Rootstock induced epigenetic variation in *Hevea brasiliensis*

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ABSTRACT: *The heritable variation within a species is the consequence of the difference in the primary DNA sequence of different individuals. The term epigenetics is generally used to refer the study of heritable change in gene expression that is independent of DNA sequence. Epigenetic changes can result in altered gene transcription and serves as an important mechanism in regulating gene expression during development and in response to stimulus. It is reported that heritable epigenetic marks persists through meiosis and are stably transferred to the next generation resulting in trans-generational epigenetic inheritance. Rubber tree is commercially propagated by bud grafting and the effect of rootstock on the performance of scion is still debated. In the present study we made an attempt to identify the impact of stock scion interaction on the epigenome of rubber by comparing the methylation pattern of uniform, own rooted seedlings and their bud grafted counter parts. Multiple uniform seedlings of Hevea brasiliensis were developed through half ovulo embryo culture method. Genetic as well as epigenetic uniformity of these seedlings was confirmed by RAPD as well as MSAP (Methylation Sensitive Amplified Length Polymorphism). One among the uniform seedlings was multiplied by bud grafting to divergent rootstocks and were again tested for their genetic and epigenetic uniformity. The study revealed that there is no genetic variation among the multiple seedlings and among the bud grafted counterparts. However, epigenetic variation was observed among the bud grafted plants which were maintained under uniform environmental conditions. Since rootstock is the only source of variation here, it is presumed that the variation was induced by rootstock. The study assumes importance in Hevea because accumulation and maintenance of epigenetic changes during various cycles of vegetative propagation may eventually lead to an altered phenotype and can result in intraclonal variability.*

Key words*: Hevea brasiliensis, RAPD, MSAP, 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. Commercial planting materials are raised through bud grafting 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 many workers by comparing different types of rootstocks on the same scion (Combe & Gerner 1977; Negi et al. 1981; Sobhana et al. 2001; Gonçalves & Martins 2002; Cardinal et al. 2007). Though factors like soil heterogeneity and G x E interactions are partly responsible for these variations, it is mainly attributed to the genetically divergent nature of the rootstocks used for propagation (Nayanakantha and

Seneviratne 2007). The key mode by which the rootstock controls growth and properties of scion is yet to be unravelled. 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). 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). In the present study we made an attempt to identify whether rootstock influences the epigenome of the scion, by comparing the methylation pattern of uniform, own rooted seedlings and their bud grafted counter parts.

Materials and methods

Induction of multiple, uniform, own rooted seedlings

Uniform own rooted multiple seedlings were developed through the induction of zygotic polyembryony, by *in vitro* culture of immature fruits following half *ovulo* embryo culture technique (Rekha et al. 2015). The well-developed plantlets were transferred to small polythene bags filled with sand, soil, and soil rite mixture and kept in environmentally controlled growth chamber for 2–3 weeks, for hardening. Acclimatized plantlets were field planted. Among the multiple seedlings, eye buds from one of the plantlet was bud grafted to assorted seedlings for comparison.

Molecular analyses

DNA extraction

Genomic DNA was isolated from own rooted seedlings and their bud grafted counter parts following a modified CTAB method of Doyle and Doyle (1990). The DNA concentration and purity were determined spectrophotometrically. Integrity of DNA samples was also checked on 0.7 % agarose gel (Sigma). Working solutions of DNA stock for PCR were adjusted to 10ng/µl and stored at 4 °C.

RAPD analysis

Four arbitrary decamer primers, OPA10 OPA7, OPA1 and OPA15 primers (Operon Technologies, Inc., USA), were used for this study. Amplifications were performed in a DNA Thermal Cycler (Gene Amp 9600 PCR system, Perklin Elmer, USA) in a reaction mix containing 50 ng of DNA template, 20 pmol of primer, 2 mM MgCl2, 200 µM dNTPs, 0.7 units of *Taq* polymerase with 1X PCR buffer (Promega), in a final volume of 25 µl for each reaction. The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 1 min at 36 °C and 2 min at 72 °C with a final extension for 10 min at 72 °C. The amplified products were analyzed by electrophoresis in 1.4 % agarose (Sigma-Aldrich, India) gels, stained with ethidium

bromide (0.5 µg/ml of TAE buffer), and photographed using a Gel Documentation system.

MSAP analysis

To detect MSAP, two reactions were set up at the same time. In the first reaction, 1 µg of genomic DNA of the three polyembryony derived plants was digested with 10 U of *EcoR*I plus 10Uof *Msp*I in a final volume of 50 µl containing 50 mMTris–HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl by incubating overnight at 37 °C. The second digestion reaction was carried out as above with the exception that *Hpa*II was used in place of *Msp*I. Following ligation with *EcoR*I 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 *EcoR*I 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 *EcoR*I primer with three selective bases (E+AAG/E+AGT/E+AGC/E+AGA/E+ AGG) and *Msp*I-*Hpa*II primer with three selective bases (Met+TAC/Met+TAG) respectively. Adaptor ligation, pre amplification and selective amplifications were performed as per standard AFLP procedure. The selectively amplified products were mixed with an equal volume of formamide gel loading buffer and denatured and electrophoresed on 6 % (w/v) denaturing polyacrylamide gel containing 7 M urea and 1×TBE. Gels were run at 1,200 V for 4 h and stained by the silver staining method. The same procedure was followed for analysing the bud grafted plants also.

Results

Induction of multiple embryos and plant regeneration

Multiple uniform seedlings of single zygotic origin were successfully developed by *in vitro* culture of immature fruits (Fig. 1a). All the plants were phenotypically similar (Fig. 1b). The regenerated plants were acclimatised and field planted. Growth of the plants was found to be on par with seedlings raised from assorted seeds. Among the uniform seedlings three field established plants of single zygotic origin was utilized for further studies.

RAPD analysis

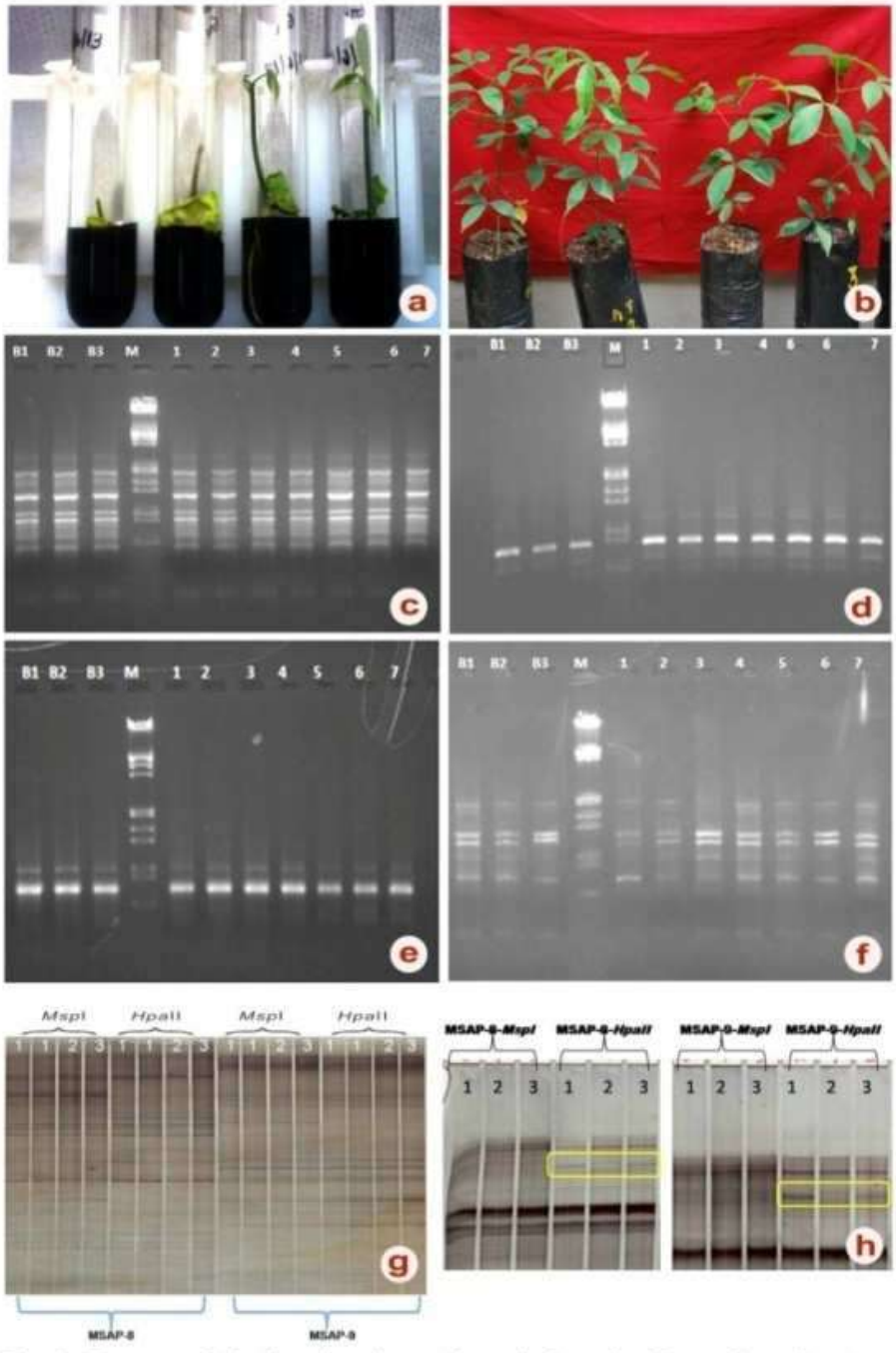
RAPD experiments revealed the own rooted plants as well as the bud grafted counterparts showed similar banding pattern with the tested primers (Fig. 1.c-f). Lane B_1, B2 and B_3 in the figure show the RAPD profile of own rooted seedlings of single

zygotic origin. Lane 1-7 shows the RAPD profile of bud grafted plants. The same banding pattern obtained with the same primers ensures the genetic fidelity.

MSAP analysis

Similarity/dissimilarity in the epigenome of the three own rooted plants and their bud grafts was determined by assessing their global genomic DNA methylation pattern using the MSAP technique. Notable variations were not observed among the three plants in the *Msp*I as well as *Hpa*II digest sets for all the primer combinations analysed (Fig. 1g). In the figure, the amplification of DNA samples with 2 primers after digestion with methylation insensitive (*Msp*I) methylation sensitive (*Hpa*II) are given. This indicates that there is no epigenetic variation among the own rooted seedlings. All the primer combinations analysed showed clear variation in the banding pattern between the *Msp*I and *Hpa*II digests. However the three plants showed same banding pattern with both the digests. When bud grafted plants were subjected to MSAP analysis, variations were observed with two primer combinations in the methylation sensitive *Hpa*II digest set indicating epigenetic variations among these plants (Fig. 1h). In the MSAP digest set, the banding pattern was uniform.

Figure 1: Root stock induced epigenetic variations in *Hevea brasiliensis*. (a & b). development of multiple uniform seedlings, (c-f). RAPD analysis of uniform seedlings and their bud grafted counter parts with different primers, (g). MSAP analysis of uniform seedlings and (h). MSAP analysis of bud grafted seedlings.



Discussion

Genetically similar uniform seedlings generated *via* induction of zygotic polyembryony, and their budgrafted counterparts were used in the present study in order to minimise the error. The uniformity and single zygotic origin of polyembryony derived plants of *H. brasiliensis* has been already established (Rekha et al. 2015). Molecular analysis proved that the plants developed were genetically similar. Conventional molecular markers based on sequence polymorphisms like RAPD as well as methylation sensitive AFLP which could detect sequence structure independent DNA modification like methylation changes, were utilized for the purpose. The budgrafted plants derived from these seedlings also showed same RAPD profile with tested primers, indicating their genetic uniformity (Fig. 1.c-f). When MSAP was performed, notable variations could not be detected among the initially developed three polyemryony derived plants for all the primer combinations tested which implies that there exists no methylation variation among them i.e. they are epigenetically uniform (Fig. 1g). On the contrary, in the bud grafted plants MSAP results showed polymorphic bands among with methylation sensitive *HpaII* digest indicating the presence of epigenetic changes (Fig. 1h). Though *Hevea* genome is reported to be highly methylated (Uthup et al. 2011) the absence of the variation in the MSAP profile in polyembryony derived plants and the presence of the same among the bud grafts appears to be exciting since the only source of variation could be the divergent rootstock. Other factors like growing environment, soil properties and nutrient availability were kept uniform. Therefore the possibilities of these factors influencing the epigenome are minimal when compared to the genetically different rootstock. Epigenetic traits are heritable changes associated with chemical modification of DNA without altering the primary DNA sequence. Depending on the site as well as type of tissue in which it occurs, DNA methylation can mediate the transmission of an active or silent gene either for short-term during mitosis or for long-term across generations during meiosis (Saze 2008). Therefore, phenotypic changes induced by variations in DNA methylation patterns may either be transient or heritable in nature depending on the type of tissue in which they occur. This is important in a species like *Hevea*, where vegetative mode of reproduction is commercially accepted. The change in the DNA methylation pattern is an indication of influence of rootstock on the scion. If it happens in a coding region of DNA, that will be reflected in gene expression also. Epigenetic changes are reversible or irreversible and some irreversible changes are likely to get accumulated over generations and can alter the phenotype of the plant. There is every chance that similar changes that are observed in the present study may be perpetuated among the vegetatively propagated generations of the composite plant. During subsequent cycles of budding, changes may happen again and they may get accumulated finally altering several properties of the original rubber seedling. A series of responses due to the influence of rootstock on scion physiology, gene expression, and protein function parameters in plants are already reported in other plants (Koepke

and Dhingra 2013). Stock induced variation in DNA methylation pattern as observed in the present study can be attributed to stock scion interaction leading to intraclonal variability which is a major reason for destabilising the productivity in rubber plantations. However further extensive studies are needed in this direction for identifying the extent of variation, its stability and frequency. Thus the present study could facilitate a better understanding of the stock-scion interaction process in rubber. This attempt towards unravelling the intricacies of stock-scion interaction in *Hevea* through methylation studies is a first step in this direction.

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