., Sgorbati, S., Sala, F., Vannini, C., Ruffini, C.M., and Bracale, M. (2002). Analysis of cytosine methylation pattern in response to water deficit in pea root tips. Plant Biol. **4,** 694–699.

Lee, T., Zhai, J., and Meyers, B.C. (2010). Conservation and divergence in eukaryotic DNA methylation. Proc. Natl Acad. Sci. U S A. **107,** 9027–9028.

Lekawipat, N., Teerawatanasuk, K., Rodier-Goud, M., Seguin, M., Vanavichit, A., Toojinda, T., and Tragoonrung, S. (2003). Genetic diversity analysis of wild germplasm and cultivated clones of *Hevea brasiliensis* Muell. Arg. by using microsatellite markers. J. Rubber Res. **6,** 36–34.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., and Rombauts, S. (2002). PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Res. **30,** 325–327.

Li, L., and Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. Bioinformatics. **18,** 1427–1431.

Lindlöf, A., Bräutigam, M., Chawade, A., Olsson, O., and Olsson, B. (2009). *In silico* analysis of promoter regions from cold-induced genes in rice (*Oryza sativa* L.) and *Arabidopsis thaliana* reveals the importance of combinatorial control. Bioinformatics. **25,** 1345–1348.

Lippman, Z., et al. (2004). Role of transposable elements in heterochromatin and epigenetic control. Nature. **430,** 471–476.

Lister, R., et al. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature. **462,** 315–322.

Lukens, L.N., and Zhan, S. (2007). The plant genome's methylation status and response to stress: implications for plant improvement. Curr. Opin. Plant Biol. **10,** 317–322.

Maloisel, L., and Rossignol, J.L. (1998). Suppression of crossing-over by DNA methylation in *Ascobolus*. Genes Dev. **12,** 1381–1389.

McClelland, M., Nelson, M., and Raschke, E. (1994). Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. Nucleic Acids Res. **22,** 3640–3659.

Meijón, M., Feito, I., Valledor, L., Rodríguez, R., and Cañal, M.J. (2010). Dynamics of DNA methylation and histone H4 acetylation during floral bud differentiation in azalea. BMC Plant Biol. **10,** 10.

Munné-Bosch, S., Falarab, V., Paterakib, I., Marta, L.C., Celaa, J., and Kanellis, A.K. (2009). Physiological and molecular responses of the isoprenoid biosynthetic pathway in a drought-resistant Mediterranean shrub, *Cistus creticus* exposed to water deficit. J. Plant Physiol. **166,** 136–145.

Okamoto, H., and Hirochika, H. (2001). Silencing of transposable elements in plants. Trends Plant Sci. **1,** 527–534.

Palmer, L.E., Rabinowicz, P.D., O'Shaughnessy, A.L., Balija, V.S., and Nascimento, L.U. (2003). Maize genome sequencing by methylation filtration. Science. **302,** 2115–2117.

Peredo, E.L., Revilla, M.A., and Arroyo-García, R. (2006). Assessment of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organogenic calli. J. Plant Physiol. **10,** 1071–1079.

Pradhan, S., and Adams, R.L. (1995). Distinct CG and CNG DNA methyltransferases in *Pisum sativum*. Plant J. **3,** 471–481.

Priyadarshan, P.M., Hoa, T.T.T., Huasun, H., and de Gonalves, P.S. (2005). Yielding potential of rubber (*Hevea brasiliensis*) in suboptimal environments. J. Crop Improvement. **14,** 221–247.

Rogers, J.C., Lanahan, M.B., and Rogers, S.W. (1994). The *cis*-acting gibberellin response complex in high pI alpha-amylase gene promoters: requirement of a coupling element for high-level transcription. Plant Physiol. **105,** 151–158.

Rouster, J., Robert, L., Mundy, J., and Cameron-Mills, V. (1997). Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. Plant J. **11,** 513–523.

Roy, C.B., Nazeer, M.A., and Saha, T. (2004). Identification of simple sequence repeats in rubber (*Hevea brasiliensis)*. Current Sci. **87,** 807–811.

Saha, T., Roy, C.B., Ravindran, M., Bini, K., and Nazeer, M.A. (2006). Existence of retroelements in rubber (*Hevea brasiliensis*) genome. J. Plantation Crops. **34,** 546–551.

Saraswathyamma, C.K., Licy, J., and Marattukalam, G. (2000). Planting materials. In Natural Rubber: Agromanagement and Crop Processing, George P.J., and Jacob C.K., eds (Kottayam: Rubber Research Institute of India), pp. 59–74.

Saze, H. (2008). Epigenetic memory transmission through mitosis and meiosis in plants. Seminars Cell Dev. Biol. **19,** 527–536.

Seguin, M., Besse, P., Lespinasse, D., Lebrun, P., Rodier-Goud, M., and Nicolas, D. (1995). Characterization of genetic diversity and *Hevea* genome mapping by biochemical and molecular markers. In IRRDB symposium on physiological and molecular aspects of the breeding of. Hevea brasiliensis, Brickendonbury, UK.

Seong-Ryong, K., Younghee, K., and Gynheung, A. (1993). Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (nos) promoter. Plant Physiol. **103,** 97–103.

Soppe, J.W., Jacobsen, E.S., Alonso-Blanco, C., Jackson, P.J., Kakutani, T., Koornneef, M., and Peeters, J.A. (2000). The late flowering phenotype of fwa mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. Mol. Cell. **6,** 791–802.

Suzuki, M.M., and Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. **9,** 465–476.

Tierney, R.J., Kirby, H.E., Nagra, J.K., Desmond, J., Bell, A.I., and Rickinson, A.B. (2000). Methylation of transcription factor binding sites in the Epstein–Barr virus latent cycle promoter Wp coincides with promoter down-regulation during virus-induced B-cell transformation. J. Virol. **74,** 10468–10479.

Turker, M.S. (2002). Gene silencing in mammalian cells and the spread of DNA methylation. Oncogene. **21,** 5388–5393.

Vaughn, M.W., et al. (2007). Epigenetic natural variation in *Arabidopsis thaliana*. PLoS Biol. **5,** e174.

Villar, C.B.R., Erilova, A., Makarevich, G., Trösch, R., and Köhler, C. (2009). Control of PHERES1 imprinting in *Arabidopsis* by direct tandem repeats. Mol. Plant. **2,** 654–660.

Vyroubalová, S., Václavíková, K., Turecková, V., Novák, O., Smehilová, M., Hluska, T., Ohnoutková, L., Frébort, I., and Galuszka, P. (2009). Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. Plant Physiol. **151,** 433–447.

Wahid, A., Gelani, S., Ashraf, M., and Foolad, M.R. (2007). Heat tolerance in plants: an overview. Environ. Exper. Bot. **61,** 199–223.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Pääbo, S., Rebhan, M., and Schübeler, D. (2007). Distribution, silencing

potential and evolutionary impact of promoter DNA methylation in the human genome. Nat. Genet. **39,** 457–466.

Whitelaw, C.A., Barbazuk, W.B., Pertea, G., Chan, A.P., Cheung, F., Lee, Y., Zheng, L., Van Heeringen, S., Karamycheva, S., and Bennetzen, J.L. (2003). Enrichment of gene-coding sequences in maize by genome filtration. Science. **302,** 2118–2120.

Wong, A.H.C., Gottesman, I.I., and Petronis, A. (2005). Phenotypic differences in genetically identical organisms: the epigenetic perspective. Human Mol. Genet. 14. Review Issue. **1,** R11–R18.

Xie, D.X., Feys, B.F., James, S., Manuela, N.R., and Turner, J.G. (1998). COI1: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science. **280,** 1091–1094.

Yamaguchi, S., and Kamiya, Y. (2000). Gibberellin biosynthesis: its regulation by endogenous and environmental signals. Plant Cell Physiol. **41,** 251–257.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (2005). Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci. **10,** 88–94.

Zemojtel, T., Kielbasa Szymon, M., Arndt, P.F., Chung, H.R., and Vingron, M. (2008). Methylation and deamination of CpGs generate p53-binding sites on a genomic scale. Trends Genet. **25,** 63–66.

Zhang, H., and Ogas, J. (2009). An epigenetic perspective on developmental regulation of seed genes. Mol. Plant. **2,** 610–627.