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Note

Characterization of *cis*-prenyltransferases from the rubber producing plant *Hevea brasiliensis* heterologously expressed in yeast and plant cells

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Abstract A precise mechanism for the biosynthesis of natural rubber has not yet been elucidated. The *cis*-prenyltransferase (cPT), HRT2, identified from latex of *Hevea brasiliensis*, is thought to be a key enzyme in the biosynthesis of natural rubber. This is due to the observation that recombinant HRT2, expressed in *Escherichia coli*, is significantly activated in the presence of a centrifuged latex fraction, resulting in the formation of polyisoprenes corresponding to natural rubber. The precise enzymatic characterization of cPT function for HRT2, however, has not been investigated because HRT2 expressed in *E. coli* does not exhibit significant activity independently. Herein, the enzymatic characterization of HRTs expressed in eukaryotic cell systems is reported. Both HRT2 and HRT1, another cPT from Hevea latex, expressed in *Saccharomyces cerevisiae* and *Arabidopsis* T87 cultured cells showed distinct cPT activity, producing polyisoprenoids with chain-lengths of C_{80-100}, although failing to catalyze the formation of natural rubber. The chain lengths of the HRT1/HRT2 products were not altered by the addition of centrifuged latex fractions, and the HRT1/HRT2 expressed in yeast competed with the rubber transferase activity of the latex fraction. These results indicate that HRT1/HRT2 requires additional co-factors from the eukaryotic cells to produce distinct cPT activity, and that latex specific co-factor(s) may be required to enable HRT1/HRT2 rubber transferase activity.

Key words: *Hevea brasiliensis*, isoprenoid, latex, natural rubber, *cis*-prenyltransferase

Natural rubber consists mainly of *cis*-1,4-polyisoprene and is the most important natural polymer produced by plants, due to its unique physical properties including: resilience, elasticity, abrasion and impact resistance, efficient heat dispersion and malleability at cold temperatures (Cornish 2001). Of the known 2,500 or more natural rubber producing higher plants (Mooibroek and Cornish 2000), only the Para rubber tree (*Hevea brasiliensis*) has been established as a key commercial source because of its high yield and the excellent physical properties of its natural rubber. To meet the continuously increasing demand for natural rubber, especially for tire manufacturing, metabolic engineering of *H. brasiliensis* is required to improve natural rubber production. However, the precise structure and biosynthetic pathway of natural rubber production in *H. brasiliensis* has not yet been reported.

Natural rubber is obtained from latex, which is the cytoplasm of highly specialized cells, know as laticifers, in the vascular tissues of plants. Using ultracentrifugation, fresh latex from *H. brasiliensis* can be fractionated into the four major fractions (Moir 1959; Wititsuwannakul and Wititsuwannakul 2005) including the top rubber layer consisting of rubber particles (RPs), an aqueous cytosolic fraction termed C-serum, a sedimented bottom fraction (BF) consisting mainly of membrane-bound lutoid particles, and a yellow-colored complex consisting of a category of plastid Frey-Wyssling particle. RPs are mainly composed of rubber molecules, surrounded by a lipid monolayer and membrane proteins (Cornish et al. 1999). Based on the particle size and molecular weight distribution of the rubber, RPs can be categorized into large rubber particles (LRPs) and small rubber particles (SRPs). *In vitro* assays of rubber biosynthesis using ultracentrifugation fractions revealed that SRPs showed higher levels of activity than LRPs (Ohya et al. 2000). Rubber biosynthesis activity was also observed in the BF of fresh latex (Tangpakdee et al. 1997;

Abbreviations: BF, bottom fraction; cPT, *cis*-prenyltransferase; *E*,*E*-FPP, *E*,*E*-farnesyl diphosphate; *E*,*E*,*E*-GGPP, *E*,*E*,*E*-geranylgeranyl diphosphate; *Z*,*E*,*E*-GGPP, *Z*,*E*,*E*-geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; LRP, large rubber particle; RP, rubber particle; SRP, small rubber particle; WBP, washed bottom fraction particle.

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Wititsuwannakul et al. 2003), suggesting localization of rubber biosynthesis enzymes on SRPs as well as on lutoid particles, which are considered to be polydispersed lysosomal vacuoles.

The basic backbone structure of natural rubber, which is composed of *cis*-1,4-polyisoprene with two or three *trans*-isoprene units at the *ω*-terminus (Tanaka 1989; Tanaka et al. 1995, 1996), suggests that its biosynthesis involves sequential condensation of the C_5 isoprene unit, isoprentenyl diphosphate (IPP), onto an all-*trans* short-chain prenyl diphosphate, such as *E*,*E*-farnesyl diphosphate (FPP, C_{15}) or *E*,*E*,*E*-geranylgeranyl diphosphate (GGPP, C_{20}), with *cis*-configuration. Although the precise mechanism for the biosynthesis of natural rubber molecules has not yet been elucidated, the enzyme responsible for catalyzing *cis*-1,4-condendsation in natural rubber biosynthesis has been identified as an RP-bound rubber transferase [EC.2.5.1.20] (Archer and Cockbain 1969). The key enzyme responsible for the biosynthesis of natural rubber is believed to be a member of the *cis*-prenyltransferase (cPT) enzyme family. These ubiquitous enzymes are involved in the formation of *Z*,*E*-mixed isoprenoids, such as polyprenols and dolichols. Using the sequence information from the five conserved regions of cPTs (Koyama 1999; Takahashi and Koyama 2006), two cPT homologues, designated *HRT1* and *HRT2*, were cloned from the *H. brasiliensis* latex (Asawatreratanakul et al. 2003). Predominant expression of these genes in latex suggested a specific function in latex. The cPT activity of recombinant HRT2 expressed in *E. coli* was significantly activated by the addition of washed bottom fraction particles (WBPs) from *H. brasiliensis* latex, resulting in the formation of polyisoprenes with approximate sizes in the range of 2×10^5 to 10^6 Da, which correspond well with the natural rubber in *Hevea* latex. Therefore, it was suggested that HRT2 functions as a key enzyme in the biosynthesis of natural rubber, coordinated with essential co-factor(s) in WBPs of fresh *Hevea* latex. However, enzymatic characterization of HRT1 and HRT2 as a cPTs has not been investigated because the purified recombinant proteins expressed in *E. coli* do not exhibit significant activity in isolation. In this study, we conducted enzymatic characterization of HRTs expressed in eukaryotic cell systems.

To investigate the cPT activity of HRT1 and HRT2 expressed in *Saccharomyces cerevisiae* and cultured plant cells, the coding cDNAs for HRT1 and HRT2, fused in frame with a C-terminal His_6 tag sequence, were amplified using KOD-plus DNA polymerase (TOYOBO Osaka, Japan) from *E. coli* expression plasmids (pETHRT1 and pETHRT2; Asawatreratanakul et al. 2003) with the following primers: HRTXb (5′-gacgtctagaaggccatggaa-3′, *Xba*I site underlined and the initiation codon for *HRT1* or *HRT2* double underlined) and HRTSm (5′-cagcttcctcccgggctttg-3′, *Sma*I site underlined). The PCR products were sequenced, digested with *Xba*I and *Sma*I and ligated into the *Xba*I–*Sma*I sites of a plant binary vector harboring a CaMV35S promoter, pBE2113Not (Mitsuhara et al. 1996), resulting in pBE-HRT1 and pBE-HRT2. To construct an expression plasmid for yeast, the *Xba*I–*Sma*I fragments of His_6-tagged *HRT1* or *HRT2* were ligated into the *Sma*I site of pJR1133, a yeast expression vector harboring the strong *GPD* promoter and *URA3* (Cunillera et al. 2000). The *Sma*I and *Xba* I incompatible ends were blunted and ligated, resulting in pJR-HRT1 and pJR-HRT2.

The plasmids, pJR-HRT1, pJR-HRT2 and pJR1133, were introduced into the yeast mutant strain SNH23-7D (*MATα rer2-2 mfα1::ADE2 mfα2::TRP1 bar1::HIS3 ade2 trp1 his3 leu2 ura3 lys2*), which has defects in the cPT activity of a Rer2p (Sato et al. 1999). This system has been used to analyze the products from an exogenous cPT (Cunillera et al. 2000; Endo et al. 2003; Kharel et al. 2006) because the activity of the other yeast cPT, Srt1p (Sato et al. 2001), is insufficient to be detected until the stationary phase of cell growth. As a control, SNY9, a WT strain of *RER2*, harboring pJR1133 was also prepared. Each transformant was grown at 23°C in 100 ml SD/-Ura medium until the late-logarithmic phase and lysed as described by Endo et al. (2003). Cell lysates were centrifuged at 300×*g* for 5 min at 4°C to remove intact cells. The resulting supernatants were further centrifuged at 13,000×*g* for 15 min at 4°C to separate membrane and soluble fractions. Immunoblotting with anti-His HRP conjugate (Qiagen, Venlo, Netherlands) using ECL Western Blotting Detection System (GE Healthcare, Little Chalfont, UK) showed that both His_6 tagged HRT1 and HRT2 were expressed predominantly in the membrane fraction from SNH23-7D (Figure 1A). Signals for His_6 tagged HRT1 and HRT2 were detected at approximately 41 kDa, which is higher than the molecular weight calculated from their amino acid sequences (35 kDa), suggesting post-translational modification of the expressed proteins in yeast.

The cPT activities of HRT1 and HRT2 expressed in yeast were examined in reactions containing 50 *μ*g of crude protein in membrane or soluble fraction at 30°C for 120 min in an assay mixture composed of 25 mM potassium phosphate buffer (pH 7.5), 20 mM *β*-mercaptoethanol, 20 mM KF, 4 mM $MgCl_2$, 14 *μ*M *E*,*E*-FPP and 50 *μ*M [1-^{14}C]IPP (29 Ci/mol, GE Healthcare) in a final volume of 100 *μ*l. After the addition of 200 *μ*l of saturated NaCl solution into the assay mixture, ^{14}C-labelled isopentenol occurred from ^{14}C-IPP by the action of phosphatases included in the crude proteins were removed by extraction with 1 ml of diethylether. Then, the resulting polyprenyl products apparently smaller than natural rubber molecular weight range

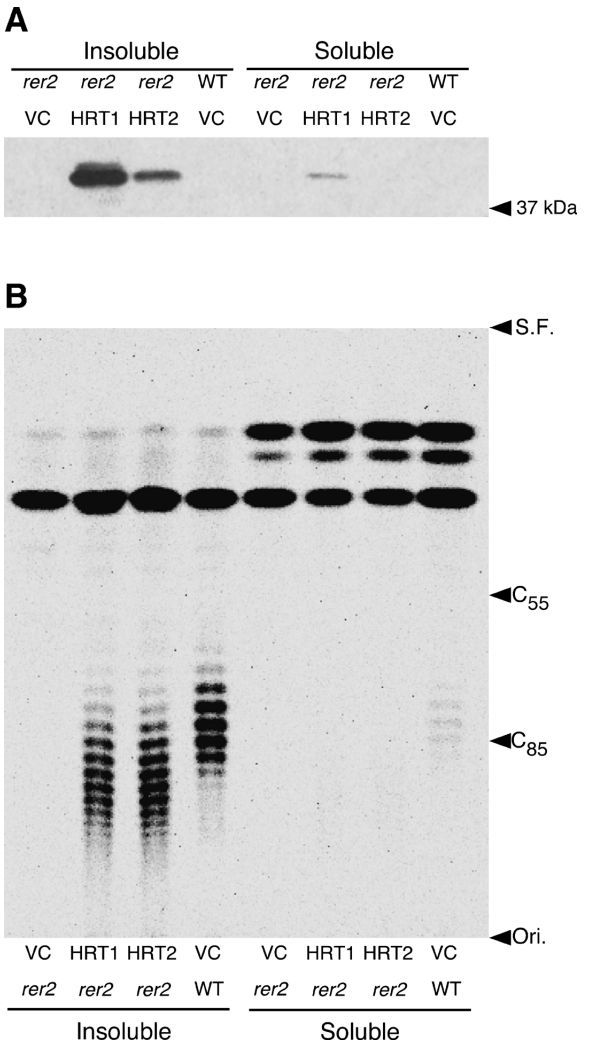

Figure 1. Prenyltransferase assays with crude proteins from yeast strains expressing HRT1 or HRT2. (A) Immunodetection of His_6 tagged HRT1 and HRT2 with an anti-His antibody. In each lane, 40 μg of crude proteins from the insoluble and soluble fractions were transferred to a PVDF membrane after SDS-PAGE. WT and *rer2*, shown above the lanes, indicates the host strains used, SNY9 and SNH23-7D, respectively. VC (vector control), HRT1 and HRT2 indicate constructs introduced into each strain, pJR1133, pJR-HRT1 and pJR-HRT2, respectively. (B) TLC analysis of the products synthesized by crude proteins from the insoluble and soluble fractions of the strains indicated below each lane, as in panel (A). Positions of authentic standards, *Z*,*E*-mixed undecaprenol (C_{55}) and *Z*,*E*-mixed polyprenol (C_{85}), are indicated. Three major radioactive products migrated faster than *Z*,*E*-mixed undecaprenol were formed by endogenous enzymes and were identified, by an independent TLC analysis, as *E*,*E*-farnesol, *E*,*E*-geranylgeraniol and all-*E*-hexapenol (data not shown). Ori.: origin, S.F.: solvent front.

were extracted from aqueous phase with 1 ml of water saturated 1-butanol. Polyisoprenoids with high molecular weights corresponding to natural rubber were subsequently extracted from the aqueous phase with 1 ml of a toluene/hexane (1 : 1) mixture (T/H). The incorporation of ^{14}C-IPP into the 1-butanol and T/H extracts was measured with an Aloka LSC-1000 liquid scintillation counter to determine prenyltransferase and rubber transferase activity, respectively. In this assay system, significant incorporation of [1-^{14}C]-IPP into the T/H extracts was not detected in any of the reactions with either crude soluble or membrane proteins from SNH23-7D/pJR1133, SNH23-7D/pJR-HRT1, SNH23-7D/pJR-HRT2 and SNY9/pJR1133. In contrast, incorporation into the 1-butanol extracts, corresponding to prenyltransferase activity, was detected in all samples tested. To analyze the chain lengths of the reaction products from HRT1 and HRT2, the 1-butanol extractable polyprenyl diphosphates were hydrolyzed with potato acid phosphatase, according to a published method (Fujii et al. 1982), to their corresponding alcohols, extracted with pentane, and analyzed by reversed-phase TLC (LKC-18, Whatman, Maidstone, Kent, UK) using an acetone/water (39 : 1) solvent system. Radioactive compounds on the TLC plate were analyzed using a BAS 1000 Mac Bioimage Analyzer (FUJIFILM, Tokyo, Japan). The insoluble membrane fraction prepared from the wild-type strain SNY9/pJR1133 synthesized ^{14}C-labeled polyprenyl products with chain lengths from C_{70} to C_{95}, with C_{75}, C_{80} and C_{85} being the predominant species (Figure 1B), which is consistent with dolichol chain-lengths in *S. cerevisiae* (Sato et al. 2001); whereas those from the cPT mutant strain SNH23-7D/pJR1133 resulted in very low levels of the corresponding products. In contrast, the insoluble membrane fractions from SNH23-7D/pJR-HRT1 and SNH23-7D/pJR-HRT2 synthesized polyprenyl products with chain lengths between C_{80} and C_{100}, with C_{95} and C_{100} being the predominant species (Figure 1B), indicating that HRT1 and HRT2 expressed in yeast exhibit distinct prenyltransferase activity. The chain length distribution of the products was shown to be independent of the type of the allylic primer substrate tested (i.e. *E*,*E*-FPP, *E*,*E*,*E*-GGPP and *Z*,*E*,*E*-GGPP; data not shown). These results indicate that HRT1 and HRT2 expressed in yeast do not show rubber transferase activity, but act as long-chain cPTs functioning in the general biosynthesis of *Z*,*E*-mixed polyisoprenoids, polyprenols and dolichols (Takahashi and Koyama 2006).

It has been reported (Asawatreratanakul et al. 2003) that only HRT2 can suppress a temperature-sensitive phenotype of *rer2-2* at 37°C, due to dolichol deficiency (Sato et al. 1999); suggesting that HRT1 does not have significant activity in yeast. In comparison, HRT1 can suppress growth retardation at a permissive temperature of 23°C (Figure 2) in *rer2-2*, which is concordant with the similar cPT activities of HRT1 and HRT2 *in vitro* (Figure 1B). However, weak suppression of the temperature sensitive growth phenotype at 37°C by HRT1 (Figure 2), in spite of the higher expression level of HRT1 relative to HRT2 (Figure 1A), indicates that the cPT properties of HRT1 in yeast were different from those of HRT2.

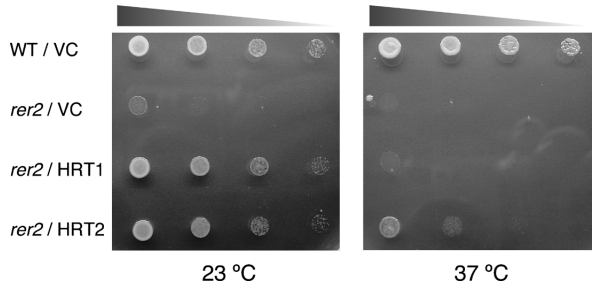

Figure 2. Functional complementation of *rer2-2* by HRT1 and HRT2. Yeast strains, SNY9 harboring pJR1133 (WT/VC) and SNH23-7D harboring pJR1133 (*rer2*/VC), pJR-HRT1 (*rer2*/HRT1) and pJR-HRT2 (*rer2*/HRT2), were cultured on SD/-Ura media at 23 or 37°C. Black slopes indicate the gradation of culture applied on plates (serial 5-fold dilutions).

To investigate the enzymatic functions of HRT1 and HRT2 expressed in plant systems, *Arabidopsis thaliana* T87 cells (Axelos et al. 1992) were transformed by infection with *Agrobacterium tumefaciens* GV3101(pMP90) harboring the binary vectors pBE-HRT1 and pBE-HRT2, according to a reported procedure (Suzuki et al. 2006). Due to significant levels of non-specific signals detected by immunoblotting against T87 crude extracts with the anti-His antibody, expressions of HRT1 and HRT2 in the transgenic T87 cell lines were confirmed (Figure 3A) by semi-quantitative RT-PCR with ReverTra-Plus (TOYOBO), in which first strand cDNAs, synthesized with oligo(dT)$_{20}$ primer and 50 ng of total RNA from each transgenic line, were used as templates to amplify the transgene by the PCR reaction, using HRT-S1 (5′-ttaggatccatggaattatacaacgg-3′) and HRT-A1 (5′-aacggatccttttaagtattccttatg-3′) primers, with 30 cycles of programmed temperature control of 98°C for 10 s, 55°C for 30 s and 68°C for 1 min. For the cPT assay, the transgenic T87 cell lines were grown in JPL medium (Jouanneau and Péaud-Lenoël 1967) with gentle agitation (120 rpm) under continuous illumination of ≈60 μmol/s/m^2 at 22°C for 7 days after subculturing. The cells were subsequently harvested, disrupted in a mortar and pestle under liquid nitrogen, and homogenized in a protein extraction buffer [20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 5% (w/v) glycerol, 3 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A]. Cell lysates were centrifuged at 300×g for 5 min at 4°C to remove intact cells and the supernatants further centrifuged at 20,000×g for 15 min at 4°C to separate the membrane and soluble fractions. The prenyltransferase and rubber transferase activities of T87 cells expressing HRT1 or HRT2 were examined in reactions containing 50 μg of crude protein from each fraction at 30°C for 120 min in assay mixture (described above). As with HRT1 and HRT2 expressed in yeast, significant incorporation of [1-^{14}C]IPP into

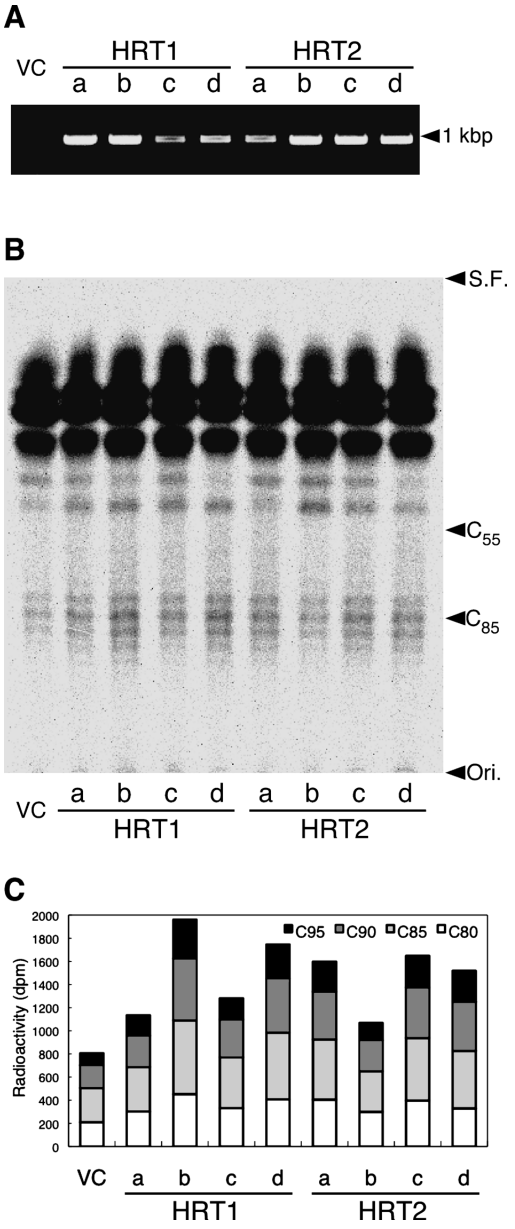

Figure 3. Prenyltransferase assays with crude proteins from T87 cells expressing HRT1 and HRT2. Four transgenic lines (a to d) harboring pBE-HRT1 or pBE-HRT2 were analyzed. VC: vector control line (T87/pBE2113Not). (A) Semi-quantitative RT-PCR analyses of *HRT1* and *HRT2* in the transgenic T87 cell lines. The PCR products were analyzed by agarose gel electrophoresis. (B) TLC analysis of the products synthesized by the membrane fractions from the transgenic T87 cell lines. *Z*,*E*-mixed undecaprenol (C_{55}) and *Z*,*E*-mixed polyprenol (C_{85}) were used as authentic standards. Ori.: origin, S.F.: solvent front. (C) Distribution of the radioactivity in the products with chain length of C_{80-95} on the TLC shown in (B).

the T/H extracts was not detected in assays with any of the crude protein samples (data not shown); indicating the absence of rubber transferase activity in HRT1 and HRT2 expressing *Arabidopsis* cells. Whereas, reversed-phase TLC analyses of hydrolyzed 1-butanol-extracts using a BAS 1000 Mac Bioimage Analyzer and Image Gauge software (FUJIFILM) showed a moderate increase

in prenyl products with chain-lengths from C_{80} to C_{95}, composed mainly of C_{90-95} polyisoprenoids (Figs. 3B and 3C), in reactions with crude membrane fractions from cell lines expressing HRT1 or HRT2. Taken together, HRT1 and HRT2 expressed in a non-rubber producing plant may act as long-chain cPTs showing similar properties (product chain-length) to those observed in a yeast expression system.

In order to elucidate the effects of the latex components on HRT1 and HRT2 expressed in a eukaryotic system, WBPs, C-serum and SRPs were prepared from the fresh latex of *H. brasiliensis* (RRIM 600) by ultracentrifugation at 49,000×*g* for 45 min at 4°C, followed by repeated washing with 50 mM Tris-HCl (pH 7.4) containing 0.9% NaCl (w/v) according to the method of Wititsuwannakul *et al.* (2003). After protein concentration determination in each fraction, using the Bradford Protein Assay (Bio-Rad, Hercules, California, U.S.), the prenyltransferase and rubber transferase activities were examined in reactions containing 50 μg crude proteins from each latex fraction and various amounts of proteins from yeast strain membrane fractions at 30°C for 120 min (as described above). After stepwise extraction of the products with solvents, followed by measurement of ^{14}C-IPP incorporation into each extract, the 1-butanol extractable polyprenyl diphosphates were hydrolyzed, extracted with pentane, and analyzed by reversed-phase TLC (as described above). The molecular size distribution of the radioactive products in the T/H extracts was analyzed by gel permeation chromatography (GPC) using 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)] with exclusion limits of 4×10^8, 4×10^6, 2×10^4 and 10^3 Da, respectively. The chromatography was carried out at 35°C using tetrahydrofuran as the eluent, at a flow rate of 0.5 ml/min. The eluates were monitored by UV absorption at 210 nm following collection at 1 min intervals, and radioactivity was counted using a liquid scintillation counter. The molecular mass of the reaction products was estimated by comparison with the retention times of commercially available polyisoprene standards (Polymer Standards Service, Mainz, Germany).

In assays using WBPs and C-serum without yeast proteins, incorporation of [1-^{14}C]IPP into the T/H extracts was quite low and no radioactive products corresponding to natural rubber were detected by GPC analysis (data not shown). Furthermore, and in contrast to the results obtained with HRT2 expressed in *E. coli* (Asawatreratanakul et al. 2003), neither WBPs nor C-serum increased the rubber transferase activity of HRT1 or HRT2 in insoluble membrane fractions. In assays of SRPs in the absence of yeast proteins, incorporation of [1-^{14}C]IPP into the T/H extracts was much higher than those with WBPs and C-serum, whereas incorporation into 1-butanol extracts was quite low (Figure 4A). These results indicated that SRPs sustained a high level of rubber transferase activity after

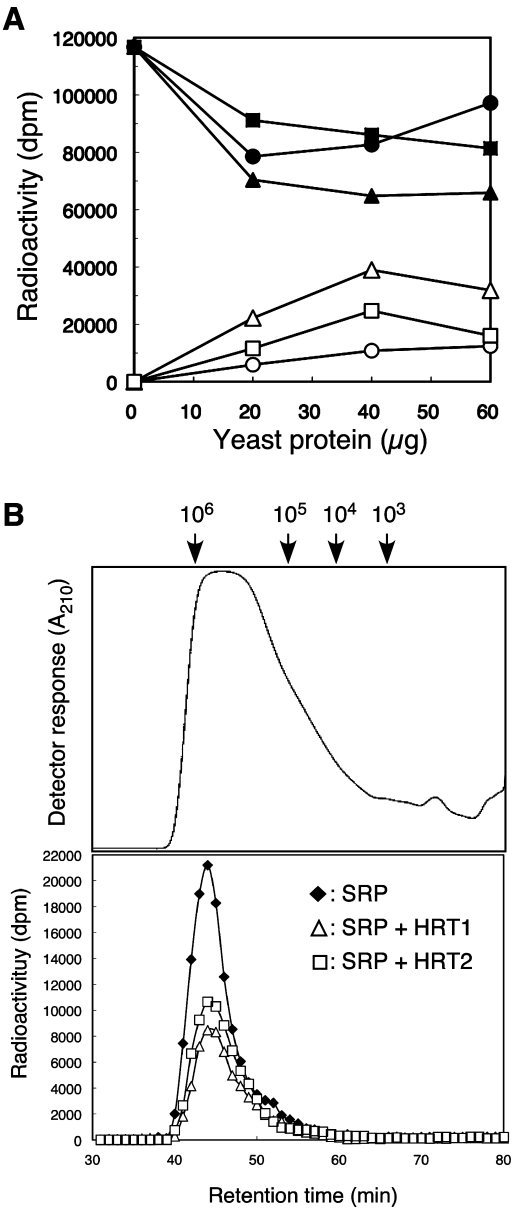

Figure 4. Assays for prenyltransferase and rubber transferase in SRPs. (A) Incorporation of ^{14}C-IPP into T/H extracts (filled symbols) and 1-butanol extracts (open symbols) from the assay with indicated amount of yeast crude proteins (membrane fractions), from SNH23-7D/pJR1133 (circles), SNH23-7D/pJR-HRT1 (triangles) and SNH23-7D/pJR-HRT2 (squares), incubated with 50 μg of SRP proteins. (B) GPC analysis of the T/H-extractable reaction products derived from the *in vitro* rubber transferase assay. Upper panel: molecular mass distribution of endogenous rubber molecules contained in SRPs, detected by UV absorption at 210 nm. Lower panel: molecular mass of ^{14}C-labeled products synthesized *in vitro* by SRPs only (SRP), SRPs supplemented with 60 μg crude protein (membrane fraction) from SNH23-7D/pJR-HRT1 (SRP+HRT1) or SNH23-7D/pJR-HRT2 (SRP+HRT2). The elution peaks of the commercially available polyisoprene standards were indicated at the top of upper panel.

separation by ultracentrifugation. However, ^{14}C-IPP incorporation into the T/H extracts was reduced by the addition of insoluble membrane fractions from pJR1133/SNH23-7D, pJR-HRT1/SNH23-7D and pJR-HRT2/SNH23-7D, which coincided with increased incorporation into 1-butanol extracts (Figure 4A). GPC analysis of the T/H extracts revealed that crude yeast proteins containing HRT1 or HRT2 affected rubber transferase activity in SRPs, but not the molecular size distribution of the radioactive products (Figure 4B Lower panel), which was consistent with results for natural rubber endogenous to SRPs (Figure 4B Upper panel). These results indicated that HRT1 and HRT2 expressed in yeast competed with SRP enzymes in the rubber transferase reaction by consuming IPP, and that HRT1 and HRT2 did not alter their rubber transferase activity through interaction with co-factor(s) present in SRPs.

In conclusion, HRT1 and HRT2, expressed in eukaryotic cells, show distinct prenyltransferase activities. However, HRT1 and HRT2 behave as long-chain cPTs rather than rubber transferases, producing C_{80-100} polyisoprenoids. Moreover, their catalytic properties are not altered to rubber transferase by the addition of latex fractions, such as WBPs, C-serum and SRPs. These results, taken together with the observation that HRT1 and HRT2 expressed in *E. coli* do not show significant prenyltransferase activity in isolation, indicate that HRT1 and HRT2 require certain co-factors from eukaryotic cells to exhibit cPT activity. As with HRT1 and HRT2, long-chain cPTs from *A. thaliana* (Cunillera et al. 2000), *S. cerevisiae* (Schulbach et al. 2000) and human (Schulbach et al. 2001) do not show significant cPT activity *in vitro* when expressed in *E. coli*. One possible factor in the activation of enzymes is eukaryotic post-translational modifications, such as glycosylation in the ER, which could account for the increase in apparent molecular weight of HRT1 and HRT2 expressed in yeast (Figure 1A). However, the involvement of HRT1 and HRT2 in rubber biosynthesis in *Hevea* latex remains to be clarified. To elucidate the precise mechanism for rubber biosynthesis, we are currently analyzing the binding partners of HRT1 and HRT2 in latex and attempting to purify active rubber transferase from *Hevea* latex.

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