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Molecular cloning of rubber elongation factor protein cDNA from *Hevea brasiliensis* Muell. Arg. and its heterologous expression in *Escherichia coli* and *Nicotiana tabacum**

Hevea brasiliensis, commonly known as rubber tree, is presently the world's sole commercial source of natural rubber (*cis*-1,4-polyisoprene). Natural rubber, an important biomacromolecule for many industrial purposes in the world, is produced by over 2000 plant species distributed among four of the six superorders of Dicotyledonae[1]. Despite the availability of petroleum-based synthetics, natural rubber is highly valued because no synthetic substitute has comparable elasticity, resilience and resistance to high temperature[2]. Rubber represents 30–50% by weight of the latex exuded by mature *Hevea* trees in regular tappings and constitutes more than 90% of the total latex solid[3].

Natural rubber from *H. brasiliensis* consists of long chains of *cis*-polyisoprene which are synthesized through the mevalonate pathway from acetyl CoA derived from glycolysis[3]. Polymerization of isopentenyl pyrophosphate by prenyltransferase assisted by the Rubber Elongation Factor (REF) gives rise to long chains of *cis*-polyisoprene which form aggregates called rubber particles surrounded by a lipoproteic membrane. Rubber biosynthesis takes place on the surface of rubber particles suspended in the latex produced in special latex vessels called laticifers[4]. Kush *et al.*[5] have shown the differential expression of several rubber biosynthesis-related genes in the latex of *Hevea*. The REF, an enzyme involved in rubber biosynthesis, is highly expressed in laticifers[6]. The primary protein structure of REF has been elucidated[7]. The amount of REF in the whole latex is proportional to the rubber content. As such, if the amount of REF protein is correlated with rubber yield, there is a possibility that this relationship could be used to select clones that are able to sustain high rubber biosynthetic efficiency. Therefore, overexpression of *REF* gene may enhance latex yield in transgenic plants by genetic engineering. To achieve this goal, it is essential to clone and characterize REF cDNA from high latex-yielding Indian *Hevea* clone. More recently, cloning and characterization of a genomic DNA coding for *REF* gene and its promoter sequence has been reported[8].

Here we report cloning and characterization of *REF* gene from latex of Indian *Hevea* clone RRII105; the cDNA was inserted into an expression vector for heterologous expression in *Escherichia coli*. In addition, this gene was also overexpressed in tobacco plants via *Agrobacterium*-mediated genetic transformation.

Total RNA was isolated from mature rubber trees of RRII 105 clone following the method of Venkatachalam *et al.*[9]. REF cDNA was synthesized by RT–PCR according to the manufacturer's instructions (Promega). PCR was carried out with *REF* gene-specific primers (5′-CGA TTA TGG CTG AAG ACG AAG ACA ACC-3′ and 5′-GGC CAA TAA TTC AAT TGG CCC TTT ATT C-3′) using the following parameters: 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 55°C for 1.30 min, 72°C for 2 min and a final extension at 72°C for 10 min. The amplified cDNA was cloned into pUC19 vector and sequenced.

For heterologous protein expression, the REF cDNA was cloned between *Bam*HI and *Eco*RI sites of pGEX-2T vector to generate a recombinant plasmid with *REF* gene designated as pGEX-REF, according to vendor's instructions (Amersham, UK). The recombinant plasmid was transformed into *E. coli* competent cells (DH5α) according to standard protocols[10] and induced with 0.1 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) to express the foreign protein. The bacterial proteins were analysed on 12% SDS–PAGE[11]. The fusion protein was purified, transferred to polyvinylidene fluoride (PVDF) membrane and detected by an enhanced chemilumeniscence system (ECL, Amersham, UK). The membrane was also incubated in buffer (TTBS) containing polyclonal antibodies raised against native REF protein followed by incubation with goat anti-rabbit IgG–ALP conjugate and visualized using BCIP/NBT.

*Hb*REF cDNA was cloned into the binary vector pBIB and the recombinant plasmid was then mobilized into *Agrobacterium tumefaciens* strain, LBA 4404

*The sequence described in this work has been deposited in the NCBI GenBank database under accession number AY120685. The first two authors contributed equally to the work.

by freeze–thaw method. Tobacco leaf discs were infected with bacteria grown in liquid LB medium containing 100 mg/l kanamycin and 25 mg/l rifampicin, co-cultivated for two days in the dark on shoot regeneration medium (MS basal salts[12], 1 mg/l BA and 0.2 mg/l NAA without antibiotics). Subsequently, they were transferred to a regeneration medium containing 100 mg/l kanamycin and 250 mg/l cefotoxime for the selection of putatively transformed shoots which were rooted on MS media containing 1.0 mg/l NAA along with 50 mg/l kanamycin. To confirm the integration of REF and *npt*II genes into the tobacco genome, PCR was performed with DNA isolated from the putative transgenic and nontransgenic tobacco plants (negative control) along with plasmid DNA (pBIB-REF) as positive control. *REF* gene-specific primers and PCR cycles used were the same as described previously. The primer sequences for *npt*II gene were: forward primer 5′-GAG GCT ATT CGG CTA TGA CT-3′ and reverse primer 5′-AAT CTC GTG ATG GCA GGT TG-3′. RT–PCR was carried out with leaf RNA isolated from transgenic plants as well as untransformed (negative control) plants, as described earlier.

A full-length cDNA coding for REF protein from the latex RNA of *H. brasiliensis* was isolated by RT–PCR and cloned into the plasmid vector. The cloned REF cDNA was sequenced and the encoded amino acid sequence of REF cDNA is shown in Figure 1. Sequence analysis revealed that the amplified REF cDNA was 622 bp long with an open reading frame of 414 bp coding for the 138 amino acid protein, with a predicted molecular mass of 14.7 kDa. The deduced REF protein is acidic with a calculated pI of 5.0. In the amplified fragment, an ATG initiation codon was located at 10 bp and a translation termination codon was identified at the 424 bp position. Analysis of the deduced amino acid sequence with SignalP program revealed the absence of a signal peptide and cytoplasmic localization of REF was predicted by pSORT. The REF cDNA isolated in this study showed significant homology with previously reported cDNAs in the database[13]. The deduced amino acid sequence of the present REF cDNA is identical to the primary REF protein sequence reported earlier by Dennis *et al.*[7]. The multiple sequence alignment of *REF* gene with other *Hevea* sequences is presented in Figure 2.

It has been suggested that rubber biosynthesis in *H. brasiliensis* is mediated by the association of a soluble *trans*-prenyltransferase with a rubber elongation factor protein, bound to the rubber particles in the laticifers[6]. Ko *et al.*[14] described that about 29% of the latex transcripts were from REF and SRPP. It is well characterized that the REF protein is tightly associated with large rubber particles, whereas a 24 kDa SRPP protein is attached with small rubber particles in *Hevea*[15]. So far, *REF* gene has been reported only from *H. brasiliensis*. Among 2000 rubber-producing plant species, *H. brasiliensis* is the only one producing commercially viable quantity of high molecular weight rubber. We presume that the presence of *REF* gene may be one of the reasons for producing large quantity of latex in this species. It was proved that the removal of REF protein inhibited rubber biosynthesis *in vitro*[6].

To confirm whether the obtained cDNA actually encodes the REF protein, we constructed an expression vector system using pGEX plasmid. REF fusion protein was detected as a major band, approximately of 40 kDa on SDS–PAGE gel. It is close to the expected size of fusion protein, GST-fusion (26 kDa) plus REF protein (14 kDa; Figure 3 *a*). This protein band did not appear in the protein fraction of the bacteria containing the empty pGEX plasmid (control). As shown in Figure 3 *b*, the REF–GST fusion protein was purified and blotted on PVDF membrane. Immunoblot results showed

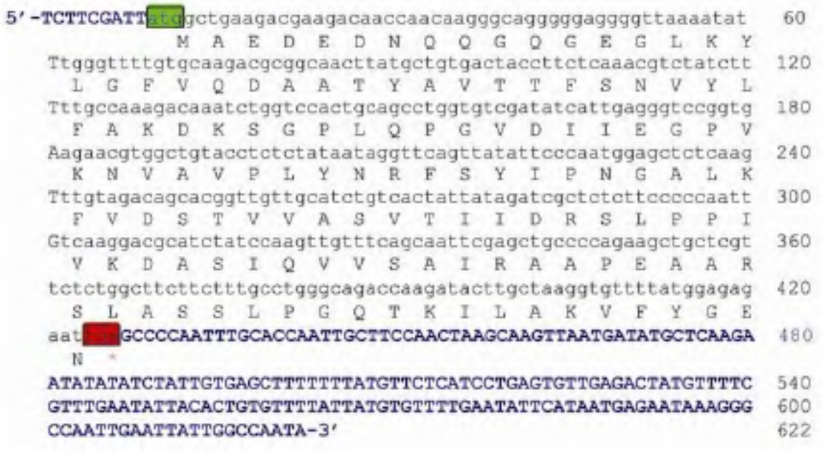

Figure 1. Nucleotide and the deduced amino acid sequence of *Hb*REF cDNA (622 bp). Upper case letters represent 5′ and 3′ UTR regions. Lower case letters represent ORFs and start as well as stop codons have been highlighted. The translated amino acid sequence is shown in single-letter code below the ORF sequences. The termination codon is marked with an asterisk.

Figure 2. Multiple alignment of predicted amino acid sequence of *Hevea brasiliensis* (*Hb*) *HbREF* gene with *Hb*SRPP (accession no. AF051317), *Hb* isoform of REF (accession no. AY430052), *Hb* stress-related REF-like protein 1 (accession no. AY221988), and *Hb* stress-related REF-like protein 2 (AY221989).

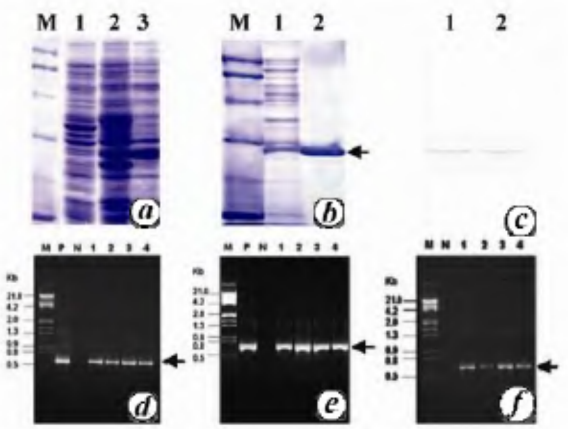

Figure 3. Heterologous expression of *HbREF* gene and molecular confirmation of *REF* gene integration into tobacco genome by PCR. M, Markers. ***a***, SDS–PAGE gel showing profiles of: Lane 1, Protein from control pGEX vector; lane 2, Uninduced REF-fusion protein and lane 3, IPTG-induced REF-fusion protein. ***b***, SDS–PAGE gel showing profiles of: Lane 1, IPTG-induced REF-fusion protein and lane 2, Purified REF recombinant protein (arrow indicates REF fusion protein). ***c***, Immunoblot analysis of recombinant REF protein with: Lane 1, Anti REF antibodies and lane 2, Anti-GST antibodies; ***d***, PCR profile of *REF* gene (arrow indicates 600 bp). ***e***, PCR profile of *npt*II gene (arrow indicates 800 bp). ***f***, Accumulation of REF transcripts by RT–PCR analysis (arrow indicates 600 bp): P, Positive control; N, Negative control; Lanes 1–4, Independent transgenic lines.

that the REF–GST fusion protein binds to the antibodies raised against REF protein (Figure 3 *c*, lane 1) and the GST-antibodies recognized the recombinant REF–GST fusion protein (Figure 3 *c*, lane 2). This result confirms that the 40 kDa protein overexpressed in *E. coli* cells was indeed the GST::REF fusion protein and the isolated cDNA encoded the REF protein of *H. brasiliensis*.

Tobacco leaf discs were transformed with *HbREF* gene under the control of a super promoter (modified version of 35CaMV). Putatively transformed shoots were selected on MS medium containing 100 mg/l kanamycin and they were successfully rooted. *HbREF* transgene integration into tobacco genome was confirmed by PCR (Figure 3 *d* and *e*). Genomic DNA from all the transgenic plants and the plasmid DNA (positive control) amplified 600 bp of *REF* as well as 800 bp of *npt*II gene fragments, while the corresponding DNA bands did not amplify in the nontransgenic plant (negative control). This result suggested that the *HbREF* gene was stably integrated into the host genome. RT–PCR results indicated the differential accumulation of REF transcripts in transgenic tobacco plants (Figure 3 *f*). Although plant regeneration and genetic transformation systems are available for *Hevea* as described previously[16], it is more time-consuming and labour-intensive. Therefore, we used tobacco transformation system for heterologous *REF* gene expression studies.

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