Genetic polymorphism and expression of the rubber elongation factor gene (*ref*) in *Hevea brasiliensis* cultivars

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Abstract

Rubber Elongation Factor (*REF*) protein is one of the most abundant proteins found in *Hevea latex*. Investigation on the organization and expression of the gene encoding *REF* protein in commercially planted *Hevea brasiliensis* cultivars is important to shed light on its possible role in rubber biosynthesis, the studies of *Hevea latex* allergy and in molecular breeding of new rubber clones. RFLP analysis of the *Hevea brasiliensis* cultivars used in this study, showed the occurrence of the polymorphic forms of the *ref* gene and its possibility to be used as an important genetic marker in *Hevea* breeding and selection. The RRIC 100 series clones produced more bands indicating the possibility of occurrence of more *ref* gene copies. The RFLP analysis of the progeny derived by crossing RRIC 110 and IAN 45/710 proved the nuclear inheritance pattern of the *ref* gene with two alleles at *ref* loci E-1 and E-3.

The gene expression studies showed the differential expression of the *ref* gene between leaf and laticiferous tissues with high abundance of *ref* gene transcripts in lattices of *Hevea brasiliensis*. The *ref* gene expression was approximately 100 fold higher in latex than in leaves. The intensities of the hybridisation signals obtained from latex RNA of different clones showed variation implying the existence of the genotypic differences for *ref* gene expression.

Key words: expression of *ref* gene, *Hevea latex*, RFLPs, RNA dot-blot, Rubber Elongation Factor gene

Introduction

Rubber Elongation Factor gene (*ref*) codes for a major latex protein of *Hevea brasiliensis*. This protein was named the Rubber Elongation Factor protein (REF) by Dennis & Light (1989) and to this

protein they have attributed the ability to switch in the stereospecificity of prenyl transferases to add multiple *cis*-isoprene units to the growing rubber molecules. Cornish (1993) refuted the function attributed to REF protein by

Dennis and Light and gave experimental evidence to show the existence of separate *cis*- and *trans*- polymerizing prenyl transferases involved in rubber biosynthesis. However, being a predominant protein found in the half unit lipid membrane of the rubber particles the REF protein is obviously taking part in the biosynthesis of natural rubber but its exact function is still unclear (Berthelot *et al.*, 2012). High abundance of this protein in *Hevea* rubber on the other hand has created a serious concern at present as it has been identified as a major allergen found in *Hevea* latex.

The nucleotide sequences of the cDNA (Attanayaka *et al.*, 1991, Goyvaerts *et al.*, 1991) and the genomic clones (Priya *et al.*, 2006) of the *ref* gene have been determined. *Hevea* genome sequencing has revealed the occurrence of multiple forms of the *ref* gene (Rahman *et al.*, 2013) which suggests the possibility of occurrence of *ref* gene polymorphism between cultivars of *H. brasiliensis*. Investigations on *ref* gene polymorphism and its expression in *Hevea* cultivars are useful not only in rubber breeding and selection but also in the studies on *Hevea* latex allergy and rubber biosynthesis. This paper presents the results of an investigation on the *ref* gene organization and its expression in some selected cultivars of *H. brasiliensis*.

Materials and Methods

Plant material

Leaf material from the *H. brasiliensis* cultivars, RRIC 100, RRIC 101, RRIC

102, RRIC 103, RRIC 110, LCB 1320, PB 86, RRIM 600, GT 1, Tjir 1 and progeny individuals of the cross between RRIC 110 x LCB 1320 were obtained from the Rubber Research Institute of Sri Lanka (RRISL). Leaf and latex materials for RNA extractions were collected from plants of the same age grown under similar management conditions in the budwood nurseries of RRISL.

DNA extraction

Plant DNA was isolated as described by Dellaporta *et al.*, (1983).

RNA extraction

Leaf RNA extraction

Approximately 200 mg of leaf tissue ground with liquid nitrogen were transferred to a 2 ml microfuge tube and homogenized to form a fine paste with 750 µl of RNA extraction buffer (400 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% SDS, 10 mM DTT, 4 units/ml heparin, 10 mM ATT). It was extracted with 500 µl of phenol by centrifugation at 12 000 rpm for 5 m. The aqueous phase was re-extracted with 500 µl of phenol/chloroform. The salt concentration of the supernatant was adjusted to 2 M by addition of 12 M LiCl and RNA was precipitated overnight at 4 °C. The recovered pellet was resuspended in 600 µl of DEPC treated water and RNA was re-precipitated overnight with 2 M LiCl. The pellet recovered after centrifugation was washed twice with 70% ethanol, vacuum dried and re-suspended in 25 µl of DEPC treated sterile water.

Latex RNA extraction

Three ml of latex was collected to a Greiner tube placed on ice. Latex was immediately aliquoted (750 µl) to eppendorf tubes containing 1000 µl of RNA extraction buffer and thoroughly mixed. The serum phase was separated from rubber particles by centrifugation for 10 m at 12 000 rpm. The aqueous phase (C-serum) was then collected using a 3 ml syringe by piercing the wall of the microfuge and extracted with equal volume of phenol followed by a phenol-chloroform extraction step. The salt concentration of the supernatant was adjusted to 2 M by using 12 M LiCl and RNA was precipitated at 4 °C overnight. The pellet obtained was re-suspended in 100 µl of DEPC treated water. The RNA obtained from the 3 ml latex was pooled into a 1.5 ml eppendorf tube and used for re-precipitation with LiCl at 4 °C overnight. The RNA recovered after centrifugation was washed with 70% ethanol, vacuum dried and dissolved in 20 µl of DEPC treated water. RNA was then stored at – 70 °C.

RFLP

Genomic DNA was digested using the *EcoR* I restriction enzyme. Genomic DNA (12 µg) from each clones in 5 µl volume was digested with 20 units of enzyme (2 µl) with 10 x reaction buffer in a 40 µl reaction volume at 37 °C for three hours. Enzyme was deactivated by heating to 65 °C for 10 min.

Following electrophoresis of digested DNA in agarose gels, Southern hybridization was performed using

radioactively labeled *ref* cDNA probe with ³²P dCTP. After hybridization filters were washed at high stringency 2x SSC, 5% SDS at 65 °C for 30 min with gentle shaking and autoradiography was performed at -80 °C overnight.

RNA dot blots

Total RNA samples from leaf tissues and latex were prepared from each rubber cultivar (RRIC 100, RRIC 102, RRIC 121, RRIC 130 and RRIC 133) to give 5, 25, 50 and 100 ng in a 25 µl volume. They were blotted onto a hybond-N membrane (11 cm x 8 cm) using dot blot manifold (Biorad) as recommended by the manufacturer. Separate dot blot experiment with higher concentration of RNA was also performed. In this experiment the leaf RNA concentrations used are 100, 250, 500, 1000 and 5000 ng and the latex RNA concentrations used are 25, 50, 100, 200, and 400 ng. In both blots 25 ng of salmon sperm DNA and 100 ng of *ref* cDNA was used as negative and positive control respectively. RNA dot blot membrane was hybridized overnight with ³²P dCTP labelled *ref* cDNA probe. The hybridized filters were washed in 50 ml of 20 x SSC, 5 ml of 10% SDS, 445 ml distilled water at 65 °C for 30 minutes. More stringent washings were done as required.

Results and Discussion

RFLP analysis of Hevea cultivars

Figures 1 and 2 show the results of the RFLP analysis of *Hevea* genomic DNA of different rubber cultivars using the

ref cDNA probe. *ref* gene probe strongly hybridized with the genomic DNA of all the cultivars indicating the presence of the *ref* gene in the *H. brasiliensis* genome. The RFLP banding pattern between cultivars, are clearly polymorphic showing the presence of variation of the *ref* gene organisation in the *Hevea* genome. The number of observed bands in different genotypes ranges from 2-6 and the observed *ref* gene banding pattern is sufficiently polymorphic to group the rubber cultivars into different classes. The high

yielding cultivars, *i.e.* RRIC 100 (lane 1 in Fig. 1), RRIC 102 (lane 8 in Fig. 1 and lane 7 in Fig. 2), RRIC 103 (lane 2 in Fig. 1 and lane 11 in Fig. 2) had produced more bands with high intensity suggesting that they are distinct from the rest of the cultivars with respect to the *ref* gene copy number and sequence. The draft genome sequence of the *H. brasiliensis* assembled by Rahman *et al.* (2013) too has shown that the rubber elongation factor (REF) is represented by 12 genes.

Fig. 1. Southern analysis of genomic DNA of *Hevea brasiliensis* cultivars digested with *EcoR* I. Lanes 1-5; RRIC 100, RRIC 103, PB 86, RRIM 600, Tjir 1. Lanes 7-9; RRIC 101, RRIC 102, GT1

Fig. 2. Southern analysis of genomic DNA of *Hevea brasiliensis* cultivars digested with *EcoR*. I. Lanes 1-3; GT 1, Tjir 1, RIM 600. Lanes 7-11; RRIC 102, RRIC 101, RRIC 7, PB 86, RRIC 103

RFLP analysis of RRIC 110 x IAN 45/710 progeny

The segregation pattern of the *ref* gene was observed by RFLP analysis of a family of six individuals derived from a cross between RRIC 110 and IAN 45/710. Figure 3a shows the banding pattern of the progeny individuals (lanes 4-9) along with the parental genotypes

(lanes 3 and 10). The parental genotypes of the RRIC 110, LCB 1320 and RRIC 7 are also included in lane 1 and 2 respectively. The diagrammatic representation of the segregation pattern of the *ref* loci in the parental and the progeny individuals (lanes 3- 10 of Fig. 3a) is given in Figure 3b.

Fig. 3a. Segregation of the *ref* gene in a family of six individuals (lanes 4-9) derived by crossing RRIC 110 (lane 3) and IAN 45/710 (lane 10). Genomic DNA was digested with *EcoR* restriction enzyme. Lanes 1 and 2 represent the two parents of the RRIC 110, LCB 1320 and RRIC 7

Fig. 3b. Diagrammatic representation of segregation of the *ref* gene in a family of six individuals (lanes 1 - 6) derived by crossing RRIC 110 and IAN 45/710

The RFLP analysis of parental and progeny genotypes of the cross RRIC 110 x IAN 45/710 shows three main *ref* loci. They are depicted as E-1, E-2 and E-3 in Figures 3a and 3b. At E-1 locus *ref* gene has resulted polymorphic banding pattern with two alleles (a and b). The parental genotype IAN 45/710 (lane 10) has both 'a' and 'b' alleles and the male parent RRIC 110 is homozygous for the allele 'a'. Hence it is possible to consider the progeny as a segregating generation of a heterozygous x homozygous cross for the E-1 locus which should produce heterozygous and homozygous individuals in 1:1 ratio in the progeny. The RFLP genotypes of progeny individuals (lanes 4-9) represents the parental RFLP genotypes in 1 : 1 ratio suggesting that the parents differ at the E-1 *ref* locus with two alleles. The observed Mendelian inheritance pattern

therefore confirms the nuclear inheritance of the *ref* gene. The bands 'c' represents the alleles of a homozygous locus E-2. At the E-3 *ref* locus the band, 'd' represents an allele of a heterozygous locus with a null allele present in the homozygous condition in the IAN 45/710 which has not produced a RFLP phenotype.

***ref* gene expression in *Hevea* cultivars**

The mRNA levels of the *ref* gene in both leaf tissue and latex of some selected clones, were observed using a series of dot blot hybridisation experiments to study the *ref* gene expression. Initial dot blot hybridisation experiments involving low quantities of total RNA (25 ng – 100 ng) showed that *ref* gene transcripts in leaf tissues were not detectable even at the 100 ng level of total RNA (Fig. 4, LF). However, with respect to the latex total RNA,

except for the 5 ng level (Fig. 4, lane A) the hybridisation signals were obtained from each of the five clones with increasing intensities as the amount of the RNA increases. These results show the differential expression of the *ref* gene between leaf and laticiferous tissues with high abundance of *ref* gene transcripts in lattices of *H. brasiliensis* cultivars. The intensities of the hybridisation signals obtained for latex RNA of different cultivars showed variation. This shows the existence of

genotypic differences among the *H. brasiliensis* cultivars with respect to the *ref* gene expression in laticiferous tissues. The highest intensity of the hybridisation signals for latex RNA was observed in RRIC 121 (Fig. 4) which is recognized as a very high yielding clone with high latex dry rubber content. However accurate controlling of the plant environmental conditions was not possible in this study as the plant materials were obtained from a field planted budwood nursery.

Fig. 4. Autoradiogramme of dot blot containing dilutions of leaf and latex RNA of different *Hevea* clones probed with ^{32}P labeled *ref* gene. Lanes A-D: 5, 25, 50, 100 ng, LF; leaf RNA dilutions, LX; latex RNA dilutions, 1- 25 ng of salmon sperm DNA, 2- positive control (denatured *ref* cDNA)

The results of the dot blot experiment with increased amount of leaf and latex total RNA is given in Figure 5. As expected, signals from latex RNA samples from every cultivar were obtained. Leaf RNA samples have produced weak signals at 1000 ng and 5000 ng levels (Fig. 5, RRIC 133-LF and RRIC 130-LF). Moreover the comparison of the hybridisation intensities of the signals obtained from

leaf and latex RNA dilutions (Fig. 5, RRIC 130 and RRIC 133 LF Lane E and LX Lane A) suggests that the *ref* gene expression is approximately 100 fold higher in laticifers than in leaves. In a previous study, Kush *et al.* (1990) too has shown that laticifer RNA is 20- to 100- fold enriched in transcripts encoding enzymes involve in rubber biosynthesis.

Fig. 5. Autoradiogramme of dot blot containing dilutions of leaf and latex RNA of different *Hevea* clones probed with ^{32}P labeled *ref* gene. Lane LF; leaf RNA dilutions, Lanes LX; Latex RNA dilutions. A/LF; 100 ng, B/LF 250 ng, C/LF; 500 ng; D/LF1000 ng, E/LF 5000 ng, A/LX 25 ng, B/LX 50 ng, C/LX 100ng, E/LX 200 ng, 1- 25 ng of salmon sperm DNA 2- positive control (denatured *ref* cDNA)

Conclusions

Southern analysis of *Hevea* cultivars showed the polymorphic nature of the *ref* locus in the *Hevea* genome indicating the presence of allelic variation. The segregation analysis of the *ref* gene in parental genotypes and progeny individuals of the cross between RRIC 110 x IAN 45/710 proved the nuclear inheritance pattern of the *ref* gene and the segregation of two *ref* alleles. The *ref* gene probe could be successfully used to group the presently recommended *Hevea* cultivars with possible application as a molecular marker having meaningful genetic interpretation.

The *ref* gene showed a tissue specific gene expression having at least 100 fold more abundance of *ref* transcripts in laticifers than in leaves. Cultivar differences were observed for *ref* gene expression in *Hevea* latex.

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