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ROOTSTOCK INFLUENCES GENE EXPRESSION IN SCION IN *HEVEA BRASILIENSIS*

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Bud-grafting is the most popular propagation method to produce a large number of "true-to-type" plants of desirable clones of *Hevea brasiliensis*. However, variability can exist among bud-grafted plants of the same clone due to the influence of genetically heterogeneous rootstocks used for budding. Two groups of plants of the same clone RRII 105, *viz.* mature trees in the field raised through shoot tip culture (devoid of any rootstock) and normal bud-grafted trees of the same age (with genetically different rootstocks) were used for the present study to determine whether the rootstock has influenced gene expression in the scion. DDRT-PCR analysis showed transcript-level changes in the latex collected from the scion of the tissue-cultured and bud-grafted trees. The similarity index (SI) was 57% for bud-grafted trees and 80% for tissue-cultured trees. Less similarity in transcript levels among the bud-grafted trees could be attributed to the influence of heterogeneous rootstocks on gene expression in scions. Greater similarity in gene expression among the tissue-cultured trees may be due to the absence of rootstocks.

Keywords: DDRT-PCR, Gene expression, Genetic heterogeneity, *Hevea brasiliensis*, Rootstock influence, Rootstock-scion interaction

INTRODUCTION

Natural rubber, *Hevea brasiliensis*, is propagated commercially through bud-grafting elite scions (clones) on genetically heterogeneous rootstock plants derived from polyclonal seeds. The heterogeneous rootstocks used for bud-grafting may impart intraclonal (tree-to-tree) variation. Rootstock-scion interactions have been reported as a complex phenomenon in several species of bud-grafted plants (Hartman and Kester, 1976) including *H. brasiliensis* (Sobhana, 1988; Krishnakumar *et al.*, 1992; Sobhana *et al.*, 2001). Graft-induced changes in fruit yield and quality have been reported in apple (Lord *et al.*, 1985), *Capsicum annuum* (Yagishita and Hirata, 1987) and *Anona* sp. (George and Nissen, 1987).

The metabolic activities of bud-grafted plants may be different from the two graft partners (*i.e.* scion and rootstock) and when the genetic constitution of these two partners is different, there are chances of incompatibility (Andrews and Marquez, 1993). Rootstock influence on growth and yield in *Hevea* was reported earlier (Templeton, 1960; Buttery, 1961; Ng *et al.*, 1981; Cardinal *et al.*, 2007). In rubber, subtle symptoms of rootstock-scion interaction are often reflected in many physiological and biochemical characteristics of the scion (Sobhana, 1998; Ahmed, 1999). Isozymes, the biochemical markers of gene expression, were also reported to be influenced by rootstocks in natural rubber (Krishnakumar *et al.*, 1992; Sobhana *et al.*, 2001) and mango

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(Degani *et al.*, 1990). In citrus, the peroxidase isozyme exhibited polymorphism depending upon the rootstock-scion genomic diversity and degree of compatibility between rootstock and scion (Protopapadakis, 1988).

Tapping panel dryness (TPD), a metabolic disorder in *Hevea*, is suspected to be influenced by incompatible rootstocks apart from other factors. A wider genetic difference between the rootstock and scion may lead to TPD incidence (Sobhana *et al.*, 2005). It was reported that specific species of mRNA could migrate from the rootstocks to scion in grafted tomatoes (Kim *et al.*, 2001), whereas in grafted potatoes they migrated from the scion to rootstock (Peres *et al.*, 2005). These reports indicated the possible rootstock influence on the scion and *vice versa*. It had been reported that rootstock effect on scion was stable and heritable. A low capsicin variety of red pepper grafted onto a high capsicin rootstock gradually produced red peppers which were very hot and this character was inherited by the next generation as in the case of genetic transformation (Ohta and Chuong, 1975 a & b; Hirata *et al.*, 1986). There are no reports available on graft-induced changes at molecular level in *H. brasiliensis*. The present study reports for the first time the influence of rootstock on expression of genes in the scion of bud-grafted *H. brasiliensis* trees.

MATERIALS AND METHODS

Plant material

Five 18-year-old trees of the clone RRII 105 developed through shoot tip culture and 10 normal bud-grafted trees of the same age and clone from the experimental farm of Rubber Research Institute of India, Kottayam, were used for this study. Latex samples were collected from the scion portion of the trees by making controlled wounding of the bark.

Total RNA isolation

Total RNA was isolated from the latex samples as described by Venkatachalam *et al.* (1999). Genomic DNA contamination was removed from total RNA by treating 1µg RNA with 1 U RNase-free DNase I (Sigma Chemicals). Purity and concentration of the RNA extracted were determined spectrophotometrically (NanoDrop Technologies, USA). RNA integrity was evaluated by separation of an aliquot of total RNA on 1.2% formaldehyde agarose gel.

mRNA differential display

Differential display RT-PCR reactions were carried out using RNA image kit (GenHunter Corporation, USA) as per the manufacturer's instructions. Total RNA (0.2 µg) was reverse-transcribed using an oligo-dT primer H-T_{11}G, which anchored to the poly (A^+) tail. Moloney murine leukemia virus reverse transcriptase was added after pre-incubation at 65 °C for 5 min and further at 37 °C for 10 min. Reverse transcription was performed by incubating at 37 °C for 50 min followed by 75 °C for 5 min. The products of reverse transcription were stored at -20 °C.

PCR amplification of reverse transcription products (cDNA) was carried out separately with eight arbitrary primers (Table 1). PCR was carried out as follows: 94 °C for 30 s; 40 °C for 2 min; 72 °C for 30 s for 40 cycles and finally 72 °C for 5 min. The amplified products were size-fractionated on 6% denaturing polyacrylamide gel electrophoresis using Bio-Rad Mini-Protean Electrophoresis Cell. PCR samples (4 µl)

Table 1. **Arbitrary primers used for mRNA differential display**

Sl. No.	Primer	Sequence
1.	H-AP1	5′-AAGCTTGATTGCC-3′
2.	H-AP2	5′-AAGCTTCGACTGT-3′
3.	H-AP3	5′-AAGCTTTGGTCAG-3′
4.	H-AP4	5′-AAGCTTCTCAACG-3′
5.	H-AP5	5′-AAGCTTAGTAGGC-3′
6.	H-AP6	5′-AAGCTTGCACCAT-3′
7.	H-AP7	5′-AAGCTTAACGAGG-3′
8.	H-AP8	5′-AAGCTTTTACCGC-3′

were mixed with 2µl loading dye (95% formamide, 10mM EDTA, pH 8.0, 0.01% bromophenol blue) and incubated at 80 ^{0}C for 2 min before loading. The samples were electrophoresed at constant volt (150 volt) until xylene dye reached the bottom of the gel.

Silver staining

The gels were silver-stained using the method of Benbouza *et al.* (2006) with the following modifications. After electrophoresis, the gel was fixed in 50 ml cold (10-12 ^{0}C) fixing solution (10% absolute ethanol and 0.5% acetic acid) for 5 min. The fixed gel was soaked for 3 min at room temperature (37 ^{0}C) in 100 ml solution of 0.12% silver nitrate ($AgNO_3$) and 150 µl of 37% formaldehyde (HCHO). The gel was rinsed quickly (10-15 s) once with 200 ml distilled water and then soaked in 100 ml developing solution (1.2% NaOH and 200 µl 37% HCHO) at room temperature until the bands appeared with sufficient intensity (3-5 min). Further staining was stopped by incubating the gel in 100 ml stop solution (10% absolute ethanol and 0.5% acetic acid) for 2 min. The gel was washed several times thoroughly with distilled water and the visible cDNA bands were photographed using Genius Bio Imaging system, Syngene, Cambridge, UK. The entire differential display procedure was repeated three times using fresh latex samples every time for confirming the results.

Data analysis

Differences in mRNA transcripts profiles between the samples were observed for each primer combination. Similarity index (SI) among the samples was calculated using the modified Jaccard Index (Jackson *et al.*, 1989): $SI_{ij} = (B_{ij}/M_{ij})*100$; where SI_{ij} is the similarity index between the samples I and J; B_{ij} is the number of similar bands between I and J and M_{ij} is the total number of bands in I and J. For each sample, the presence or absence of bands was recorded as 1 or 0, respectively. Only the cDNA bands that reproduced under repeated amplifications were considered for estimating the SI.

RESULTS AND DISCUSSION

In the present study, differential display RT-PCR technique was employed for identifying differentially expressed mRNA in the latex collected from the scion of *Hevea* trees. Figure 1 shows the good quality and intactness of total RNA isolated from the latex.

Out of the eight primer combinations used, only three combinations, *i.e.* H-T_{11}G + primer 2, H-T_{11}G + primer 5 and H-T_{11}G + primer 7, gave significantly good results. The total number of cDNA bands resolved on the gel with each primer combination was recorded in every sample. Differences in the number of bands were observed between different primer combinations. The number of cDNA bands ranged from 18 to 23 in tissue-cultured and 37 to 40 in the bud-grafted trees (Table 2). Most of the bands

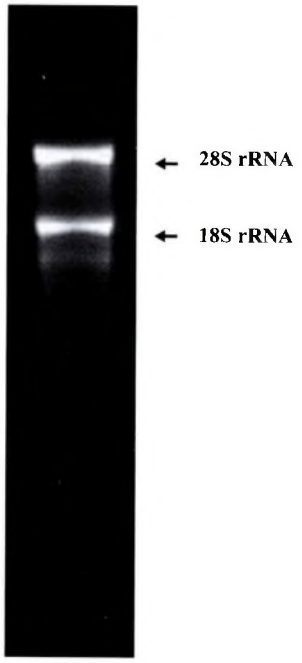

Fig. 1. Total RNA isolated from latex on a denaturing 1.2% agarose formaldehyde gel

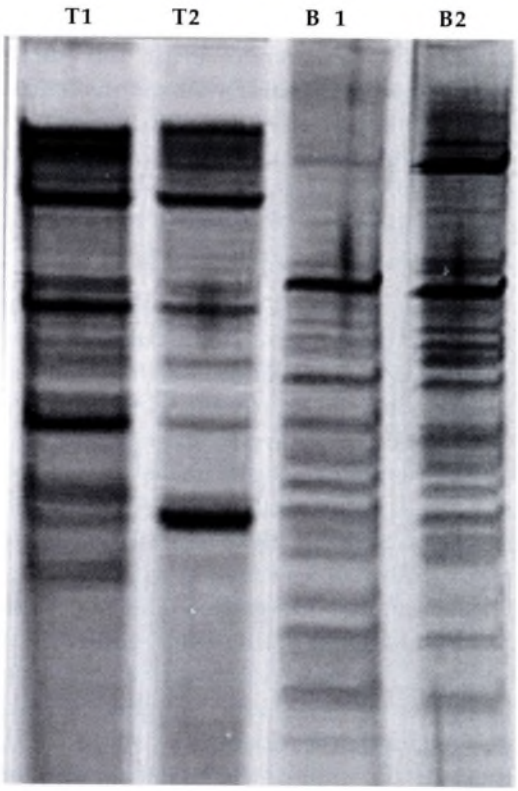

Fig. 2. DDRT-PCR profile of tissue-cultured (T1 and T2) plants and bud-grafted (B1 and B2) plants of *H. brasiliensis* with the primer combination (H-T_{11}G + H-AP2)

were amplified at equal levels in both the latex samples, but a few bands were amplified with more intensity (Fig. 2). In addition, some cDNA bands were found unique either to tissue-cultured or bud-grafted tree latex samples. Percentage of similarity index was calculated for tissue-cultured and bud-grafted trees as described earlier. With the primer combination of H-T_{11}G + primer 2, 14 similar bands were detected among tissue-cultured trees, whereas 19 similar bands were noticed among bud-grafted trees (Fig. 2). A total of 14 similar bands were observed among tissue-cultured trees when the combination

Table 2. **Similarity index (%) in tissue-cultured and bud-grafted trees**

Primer	Tissue-cultured			Bud-grafted		
	Total bands	Similar bands	Similarity index (%)	Total bands	Similar bands	Similarity index (%)
H-AP2	19	14	73.68	38	19	50.00
H-AP5	18	14	77.77	37	20	54.05
H-AP7	23	20	86.95	40	27	67.50
Mean	20	16	79.50	38	22	57.00

of H-T_{11}G -anchored primer and arbitrary primer 5 were used. With the same combination, 20 similar cDNAs were identified within bud-grafted trees. With the primer combination of H-T_{11}G + primer 7, a total of 20 similar cDNAs were detected among tissue-cultured, whereas 27 similar mRNAs were noticed among bud-grafted trees.

On an average, 16 similar cDNA bands were found among the tissue-cultured trees whereas 22 similar bands were found in the bud-grafted trees for the three primer combinations. Tissue-cultured trees showed a greater similarity index than the bud-grafted trees in all primer combinations (Table 2). The average similarity index was 80% among the tissue- cultured tree samples, whereas it was 57% among the bud-grafted (Table 2). Less SI (more dissimilarity) in the bud-grafted trees suggests the likely influence of rootstock heterogeneity on scion. A higher SI (less dissimilarity) in the tissue trees culture a trees indicates the uniformity in gene expression in the absence of rootstocks. More number of cDNA bands observed in bud-grafted than in tissue-cultured trees (Table 2) may be due to the influence of genetically different rootstocks. These observations clearly indicate that rootstocks are responsible for inducing variations in the gene expression pattern of the scion.

In *H. brasiliensis*, bud-grafting is practised as a propagation technique for the past several decades to obtain true-to-type plants. Though propagating the same scion, the tree-to-tree phenotypic variations could not be avoided completely. These tree-to-tree variations are often reflected at morphological (growth, yield, root system, *etc.*), physiological (photosynthesis, effects of biotic and abiotic stresses, mineral nutrition, *etc.*) and biochemical levels (Shobhana *et al.*, 2007).

In the present study, more variations at transcript/gene expression level was found among mature bud-grafted trees than the mature tissue-cultured trees. These results indicate the likely influence of rootstocks on gene expression in the scion. Several studies have suggested that specific traits of the scions can be dramatically altered as a result of grafting to suitable stocks (Beveridge *et al.*, 1996; Napolo, 1996; Salm *et al.*, 1998; Pantalone *et al.*, 1999). Recently, Zhang *et al.* (2008) used eggplant as scion and tomato as rootstock to study the effects of rootstocks on scions from a molecular perspective. Variations were noticed when eggplant scions were grafted onto tomato rootstocks when compared to the un-grafted and self-grafted eggplant controls.

Jenson *et al.* (2003) demonstrated rootstock influence on gene expression patterns in apple tree using cDNA-AFLP technique. In this study, scions grafted to a particular rootstock (M9T337) showed elevated expression of several genes related to photosynthesis, transcription/translation, and cell division, while scions grafted to another rootstock (M7EMLA) showed increased expression of some stress-related genes. Recently Jenson *et al.* (2010) studied the effects of seven different rootstocks on the scion gene expression patterns at the whole genome level using a DNA microarray based on the available apple expressed sequence tag (EST) and mRNA collection and demonstrated that rootstocks modulated various traits in the clonal apple scions.

There were dissimilarities in the gene expression pattern both in tissue-cultured and bud-grafted trees of rubber in this study. However, the differences were significantly more among bud-grafted than the tissue-

cultured trees. The minor dissimilarity observed in the gene expression pattern among the tissue-cultured trees could be due to the variations in the explants used for tissue culture that were originated from different bud-grafted trees. In conclusion, this study is the first report on rootstock influenced gene expression in the scion in *H. brasiliensis*. Significant transcript-level changes were noticed in bud-grafted compared to tissue-cultured plants, indicating changes in the mRNA pattern in the scion of bud-grafted *H. brasiliensis* influenced by rootstocks.

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