Molecular cloning, expression and characterization of cDNA encoding cis-prenyltransferases from *Hevea brasiliensis*

A key factor participating in natural rubber biosynthesis

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Natural rubber from *Hevea brasiliensis* is a high molecular mass polymer of isoprene units with cis-configuration. The enzyme responsible for the cis-1,4-polymerization of isoprene units has been identified as a particle-bound rubber transferase, but no gene encoding this enzyme has been cloned from rubber-producing plants. By using sequence information from the conserved regions of cis-prenyl chain elongating enzymes that were cloned recently, we have isolated and characterized cDNAs from *H. brasiliensis* for a functional factor participating in natural rubber biosynthesis. Sequence analysis revealed that all of the five highly conserved regions among cis-prenyl chain elongating enzymes were found in the protein sequences of the *Hevea* cis-prenyltransferase. Northern blot analysis indicated that the transcript(s) of the *Hevea* cis-prenyltransferase were expressed predominantly in the latex as compared with other *Hevea* tissues examined. In vitro rubber transferase assays using the recombinant gene product overexpressed in *Escherichia coli* revealed that the enzyme catalyzed the formation of long chain polyisoprenyl products with approximate sizes of \(2 \times 10^3\)–\(1 \times 10^4\) Da. Moreover, in the presence of washed bottom fraction particles from latex, the rubber transferase activity producing rubber product of high molecular size was increased. These results suggest that the *Hevea* cis-prenyltransferase might require certain activation factors in the washed bottom fraction particles for the production of high molecular mass rubber.

*Keywords:* prenyltransferase; rubber transferase; *Hevea brasiliensis*; isoprenoid.

Although over 2000 species of higher plants are recognized for producing latex with polyisoprenes [1], only the rubber tree (*Hevea brasiliensis*) has been established as a key commercial rubber source due to its good yield of rubber and the excellent physical properties of the rubber products. *Hevea* rubber is a high molecular mass polymer of isoprene units in cis-configuration. Rubber molecules are produced and aggregated or packaged as rubber particles in latex vessels of the rubber tree [2]. Although natural rubber is synthesized and made almost entirely of isoprene units derived from isopentenyl diphosphate (IPP), an allylic diphosphate is also required as the priming cosubstrate to initiate the subsequent extensive prenyl chain elongation process for the formation of rubber macromolecules [3–5]. Synthesis of the allylic prenyl diphosphates are catalyzed by IPP isomerase and *trans*-prenyltransferase enzymes, the enzymatic activities of which were found in both the bottom fraction and the supernatant cytosol (C-serum) of centrifuged fresh *Hevea* latex [6–8]. The enzyme responsible for cis-1,4-polymerization of isoprene units from IPP onto the allylic primer has been identified as a particle-bound rubber transferase (EC 2.5.1.20) [3,9,10]. The particle-bound rubber transferase activities were demonstrated in various rubber producing plants; guayule [4,5], *Ficus elastica* [11] and *Ficus carica* [12]. It has been shown that IPP is incorporated into rubber at the surface of the rubber particles in latex, by reaction with a terminal allylic diphosphate group of the rubber molecules [13–15]. This indicates that rubber transferase is bound to the rubber particles and is still present even after the particles are washed repeatedly. However, the precise mechanism for the biosynthesis of rubber molecules has not yet been established. Moreover, the exact site of the formation of new rubber molecules still remains unknown. It has been suggested that the bottom fraction membrane could possibly serve as the site for initiation of new rubber formation [16,17].
In the biosynthesis of polyisoprenoid compounds, the prenyl chain elongation, catalyzed by prenyltransferases, proceeds consecutively and terminates precisely at certain chain lengths according to the specificities of individual enzymes [18–20]. These enzymes have been classified into two major groups, referred to as trans- or (E)-prenyl diphosphate synthases (trans-prenyltransferases) and as cis- or (Z)-prenyl diphosphate synthases (cis-prenyltransferases), depending on the stereochemistry of the condensation reaction of IPP with the corresponding allylic prenyl diphosphate initiator. During the past 16 years, many different genes encoding trans-prenyltransferases have been cloned and characterized [19,20]. On the other hand, very limited information was available on cis-prenyltransferases until the recent cloning and characterization of the genes encoding cis-prenyltransferases from Micrococcus luteus B-P 26, Escherichia coli, Haemophilus influenzae, Streptococcus pneumoniae, Saccharomyces cerevisiae and Arabidopsis thaliana [21–25].

Natural rubber has been thought to be made almost entirely of cis-isoprene units derived from IPP, and the enzyme responsible for polymerization is believed to have characteristics similar to the cis-prenyl diphosphate synthases. However, the genes encoding Hevea cis-prenyltransferases or rubber transferase (HRT) from H. brasiliensis have not yet been reported. It was suggested that rubber biosynthesis in H. brasiliensis is mediated by the association of a soluble trans-prenyltransferase with a rubber elongation factor, a 14.6 kDa protein, tightly bound to the rubber particles in the laticifers [26]. However, Cornish [10] has demonstrated that the soluble trans-prenyltransferase functions as farnesyl diphosphate synthase, and almost certainly plays no direct role in the cis-1,4-polyisoprene elongation.

A detailed understanding of rubber biosynthesis processes at the molecular level is important for genetic manipulation of the isoprenoid biosynthesis pathway enzymes. In this study, by using the sequence information from the conserved regions of the cis-prenyl chain elongating enzymes [20–22], we isolated and characterized two Hevea cis-prenyltransferases cDNAs designated as HRT1 and HRT2 from the H. brasiliensis latex. Sequence analysis showed that all five regions that are conserved among cis-prenyl chain elongating enzymes were present in each of the deduced amino acid sequences encoded by the two cDNAs. In vitro assay of the rubber transferase activity of the recombinant HRT proteins was carried out in the presence of washed bottom fraction particles (WBP) of fresh Hevea latex. These results suggest that the HRT2 protein catalyzes the synthesis of new rubber molecules with the active involvement of a number of factors in WBP of fresh Hevea latex.

Materials and methods

Plant materials and RNA isolation

Latex and various tissue samples were obtained from ten-year old rubber plants (H. brasiliensis clone RRIM 600) being grown at the Rubber Research Center of Songkla, Thailand. Latex collection was performed as described by Kush et al. [27]. The latex total RNA was extracted by using RNAagents Total RNA Isolation System (Promega).

Total RNAs of rubber leaf and other tissues were obtained using QuickPrep Total RNA Extraction kit (Amersham Biosciences) and RNA Isolation kit (Qiagen), respectively. Poly(A)+ RNA was isolated from the total RNA with Oligo(dT)30 mRNA Purification kit (TaKaRa, Ohtsu, Japan).

RT-PCR amplification of Hevea cis-prenyltransferase cDNA fragment

RT-PCR was carried out using Ready-To-Go™ RT-PCR Beads (Amersham Biosciences). First strand cDNA synthesis was performed by reverse transcription with 100 ng of poly(A)+ RNA isolated from latex using poly-d(T)12–18 primer. Two oligonucleotide degenerate primers were designed to amplify the Hevea cis-prenyltransferase cDNA fragment according to the highly conserved regions among cis-prenyl chain elongating enzymes; sense primer, P1 (AFIMDGN, region I) 5'-GCTTTTATGAAVG GHAA-3' and antisense primer, P2 (IRTSGE, region V) 5'-CTCACAGAGTCKWTCKAT-3', where H is A, C or T; K is G or T; W is A or T and Y is C or T. PCR was performed in a final volume of 50 μL containing 50 pmol of amplification primer pair for 45 cycles of 30 s at 95 °C, 30 s at 45 °C and 1 min at 72 °C with a 5 min preheat and a 10 min final extension at 72 °C. The resulting band of PCR products were extracted from agarose gel and subcloned into pT7Blue T vector (Novagen) for sequencing. One of the resulting clones, which showed homology to cis-prenyltransferases, was termed LT600.

3’- and 5’-RACE reaction and cloning of Hevea cis-prenyltransferase cDNA

Cloning of the full-length cDNA of Hevea cis-prenyltransferase, used poly(A)+ RNA from latex as the template for performing both 3’- and 5’-RACE reactions based on the cDNA sequence of LT600. The procedures applied for 3’- and 5’-RACE reaction were according to the manufacturers instructions (3’-Full RACE Core Set, TaKaRa; 5’-RACE kit, Roche). The primer, F1, used for 3’-RACE was 5’-AGGGCTACTGCCAACAATTCC-3’ and the primers, R1 and R2, used for 5’-RACE reaction were 5’-GCTTCAGTGCTTTGCTTCCTCC-3’ and 5’-GC TAAAGCGCATAGATAGTGCGC-3’ respectively. According to the sequence information obtained by the 5’- or 3’-RACE reaction, the cDNA was amplified by RT-PCR with the latex poly(A)+ RNA as the template and then sequenced. Finally, two cDNAs were obtained and designated HRT1 and HRT2, respectively.

DNA sequencing analysis

Sequencing reactions were performed using Thermo Sequenase Cycle Sequencing kit (Amersham Pharmacia Biotech) with fluorescent labeled primers. Nucleotide sequences were determined by the dideoxy chain termination method [28] with a DNA sequencer (LI-COR, model 4200, LI-COR Inc., Lincoln, NE, USA). Computer analysis and comparison of DNA sequences were carried out using Genetyx genetic information processing software (Genetyx Corp., Tokyo, Japan).
Analysis of HRT gene expression

For Northern hybridization, total RNAs (15 µg) from various tissues were subjected to electrophoresis on 0.8% agarose gel containing 1% formaldehyde, and blotted onto a positively charged nylon membrane (Roche). The membranes were hybridized with 32P-labeled HRT cDNAs for 1 h at 68 °C in ExpressHyb solution (Clontech). High stringency washes were performed twice at 50 °C in 2 × NaCl/Cit, 0.05% SDS and twice in 0.1 × NaCl/Cit, 0.1% SDS. The hybridized membranes were exposed for 12 h on a Fuji BAS 1000 Mac Bioimage analyzer. RT-PCR for the analysis of HRT expression was performed by using total RNAs (2 µg) from various Hevea tissues, amplified with HRT1 or HRT2 specific primers. The PCR reaction was performed with a 5 min preheat at 95 °C and a 10 min final extension at 72 °C using primers, S1 (5'-GGAATTCCTCTAAGGATAA-3') and A1 (5'-ACAGCCTGCTGACGAAGAGGGG-3') for amplification of HRT1, and primers S2 (5'-GAAGAATCCCTAAGGATAA-3') and A2 (5'-TACGAAGGATTATCCCTGC-3') for amplification of HRT2. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Construction of expression vector systems in E. coli, and purification of Hevea cis-prenyltransferase

Expression vector systems for the HRTs were constructed using pET32b (+) vector (Novagen), which is designed to express the gene product as a thioredoxin- and His-tagged fusion protein, suitable for production of soluble protein in E. coli cytoplasm and rapid purification. The restriction enzyme recognition site for NcoI or BamHI was introduced by PCR at either the 5'-end or 3'-end of the coding regions of the two HRT cDNAs. The resulting fragments were sequenced, digested with NcoI and BamHI, and ligated into the NcoI–BamHI vector of pET32b (+), yielding the expression plasmids pETHRT1 and pETHRT2. Each of the expression plasmids was used for transformation of E. coli BL21(DE3), and 1 mL of an overnight culture of the transformant in Luria–Bertani medium containing 50 µg/mL ampicillin was inoculated into 200 mL of M9YG medium [29] containing 50 µg/mL ampicillin. The cells were grown at 37 °C to an A600 value of 0.4. Isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 0.5 mM, and then a further incubation at 30 °C for 4 h was carried out. Overproduction of the proteins was confirmed by SDS/PAGE according to the standard method of Laemmli [30].

The cells were harvested by centrifugation (5000 g for 10 min) and then disrupted by sonication. The cell homogenates were fractionated into soluble and insoluble proteins by centrifugation at 8000 g for 10 min. The expressed proteins were purified essentially according to the protocol of Xpress Protein Purification System (Invitrogen), using a Ni2+ nitrilotriacetic acid-agarose column. The soluble proteins were applied to a Ni2+ resin column, and the His-tagged fusion protein was eluted with a gradient of 50–500 mM imidazole in 20 mM phosphate buffer, pH 6.0. The insoluble proteins were solubilized with 6 M guanidine hydrochloride lysis buffer, pH 7.8 and subjected to a Ni2+ resin column. The column was washed and the tagged protein was eluted with 8 M urea in 20 mM phosphate buffer, pH 4.0. The purified protein was renatured by removal of urea via stepwise dialysis. The portion of purified fusion protein was treated with enterokinase to remove the N-terminal fused thioredoxin. The digested protein was used for the rubber transferase activity assay and for product analysis. Protein concentration was measured by the Bradford method [31].

In vitro rubber transferase activity assay

The washed bottom fraction particles (WBP) were prepared by ultracentrifugation (49 000 g, 45 min, 4 °C) of fresh Hevea latex followed by repeated washing of the fresh bottom fraction with 50 mM Tris/HCl buffer, pH 7.4 containing 0.9% NaCl (w/v) according to the method of Wititsuwannakul et al. [17]. The rubber transferase activity assay was performed by the modified method of Tangpakdee et al. [16]. The reaction mixture contained, in a final volume of 0.2 mL, 50 mM Tris/HCl buffer (pH 7.4), 30 mM KCl, 2 mM MgCl2, 5 µM ZnCl2, 5 µM dithiothreitol, 20 mM KF, 0.1 mM deoxycholate, 0.5 mg of WBP, 15 µM farnesyl diphosphate, 50 µM [1-14C]IPP (2.15 GBq/mmol−1, Amersham Pharmacia Biotech), and a suitable amount of cell-free homogenate or purified protein. After incubation for 4 h at 30 °C, short and medium chain polyisoprenyl diphosphate products were extracted with 1-butanol, and then residual radioactive rubber in aqueous phase was extracted three times with 0.6 mL of toluene/hexane mixture (1 : 1 v/v). The extracts were concentrated to a small volume and the radioactivity was measured with an Aloka LSC-1000 liquid scintillation counter (Tokyo, Japan). The rubber transferase activity was determined by measuring the amount of [14C]IPP incorporated into rubber in the toluene/hexane extracts.

Analysis of HRT Reaction Products

The radioactive products extracted with the toluene/hexane mixture were treated with potato acid phosphatase according to the method reported previously [32]. The radioactive products were extracted with toluene and analyzed by TLC on a reversed phase RP-18 plate (Merck) with a solvent system of acetonitrile/water (39 : 1 v/v). The positions of authentic standards were visualized with iodine vapor, and the distribution of radioactive products on the TLC plate was analyzed with a Fuji BAS-1000 Mac Bioimage analyzer.

Distribution of molecular size of rubber products were analyzed by gel permeation chromatography (GPC) which was carried out with a Tosoh high performance liquid chromatography system, equipped in tandem with a series of four TSK gel GPC columns, G7000H, G5000H, G2500H, and G1000H (Tosoh Corp., Tokyo, Japan), each of which has an exclusion limit of 4 × 105, 4 × 106, 2 × 108 and 1 × 109 Da, respectively. The chromatography was carried out at 35 °C using tetrahydrofuran as eluent, at a flow rate of 0.5 mL·min−1. The eluate was monitored by UV absorption at 210 nm following collection at 1 min
intervals, and assayed for radioactivity. The molecular mass of the reaction products were estimated by comparing them with the elution volumes of radioactively labeled standard polystyrenes.

Expression of HRT in yeast strain SNH23-7D

To express the HRT cDNA in the yeast mutant strain SNH23-7D (MATa rer2-2 mfa1::ADE2 mfa2::TRP1 bar1::HIS3 ade2 trpl his3 leu2 ura3 lys2) according to the study of Sato et al. [23], the BglII–SalI fragments containing HRT1 or HRT2 cDNA were cloned into the corresponding sites of plasmid pJR1133, which contain the URA3 marker gene and yeast glyceraldehyde phosphate dehydrogenase promoter [25]. The resulting plasmids designated pJRHRT1 and pJRHRT2, contained HRT1 and HRT2 respectively. The SNH23-7D yeast strain was transformed with plasmid pJRHRT1 and pJRHRT2 according to the protocol of Fast™ Yeast Transformation kit (Geno Technology Inc., St. Louis, USA). Ura+ transformants were selected at 23 °C on agar plates containing minimal medium [0.67% (w/v) yeast nitrogen base without amino acid, 2% glucose, supplemented with 60 µg·mL⁻¹ leucine and 30 µg·mL⁻¹ lysine]. Selected Ura+ colonies and yeast strain SNY9 (MATα mfa1::ADE2 mfa2::TRP1 bar1::HIS3 ade2 trpl his3 leu2 ura3 lys2), which contains the wild type RER2 gene, were streaked on agar plates containing YPD medium and incubated at 23 °C or 37 °C.

Results

Isolation and characterization of HRT cDNAs

A pair of degenerate primers designed from two highly conserved regions (I and V) of the known cis-prenyl chain elongating enzymes [20–22,33–35], were used to amplify of a possible cDNA encoding cis-prenyltransferase(s) in Hevea latex that might be responsible for the prenyl chain elongation of natural rubber. RT-PCR with these primers yielded amplified products of ~600 bp in length. These products were extracted and cloned into pT7Blue T vector. One of the cDNA fragments, which contained regions homologous to those of the conserved regions II, III and IV of cis-prenyltransferase was designated as LT600. The

Fig. 1. Nucleotide and deduced amino acid sequences of HRT1 (A) and HRT2 (B). Numbers of nucleotide sequence and amino acid sequence are indicated on the left and right, respectively. The underline in HRT1 indicates the sequence corresponding to the LT600 fragments.
nucleotide sequence of the fragment was used to design the primers to amplify unknown 3'- and 5'-end sequences of HRT cDNAs by using 3'- and 5'-RACE strategies. The amplified products of the 3'-end gave two distinct cDNA fragments which were 689 and 557 bp in length, with a sequence identity of 85% having identical sequence near the 3'-end of the possible open reading frame. When the 5'-cDNA was amplified, only a 424 bp cDNA fragment was obtained. To obtain full-length sequences of these ORFs, a pair of primers was designed according to the sequence information from the RACE analysis. After amplification by RT-PCR from latex poly(A)+ RNA, we sequenced a number of fragments and obtained two distinct cDNAs that contain sequences identical to those obtained by 3'-RACE.

General cDNAs containing 5'- and 3'-untranslated regions were also amplified by RT-PCR and sequenced [Fig. 1]. These clones were designated as HRT1 and HRT2. The cDNA of HRT1 was 1282 bp long containing an 870 bp ORF, flanked by a 155 bp 5'-UTR and a 254 bp 3'-UTR including a poly(A) tail of 15 bp (Fig. 1A). The cDNA of HRT2 was 1051 bp in length containing an 852 bp ORF (Fig. 1B) and with 92% sequence homology to that of HRT1. The complete cDNA sequences of HRT1 and HRT2 cloned in this study are available from the DDBJ/GenBankTM/EMBL database under accession numbers AB061234 and AB064661 respectively. The ORFs of HRT1 and HRT2 encode 290 and 284 amino acid residues (87.3% identity) with predicted molecular masses of 33.2 and 32.8 kDa respectively. Hydropathy and transmembrane motif analysis of the deduced amino acid sequences (TOPPRED2 program; http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) predicted that both gene products, HRT1 and HRT2, are hydrophilic proteins having a putative N-terminus membrane-spanning segment (amino acid residues 22–42). The deduced amino acid sequences of HRT1 and HRT2 showed high identities of 31.6, 32.0, 31.6, 29.9 and 30.1% to those of cis-prenyl chain elongating enzymes from M. luteus B-P 26 [21], S. cerevisiae (Rer2p [23], Srt1p [36]), E. coli [22,37] and A. thaliana [24,25] respectively. Moreover, all of the five highly conserved regions of cis-prenyl chain elongating enzymes [20–22,34,35] are found in both the HRT1 and HRT2 sequences [Fig. 2]. These conserved regions are proposed to be important for the catalytic function, in addition to substrate binding, for cis-prenyl chain elongating enzymes [33–35,38,39].

Expression analysis of HRT mRNAs

To examine the HRT expression in various Hevea tissues, Northern blot analysis was carried out by using 32P-labeled HRT1 or HRT2 cDNA as the probes. As shown in Fig. 3 (A1 and A2) both probes gave specific hybridization bands of 1 kb which are consistent with the sizes of each of the cDNAs. The expression patterns of HRT1 and HRT2 mRNAs among the examined Hevea tissues seem predominant in latex, whereas little expression was detected in leaves and shoot tips. However, it is possible that the probes of HRT1 and HRT2 cross-hybridize with each other because of the high level of sequence identity. For the study of the specific expression patterns, RT-PCR analyses were carried...
out by designing specific primers from the low homology regions of HRT1 and HRT2 coding sequences and each of the 3′-UTRs. As shown in Fig. 3B, specific bands of the RT-PCR products with HRT1 and HRT2 specific primers could be detected only in the reactions with latex mRNA. These results indicate the specific expression of HRT1 and HRT2 in latex, supporting the proposal that the HRT mRNAs probably function in *Hevea* latex where natural rubber is produced.

**Overproduction of recombinant HRT proteins in E. coli cells**

In order to obtain HRT gene products, the cDNAs were expressed in *E. coli* by means of a pET32b(+) expression system that contains a thioredoxin fusion sequence suitable for production of a soluble protein in *E. coli* cytoplasm. By induction with IPTG the *E. coli* cells harboring HRT1 or HRT2 produced recombinant proteins at 52 kDa in the pellet fraction (Fig. 4, lanes P1 and P2). However, a detectable amount of HRT2 could be found also in the soluble fraction of cell-free extract (Fig. 4, lane S2). The soluble and pellet fractions of HRT2 were subjected to a metal affinity column for purification of the His-tagged fusion protein, under native and denatured conditions respectively. As shown in Fig. 4, the affinity-purified HRT2 protein was the major protein band of 52 kDa (lane P), corresponding to the His-tagged fusion protein, and when the fused N-terminal thioredoxin sequence was removed by enterokinase digestion, a protein of 33 kDa (lane E), was produced which is attributable to the predicted molecular mass of the HRT2 protein.

**Enzymatic activity of HRT2 proteins**

The cell-free homogenates of *E. coli* BL21(DE3)/pETHRT1 and *E. coli* BL21(DE3)/pETHRT2 were examined for *in vitro* rubber transferase activity. Rubber materials were extracted with a solvent mixture of toluene and hexane (1:1 v/v) after the extraction of medium chain polyprenyl diphosphates with 1-butanol. The crude homogenate from HRT2-overexpressing cells showed a slight increase in the production of polyprenyl diphosphates able to be extracted with butanol compared to the crude homogenate of the host cells (Table 1), whereas the HRT1 protein overproduced in *E. coli* cells showed no significant increase in enzymatic activity. Similarly, a slight increase in the radioactivity of the toluene/hexane extracts was detected after the reaction with.*
the crude homogenate of HRT2-overexpressed cells, but not that of HRT1-overexpressed cells (Table 1).

Because the rubber transferase activities of HRT1 and HRT2, which were determined by measuring the amount of rubber material extractable with the toluene/hexane mixture, were not particularly significant, an *in vitro* rubber transferase assay was carried out with the addition of fresh WBP because it has been suggested that these serve as the site for the initiation of new rubber formation [16,17]. The amount of [1-14C]IPP incorporation in the toluene/hexane extracts of 2500 dpm that was observed in the control reaction with WBP is attributable to the endogenous rubber transferase activity in the WBP. When the homogenates of BL21(DE3)/pETHRT1 were coincubated with WBP, the amount of IPP incorporation into the toluene/hexane extracts was similar to that of the control experiment with WBP alone. On the other hand, addition of WBP to the homogenate of BL21(DE3)/pETHRT2 resulted in a remarkable increase in the amount of IPP incorporation in the toluene/hexane extracts (Table 1). The increase in rubber transferase activity over the additive effect of endogenous activity in WBP clearly indicates that the rubber transferase activity of HRT2 was enhanced by various factors in the WBP. The endogenous rubber transferase activity in the WBP could be denatured most effectively by heat treatment at 100 °C for 20 min (Table 1). However, the rubber transferase activity of HRT2 could be induced partially, even by the addition of boiled WBP, implying the presence of heat-stable activator(s) in the WBP, in addition to heat-sensitive ones.

Apparent rubber transferase activity could be obtained when the purified HRT2 protein was used. Figure 5A shows the effect of the HRT2 protein on rubber transferase activity in coincidence with WBP. The activity increased as the concentration of HRT2 was raised, until the activity reached saturation. The effect of WBP on the rubber transferase activity of HRT2, which was assayed in the presence of 5 µg of HRT2 protein, is shown in Fig. 5B. The rubber transferase activity increased proportionally

### Table 1. *In vitro* rubber transferase activities in the cell-free homogenates of HRT transformants. The prenyl chain elongating enzyme activity were assayed in the absence of WBP, in the presence of WBP, and in the presence of boiled WBP as described in Materials and methods. The amounts of [14C]IPP incorporation are averaged from triplicated measurements.

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<tr>
<th>Cell-free homogenate</th>
<th>[14C]IPP incorporation (d.p.m.)</th>
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<tr>
<td></td>
<td>BuOH extract</td>
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<tr>
<td>In the absence of WBP</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pET32b</td>
<td>2760 ± 225</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pETHRT1</td>
<td>2820 ± 253</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pETHRT2</td>
<td>3210 ± 244</td>
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<tr>
<td>In the presence of WBP</td>
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<tr>
<td>WBP</td>
<td>2810 ± 298</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pET32b + WBP</td>
<td>3150 ± 173</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pETHRT1 + WBP</td>
<td>3700 ± 340</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pETHRT2 + WBP</td>
<td>5190 ± 414</td>
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<tr>
<td>In the presence of boiled WBP</td>
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<tr>
<td>Boiled WBP</td>
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<tr>
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<tr>
<td><em>E. coli</em> BL21(DE3)/pETHRT2 + boiled WBP</td>
<td>4080 ± 387</td>
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Fig. 5. Effect of HRT2 and WBP amounts on rubber transferase activity of HRT2. (A) Effect of HRT2 amount on rubber transferase activity. *In vitro* rubber transferase assay was performed in 0.2 mL reaction volume containing 0.5 mg of WBP and the indicated amount of purified HRT2 (●) or *E. coli* BL21(DE3)/pETHRT2 cell-free homogenate without IPTG (■). After extraction of polyprenyl diphosphate products with 1-butanol, rubber transferase activity was measured as described in Materials and methods. (B) Effect of WBP amount on rubber transferase activity. The rubber transferase was assayed in the presence of 5 µg of purified HRT2 and the indicated amount of WBP (●). The control reaction (■) was assayed under similar conditions without the addition of HRT2.
to the amount of added WBP and the activity became saturated when the WBP exceeded 1 mg.

Product analysis of the rubber materials in the toluene/hexane extracts of the HRT2 reactions that were coincubated with WBP, by reversed phase TLC, showed a large spot of radioactivity at the origin of the TLC plate indicating a high molecular mass rubber product (data not shown). Conversely, the reaction of WBP or HRT2 alone gave a small spot at the origin of the TLC plate. In order to determine the molecular masses of the rubber products from the reaction of HRT2 coincubated with WBP, the radioactive products detected at the origin of the reversed phase TLC were analyzed by GPC (Fig. 6B). The major radioactivity eluted in the range of molecular mass of 2 × 10^5–1 × 10^6 Da (corresponding to the rubber polymer of 3000–15 000 isoprene units), with minor peaks of radioactivity at 3 × 10^5, 2 × 10^5 and 1 × 10^5 Da, equivalent to ~400, 30 and 15 isoprene units respectively, showing similar distribution of endogenous rubber materials in WBP (Fig. 6A). When HRT2 was assayed in the absence of WBP, only a small amount of medium chain polyisoprene intermediates of the size ~2000 to 10^6 Da were produced (Fig. 6C). The products of the control reaction with WBP alone (Fig. 6D), show very low quantities of long chain rubber products whose molecular size distributions are similar to those in Fig. 6B indicating the low endogenous rubber transferase activity in WBP. These results suggest that the long chain rubber molecules (approximate size of 10^5–10^6 Da) can be produced in larger amounts when the HRT2 protein is coincubated with WBP.

Functional complementation of the yeast mutant strain SNH23-7D by HRT2s

To determine whether HRT1 and HRT2 cDNAs encode functional enzymes related to the cis-prenyl chain elongating enzyme in vivo, the cDNAs were expressed in the yeast mutant strain SNH23-7D, which is deficient in the activity of dehydrodolichyl diphosphate (dedol-PP) synthase [24]. SNH23-7D shows a temperature-sensitive growth phenotype at 37 °C. As shown in Fig. 7, overexpression of HRT2 suppresses the temperature-sensitive growth phenotype of strain SNH23-7D, whereas the HRT1 showed no effect on this phenotype of the yeast mutant strain.

Discussion

In this study, we isolated two cDNA clones termed HRT1 and HRT2 that possibly encode the latex cis-prenyltransferases of the rubber tree H. brasiliensis. Both of the deduced amino acid sequences, having all of the five highly conserved regions among cis-prenyl chain elongating enzymes, showed high homology (87% identity). These regions have been found to construct the major part of the hydrophobic cleft in the three-dimensional structure [38], and are important for the catalytic function as well as the substrate binding of the enzymes [33–35,39,40]. Therefore, it is reasonable to assign both HRT1 and HRT2 to a family of cis-prenyl chain elongating enzymes present in the laticifers of H. brasiliensis.

Recently, an A. thaliana gene encoding dedol-PP synthase, was identified and shown to be highly expressed in
roots and leaves [24,25]. On the other hand, in the present study, Northern blot and RT-PCR analyses of the HRT genes in various Hevea tissues showed predominant expression of these genes in the latex. As it has been suggested previously that rubber biosynthesis takes place only in the laticifers, however, the specific expression in the latex strongly suggests a possible functional role of both HRT1 and HRT2 in the Hevea latex as a cis-prenyl chain elongating enzyme, i.e. rubber transferase.

The majority of HRT1 and HRT2 were expressed as insoluble fusion proteins in E. coli, which suggests that these proteins might each be associated with the membrane as a particle-bound enzyme in a similar manner to rubber transferase [3,10,40]. Furthermore, hydropathy and transmembrane motif analysis revealed that HRT1 and HRT2 sequences have a possible membrane-spanning segment at amino acid residues 22–42, which is very similar to those in the dedol-PP synthases cloned from yeast [23] and A. thaliana [24,25].

Although rubber transferase has been reported previously, to be bound to rubber particles [13–15], the detailed mechanism of rubber biosynthesis was not clearly understood. More recently, it has been found that the WBP of centrifuged fresh Hevea latex is active for in vitro rubber biosynthesis [16,17], suggesting that the WBP might have the necessary enzyme systems that are responsible for rubber biosynthesis. In the current study, we demonstrated that the recombinant HRT2 protein coincubated with WBP could synthesize medium chain polyprenyl diphosphate intermediates as well as long chain rubber, although HRT2 showed a low cis-prenyl chain elongating enzyme activity when assayed in the absence of WBP. HRT2 may possibly function as a cis-prenyl chain elongating enzyme that cooperates with other activation factors in the WBP for the biosynthesis of long chain rubber molecules. Furthermore, the partial enhancement of HRT2 activity by the addition of the boiled WBP suggests that some heat-stable compounds in WBP could act as the activator of rubber transferase. In addition to heat-stable compounds, HRT2 may require other heat-unstable factors in WBP for complete activation.

Suppression of HRT2 on the temperature-sensitive growth of yeast mutant strain SNH23-7D shows clearly that HRT2 functions as a cis-prenyltransferase and produces dedol-PP, whose prenyl chain length is compatible with that of yeast dolichols (C_{90}–C_{105}: 1.1–1.3 kDa). GPC analysis of the rubber material synthesized by the action of purified HRT2 in the absence of WBP indicated the production of a small amount of medium chain polyprenosoprene intermediates of molecular size between 10^{3} and 10^{4} Da, which are smaller than those produced by the coincubation of HRT2 with WBP (2 \times 10^{5}–1 \times 10^{6} Da) but similar to the molecular size of dolichols in yeast (Fig. 6C). Furthermore, HRT2 showed a little activity for the production of prenyl diphosphates extractable with butanol. Taken altogether, two probable mechanisms of natural rubber biosynthesis including HRT2 as well as the other factor(s) in WBP could be deduced as follows (a) HRT2 synthesizes medium chain polyprenyl diphosphate whose chain lengths are comparable to those of dolichols (\approx 10^{3} Da), then various factors in WBP combine the medium chain polyprenyl products to synthesize high molecular mass rubber materials, and (b) HRT2 catalyzes further prenyl chain elongation to synthesize linear high molecular mass polyprenyl products with the cooperation of various factors in WBP that remarkably enhance HRT2 activity.

We were not able to detect the rubber transferase activity in any fraction of HRT1 protein overproduced in E. coli. These results led us to speculate that the 35 amino acids mismatch between HRT1 and HRT2 may be effective on their native structures and also important for their enzymatic functions. The high similarity between HRT1 and HRT2 suggests the possibility that HRT1 may encode a cis-prenyl chain elongating enzyme such as dedol-PP synthase whose function in the rubber tree is still unknown. Tateyama et al. analyzed the polyprenoid alcohols of H. brasiliensis using a two-plate TLC method [41], and established that polyprenols and dolichols are found with prenyl chain-length distribution of around C_{50}–C_{105} in several tissues of H. brasiliensis. In addition, we found many protein sequences that originated from H. brasiliensis in protein databases, showing high homology to cis-prenyltransferase, such as AAM92880 (AAM92889, AAM92890), AAM92881, AAM92879, BAB92023 (AAM92883, AAM92884, AAM92885, AAM92887, AAM92888), BAB92024 and AAM92882 (AAM92886) (submitted to Genbank™ and DDBJ by Coldren et al. and Sando et al. respectively). However, most of these amino acid sequences, except for that of AAM92882, are similar to that of HRT1 (more than 98% identity) but not to HRT2. None of them have been proved to show rubber transferase activity.

The precise mechanisms of rubber biosynthesis in H. brasiliensis are still not well understood. The results of our study of Hevea rubber transferase represent an important step in understanding the process of rubber biosynthesis in rubber-producing plants at the molecular level and provide a basis for further investigation of the molecular mechanism of HRT reaction. More biochemical studies with the purified HRT are required to further characterize the involvement of this enzyme in rubber biosynthesis, especially on the molecular analysis of the activator(s) in WBP.

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References


