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Research article

# Identification and subcellular localization analysis of two rubber elongation factor isoforms on *Hevea brasiliensis* rubber particles

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ABSTRACT

Rubber elongation factor (REF) is the most abundant protein found on the rubber particles or latex from *Hevea brasiliensis* (the Para rubber tree) and is considered to play important roles in natural rubber (*cis*-polyisoprene) biosynthesis. 16 BAC (benzyldimethyl-*n*-hexadecylammonium chloride)/SDS-PAGE separations and mass spectrometric identification had revealed that two REF isoforms shared similar amino acid sequences and common C-terminal sequences. In this study, the gene sequences encoding these two REF isoforms (one is 23.6 kDa in size with 222 amino acid residues and the other is 27.3 kDa in size with 258 amino acid residues) were obtained. Their proteins were relatively enriched by sequential extraction of the rubber particle proteins and separated by 16 BAC/SDS-PAGE. The localization of these isoforms on the surfaces of rubber particles was further verified by western blotting and immunogold electron microscopy, which demonstrated that these two REF isoforms are mainly located on the surfaces of larger rubber particles and that they bind more tightly to rubber particles than the most abundant REF and SRPP (small rubber particle protein).



## 1. Introduction

Rubber elongation factor (REF, 138 amino acid residues, 14.6 kDa) and small rubber particle protein (SRPP, 204 amino acid residues, 22.4 kDa) are the most important proteins in *Hevea brasiliensis* (the Para rubber trees) latex and have attracted the attention of researchers because of their allergenicity in natural rubber products and their high contents in rubber latex. REF and SRPP were first detected as natural latex allergens in natural rubber products and named Hev b 1 and Hev b 3, respectively (Posch et al., 1997). These two proteins account for more than 75% of the proteins found on rubber particles (Dai et al., 2012), which are laticifer-specific organelles accounting for 30%–45% of the latex volume and more than 90% of the latex dry weight in rubber trees (Jacob et al., 1993).

In rubber trees, REF and SRPP are thought to play positive roles in natural rubber biosynthesis (Light et al., 1989). However, the exact functions of these two proteins are unclear. For example, Light et al. (1989) suggested that REF helps a "rubber transferase" in the rubber latex to switch between *cis*-prenyl transferase and *trans*-prenyl transferase activities, but Cornish (1993) refuted this.

The amino acid sequences for REF and SRPP share 72% similarity (Oh et al., 1999). Many REF and SRPP analogues in rubber trees have been reported. Rahman et al. (2013) found 10 REF genes and 12 SRPP genes in the genome of rubber tree, Tang et al. (2016) recently characterized 8 and 10, and Lau et al. (2016) found 9 and 8, respectively. They constitute the REF superfamily and are usually divided into REF or SRPP subfamilies (Chow et al., 2007; Rahman et al., 2013). However, when more REF/SRPP protein isoforms in *H. brasiliensis* were found through genome sequencing and analyzed phylogenetically, no distinct REF and SRPP subfamilies were found (Tang et al., 2016).

Laibach et al. (2015) found that TbREF, a HbREF analogue, contained two conserved REF domains in *Taraxacum brevicorniculatum*, and that TbREF-silenced plants showed lower TbCPT protein levels and less TbCPT activity in the latex, and therefore lower rubber yielding, but the molecular weight of rubber and colloidal stability of rubber particles was not affected. According to these results,

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Laibach et al. (2015) suggested that TbREF may play a role in rubber particle biogenesis and affect the biosynthesis of natural rubber. SRPP analogues have been found in other plant species, including non-rubber-producing plants, such as *Arabidopsis* (Kim et al., 2016) and *Capsicum annuum* (Kim et al., 2010), and rubber-producing plants, such as guayule (*Parthenium argentatum* Gray) (Kim et al., 2004), Russian dandelion (*Taraxacum kok-saghyz*) (Schmidt et al., 2010), and lettuce (*Lactuca sativa*) (Chakrabarty et al., 2015). Previous investigations demonstrated that SRPP homologues in *Arabidopsis* (Kim et al., 2016) and in *Capsicum annuum* (Kim et al., 2010) were associated with tissue growth and development, and with drought stress responses, and that an SRPP homolog in guayule can enhance the biosynthesis of natural rubber (Kim et al., 2004).

In addition to the stress-related functions associated with REF superfamily proteins, the relationship between the proteins and the integrity of rubber particles has been noticed by several researchers. Dai et al. (2012) suggested the REF superfamily proteins might act as structural proteins in rubber particles in a way similar to that oleosin does in lipid droplets. Hillebrand et al. (2012) found that in *T. brevicorniculatum*, down-regulation of SRPP homologues affected rubber particle integrity and consequently rubber biosynthesis. However, Chakrabarty et al. (2015) found that silencing the lettuce (*Lactuca sativa*) SRPP homologues did not interfere with natural rubber biosynthesis. Biophysical studies on the interactions between REF/SRPP and lipid monolayers were carried out (Berthelot et al., 2014a,b). Interestingly, lipid-droplet associated proteins (Gidda et al., 2013; Horn et al., 2013), which are SRPP homologues, act as structural proteins in avocado mesocarp lipid droplets, whereas oleosins are structural proteins in plant seed lipid droplets.

Immunogold electron microscopy has shown that REF and SRPP locate on the surface of rubber particles (Shamsul Bahri and Hamzah, 1996), but there have been no similar studies to show the localization of other REF/SRPP analogues in *H. brasiliensis*. In our previous study, 16 BAC/SDS-PAGE separations and mass spectrometry revealed two REF isoforms that had not been reported in the literature at that time, and they had common C-terminal sequences (Dai et al., 2012). However, it was uncertain whether the difference between their apparent molecular weights was due to the different amino acid sequences or merely some kind of post-translational modification, such as glycosylation. In this study, the individual gene sequences encoding these two REF isoforms were obtained, the two REF isoforms were enriched by sequential extraction and separated by 16 BAC/SDS-PAGE, and their localizations on the surfaces of rubber particles were verified by western blotting and immunogold electron microscopy.

## 2. Materials and methods

### 2.1. Plant materials

Ten-year-old regularly tapped rubber trees (*H. brasiliensis* Reyan 7-33-97 clones) from an experimental farm operated by the Chinese Academy of Tropical Agricultural Sciences (CATAS) in Hainan, China, were used for the experiments. Fresh latex was collected and preserved as previously described (Dai et al., 2016).

### 2.2. Extraction, electrophoretic separation, and mass spectrometric identification of rubber particle proteins

The fresh latex was centrifuged at 11,000 rpm (9880×*g*) and 4 °C for 1 h, and the creamy rubber particle layer was removed and washed three times with a solution of 10 mM Tris and 250 mM sucrose, pH 7.0. The rubber particles were collected by centrifugation at 12,000 rpm (11,800×*g*) and 4 °C for 20 min and extracted for 30 min with a solution based on a mixture used by Wittig et al. (2006) (1 g solution for 1 g rubber particle cream) that contained 1% Triton X-100, 50 mM sodium chloride, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and a protease inhibitor cocktail (1 tablet per 10 mL, Roche Applied Science, Mannheim, Germany), pH 7.0, in a clean tube that was on ice on a horizontal shaker. The extraction mixture was centrifuged at 18,000 rpm (26,400×*g*) and 4 °C for 1 h and the aqueous phase was transferred to a clean tube for further clarification by centrifugation at 35,000 rpm (105,000×*g*) and 4 °C for 35 min. The creamy phase was removed and further extracted for 30 min with a solution (1 g solution for 1 g rubber particle cream) that contained 2% SDS, 50 mM sodium chloride, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and a protease inhibitor cocktail (1 tablet per 10 mL, Roche Applied Science), pH 7.0, in a clean tube that was on ice on a horizontal shaker. The mixture from the second round extraction was centrifuged and further cleaned up in the same manner as the first round extraction. Proteins in the extracts were precipitated with a 10% TCA (trifluoroacetate)/90% acetone/0.2% DTT solution overnight and collected by centrifugation at 13,000 rpm (13,800×*g*) at room temperature for 10 min, washed with acetone three times, and dissolved in a solution of 7 M urea and 2 M thiourea. Protein concentrations in the samples were determined using a Bradford kit (Bio-Rad, Hercules, CA, USA). Protein samples (700 μg/sample) were extracted with Triton X-100 or SDS containing buffer and were separated using a 16-BAC/SDS PAGE approach according to Nothwang and Schindler (2009). Protein spots were cut out and proteins were in-gel digested with trypsin. The resulting tryptic peptides were subjected to MALDI TOF-TOF mass spectrometric analysis and a database search. Peak lists extracted from mass spectrometric raw data were first used to search against the NCBI non-redundant protein database. Then peak lists for the protein spots that were not successfully matched with proteins in the NCBI nr (National Center for Biotechnology Information non-redundant protein) database were used to search against a peptide database deduced (using the EMBOSS getorf tool, http://emboss.bioinformatics.nl/cgi-bin/emboss/getorf, to translate nucleotide sequences between start and end codons into amino acid sequences) from transcriptome shotgun assembly (TSA) sequences submitted by Rahman et al. (2013). The matched peptide sequences were then used to carry out a BLASTP search against the NCBI nr protein database. Functional descriptions were manually assigned to the query peptide sequences according to the superfamilies the protein hits belonged to and the descriptions of hits with relatively high scores.

For western blot analysis, latex fractions were collected as follows: (1), about 40 mL fresh latex was centrifuged at 4950 rpm (2000×*g*) and 4 °C for 45 min to scoop out the creamy layer as the large rubber particle sample, the remaining part was further concentrated at 26,400×*g* and 4 °C for 1 h to scoop out the creamy layer as the small rubber particle sample; (2), another 40 mL fresh latex was concentrated at 26,400×g and 4 °C for 1 h to scoop out the creamy layer as the total rubber particle sample and the bottom fraction sample (which includes mainly lutoids, the heaviest organelles in rubber latex); (3) the remaining aqueous part of process (2) was then centrifuged at 26,400×*g* and 4 °C for one additional hour to scoop out the creamy layer as the very small rubber particle sample; and (4) the remaining aqueous part of process (3) was further cleaned up by centrifugation at 35,000 rpm (105,000×*g*) and 4 °C for 35 min to obtain the C-serum sample. The total, large, small, and very small rubber particle samples were extracted with the 2% SDS buffer mentioned above. The proteins in the extraction mixtures (after the rubber phases were removed by centrifugation at 16,000 rpm/26,400×*g* and further centrifugation at 35,000 rpm/