

Isolation, Characterization, and Functional Analysis of a Novel cDNA Clone Encoding a Small Rubber Particle Protein from *Hevea brasiliensis**

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Biochemical evidence reported so far suggests that rubber synthesis takes place on the surface of rubber particles suspended in the latex of *Hevea brasiliensis*. We have isolated and characterized a cDNA clone that encodes a protein tightly bound on a small rubber particle. We named this protein small rubber particle protein (SRPP). Prior to this study, this protein was known as a latex allergen, and only its partial amino acid sequence was reported. Sequence analysis revealed that this protein is highly homologous to the rubber elongation factor and the *Phaseolus vulgaris* stress-related protein. Southern and Northern analyses indicate that the protein is encoded by a single gene and highly expressed in latex. An allergenicity test using the recombinant protein confirmed that the cloned cDNA encodes the known 24-kDa latex allergen. Neither ethylene stimulation nor wounding changed the transcript level of the SRPP gene in *H. brasiliensis*. An *in vitro* rubber assay showed that the protein plays a positive role in rubber biosynthesis. Therefore, it is likely that SRPP is a part of the rubber biosynthesis machinery, if not the rubber polymerase, along with the rubber elongation factor.

Rubber (*cis*-1,4-polyisoprene), an isoprenoid polymer with no known physiological function to the plant, is produced in about 2000 plant species with varying degrees of quality and quantity (1). Rubber is the raw material of choice for heavy duty tires and other industrial uses requiring elasticity, flexibility, and resilience. *Hevea brasiliensis* has been the only commercial source of natural rubber mainly because of its abundance in the tree, its quality, and the ease of harvesting. The diminishing acreage of rubber plantations and life-threatening latex allergy to *Hevea* rubber, coupled with an increasing demand, have

prompted research interests in the study of rubber biosynthesis and the development of alternative rubber sources.

In *H. brasiliensis*, rubber synthesis takes place on the surface of rubber particles suspended in the latex (the cytoplasm of laticifers). The laticifers are specialized vessels that are located adjacent to the phloem of the rubber tree. When severed during tapping, the high turgor pressure inside the laticifers expels latex containing 30–50% (w/w) *cis*-1,4-polyisoprene. The latex can be fractionated by centrifugation into three phases: the top fraction containing mostly rubber particles, the metabolically active middle fraction (called C-serum), and the bottom fraction of mainly vacuole-like organelles called lutoids. More than 240 expressed sequence tags (ESTs)¹ have been identified from the latex of *H. brasiliensis*² Kush *et al.* (2) have shown differential expression of several rubber biosynthesis-related genes in latex. The rubber elongation factor (REF), an enzyme involved in rubber biosynthesis (3), is highly expressed in laticifers (4). Laticiferous cells actively translate the transcribed genes into proteins. About 200 distinct polypeptides are present in the latex of *H. brasiliensis* (5). Arokiaraj *et al.* (6) observed that the *GUS* reporter gene introduced by *Agrobacterium*-mediated transformation was expressed in the latex of the transgenic *Hevea* plant. Genes expressed in the latex of *Hevea* can be divided into three groups based on the proteins they encode: 1) defense-related proteins such as hevein (7), chitinase (8), β -1,3-glucanase (9), and HEVER (10); 2) rubber biosynthesis-related proteins such as REF (4), hydroxymethylglutaryl-CoA reductase (11), and farnesyl diphosphate synthase (12); and 3) latex allergens (proteins) such as Hev b 3 (13), Hev b 4, Hev b 5 (14, 15), and Hev b 7 (16). Biological functions of the allergenic proteins are largely unknown.

Rubber particles are essential components in rubber synthesis *in vitro* (17). Most of the experiments showing *in vitro* rubber synthesis with labeled isopentenyl pyrophosphate (IPP) have always required rubber particles. Dennis and Light (3) showed that REF, a rubber particle-associated protein (RPP), was necessary for rubber elongation *in vitro*. Yeang *et al.* (13) identified a 24-kDa RPP (Hev b 3) (later revised to 22 kDa (18))

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ223388, AF051317, and 1326163.

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¹ The abbreviations used are: EST, expressed sequence tag; GST, glutathione *S*-transferase; IPP, isopentenyl diphosphate; PAGE, polyacrylamide gel electrophoresis; REF, rubber elongation factor; RPP, rubber particle protein; RT-PCR, reverse transcriptase-polymerase chain reaction; SRPP, small rubber particle protein; PvSRP, *Phaseolus vulgaris* stress-related protein; WRP, washed rubber particle; bp, base pair(s); IPTG, isopropyl-1-thio- β -D-galactopyranoside; FPP, farnesyl pyrophosphate; PBS, phosphate-buffered saline; UTR, untranslated region; kb, kilobase.

² K.-H. Han, D. H. Shin, J. Yang, I. J. Kim, S. K. Oh, and K.-S. Chow, submitted for publication.

tightly bound on small rubber particles as latex allergen. Pan *et al.* (19) identified the most abundant RPP (53 kDa) in guayule, another rubber-producing plant, as a cytochrome P450 known as allene oxide synthase. A United States patent (No. 5,633,433) claims that this protein is necessary for rubber biosynthesis. In light of these observations, it is plausible that the surface of the rubber particle is the appropriate location for a rubber transferase, which polymerizes a hydrophobic polymer into the particle interior while obtaining hydrophilic substrates, like IPP, from the cytosol. As major RPPs, both REF and Hev b 3 could well be the integral components of the rubber biosynthesis machinery.

We are interested in understanding the molecular and biological aspects of rubber biosynthesis. In a separate study to investigate the gene expression profile in the rubber-producing tissue, we generated a number of ESTs from latex. One of the genes most abundantly expressed in latex was found to be a cDNA clone encoding a major rubber particle protein, Hev b 3. This protein causes allergenic response in spina bifida patients, and only its partial amino acid sequence was determined (13, 18). The gene encoding this protein has not been cloned. In this study, we isolated and characterized a full-length cDNA encoding the allergen Hev b 3. We named the gene small rubber particle protein (SRPP) for a common name. The amino acid sequence of SRPP is highly homologous to that of REF, suggesting its potential involvement in rubber biosynthesis. However, there has been no experimental evidence for the function of SRPP. SRPP has also high sequence homology to *Phaseolus vulgaris* stress-related protein (PvSRP) that was reported in the data base entry to be induced by heavy metals, wounding, and virus infection. The present study shows that SRPP is not induced by wounding or ethylene but plays a positive role in rubber biosynthesis.

MATERIALS AND METHODS

Plant Material and RNA Isolation—Latex and leaf samples were obtained from mature rubber plants (*H. brasiliensis* clone RRIM 600) growing at the Rubber Research Institute of Malaysia (RRIM), Selangor, Malaysia. Latex collection and RNA extraction from the latex were performed as described in Kush *et al.* (2) using the Qiagen Rneasy Plant Minikit (Qiagen Inc., Chatsworth, CA). Poly(A)⁺ RNA was isolated using Oligotex-dTTM mRNA kit (Qiagen Inc.).

Wounding and Ethephon Treatment—For the effect of wounding, five *H. brasiliensis* (clone RRIM 600) trees were wounded with six nails above and along the slope of the tapping cut while another five control trees were not punctured. Latex and leaf samples were collected from each tree about 16 h after puncturing. For the effect of ethylene stimulation, five *H. brasiliensis* (clone RRIM 600) trees were stimulated with an ethylene-releasing agent (ethephon, 2.5%, v/v) while five control trees were not. Latex and leaf samples were collected from each tree 2 days after stimulation.

RT-PCR Amplification of SRPP—First strand cDNA synthesis was performed by reverse transcribing 0.5 µg of total latex RNA using a modified oligo-dT primer, T₂₅NN (5'-GGAGAAGGA(T)₂₅(A/G/C)N-3'). For PCR, 10 µl of the first strand cDNA synthesis was used to amplify part of the coding sequence for SRPP. A degenerate primer, BW1, was designed according to sequence similarities between Hev b 1 (commonly known as REF) and previously published peptide fragments of Hev b 3 (13). The peptide sequence KYLDFV was chosen (see Fig. 1, *underlined*). The primers used to amplify a fragment of the SRPP (Hev b 3) cDNA were BW1 (5'-AAGCTTAA(A/G)TA(C/T)TIGA(C/T)TT(C/T)GT-3', where I stands for inosine) and T₂₅NN. The PCR product was excised from an agarose gel and purified with the QIAEX II agarose gel extraction kit (Qiagen, Hilden, Germany). Purified PCR products were ligated into the pCRTM 2.1 vector (TA cloning kit, Invitrogen, NV Leek, Netherlands). Plasmids of transformed *Escherichia coli* strain XL1-Blue were isolated and sequenced by the dideoxyribonucleotide chain termination method using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech). To complete the missing 5'-portion of the cDNA, the 5'-AmpliFINDERTM RACE kit (CLONTECH, Palo Alto, CA) was used according to the vendor's instructions. cDNA synthesis was performed with the T₂₅NN primer and

PCR after anchor ligation with the kit's AmpliFINDER anchor primer and the gene-specific internal primer BW4 (5'-AGGTAATGACCG-CATATTGCTCAGC-3'). The complete cDNA sequence of SRPP cloned by this method is available from the GenBankTM/EBI data base under accession number AJ223388.

Construction and Screening of Latex cDNA Library—A cDNA library was constructed in a Uni-ZAP II vector according to the supplier's instructions (Stratagene, La Jolla, CA) using poly(A)⁺ RNA prepared from latex. In a separate study using a subtracted cDNA library (latex - leaf), we identified an EST whose deduced amino acid sequence matched with the known partial sequence of Hev b 3.2 From the sequence information of the EST clone, we designed a PCR primer corresponding to the sequences from +185 to +211 bp in SRPP (see Fig. 1). This primer (upstream) and T7 primer (downstream) were used to amplify SRPP from the latex cDNA library. PCR was performed for 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C, with a 5-min preheat and a 10-min final extension at 72 °C. The PCR product was used to screen 5 × 10⁵ plaques of the latex cDNA library. The cDNA clones hybridized to the probe were subjected to *in vivo* excision according to the protocol provided by the cDNA library kit and sequenced. One clone carrying a full-length cDNA insert was designated as pSRPP and chosen for further study.

Sequencing of cDNA Clones—Plasmid DNA for sequencing reactions was prepared by the alkaline lysis method (20) using WizardTM Plus SV Minipreps DNA purification system kit (Promega). The sequencing reaction was performed with the ALFexpress AutoRead sequencing kit (Amersham Pharmacia Biotech) using the fluorescent dye-labeled M13 universal or reverse primer (provided by the kit). The nucleotide sequences were obtained by electrophoresis on an ALF automatic sequencer (Perkin Elmer Corp.).

Heterologous Expression of SRPP Protein in *E. coli*—The SRPP gene was cloned either in the *EcoRI-XhoI* site of pGEX (Amersham Pharmacia Biotech) or in the *BamHI-PvuII* site of pRSET (Invitrogen) to construct pGEX-SRPP or pRSET-SRPP. The *E. coli* XL1-Blue transformed with either pGEX-SRPP or pRSET-SRPP was grown to midstationary phase in Luria broth (LB) containing 50 mg/liter ampicillin at 30 °C with vigorous aeration. The cultures were induced by adding IPTG to a concentration of 0.1 mM and then incubated for another 6 h. All subsequent steps were carried out at 4 °C. The cells were harvested, washed with 0.1 M potassium phosphate (pH 7.4) by centrifugation (5000 × g, 10 min), and then disrupted by sonication. The homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant was used to determine rubber synthesis activity. The lysate was subjected to SDS-PAGE according to the standard method of Laemmli (21).

Southern and Northern Blot Analysis—Genomic DNA from leaf tissues of a mature *H. brasiliensis* RRIM 500 was prepared, digested, and blotted as described in Han *et al.* (22). For Northern blot, total RNA was electrophoresed on a 1.4% agarose-formaldehyde gel and blotted as for Southern blot. A ³²P-labeled 175-bp fragment (from 480 to 655 bp in Fig. 1) from SRPP cDNA was used as a probe. The hybridization was performed with high stringency (22).

In Vitro Rubber Biosynthesis Assay—Washed rubber particles (WRP) were prepared by a repeated centrifugation/flotation procedure as described (23, 24). C-serum or WRP was incubated in 50 µl of reaction mixture containing 100 mM Tris-HCl, pH 7.5, 80 µM [¹⁴C]IPP (55 mCi mmol⁻¹, Amersham Pharmacia Biotech), 20 µM FPP, 1 mM MgSO₄, and 1 mM dithiothreitol for 6 h at 25 °C. For control experiments, 25 mM EDTA was added to the reaction mixture to chelate Mg²⁺ ion necessary for rubber transferase activity. The reaction was stopped by adding 25 mM EDTA. The resulting [¹⁴C]IPP-incorporated rubber was quantified by using either a filtration or a benzene extraction method. For the filtration method, the reaction mixture was filtered through either 0.02 or 0.1 µm anodisc membrane (Whatman). The filter was subjected to repeated washing with 1 M HCl and 95% ethanol (23), and the remaining radioactivity on the washed filters was determined by a liquid scintillation counter (Beckman). For the benzene extraction method, the reaction mixture was extracted three times with 2 volumes of benzene. The benzene extract was mixed with a Ready Solv HP scintillation mixture (Beckman), and the radioactivity was determined by a liquid scintillation counter.

Inhibition of Patient Serum with Native SRPP—To inhibit IgE specific to SRPP in the serum, 170 µl of native SRPP (100 µg ml⁻¹) was added to a mixture containing 0.35 ml of patient serum, 1.3 ml of 5% nonfat milk in phosphate-buffered saline (PBS), and 90 µl of 1% sodium azide. A sample of uninhibited patient serum was similarly treated, but SRPP was replaced with water. The serum samples were incubated overnight at 7 °C.

Western Blot Analysis—Native and recombinant SRPP proteins were

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2   cagtgtttccgaaaggcaaatcttttttcaacttcagcgactgcgttttgaatttgt
60  gatttttaaaggaaattttcaattatggctgaagaggtggaggaagagaggctaagat
      M A E E V E E E R L K Y

119 ttggattttgtgcgagcggtggagtttatgctgtagattctttctcaactctctacct
      L D F V R A A G V Y A V D S F S T L Y L

179 tatgccaaggacatatctggtccattaaaacctggtgctgatactattgagaatgtgggtg
      Y A K D I S G P L K P G V D T I E N V V

239 aagaccgtggttactcctgtttattatattccccttgaggctgtcaagttttagacaaa
      K T V V T P V Y Y I P L E A V K F V D K

299 acggtggatgtatcggctcactagcctagatggcgtttgttccccagttatcaagcaggtg
      T V D V S V T S L D G V V P P V I K Q V

359 tctgccccaaacttactcggtagctcaagatgctccaagaattggttcttgatgtggcttct
      S A Q T Y S V A Q D A P R I V L D V A S

419 tcagttttcaactggtgtgcaggaaggcgcaaaagctctgtacgctaattcttgaacca
      S V F N T G V Q E G A K A L Y A N L E P

479 aaagctgagcaatatgcggtcattacctggcgtgcctcaataagctgccactagttcct
      K A E Q Y A V I T W R A L N K L P L V P

539 caagtgccaaatgtagttgtgccaaccgctgtttatttctctgaaaagtacaacgatgtt
      Q V A N V V V P T A V Y F S E K Y N D V

599 gttcgtggcactactgagcagggatagagtgctcctctatttgcctttgttcccact
      V R G T T E Q G Y R V S S Y L P L L P T

659 gagaaaattactaagggtgtttggagatgaggcatcataatctgcactggatttggttatt
      E K I T K V F G D E A S *

719 tatctattgtgagcttttttatatgtacttattcagtgtttagaataagtctttggtggt
779 gtgttttggatgtggaataaaggccaattgcattggtgcaatatataattatgtata
839 acatttcgtgatttgagttggaatctaaaggttttattaagaatggatggactaaaaaaaa
899 aaaaaaaaaa

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FIG. 1. Nucleotide (GenBank™/EBI accession no. AF051317) and deduced amino acid sequences of SRPP. Numbers of the nucleotide sequence are indicated on the left margin, and those of the amino acid sequence are indicated on the right margin. The underlined region is the sequence from which the degenerate primer was designed for RT-PCR amplification of the SRPP gene.

separated by SDS-PAGE on 15% gels (21). The gels were subsequently stained with Coomassie Blue or used for Western blotting where the proteins in the gel were transferred electrophoretically to a nitrocellulose membrane. To detect allergen-IgE binding, the nitrocellulose membrane was blocked with PBS-milk and then incubated overnight with patient serum inhibited or not inhibited with SRPP as described above. After three cycles of washing with PBS-milk, the nitrocellulose membrane was incubated for 1 h with anti-IgE antiserum conjugated to alkaline phosphatase. After a further three cycles of washing with PBS-milk, the nitrocellulose membrane was incubated for 10 min in Tris-buffered saline before being immersed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate to generate the colored alkaline phosphatase reaction product.

RESULTS

Isolation and Characterization of SRPP Gene—Two approaches, RT-PCR amplification and cDNA library screening, were employed to clone the gene. Sequence analysis showed that the genes isolated by the two different methods were identical except for minor differences in the untranslated regions (UTRs). The RT-PCR-amplified gene has an additional 21 bp (ATAATCAGTTGATAGCTTCCA) before nucleotide 1 at the 5'-UTR, whereas the cDNA library-derived gene has an additional 9 bp before the poly(A) tail.

The primary latex cDNA library used for screening contained 5×10^7 recombinant phages. Using the PCR-generated SRPP sequences, 10 positive clones with cDNA inserts (0.8–1.0 kb) were detected and isolated from 5×10^5 plaques of the cDNA library. A cDNA clone containing an insert of approximately 1.0 kb in size was selected and subjected to *in vivo* excision for

further study. The resulting phagemid was designated pSRPP. Sequence analysis showed that the cDNA insert was 910 bp long (Fig. 1) and contained a 612-bp open reading frame flanked by a 63-bp 5'-UTR and a 181-bp 3'-UTR including a poly(A) tail of 18 bp. A putative polyadenylation signal (AATAAA) beginning at bp 794 was identified. The open reading frame encodes for a 204-amino acid polypeptide with a predicted molecular mass of 22.4 kDa. The deduced protein is acidic with an isoelectric point of 4.8, which is similar to that of REF (pI = 5.04). Hydropathy analysis of the deduced amino acid sequence showed that SRPP is hydrophobic (Fig. 2). Transmembrane region analysis using the TMpred algorithm indicated that the SRPP does not have transmembrane helices large enough to span the lipid bilayers. A computer analysis using the PSORT program (K. Nakai, Osaka University, Japan) for protein localization sites suggested that SRPP is localized in cytoplasm.

The predicted amino acid sequence of the SRPP has high similarity to those of REF (72% in the paired sequence region), PvSRP (68%), and *Arabidopsis thaliana* F1N21.4 (48%). PvSRP is believed to be involved in the defense mechanism and induced by stress or wounding. The function of the *Arabidopsis* gene F1N21.4 is not known. Sequence alignment indicated that there is a highly conserved region from amino acids 38 to 65 with no match to any known functional domains (Fig. 3).

SRPP Is a Single-copy Gene—To determine the copy number of the SRPP gene, Southern blot hybridization analysis was performed using a random primed 175-bp fragment (Fig. 1,

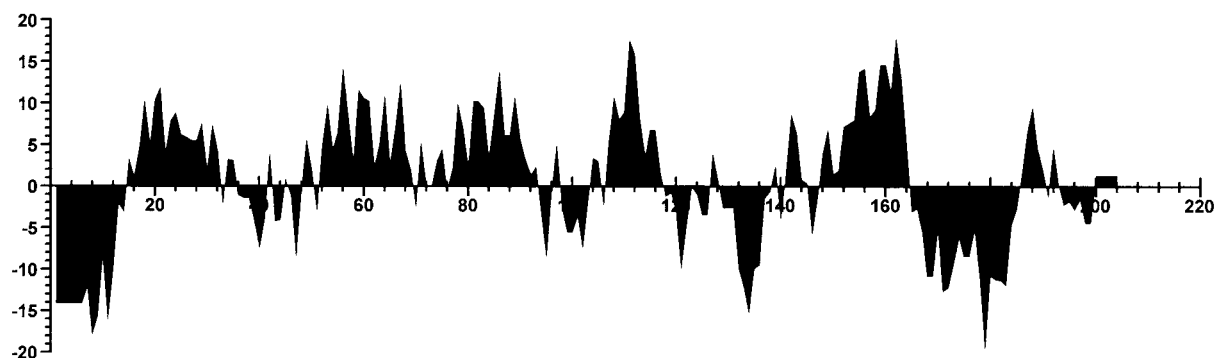


FIG. 2. Hydropathy plot calculated from the deduced amino acid sequence of SRPP. The analysis was performed according to Kyte and Doolittle (34).

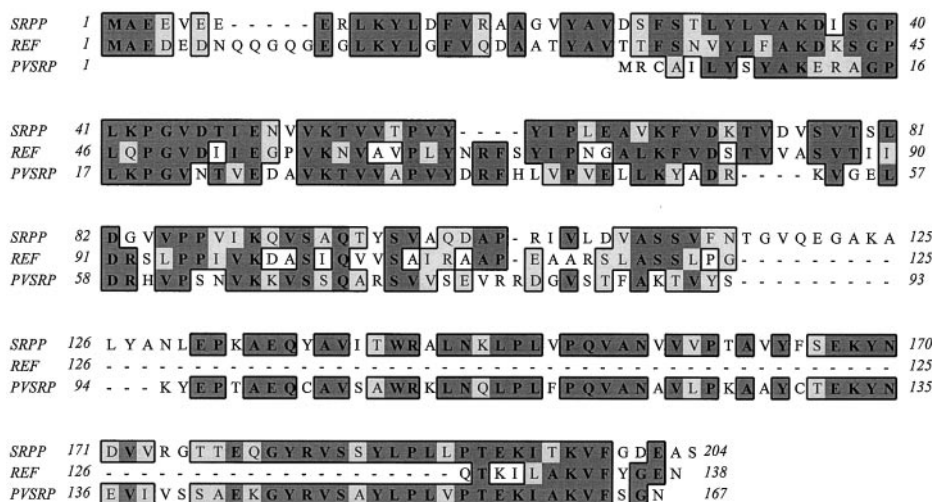


FIG. 3. Comparison of the deduced amino acid sequence of SRPP with REF (GenBank™/EBI accession no. P15252) (see Ref. 36) and PvSRP (GenBank™/EBI accession no. 1326163). The alignment was made using the ClustalW program. Gaps in the sequences are indicated by dashes. Numbers of amino acids are indicated on the right margin.

from 480 to 655 bp) from SRPP cDNA as a hybridization probe. This sequence is the least homologous region in the sequence alignment with other related genes (Fig. 3). A single hybridizing band was observed in each restriction digest (Fig. 4), suggesting that SRPP is most likely encoded by a single gene per haploid genome. However, when the entire SRPP cDNA sequence was used as a probe, four bands were detectable on the blot (data not shown).

Immunoblots and Inhibition Experiments—To confirm that the cDNA we cloned encodes for the previously known 24-kDa protein (Hev b 3), immunoblot analyses of natural and recombinant SRPP were performed with antisera from spina bifida patients. This 24-kDa protein is known to be tightly associated with the small rubber particle where rubber synthesis is actively occurring. Natural and recombinant SRPP proteins were equivalent in their ability to bind IgE (Fig. 5). The IgE reactivity to both natural and recombinant SRPP proteins could be equally inhibited by preincubation of the sera with native SRPP protein.

Expression of the SRPP Gene—Northern blot analysis showed a single band of 1.0 kb for SRPP from both leaf and latex samples. The level of transcripts was most abundant in latex (Fig. 6). The expression of SRPP in responses to wounding and ethylene stimulation was investigated at both transcription and translation levels. We found that neither wounding nor ethephon treatment changed the expression of SRPP at both transcriptional (Fig. 6) and translational levels (data not shown). This result suggested that the function of this rubber particle-associated protein is likely in areas other than defense.

SRPP Plays a Positive Role in Rubber Biosynthesis in Vitro—To determine whether SRPP is involved in rubber biosynthesis, *in vitro* rubber biosynthesis assays using *Hevea* C-

serum and WRP were performed by adding SRPP protein to the reaction mixture. SRPP was expressed in *E. coli* as a glutathione *S*-transferase (GST)-SRPP fusion protein under the control of the IPTG-inducible *tac* promoter (Fig. 7). The bacterial extracts of the culture after IPTG induction were used as a source of SRPP recombinant protein. As a control, either the same bacterial extract without IPTG induction or the culture of *E. coli* harboring pGEX vector with no insert was used. Both C-serum and WRP contain native SRPP as well as REF and exhibited a fairly high level of rubber biosynthetic activity (about 8,000 cpm) compared with control reaction (about 900 cpm) (Fig. 8). Rubber transferase in the control was inactivated by the addition of 25 mM EDTA that chelates Mg^{2+} ion necessary for the activity of the enzyme. As shown in Fig. 8, the amounts of [^{14}C]IPP incorporated into rubber were substantially increased with the addition of recombinant SRPP. The increase of [^{14}C]IPP incorporation was dependent upon the amounts of SRPP protein added to the reaction mixture. [^{14}C]IPP incorporation was increased about 4-fold by the addition of 15 μ l of bacterial extract expressing GST-SRPP fusion protein to the reaction mixture containing either 5 μ l of C-serum or 10 mg of WRP. In contrast, no increase of [^{14}C]IPP incorporation was observed in the control reaction containing bacterial extracts from either the culture without IPTG induction or the culture expressing only the GST protein.

To further confirm that SRPP plays a role in rubber biosynthesis, polyclonal antibodies from SRPP-challenged rabbit serum and monoclonal antibodies from mouse were prepared (13), and their ability to inhibit rubber biosynthesis in C-serum was tested. The untreated blood serum and the culture supernatant were used as a control without further purification. Fig. 8 shows that the polyclonal and monoclonal antibodies gener-

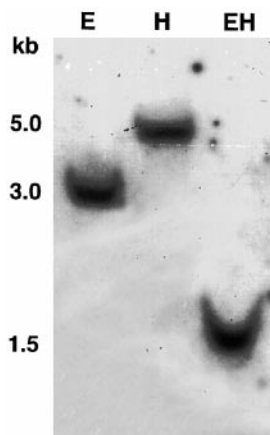


FIG. 4. **SRPP is encoded by a single gene.** Ten micrograms of genomic DNA isolated from leaf tissues was digested with *Eco*RI (*E*), *Hind*III (*H*), and *Eco*RI + *Hind*III (*EH*). These enzymes do not have restriction sites inside the coding region of SRPP. The digested DNA was size-fractionated on 0.8% agarose gel, blotted, and hybridized with the same probe used in Northern blot analysis. Sizes of the hybridizing bands are indicated on the left margin.

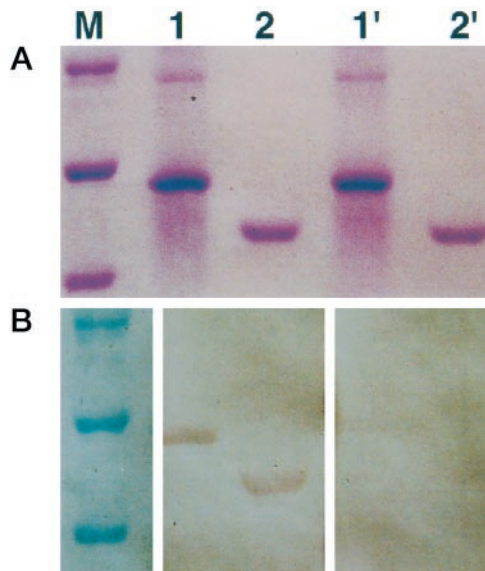


FIG. 5. **Electrophoretic separation and immunodetection of SRPP proteins.** A, Coomassie Blue staining. Lanes 1 and 1', recombinant SRPP protein; lanes 2 and 2', native SRPP protein. B, Western blot of a matching gel to show binding of SRPP protein with IgE in plasma from latex-allergic (spina bifida) patients. Lanes 1 and 2, serum not inhibited; lanes 1' and 2', serum inhibited with native SRPP. Molecular weight standards are shown in lane M.

ated against SRPP protein inhibited rubber biosynthesis to a much greater extent than the control serum. At higher concentrations of the control serum, some inhibition of rubber biosynthesis was observed. A more severe reduction in rubber biosynthesis was observed with polyclonal antibodies than with monoclonal antibodies. This differential inhibitory effect could be because of the immuno-cross-reactivity of the polyclonal antibody with REF (data not shown) because of the high sequence homology to SRPP (Fig. 3). It is also possible that the polyclonal antibody binds to both SRPP and REF, and possibly to other proteins with sequence homology with SRPP, and inhibits rubber biosynthesis to a greater extent than monoclonal antibody that binds to only SRPP. These results suggest that SRPP is involved in rubber biosynthesis *in vitro*.

SRPP Catalyzes the Formation of Polyisoprenes without the Presence of WRP—We tested whether SRPP can catalyze the

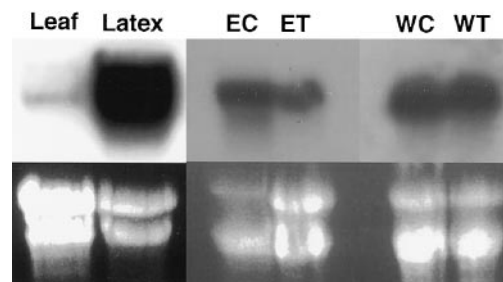


FIG. 6. **Northern blot analysis of SRPP transcript.** Fifteen micrograms of total RNA isolated from leaf and latex of *H. brasiliensis* were hybridized with a 32 P-labeled 175-bp fragment (from 480 to 655 bp in Fig. 1) from SRPP cDNA. The SRPP transcript is more abundant in latex than in leaf tissue. The same 175-bp probe was used to hybridize 15 μ g of total RNA isolated from the latex of *H. brasiliensis* trees with no treatment (*EC* and *WC*), 2.5% ethephon stimulation (*ET*), and wounding (*WT*) as described under "Materials and Methods." The lower panel shows the ethidium bromide-stained rRNA under UV light before blotting, indicating the loading of a similar amount of total RNA.

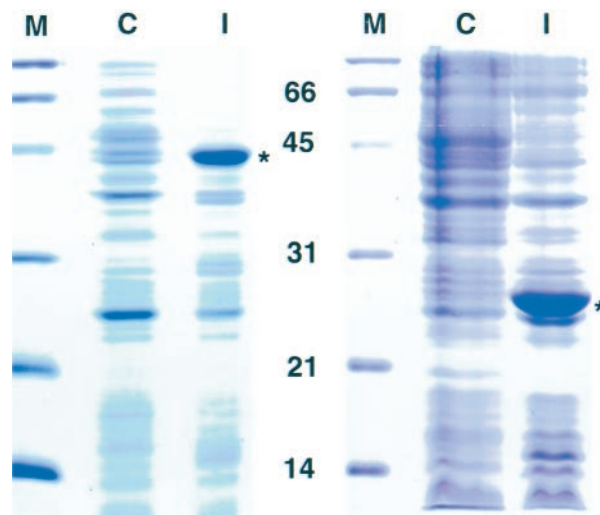


FIG. 7. **Overexpression of SRPP in *E. coli*.** Expression vectors (pGEX-SRPP and pRSET-SRPP) were constructed in which the fusion protein was driven by the T7 promoter, made IPTG-inducible, and transformed into *E. coli* XL1-Blue. Expression was induced by the addition of 0.1 mM IPTG, and total cell proteins were analyzed after 4 h by SDS-PAGE as described under "Materials and Methods." Left panel, SRPP expression from *E. coli* cells harboring pGEX-SRPP; Right panel, SRPP expression from *E. coli* cells harboring pRSET-SRPP. M, molecular markers; C, *E. coli* cells harboring pGEX-SRPP not induced; I, *E. coli* cells harboring pGEX-SRPP after 4 h of induction. The molecular mass (kDa) of the markers is indicated in the middle. An asterisk in the right margin indicates the migration of SRPP.

successive condensing reactions of IPP to the rubber-initiating molecule FPP without the presence of WRP. The *in vitro* rubber assay was carried out with or without the addition of WRP using bacterial extracts of the cells expressing only GST protein, bacterial extracts without IPTG induction, and bacterial extracts of the cells expressing GST-SRPP fusion protein with IPTG induction. The reaction products of the rubber assay were analyzed by reverse phase TLC. The radiolabeled products synthesized by WRP were retained at the origin, indicating long-chain length rubber (Fig. 9). Without the presence of WRP, reactions with GST protein alone or uninduced extracts showed no detectable IPP incorporation activity (Fig. 9, lanes a and b). Only the reaction with the bacterial extracts of cells expressing GST-SRPP fusion protein produced polyisoprene (lane c). Most of the reaction products synthesized by GST-SRPP fusion protein were retained at the origin, but a distribution of weak bands migrating with the solvent was detected above the origin (lane c). These results suggest that the reac-

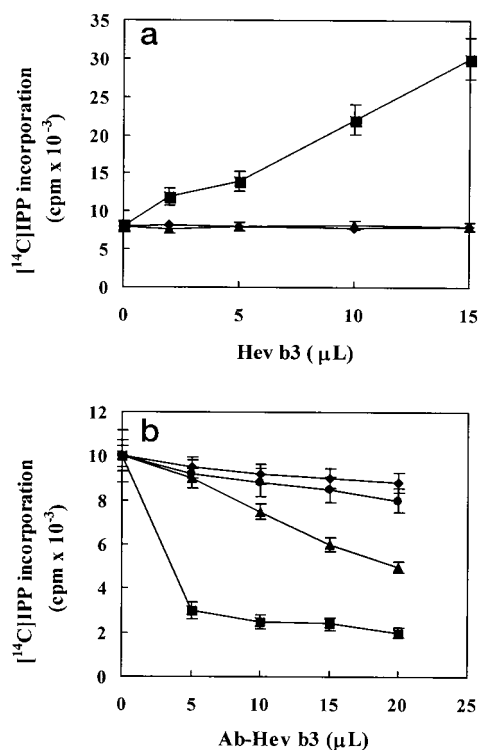


FIG. 8. SRPP plays a positive role in rubber biosynthesis *in vitro*. *a*, [¹⁴C]IPP incorporation into rubber as a function of SRPP concentration. The rubber elongation assay was performed in 50 μl of reaction mixture containing 10 mg of WRP and the indicated amounts of SRPP. Each value is the mean of three experiments for bacterial extracts of the cells expressing GST-SRPP fusion protein with IPTG induction (■), bacterial extracts without IPTG induction (◆), and bacterial extracts of the cells expressing only the GST protein (▲). *b*, concentration dependence of antibody-SRPP inhibition of rubber biosynthesis. The indicated amounts of antibody raised against SRPP were added to 50 μl of reaction mixture containing 5 μl of C-serum. Each value is the mean of three experiments for control rabbit (◆) and mouse (●) serum without antibody formation, monoclonal antibody (▲), and polyclonal antibody (■).

tion products synthesized by GST-SRPP fusion protein are mainly a mixture of long-chain length polymers. We are currently examining the nature of these long-chain length polymers.

DISCUSSION

Because rubber biosynthesis takes place in laticifers, genes highly expressed in such tissues may code for the enzymes involved in rubber synthesis. Kush *et al.* (2) reported a number of genes highly expressed in latex compared with leaves. They found that latex RNAs are highly enriched in transcripts encoding rubber biosynthesis-related enzymes (20–100-fold) as well as defense-related proteins (10–50-fold). In a separate study we have identified about 245 ESTs from the latex cDNA library.² Rubber biosynthesis-related genes (*e.g.* REF and SRPP) along with defense genes are among the most abundant transcripts. The latex has been suggested to play some protective roles because of the high concentration of defense-related proteins in latex (26). However, it remains to be answered why *Hevea* species allocate excessive energy and resource to rubber synthesis.

Rubber particles of *H. brasiliensis* latex can be separated by ultracentrifugation into two distinct fractions: Zone 1 fraction (large rubber particles) and Zone 2 fraction (small rubber particles). The size of rubber particles ranges from about 10 μm to below 0.2 μm (27, 28). Each rubber particle contains hundreds to thousands of rubber molecules within this enclosing inter-

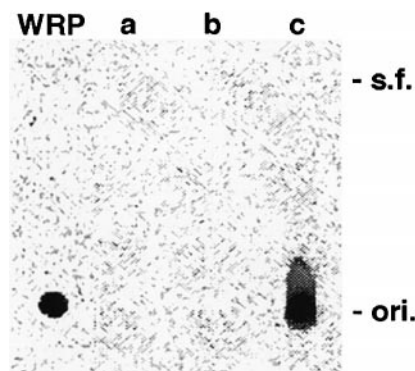


FIG. 9. Reverse phase TLC radiochromatograms of the reaction products. We tested whether SRPP can catalyze the successive condensing reactions of IPP to the rubber-initiating molecule FPP without other RPPs. *In vitro* rubber assay was carried out with (lane WRP) or without the addition of WRP using bacterial extracts of the cells expressing only GST protein (lane *a*), bacterial extracts without IPTG induction (lane *b*), and bacterial extracts of the cells expressing GST-SRPP fusion protein with IPTG induction (lane *c*). The radioactive reaction products were treated with potato acid phosphatase according to the method of Fujii *et al.* (35), extracted with hexane/benzene, and analyzed by TLC on reverse phase RP-18 in a solvent system of acetone: water (9:1, v/v). The distribution of ¹⁴C-labeled reaction products on the TLC plate was analyzed by a BAS 1500 PhosphorImager (Fuji). *s.f.*, solvent front; *ori.*, origin.

face (28). Analysis using gel permeation chromatography revealed that the small rubber particles contained rubber of higher molecular weight than the large rubber particles. Rubber particles are not simply an inert ball of rubber. Siler *et al.* (27) analyzed the composition of rubber particles of four rubber-producing plants including *H. brasiliensis* and suggested that the rubber particle surface is a mosaic of protein, conventional membrane lipids, and other components. Among many latex proteins, two main proteins remain associated with rubber particles after repeated washing: REF (14 kDa) with large rubber particles and SRPP (23 kDa) with small rubber particles (13).

Rubber transferase (*cis*-prenyltransferase) activity has been reported from the rubber particle-bound proteins of three rubber-producing plants, *H. brasiliensis* (17, 29), *Parthenium argentatum* (23, 30), and *Ficus elastica* (24). Although at least two rubber particle-associated proteins, a REF and a rubber transferase, have been suggested to be involved in rubber biosynthesis in *H. brasiliensis* (3, 31, 32), the detailed mechanism of rubber biosynthesis has not been investigated. Although the gene coding for rubber transferase has not been cloned, a full-length cDNA encoding REF has been cloned (4). It has been established that REF plays a functional role in rubber polymerization (3, 31). However, the actual role of REF and the nature of rubber transferase in *cis*-1,4-polyisoprene elongation has not been fully assessed. It is possible that SRPP is involved in rubber biosynthesis, as the rubber particle is the site of rubber synthesis and SRPP is one of the two major RPPs. In the current study, we established that SRPP has high amino acid sequence homology to REF and plays a positive role in rubber synthesis. Furthermore, we showed that SRPP synthesized long-chain polyisoprene without other rubber particle proteins (Fig. 9, lane *c*). These findings, along with the fact that SRPP is tightly associated with small rubber particles and is abundantly expressed in latex, suggest that SRPP plays a similar role in rubber synthesis as REF does and could potentially be a rubber transferase.

In addition to the postulated role in rubber biosynthesis, its high sequence homology to PvSRP suggests that SRPP is involved in defense mechanism. PvSRP is believed to play a role in plant defense mechanism and is regulated by heavy metal

stress, wounding, and virus infection. We tested to see if SRPP is induced by ethephon treatment or wounding. Ethylene regulates fruit ripening, germination, senescence, and the expression of the response to environmental stresses such as mechanical wounding, infection, and waterlogging (33) by stimulating changes in gene expression. Ethephon treatment increased (approximately 5–20 times) several defense genes including chitinase, pathogenesis-regulated protein, and phenylalanine ammonia-lyase in *H. brasiliensis* (2). Northern blot analyses indicated that the SRPP gene is not induced by wounding or ethephon treatment. These results showed that the expression pattern of the gene is different from the kidney bean stress-related protein (PvSRP). Furthermore, the calculated isoelectric point of SRPP ($pI = 4.8$) was quite different from that of PvSRP ($pI = 9.47$), suggesting that SRPP is biochemically distinct from PvSRP. These findings indicate that the function of this rubber particle-associated protein is likely in areas other than defense.

Although the biological significance of rubber to the producing plant is not obvious, natural rubber is of great commercial importance. Elucidation of a mechanism for rubber biosynthesis may provide crucial knowledge bases for genetic manipulation of the *Hevea* rubber tree to improve rubber productivity and quality and to aid the development of alternative rubber crops by transgenic expression of the gene(s) responsible for rubber formation. The present study identified SRPP as a potential candidate for rubber transferase. Although SRPP seems to catalyze the formation of long-chain length rubber, it remains to be determined whether SRPP plays a direct role in elongating the rubber chain or contributes indirectly by synthesizing the initiating molecules necessary for rubber biosynthesis. More biochemical studies with the purified SRPP are required to further characterize the exact nature of the involvement of this protein in rubber biosynthesis. Unfortunately, the *E. coli* expression system we used in the present study produced most of the fusion protein in insoluble form. We therefore are attempting to obtain large quantities of soluble protein using either *Pichia* or baculovirus expression systems. Furthermore, we are currently investigating the possible role of SRPP as a rubber transferase in transgenic plants expressing SRPP and REF.

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