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ORIGINAL ARTICLE

Heterografting induced DNA methylation polymorphisms in *Hevea brasiliensis*

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Abstract

Main conclusion **Heterografting induced intraclonal epigenetic variations were detected among rubber plants. Interaction between genetically divergent root stock and scion tissues might have triggered these epigenetic changes which may eventually lead to intraclonal variability in rubber.**

DNA methylation in response to stress may be associated with the alteration in gene transcription leading to morphological changes in plants. Rubber tree is commercially propagated by bud grafting where the scion of a high yielding variety is grafted on to a genetically divergent root stock. Still, significant levels of intraclonal variations exist among them. Epigenetic changes associated with heterografting may be partly responsible for this conundrum. In the present study, an attempt was made to identify the impact of divergent root stock on the epigenome of scion in grafted rubber plants. Heterografts were developed by grafting eye buds from a single polyembryony derived seedling on to genetically divergent root stocks of unknown parentage. The plants were uniformly maintained and their DNA was subjected to MSAP analysis. Polymorphic DNA methylation bands corresponding to CG as well as the plant-specific CHG types of methylation were observed. Cloning of selected polymorphic regions and bisulfite sequencing confirmed the presence of methylation in the promoter and coding region of important genes including an LRR receptor kinase gene. Since divergent root stock is the major factor differentiating the grafted plants, the changes in DNA methylation patterns might have been triggered by the interaction between the two genetically different tissues of stock and scion. The study assumes importance in *Hevea,* because accumulation and maintenance of epigenetic changes in functional genes and promoters during subsequent cycles of vegetative propagation may contribute towards intraclonal variability eventually leading to altered phenotypes.

Keywords DNA methylation · *Hevea brasiliensis* · Stock–scion interaction · Epigenetic changes · Intraclonal variability

Introduction

Hevea brasiliensis (Willd. ex A, Juss.) Muell. Arg. (Para rubber tree), a tropical tree species belonging to the family *Euphorbiaceae*, is preferred over alternative sources of natural rubber worldwide due to the quality as well as quantity of latex produced. High yielding varieties of *Hevea* are developed by hybridisation programs and released for commercial planting after several years of extensive field evaluation trials. To preserve the desirable characters of a high yielding variety, planting materials are raised through budgrafting technique where the desired scion is grafted on to assorted rootstocks grown from cross-pollinated seeds. Despite this vegetative mode of propagation, large tree-to-tree variation in growth and yield among bud-grafted *Hevea* trees was reported (Combe 1975; Omokhafe 2004). Strong rootstock effect on scion yield was demonstrated by comparing different types of root stocks on the same scion (Combe and Gerner 1977; Ng et al. 1981; Sobhana et al. 2001; Gonçalves and Martins 2002; Cardinal et al. 2007). Though factors like soil heterogeneity and G×E interactions are partly responsible for these variations, it is mainly attributed to the genetically divergent nature of the rootstocks

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used for propagation (Nayanakantha and Seneviratne 2007). The key mode by which the rootstock controls growth and properties of scion is not yet fully understood and is still debated. In Apple and Pyrus, the possibility of epigenetic modifications like RNA-directed DNA methylation in the scion which may result in divergent phenotypic characters was suggested by different researchers (Kanehira et al. 2010; Zhang et al. 2012). Graft-transmissible RNA gene silencing signals have been demonstrated in both upward and downward directions in Arabidopsis (Brosnan et al. 2007; Molnar et al. 2010). The signals emanating from the rootstock by the above modes have their impact on target genes resulting in the reprogramming of their expression profiles based on the site of action (Koepke and Dhingra 2013).

DNA cytosine methylation is an epigenetic phenomenon which plays an important role, in gene regulation, genomic imprinting, X-chromosome inactivation, activation of transposon, as well as expression of endogenous genes. Though major portion of the methylation is conserved in most eukaryotic groups, including many plants, animals, and fungi, some portion is highly dynamic in nature and very sensitive to external and internal physiochemical changes which the organism encounters. In general, DNA methylation is categorized into three types according to the sequence context of the cytosines, namely CG, CHG, and CHH (H = A, C, or T). The conserved MET1 DNA methyltransferase enzymes are responsible for the maintenance of CG methylation, whereas the conserved DRM2 methyltransferases maintain CHH methylation, and, to some extent, CHG methylation. Earlier studies have also shown that the plant-specific methyltransferase CMT3 is responsible for the maintenance of high levels of CHG methylation seen in plants (Chan et al. 2005; Goll and Bestor 2005). Though there exist several physiological evidences for the existence of stock–scion interaction in plants, molecular evidences at the genetic and epigenetic levels revealing the influence of stock scion interaction in plants are scarce. For example, partially heritable, locus-specific alteration of DNA methylation patterns has recently been found in scions of interspecific grafts of *Solanaceae* (Wu et al. 2013). Even though these studies provide some clues on the much debated graft-hybrid controversy, extensive and rigorous research is desperately needed to unequivocally elucidate the graft-hybrid–graft transformation issues.

In the present study, we made an attempt to identify the influence of root stock on the epigenome of the scion, by comparing the epigenome of uniformly maintained bud-grafted plants developed using eye buds from a single poly-embryony derived rubber plant. Our earlier studies established that poly-embryony derived own rooted rubber seedlings are highly uniform and stable both genetically as well as epigenetically (Rekha et al. 2015). Therefore, these plants were selected as the source plant for the study. We hypothesize that the epigenetic changes detected among the bud-grafted plants are reflection of the level of divergence of the root stock used. It is anticipated that the grafting process between genetically divergent cells of the root stock and scion (heterografting) might generate novel epigenetic marks in the scion, and the accumulated effect of several such repeated grafting cycles may result in the often-observed graft-hybrid phenotypes. To test this, we used methylation-sensitive-amplified polymorphism (MSAP) for screening DNA methylation variations among bud-grafted plants with divergent root stocks and same scion material. Analysis of the methylation pattern in the bud-grafted plants revealed the existence of divergent methylation patterns among them despite of their similar genetic makeup. Bisulfite sequencing of selected regions revealed that significant methylation changes exist in the coding and regulatory region of important genes associated with stress and signal transduction. The results based on a comparative analysis indicate that bud-grafted plants may show significant epigenetic changes which may even have an impact at the functional level.

Materials and methods

Bud grafting of poly-embryony derived plants to assorted root stock

Assorted seedlings were raised from open pollinated seeds as follows. Open pollinated seeds were collected from the field and germinated in seed beds. After the emergence of radicle, they were transferred to polybags of size 55 × 25 cm. When the seedlings attained a girth of about 7–8 cm, they were used as stock-seedlings for budding. Eye buds were collected from one of the field planted own rooted plants of single zygotic origin derived by half *ovulo* embryo culture technique (Rekha et al. 2015) and grafted to the aforementioned genetically divergent rootstocks following the standard procedure. The plants were allowed to grow in poly-bags filled with sand, soil, and soil rite mixture maintaining maximum uniformity in growth conditions like temperature, humidity, etc. After 1 year growth, five such bud-grafted plants were used for a comparative analysis with the source own rooted seedling plant using genetic and epigenetic molecular markers. Poly-embryony derived source own rooted plant in the field (5th year) from which eye buds were collected and the grafted plants in poly-bags are shown in Fig. 1.

DNA extraction

Genomic DNA was isolated from young, expanded, and light green healthy leaves from the five bud-grafted plants following a modified CTAB method of Doyle and Doyle (1990). The DNA concentration and purity was determined

Fig. 1 Own rooted source plant and bud-grafted plants. Left: poly-embryony derived source own rooted plant in the field (5th year) from which eye buds were collected and grafted to divergent rootstock. Right: the five grafted plants (Plant No. 10, 9, 8, 7, and 5 from left to right) maintained in poly-bags

spectrophotometrically. Integrity of DNA samples was also checked on 0.7% agarose gel (Sigma).

Marker analysis

RAPD analysis

Even though genetic uniformity of the scion of all the plants was assured because of their common origin from the same plant, DNA from this single source plant and the five bud-grafted plants were analyzed together by a few established polymorphic RAPD markers in rubber to eliminate the possibility of errors occurring during the grafting procedure.

MSAP analysis

To detect methylation variations associated with bud grafting in rubber, MSAP technique was employed. Two reactions were set up at the same time. In the first reaction, 1 µg of genomic DNA of the five poly-embryony-derived bud-grafted plants was digested with 10 U of *Eco*RI (NEB) plus 10 U of *Msp*I (NEB) in a final volume of 50 µl followed by incubation overnight at 37 °C. A second double digestion reaction was carried out with 10 U of *Eco*RI and 10 U of *Hpa*II using the One-Phor-All Buffer PLUS (Amersham Pharmacia Biotech) in 50 µl. Following ligation with *Eco*RI adaptor [(5′-CTCGTAGACTGCGTACC-3′/3′-CATCTGACGCATGGTTAA-5′)] and *Msp*I–*Hpa*II adaptor [(5′-ACGATGAGTCTAGAA-3′/3′-CTACTCAGATCTTGC-5′)] pre-amplification reactions were performed with *Eco*RI primer (E + A primer: 5′-GACTGCGTACCATTCA-3′) and *Msp*I–*Hpa*II primer (Met + T primer: 5′-ACGATGAGTCTAGAACGGT-3′) with one selective base each. Pre-amplified mixtures were diluted 1:50 from their original volume with sterile Milli Q water. Selective amplifications were conducted with *Eco*RI primers having three selective bases (E + AAG/E + AGT/E + AGC/E + AGA/E + AGG) and *Msp*I–*Hpa*II primers with three selective bases (Met + TAC/Met + TAG), respectively. (Primers used are listed in Table 1) Adaptor ligation, pre-amplification, and selective amplifications were performed as per the standard AFLP procedure. The selectively amplified products were mixed with an equal volume of formamide gel loading buffer, denatured and electrophoresed on 6% (w/v) denaturing polyacrylamide gel containing 7 M urea and 1× TBE. Gels were run at 1200 V for 4 h and stained by the silver staining method.

In the gel, each DNA sample is represented by two lanes of fragments (labelled) resulting from *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II-digested DNA (M and H lanes, respectively). Since the isoschizomers *Msp*I and *Hpa*II recognizes the

Table 1 Combination of selective primers used for MSAP analysis

Combination	Primer E + A	Primer MH + T
1	EC-6 E + AAC	Met + TAC
2	EC-7 E + AAG	Met + TAC
3	EC-7 E + AAG	Met + TAG
4	EC-8 E + ACC	Met + TAG
5	C17 E + AGT	Met + TAC
6	C17 E + AGT	Met + TAG
7	C18 E + AGC	Met + TAG
8	C18 E + AGC	Met + TAG
9	C19 E + AGA	Met + TAC
10	C19 E + AGA	Met + TAG
11	C20 E + AGG	Met + TAC
12	C20 E + AGG	Met + TAG
13	EC-9 E + ACT	Met + TAC
14	EC-9 E + ACT	Met + TAG

E, *Eco*RI primer sequence based on *Eco*RI adaptor (GACTGCGTA CCAATTC); MH (Met), *Msp*I–*Hpa*II primer sequence based on *Msp*I–*Hpa*II adaptor (ACGATGAGTCTAGAACGG)

same tetra-nucleotide sequence, 5′-CCGG, but have different sensitivities to methylation of the cytosines, *Hpa*II will not cut if either of the cytosines in the double strand is methylated, whereas *Msp*I will not cut if the external cytosine is fully or hemi (single-strand) methylated. Thus, for a given DNA sample in the MSAP analysis, full methylation of the internal cytosine (designated as CG methylation) or hemimethylation of the external cytosine (designated as CHG hemimethylation) at the assayed 5′-CCGG sites is revealed as the presence of a band in only one of the enzyme digests and absence from the other. The (+, +) pattern (a fragment of definite length visualized in both the M and H lanes) is attributed to digestion by both enzymes at a nonmethylated CCGG site. The (−, −) pattern indicates inhibition of digestion with both enzymes at a fully methylated mCmCGG site when another treated sample shows the presence of a fragment at that position. The (−, −) pattern may also represent a mutated site when genetically distinct samples are compared. The (+, −) pattern corresponds to digestion with *Msp*I but not *Hpa*II and refers to the presence of a CmCGG site. The (−, +) pattern is designated as CHG hemimethylation which is common in plant genomes. Scoring of bands from the MSAP gel was done as described in Fulneček and Kovařík (2014). By tabulating the number of bands representing the various types of MSAP patterns, the relative CG, CHG, and total (adding up the two) methylation levels were determined in the source plant and the five bud-grafted plants.

Elution, cloning, and sequencing of the polymorphic bands

DNA methylation variations were identified among the five bud-grafted plants using the following primer combinations: C-19/Met + TAC, EC-8/Met + TAG, and EC-9/Met + TAG. The polymorphic bands were excised from the dry polyacrylamide gel using a sterile scalpel and transferred to 100 µl of TE buffer (10:01) in 1.5 ml tube. The contents were boiled for 15 min in a hot water bath followed by centrifuging at high speeds to pellet the gel debris. After transferring the supernatant to a fresh tube, 10 µl of sodium acetate (3 Molar), 10 µl of glycogen (5 mg/ml), and 450 µl of absolute ethanol was added and kept at − 80 °C for 1 h to precipitate the DNA. After spinning, the pellet obtained was washed with 85% ethanol followed by vacuum drying and dissolving in 10 µl sterile Milli Q water. The DNA thus obtained was re-amplified using the same tracing selective primer combinations. After size verification of the respective PCR products by electrophoresis, they were gel purified and cloned into pGEMT easy vector (Promega, USA) for sequencing. The cloned fragments in pGEMT vector were sequenced from both directions using universal primers in ABI3500xl sequencer.

Sequence analysis and annotation

Advanced BLASTN and BLASTX programs of the NCBI (http://www.ncbi.nlm.nih.gov/) were used for sequence and functional homology analysis of the cloned polymorphic DNA bands obtained through MSAP analysis of the bud-grafted plants. The sequences of the polymorphic bands, C19-Met-TAG-2, C19-Met-TAG-3, EC8-Met-TAG-7, and EC9-Met-TAG-10 were blasted against the *Hevea* whole-genome shot gun sequences to identify the location of these methylation-sensitive regions. The contigs which harbored these portions were extracted and further analyzed to annotate the region of interest.

Bisulfite sequencing and PCR

Bisulfite conversion of genomic DNA from each of the five bud-grafted plants was performed using the EpiTect Bisulfite Kit (Qiagen GmbH). One microgram DNA of each plant was converted as per the manufacturer's recommendation. Purified converted DNA was stored at − 20 °C for bisulfite PCR. PCR amplification was performed as per Uthup et al. (2011). Several primers combinations were tried for each of the four genomic fragments to obtain a single clear band and the best pair which gave a unique band of expected size was selected for the final analysis. Repeated PCR reactions with various primer combinations failed to amplify the EC9TAG-10 fragment from any of the sample. Details of the selected

bisulfite PCR primer pairs used for each gene are as given in SS.1a. PCR products were run on 2% agarose gel along with a 100 bp DNA ladder (NEB) to estimate the size of the product. Amplified products were eluted from gel and cloned into pGEMT easy vector system (Promega, USA). Five-to-eight 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. Plasmids were isolated from the positive colonies from each plate and sequenced. The sequences were aligned with the respective master sequences (normal sequences from the same region) using DNASIS (Hitachi Software Solutions, USA) and uploaded to the web-based software CyMATE (Hetzl et al. 2007) to detect and characterize the methylation variations.

Results

Molecular marker analysis

Molecular genetic markers like RAPD were used to demonstrate that the source pant as well as all the bud-grafted plants shared the same genetic material and grafted plants are true to type. RAPD patterns of all the five bud-grafted plants and the single source plant showed that there is no genetic variation among them (SS.1b). Similarity/dissimilarity in the epigenome of the above plants was determined by assessing their global genomic DNA methylation pattern using the MSAP technique. When a comparison of the restriction digestion pattern of *Msp*I and *Hpa*II in these plants was carried out, notable variations were not observed between the five plants in the *Msp*I digest set, whereas major variations were observed among these plants in the *Hpa*II digest sets for four primer combinations analyzed (EC8-Met + TAG, EC9-Met + TAG, and C19-Met + TAC (SS.1c). The polymorphisms include a couple of methylation-sensitive bands observed in the primer combination EC8-Met + TAG and EC9-Met + TAG where two bands of minor size variation (EC8TAG-7 and EC9TAG-10) showed varying patterns in the five plants (Fig. 2a, b). The *Msp*I digest set had a single band at this position, and this was retained in the *Hpa*II set only in Plant No. 7, 8, and 10. The above band was missing in the Plant No. 5. Similarly, Plant No. 10 had two prominent unique bands in the *Hpa*II digest set for the C19-Met + TAC primer combination (Fig. 2c, d). The numerous uniform bands observed in the case of *Msp*I digest set indicate that the plants are genetically same. Even though epigenetic divergence observed among the grafted plants were minimal, a significant variation in the methylation patterns was observed between the own rooted source plant and the grafted plants when a comparative analysis of the present results with that of the earlier reports was carried out (Rekha et al. 2015).

A detailed comparative analysis of the various types of methylation banding pattern of the five plants along with their single source plant is shown in Table 2. The percentage of cytosine's that are methylated for each sample was calculated using the formula [% mC = 100 × mC/(*C* + mC)]. Though the total number of bands considered is less for the source plant, it is interesting to note that the percentage of un-methylated and methylated sites are comparable for the own rooted source plant and the grafted plants. The average of the percentage of un-methylated sites was around 81.6,

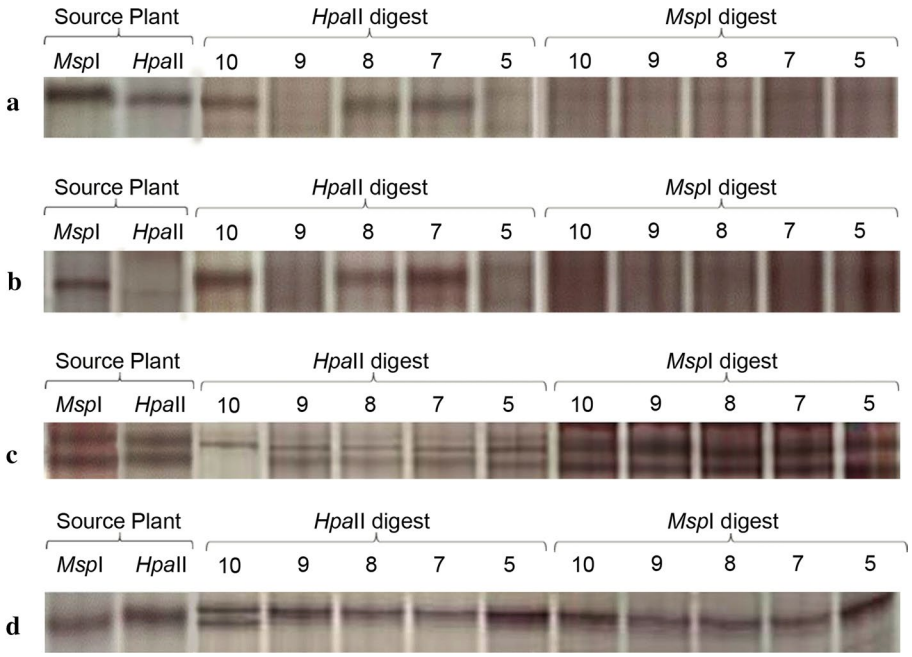

Fig. 2 Status of the four prominently polymorphic MSAP bands in the source plant and the five bud-grafted plants. **a** EC8TAG-7 band. **b** EC9TAG-10 band. **c** C19TAC-2 band. **d** C19TAC-3 band

Table 2 Levels of DNA methylation at sampled 5′-CCGG sites by MSAP in heterografted plants

Plant. no.	Total sites	Un-methylated sites	Methylated sites		
			CG full methylation	CHG hemi-methylation	Total
10	207	166 (80.19%)	32 (15.45%)	9 (4.34%)	41 (19.80%)
9	207	169 (81.64%)	29 (14.00%)	6 (2.89%)	35 (16.90%)
8	208	169 (81.25%)	30 (14.42%)	8 (3.84%)	38 (18.26%)
7	207	169 (81.64%)	29 (14.00%)	8 (3.86%)	37 (17.87%)
5	207	170 (82.12%)	28 (13.52%)	6 (2.89%)	34 (16.42%)
Source plant	181	151 (82.9%)	29 (15.93%)	1 (0.50%)	30 (16.50%)

whereas it was 17.5 for the methylated sites. Among the two major types of methylation patterns which could be discriminated using MSAP (mCG full methylation and mCHG hemimethylation), mCG type was the dominant type constituting major portion of methylation in the grafted plants as well as their source plant. CHG type constituted approximately 20% of all methylated sites in the heterografted plants. In the case of own rooted source plant, mCG type was as high as 96% of methylated sites, while CHG constituted merely 4%. Interestingly Plant No. 10 which showed the most diverse banding pattern among all the five had the highest methylation levels, as well. Altogether, the overall CHG methylation percentage was much higher in the grafted plants than the own rooted plant, whereas the CG percentage remained consistent in all plants in our study. However, extensive methylation analysis using HPLC or ELISA-based methods is required to arrive into any final conclusions as the band scoring from MSAP provides only a limited view on the global methylation landscape, taking into account only the methylation at some random CCGG sites.

Sequence analysis and annotation

To understand the functional relevance of the four prominent loci showing variations in DNA methylation patterns, the corresponding MSAP bands from the bud-grafted plants were isolated and sequenced. Based on a blastn analysis, we found that the sequence of the unique band C19TAC-2 observed in the *Hpa*II digest set of Plant No. 10 showed homology to a portion of chromosome five of *T. cacao* (LT594792.1), whereas blastx showed no significant similarity. To locate this region in the *Hevea* genome, a separate blastn with *Hevea* whole-genome sequences was performed. The sequence showed perfect match with a portion of the scaffold0064 of the *H brasiliensis* cultivar Reyan7-33-97 whole genome (Tang et al. 2016). Further analysis of the upstream and downstream of this region revealed that the portion comprised of an ORF showing homology to a gene coding for a hypothetical protein from the fungi *Schizophyllum commune* XM_003028617.1. In the case of the unique band C19TAC-3 from Plant No. 10, normal blastn, and blastx yielded no significant similarity to known sequences. Interestingly blastn with *Hevea* whole genome revealed that the region of interest lies in the scaffold1448 of *H. brasiliensis* cultivar Reyan7-33-97. Detailed sequence analysis revealed that the region of interest lies in the promoter region of a Leucine-rich Repeat Receptor Kinase (LRR-RK) gene. The entire LRR-RK gene from *Hevea* with promoters region was also extracted from the in-house *H. brasiliensis* clone RRII 105 whole-genome sequence data and was provided in the supplementary material SS.1d. Blastn analysis of the EC8TAG-7 fragment yielded no significant hit, but blastx analysis resulted in a good match with a conserved hypothetical protein in *Ricinus communis*. Blastn with *Hevea* whole genome showed high similarity with a portion of the scaffold0977 of Reyan7-33-97 cultivar. Gene prediction from this scaffold revealed that the sequence of interest is within the ORF of a sister chromatid cohesion protein gene. Similarly, the sequence of EC9TAG-10 fragment showed similarity to *Hevea* hmac3 microsatellite sequence ID: AY135658.1 during normal blast. Bastx gave no significant match to any protein sequences, but blastn with *Hevea* whole genome showed high homology of this fragment with a portion of the clone Reyan7-33-97 scaffold0884. Gene prediction from this scaffold revealed that this region does not fall in the ORF of any known genes. The nearest gene predicted was an alcohol degenerate gene positioned 1.8 kb downstream. To ensure the functional role of the four sequences obtained, they were subjected to a blastn analysis exclusively with the *Hevea* transcriptome shot gun assembly data available in NCBI. Except C19TAC-3 sequence, all the other three showed high homology ($\leq$ 80%) to *Hevea* mRNA sequences. The sequenced fragments and their annotation details are shown in Table 3.

Bisulfite sequence analysis

Bisulfite sequencing of the three fragments (C19TAC-2, C19TAC-3, and EC8TAG-7) from the five plants was performed to find out the exact location, class and extent of methylation in the samples analyzed (SS.1e). Bisulfite sequencing of the C19TAC-2 fragment revealed the presence

Table 3 Annotation of sequenced methylation-sensitive polymorphic bands

S. no.	Seq ID	Blastn with *Hevea* transcriptome shot gun assembly	Blastx (general)	Blastn with *Hevea* whole genome	Predicted ORF if any
1	C19TAC-2	*Hevea brasiliensis* contig63428, mRNA sequence JT966462.1 (79%)	Hypothetical protein *Schizophyllum commune* XM_003028617.1	*Hevea brasiliensis* cultivar reyan7-33-97 scaffold0064 LVXX01000064.1	Yes In coding region Hypothetical protein
2	C19TAC-3	No significant similarity	No	*H. brasiliensis* cultivar reyan7-33-97 scaffold1448 ID: LVXX01001448	Yes In promoter region of Leucine-rich repeat receptor kinases (LRR-RKs)
3	EC8TAG-7	*Hevea brasiliensis* contig28760, mRNA sequence (88%) JT948286.1	Hypothetical protein (*Ricinus communis*) Sequence ID: reflXP_002527301.1	*H. brasiliensis* cultivar reyan7-33-97 scaffold0977 ID: LVXX01000977	Yes In coding region of sister chromatid cohesion protein pds5 isoform X2 (*Ricinus communis*) XP_015579745.1
4	EC9TAG-10	*Hevea* contig58002, mRNA sequence JT916497.1 (100%)	No	*Hevea brasiliensis* cultivar reyan7-33-97 scaffold0884 ID: LVXX01000884.1	No 1.8 kb downstream- alcohol degenerate gene (*Jatropha curcas*) Sequence ID: XP_012073689.1

of CHH and CG methylation in the analyzed region. Interestingly the CHH methylation at position 50 was found to be uniformly present in all the plants, whereas the CG methylation at 331 was observed only in plant No. 10. CHH, CHG, and CG patterns of methylations were detected in the LRR-RK promoter region containing fragment C19TAC-3. CHH pattern was noted at position 47 of all plants, whereas CHG pattern was observed at positions 184 and 351. Unique CG methylation patterns were observed at the C19TAC-2 fragment in Plant No. 10 at positions 278 and 352. While the pattern at position 352 holds up the MSAP results by maintaining methylation at the outer cytosine of the CCGG locus (position 351) and demethylating the internal cytosine at position 352, the unique pattern noted at 278 was unforeseen and undetected in MSAP. Like the C19TAC-2 fragment, EC8TAG-7 region also had CHH and CG type of methylations. The CG methylation at position 71 seems to be specific to Plant No. 10, whereas CG at position 246 appeared to be methylated in Plant Nos. 5 and 8 and demethylated in Plant No. 7, 9, and 10. Random methylation variations were observed in the case of all the three fragments.

Discussion

Recent technological advancement in the movement, tracking, and identification of genetic elements like DNA and RNA in plants proved beyond doubt that there exist effective communication and transfer of genetic elements from the root stock to scion and vice versa. Experimental evidences from such studies suggest that there is every probability for a grafted plant to become epigenetically distinct from both the contributing partners. In the case of rubber, grafting is widely used as a propagation technique and disparity in phenotypic characters among individual plants of the same genotype grafted to divergent root stock has been reported (Premakumari et al. 2002; Omokhafe 2004; Cardinal et al. 2007). Though most of the disparities reported were in terms of biochemical and phenotypic parameters, genome-based evidences were lacking in the case of rubber. Since root stocks as well as scion are derived from less genetically divergent plant materials owing to the narrow genetic base of rubber, the chances of identifying epigenetic variations are more prominent and frequent than looking for genetic changes (Souza et al. 2015; Priyadarshan 2016; Uthup et al. 2011). Furthermore, the high sensitivity of epigenetic factors to biotic and abiotic stresses resulting in the regulation of concerned gene expression makes it more interesting (Lämke and Bäurle 2017). For example, the activation of stress-responsive genes as a result of hypomethylation due to biotic stress is a good example for the impact of biotic stress on gene regulation (Wada et al. 2004). Similarly, altered DNA methylation status in response to cold stress, salt stress, drought stress, osmotic stress, and mechanical stress were also reported in different plant species (Steward et al. 2000; Dyachenko et al. 2006; Labra et al. 2002; Kovařik et al. 1997; Galaud et al. 1993). In the present study, bud-grafted plants sourced from a polyembryony-derived own rooted plant were selected as the experimental material as polyembryony derived plants were previously shown to have high genetic and epigenetic stability (Rekha et al. 2010, 2015). Eye buds from a single own rooted polyembryony-derived

rubber plant were grafted to genetically divergent root stock, so that the variations in methylation patterns among these plants may be attributed mostly to the impact of divergent root tissues rather than the overall impact of the grafting procedure. Uniformity in growth conditions after grafting was another factor which ensured minimal impact of external factors on the rubber epigenome.

The results from the conventional molecular genetic marker analysis based on sequence polymorphisms like RAPDs ensured the genetic uniformity of the plants studied. Moreover, by re-confirming the genetic similarity of the five budgrafted plants and their source own rooted plant, the possibility of error during the budding process is eliminated. Contrary to the RAPD results, MSAP detected sequence structure independent DNA modification like methylation changes among the budgrafted plants bringing out the epigenetic variations among them. The DNA methylation polymorphisms among the bud-grafted plants whose DNA was digested with methylation-sensitive *Hpa*II enzyme indicate that, despite the similar genome content, the epigenome of each plant was modified resulting in MSAP pattern variations. This is also evident from the plant-specific unique methylation patterns observed from the bisulfite sequencing results of selected loci.

Earlier studies have reported that the degree of methylation in nuclear DNA from various plant and animal species roughly falls in the range of 20–30% (Tsaftaris and Polidoros 2000). Since the approximately 20% 5 mC content calculated from the MSAP data from five *Hevea* plants is in agreement with the above discovery, the probability of *H. brasiliensis* having an approximately 20% methylated genome is also high. Even though alterations in the DNA methylation patterns were identified in several loci, the maintenance of the relative collective level of DNA methylation in all the experimental plants suggests that methylation levels in *Hevea* are more of species or family specific than individual centric. This assumption is corroborated by whole-genome methylation studies in humans where Zhang et al. (2015) have shown that the methylation levels among individual may remain fairly constant genome-wide. However, conclusive studies involving different *Hevea* genotypes have to be carried out to ascertain this statement. As far as methylation type is concerned, the prominence of CpG methylation in *Hevea* completely agrees with the CpG methylation composition reported in the case of other plants regardless of the presence of both CpG as well as CHG patterns of the randomly sampled 5m-CCGG sites in all independent samples of graft hybrids tested (Tsaftaris and Polidoros 2000; Feng et al. 2010). Though CpG comprised of 80% methylation in the grafted plants, a comparison with the own rooted source plant suggests that this pattern of methylation is least affected by grafting as there was no significant variation in the overall CpG methylation pattern between them. On the contrary, the CHG methylation percentage significantly increased in all the five grafted plants, suggesting that CHG pattern of methylation is more responsive to grafting or stock scion interaction. Since CHG and CHH methylations are mostly associated with transposon elements (TE) and upstream region near the transcriptional start sites (TSSs), an increase of CHG methylation in heterografted plants indirectly indicates the silencing of these elements in *Hevea* due to locus-specific methylation. This argument is supported by an earlier study in *Arabidopsis* where it was established that asymmetric CpNpG methylation associated with stress may be locus-specific in nature (Cao and Jacobsen 2002). The setting up of de novo CHG type of methylation during adaptive stress response via an active CMT3 pathway reported in *Populus trichocarpa,* another tree species which shares similarity with *Hevea* genome further ascertain this statement (Liang et al. 2014; Schönberger et al. 2016). Therefore, a similar trend observed in *Hevea* may be an indication of heterografting-specific CHG methylation in rubber. Since the accurate estimation of global CHG patterns from MSAP analysis is relatively difficult, the above conclusions has to be verified by more reliable techniques like whole genome bisulfite sequencing.

The complex biochemical background behind all the changes associated with graft union formation depends on specific genetic interactions between the cells of the stock and the scion (Koepke and Dhingra 2013). At the molecular level, graft union formation presumably requires considerable reprogramming of gene expression, protein translation, and metabolism (Cohen et al. 2007; Gainza et al. 2015). Differential expression of genes related to cell cycle, metabolism, and signal transduction during the process of graft formation has been reported in *Arabidopsis thaliana* and in tree species like Hickory and *Prunus* (Zheng et al. 2010; Yin et al. 2012; Gainza et al. 2015). Grafting-induced differential gene expression was also reported to be species-specific as in the case of *Brassica rapa* (Mun et al. 2015). Being an economically important crop widely propagated by grafting, methylation changes associated with grafting in the regulatory and coding regions of genes in *H. brasiliensis* merits further understanding. Though DNA methylation in plants is a global phenomenon, depending on the type of DNA sequence, the level of each pattern of methylation may vary. Interestingly, CHG methylation identified in the methylation-sensitive locus EC9TAG-10 falls within a known repeat region in *Hevea*. Alternatively, CG methylation seems to be prominent in the body of protein-coding genes in *Arabidopsis*, Rice, and Poplar (Feng et al. 2010). The blastn data of polymorphic fragments are in congruence with the above reports, because three out of four prominent polymorphic bands screened from the MSAP analysis were found to be part of active genes in *Hevea* and CpG methylation was identified as the prominent type. The fourth

polymorphic methylation-sensitive loci identified in the promoter region of a Leucine-rich repeat receptor kinase (LRR-RK) gene also assume high relevance, because methylation variations in the promoter regions can be directly related to the downstream gene expression. Prior studies showed that receptor kinases with very divergent extracellular domains and functions exist in plant genomes and many of them were differentially expressed in response to grafting with nonself genotypes (Cookson et al. 2014). Among receptor kinases in plants, LRR proteins forms the largest group and the motif is expected to be involved in signal transduction and in mediating protein–protein interactions (Haffani et al. 2004; Sanabria et al. 2008). In this context, the differential methylation variation identified in the promoter region of an LRR receptor kinase in one of the bud-grafted *Hevea* plants could suggest that there is some degree of nonself recognition at the graft interface in heterografted plants. Moreover, the selective de-methylation of the internal cytosine at position 352 (C19TAC-3) of the above promoter while maintaining the outer cytosine methylation as such is a clear evidence for plant-specific epigenetic modifications in rubber. Since de-methylation of promoter loci is reported to be strongly associated with increased gene expression, it may be assumed that the expression rate of this LRR-RK might have been altered in the grafted plant, Plant No. 10. Simultaneously, the similar pattern of methylation seen in the C19TAC-2 locus of a hypothetical protein in the same plant is another good example for the preferential exon methylation which is reported in many plants including *Arabidopsis* (Feng et al. 2010). Based on the previous reports in animal and human cell lines, gene body methylation plays a major role in alternative splicing (AS) mechanism resulting in a single gene coding for multiple proteins (Maor et al. 2015). The crucial role of AS in the regulation of plant responses at the post-transcriptional level has been demonstrated by several studies (Shang et al. 2017). Therefore, it is tempting to speculate that the methylation of the C19TAC-2 fragment at position 331 of Plant No. 10 might have altered the expression of some splice forms, thereby modulating the post-transcriptional mechanism of the concerned gene of this plant. Based on the above unique methylation patterns observed, it is inferred that the root stock used for the Plant No. 10 was the most genetically divergent one, but further conclusive experiments by extensive genome analysis of the root tissues is required to prove the above inference. The methylation variations observed in the locus EC8TAG-7 within the coding region of a sister chromatid cohesion protein pds5 of *Hevea* is also interesting as this gene is a major regulatory subunit of the cohesin complex. Cohesin is an evolutionarily conserved, ring-shaped protein complex essential for a wide variety of intra and intermolecular DNA processes, and has important roles in sister chromatid cohesion, DNA repair, and gene regulation (Nasmyth and Haering 2009; Wendt and Peters 2009; Dorsett and Ström 2012). Since this protein is closely associated with another plant chromatin protein called DEK3 a known regulator of stress tolerance which is involved in regulating nucleosome occupancy and gene expression, there is a possibility that pd5s gene methylation may be associated with grafting-induced stress in rubber (Waidmann et al. 2014; Pradillo et al. 2015). As in the case of C19TAC-3 locus within LRR-RK gene, the varied methylation sensitivity of EC8TAG-7 locus also suggests that the divergent rootstocks used may be responsible for the variations detected. The annotation results of all the four fragments give strong indications that the methylation variations associated with grafting have functional relevance as they were all within the gene body or regulatory region of functional genes as evident from the results of *Hevea* transcriptome-specific blastn analysis.

Altogether, the results from the current study clearly indicate that heterografts in rubber are epigenetically divergent despite sharing the same scion material. The variation in DNA methylation patterns observed in the bud-grafted *Hevea* plants can be specifically attributed to stock–scion interaction, because maximum uniformity among them was maintained in terms of growing environment (temperature, relative humidity, and light intensity), soil properties, nutrient, and water availability. Therefore, the possibilities of these factors influencing the epigenome of scion are minimal when compared to the impact of a genetically divergent rootstock on it. However, extensive studies need to be undertaken in this direction for identifying the extent of methylation variation, its stability, and frequency. A prospective study in this direction involves monitoring of the same plant on a yearly basis to see the consistency of observed patterns. Further replication of each of the above plants also may be attempted to track the already identified epigenetic markers. Since epigenetic memory is transmitted through mitosis and meiosis in plants, the F1 progeny of these plants may be checked to ascertain the heritability of changes observed (Saze 2008). Molecular characterization of the root stock is also essential for validating the results from this study. Understanding how gene body DNA methylation is targeted and why exonic regions are favored over introns are also key questions for future research The significant change in the methylation pattern among the heterograft's indicates that serious epigenetic rearrangements have happened as a results of grafting in rubber and the extend of this changes in the grafted plants may depend on the level of incompatibility between the root stock and the scion. The study suggests that DNA methylation changes may accumulates in plants through successive vegetative propagation cycles (budding) and the cumulative effect may alter desirable phenotypic characters like yield which may affect the productivity of high yielding rubber clones in the future. Furthermore, the present study paves the way for better understanding of the

stock–scion interaction process in rubber and it is anticipated that unravelling the mystery behind this complicated process may lead to a solution for the intraclonal variation among popular rubber clones.

Author contribution statement TKU and RK conceived and designed the research and wrote the manuscript, TKU, RK, and MR conducted the experiments. TKU and TS analyzed the data.

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