cDNA, from *Hevea brasiliensis* latex, encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase

Kruecawan Yoonram[1], Seiji Takahashi[2], Atiya Rattanapittayaporn[1], Tanetoshi Koyama[2], Dhirayos Wititsuwannakul[3], Rapepun Wititsuwannakul[1,*]

[1] Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat -Yai, Songkhla 90112, Thailand

[2] Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai, Japan

[3]Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

[*]Corresponding Author: rapepun.w@psu.ac.th; wrapepun@yahoo.com

Department of Biochemistry

Faculty of Science

Prince of Songkla University

Hat-Yai, Songkhla 90112, Thailand

Tel: 66-74-288-266

Fax: 66-74-446-656

Abstract

Natural rubber from *Hevea brasiliensis* is synthesized by enzymic polymerization of isopentenyl diphosphate (IDP) units. This has been proposed to occur inside the latex vessel in a thread-like tubular reticulum, connecting rubber particles to single- (lutoid) and double- (Frey-Wyssling, FW) membrane-bound organelles. We show that a membrane free preparation from FW particles converted [^{14}C] glucose into radio labeled prenyl products and this was only partially inhibited by mevinolin an inhibitor of the MVA cytosol associated pathway for IDP synthesis This implicated the alternative plastid associated MEP pathway for IDP synthesis. We then identified a cDNA clone (*Hbdxr*) from a *Hevea* rubber latex cDNA library, encoding for 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), a key enzyme of the MEP pathway for the IDP biosynthesis. Sequence analysis and the deduced amino acid sequence had >80% homology to other plant DXR enzymes with an ORF consisting of 1413 bp capable of encoding a 50.97 kDa polypeptide. A highly conserved binding site for NADPH was identified and an N-terminal transit peptide with a putative conserved cleavage site. The *Hbdxr-gfp* gene, transformed into *Arabidopsis* cells was located in the chloroplasts, thus *Hbdxr* may be expressed and localized in the FW plastids. The levels of *Hbdxr* mRNA detected in young latex containing tissues, inflorescence and seedling stems, were higher than those found in the latex from the tree and were barely detectable in the mature leaves. We therefore suggest that one function of the FW particles could be to supply IDP for rubber biosynthesis through the tubular thread-like reticulum.

Keywords

Hevea brasiliensis; Euphobiaceae; 1-Deoxy-D-xylulose-5-phosphate reductoisomerase; Frey-Wyssling particles; Rubber latex; Rubber biosynthesis

1. Introduction

Hevea rubber latex is obtained as the white-colored fluid that exudes from the trunk of rubber *(Hevea brasiliensis)* trees after tapping. The tapping severs a number of latex vessel rings, allowing the latex to flow out. All the components present in the vessel ring, at their different stages of development, can be found in the tapped latex. The latex contains particles the majority of which are rubber, lutoids, and Frey-Wyssling [1-3].The rubber particles consist mainly of a highly hydrophobic rubber core surrounded by a lipid monolayer containing a few embedded proteins [4].The lutoids are vacuoles enclosed by a single membrane and because of their high acid hydrolase content [5] are considered to be phytolysosomes [6]. The Frey-Wyssling (FW) particles are surrounded by a double membrane and contain many membranes or tubular structures. They are specialized chromoplasts containing plastochromonol, plastoquinone and β-carotene with characteristic orange to yellow colors and assumed to be modified plastids [7-12]. The highly complicated structure of FW particles indicates that they have important functions in the metabolism of *Hevea* latex [8]. When viewed with a phase contrast microscope, these three major latex particles seen in fresh latex after being collected at a low temperature, seem to be attached to a network of threads or a thread-like reticulum,. The yellow FW particles were found in grape-like clusters enclosed within a bag-like membrane. The lutoids were strung along the reticulum thread, like beads, and the rubber particles were attached through their stalks [13]. The different particulate materials in fresh latex can be separated by centrifugation with the rubber particles in the top layer, the middle fraction composed of the aqueous C serum, and the sedimented bottom fraction of lutoid and FW particles. It has been suggested that the thread-like structures, commonly observed throughout the centrifuged fresh latex, represent the remnants of an extensive reticulum of hollow thread-like processes, within the latex vessel to which the various particles attach and through which they interact. It has been proposed that this thread-like reticulum could serve as the site for the biosynthesis of the natural rubber hydrocarbon [13]. In support of this possibility we have recently provided evidence for an active involvement of the latex

bottom fraction, containing the sedimented lutoid and FW particles, in rubber biosynthesis [14, 15]. The rubber is found in the top layer of rubber particles so it is possible that the tube –like reticulum acts as a conduit between these particles.

Natural rubber is synthesized and made almost entirely of *cis*-isoprene units derived from IDP. An allylic diphosphate is, however, required as the priming co-substrate to initiate the subsequent extensive prenyl chain elongation process for the formation of the rubber molecule [16-18]. Synthesis of the allylic prenyl diphosphates are catalyzed by *trans*-prenyl transferase enzymes, that were found in both the bottom fraction and the cytosol C-serum of centrifuged fresh *Hevea* latex [3, 19]. The allylic diphosphates of chain length C_5-C_{20} have been shown to stimulate *in vitro* rubber formation, the efficiency of which increases with the increasing chain length of the allylic diphosphates, i.e. $C_5 < C_{10} < C_{15} < C_{20}$ [16]. A structural analysis study to characterize the initiating species of *Hevea* rubber has indicated that farnesyl diphosphate (FDP) is the preferred starting molecule for rubber biosynthesis [20]. Recently, the expression of *Hevea* FDP synthase in rubber latex was shown to be much higher than that of the geranylgeranyl diphosphate (GGDP) synthase [21]. The rubber transferase enzymes responsible for the *cis*-1, 4-polymerization of isoprene units onto the allylic primer have until now been thought to be bound to particles [22-24].

Since a laticiferous vessel is specialized in synthesizing the rubber polymer, it must have a means for a sufficient and efficient supply of IDP as this is the most important precursor required for the biosynthesis of rubber [25]. In higher plants, IDP is formed by two pathways that operate in different subcellular compartments [26-28]. The well-known mevalonate (MVA) pathway is present in the cytosol-endoplasmic reticulum while the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway is localized in the plastid. The two pathways start from different central intermediates: the MVA pathway starts from acetyl-CoA whereas the MEP pathway starts from pyruvate and glyceraldehyde 3-phosphate. Within the MEP pathway, the initial enzymatic step involves the condensation of glyceraldehyde 3-phosphate and pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DXP),

catalysed by DXP synthase (DXS), the product of the *dxs* gene. The next reaction, the conversion of DXP to MEP, is catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), encoded by the *dxr* gene. The DXP is an intermediate precursor of not only the IDP but is also a common precursor for thiamine pyrophosphate and pyridoxol phosphate [29, 30]. Thus, the reaction catalyzed by DXR is thought to represent the first committed step in the MEP pathway. Ko et al. [31] have previously reported the expression of a gene encoding DXS in the *Hevea* latex, thus the MEP pathway is initiated in the latex. The high level of latex *dxs* gene expression reported is compatible with a possible involvement of the MEP pathway in supplying the isoprene precursor for the synthesis of the major rubber (polyisoprene) product, in addition to other minor isoprenoids, in the *Hevea* latex.

To gain further insights into the MEP pathway in a non photosynthetic latex vessel, with the possibility of implicating it in rubber biosynthesis, we decided to attempt to clone the *dxr* gene from the latex of the rubber tree. Our previous detection of radio labeled prenyl products in the isolated FW particles after incubation with [^{14}C] glucose gave us some confidence that this might be possible. So far, the detailed biological function of these particles in latex, besides their possible involvement in carotenoid biosynthesis, has not been established [3]. In this report we describe the isolation and characterization of latex cDNA encoding the *dxr* gene.

2. Experimental

2.1 Materials

E. coli JM109 and pGEMT-easy vector (Promega) were used for the TA cloning. *E. coli* BL21 (DE3) and pET-32(a+) vector (Novagen) were used for expression of the recombinant protein. [1-^{14}C]Glucose was from Amersham. Precoated reverse phase TLC plates (RP-18) were from Whatman Chemical Separation. Restriction enzymes were from

Takara (Tokyo, Japan), TOYOBO (Osaka, Japan) and New England Biolabs, Inc. DXP was purchased from Echelon Research Labs (Salt Lake City, UT, USA)

2.2 Plant materials and RNA isolation

Fresh *Hevea* latex and other tissue samples were obtained from regularly tapped rubber trees at the Songkhla Rubber Research Institute, Songkhla, Thailand. *Hevea* seeds were collected during a fruit-bearing season, September–October, germinated in sterile sand and grown into 5-week-old seedlings. Total RNAs of latex, leaves, inflorescences and seedling stems were isolated by using the RNeasy Plant Mini Kit (Qiagen).

2.3 Frey-Wyssling particle isolation

FW particle isolation was carried out as described by Phatthiya *et al.* [12]. Briefly, fresh latex was subjected to a series of centrifugations. First, a low speed centrifugation (1700 × *g* at 10°C for 30 min), using swing-out bucket rotors, was employed to minimize inter-particle collisions, especially between FW and lutoid particles, to allow all the particles to travel down the centrifuge tube through the viscous latex medium according to their density gradients. The upper half of the centrifuged latex, containing the less dense rubber particles, was removed whereas the lower half was further separated, by using a fixed angle rotor centrifuge (7000 × *g* at 4°C for 15 min), into three distinct layers: a minor uppermost rubber fraction with yellowish FW particles lying beneath, a middle aqueous fraction and a bottom fraction containing the most dense lutoid particles. After careful removal of the uppermost rubber layer, the floating yellowish-orange FW particles were collected from the top layer of the aqueous phase and collected as a pellet by ultracentrifugation (59,000 × *g* at 4 °C for 20 min). The pellet was washed once by resuspending in 2 vol. of washing buffer (50 mM Tris-HCl, pH 7.4) and kept at -20 °C until used.

2.4 Assay of radioactivity incorporation from [^{14}C] glucose into prenyl products

The washed FW particles were sonicated with 10 short 10 sec bursts interspersed by intervals of 10 sec for cooling. FW membranes were removed by centrifugation at 10,000 × g at 4 °C for 10 min). An aliquot (100 µl containing 280 µg of protein) of supernatant was added to a 500 µl reaction mixture containing 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 30 mM KF, 0.15 NADPH, 74 µM mevinolin (when required) and 1 mM [^{14}C]glucose (5 Ci/mol). After the reactions were incubated at 37°C for 6 h, radioactive prenyl diphosphate products were extracted with 1-butanol saturated with water and hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously [32]. The hydrolyzed products were extracted with pentane and analyzed by TLC on a reverse LKC-18 plate (Whatman) with acetone/water (19:1, v/v). The distribution of radioactive products on TLC plate was analyzed with a Fuji BAS-100 Mac Bioimage analyzer and spots of marker prenols were visualized with iodine vapor.

2.5 Cloning of Hevea dxr fragment

The cDNA fragment containing the region corresponding to the DXR was amplified from the *Hevea* latex cDNA library that had been constructed using the ZAP-cDNA Synthesis Kit (STRATAGENE). Degenerate primers were designed to amplify the *Hevea dxr* cDNA fragment according to the most highly conserved amino acid sequences of previously cloned genes encoding for plant *dxr*; sense primers, F1 (ALAAGSNV) 5′-GCNCTNGCNGCNGGNTCNAAYGT-3′, F2 (VVTGIVGC) 5′-GTNGTNACNGGNATHGTNGGNTG-3′, and F3 (VAAIEAGK) 5′-GTNGCTGCNATHGA AGCNGGNAA-3′ and antisense primers, R1 (AQLGWPDM) 5′-CATATCNGGCCANCCNARYTGNGC-3′, R2 (TWPRLDLC) 5′-GCANAGRYCNAGNCKNGGCCANG-3′, and R3 (MTGVLSAA) 5′-GCNGCRCTNAGA ACTCCNGTCAT-3′, where R, Y, K, H and N represents A/G, C/T, G/T, A/C/T and A/C/G/T respectively. PCR was performed in a final volume of 50 µl containing 20 mM

Tris-HCl buffer (pH7.5), 25mM $MgSO_4$ 0.2 mM each dNTP mixture, 1 μM each primers, 0.1 μg template and 1 unit KOD-Plus DNA polymerase(Toyobo). The PCR program used was 35 cycles of 94°C for 15 s, 50°C for 30 s, 68°C for 1 min (Mastercycler Eppendorf). The amplified DNA fragments were purified from agarose gel by using QIAquick Gel Extraction Kit (Qiagen) and subcloned into the TOPO TA cloning® Kit (Invitrogen) for sequencing. The resulting clones were verified by homology-based database searching using the BLASTN program. An internal 705 bp DNA fragment amplified by F1 and R1 primers which showed homology to plant *dxrs* was termed HDR705.

RLM-RACE-PCR was used for cloning the full-length cDNA of *Hevea dxr*. The procedures were according to the manufacturer's instructions (GeneRacer Kit, Invitrogen). Gene specific primers for performing both 5′- and 3′- RACE reactions were designed based on the sequence of HDR705. The primers used for 5′-RACE were 5′-TCCAGCTTCTATTGCAGCCACCG-3′ (GSP1) and 5′-CTACACTGACAGCATCTGGATGG-3′ (GSP2), respectively, and those used for 3′-RACE were 5′-TGGAGCTTTCAGGGATTGGCCTG-3′ (GSP3) and 5′-CAGTGGACTCCGCTACCCTTTC-3′ (GSP4), respectively. According to the sequence information obtained by the 5′- or 3′- RACE reaction, the cDNA was amplified by RT-PCR using the total latex RNA as the template and then sequenced. The final full length cDNA obtained was designated *Hbdxr*.

All cDNA fragments were sequenced with a Model 310 Genetic Analyzer (PE Biosystems) using a BigDye Terminator Cycle Sequencing Kit. Computer analysis and comparison of DNA sequences were carried out using GENETYX genetic information software (Genetyx Corp., Tokyo, Japan).

2.6 Analysis of Hevea dxr expression

For RT-PCR, the total RNA was extracted from inflorescences, seedling stems, leaves and latex. A quantity of 1µg of total RNA was used as a template for synthesis of the first strand cDNA using Omniscript® Reverse Transcriptase (QIAGEN). PCR amplification was performed with first strand cDNA and *Hbdxr* specific primers, GSP4 and GSP5 (5'-TCATGCAAGAACAGGGCTTAGACCAG-3'). The reactions were carried out with 28 cycles of 15 s at 94°C, 30 s at 54 °C and 1 min at 68 °C with 2 min preheat at 94 °C. A 18s rRNA fragment was amplified for comparison. The amplified PCR products were analyzed by agarose gel electrophoresis.

2.7 Subcellular localization of Hevea DXR

The gateway entry clone harboring *Hbdxr* without a stop codon was applied for recombination with a Gateway destination vector. The pGWB5, kindly provided by Dr. Tsuyoshi Nakagawa (Shimane University, Japan), was used for construction of a binary vector to express HbDXR fused with sGFP at the C-terminal region in plant cells. The resulting plasmid was introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) by triparental mating. *Arabidopsis* T87 cultured cells [33] were transformed by co-cultivation with GV3101 (pMP90) carrying the resulting construct as reported [34]. A stationary phase culture of *A. tumefaciens* was washed and resuspended with the same amount of JPL medium [35]. Seven-day-old T87 cultured cells grown in JPL medium under continuous illumination at 22°C rotating at 120 rpm were filtered through a 0.5-mm nylon sieve. The filtered culture was diluted 5 times in fresh JPL medium and cultured to grow until 20-40 mg fresh weight/ml medium was obtained. Then 5 ml of the cultured cells were incubated with 50 µl of the *Agrobacterium* suspended in JPL medium in a 100 ml Erlenmeyer flask by gentle agitation (120 rpm) under continuous illumination at 22°C. After 48 h, the cells were collected, washed three times in the same amount of JPL medium supplemented with 200 mg/l claforan (Hoechst Marion Roussel, Tokyo, Japan). The cells were resuspended with 5 ml of JPL medium supplemented with 200 µg/ml claforan and cultured again for 3-4 days in a 100 ml Erlenmeyer flask. The co-cultivated cells were plated on cell growth

medium, containing 0.5 g/l MES (pH 5.7), 3.3 g/l Gamborg's B5 medium salt mixture (Wako Pure Chemical Industries, Osaka, Japan), B5 vitamin (SIGMA, MI, USA), 3% (w/v) sucrose, 0.2 mg/l a-NAA, 200 mg/l claforan and 0.6% Bact-Agar, on which a nylon membrane was over-laid, and cultured under continuous illumination at 22°C for three days. The nylon membranes were transferred to new cell growth medium supplemented with 200 mg/l claforan and 10 μg/ml hygromycin and stored until green calli formation (for about two-weeks). Small cell aggregates of calli were transferred to JPL medium containing 200 mg/l claforan. The suspension was kept under continuous illumination at 22°C rotating at 120 rpm. After 7-10 days, an aliquot of cell suspension was transferred to fresh medium containing 200 mg/l claforan and 10 μg/ml hygromycin and maintained as an independent cell line.

For GFP imaging, T87 cells expressing GFP-fused HbDXR were observed with a BX50 microscope equipped with a FLUOVIEW confocal scanning system (OLYMPUS, Tokyo, Japan). The green (GFP) and red (chlorophyll autofluorescence) emissions were detected with filters BA510IF-BA550RIF and BA585IF, respectively. The images were then pseudo-colored and combined into one image.

3. Results

3.1 [^{14}C] labeled prenyl products derived from [^{14}C] glucose

A membrane-free extract, prepared from washed FW particles, was incubated with [^{14}C] glucose in order to evaluate the presence of the MEP pathway in the putative FW plastids. An autoradiogram of the total acid phosphatase treated prenyl products, derived from [^{14}C] IDP, revealed a major component, [^{14}C] C_{20}-OH with [^{14}C] C_{15}-OH and [^{14}C] C_{45}-OH as minor products (Fig.1, lane 1). This result may indicate the presence of the MEP pathway

in the FW particles. For further confirmation, mevilonin (74 μM) was added to the incubation mixture to inhibit HMG-CoA reductase, a rate-limiting enzyme of the MVA pathway that might be present due to contamination by residual latex cytosol and the thread-like reticulum in the original isolated FW particles. The concentration of mevinolin employed was about three times higher than that reported to completely inhibit the HMG-CoA reductase in *Parthenium argentatum* bark [36]. An autoradiogram of the prenyl alcohol products produced from [^{14}C] glucose gave a similar pattern to the original without the inhibitor but the intensity was reduced by approximately one-half (Fig. 1, lane 2). This result confirmed that the MEP pathway was present in the FW particle preparation. An allylic diphosphate, especially the C_{15}-diphosphate (FDP), had been identified as a species necessary for initiating rubber formation [21]. The presence of [^{14}C] C_{15}-OH in the autoradiogram indicated that the FW particles may be also involved in providing the initiating primer for the rubber biosynthesis. This warranted an attempt to detect and isolate the gene encoding for *Hevea* 1-deoxy-D-xylulose-5-phosphate reductoisomerase (HbDXR), a rate limiting enzyme in the MEP pathway. This assists in establishing a biological function for the FW particles in *Hevea* latex synthesis.

3.2 Cloning of a full-length cDNA encoding Hevea DXR

For the cloning of *Hevea dxr*, a BLAST database search was conducted using DXR as the query. From the four previously isolated plant cDNA's encoding for *dxr* from, *Arabidopsis thaliana* [37], *Lycopersicon esculentum* [38], *Oryza sativa* [37] and *Stevia rebaudiana* [39], three sets of degenerated primers were designed to align with highly conserved regions. When PCR was conducted using a latex cDNA library as a template with the primer pair of F1 and R1, an amplified product of 705 bp was obtained and sequenced. The sequence was subsequently verified by using the BLAST program in a search of the NCBI database. This showed that it had a high sequence homology to the other plant DXRs. Based on the sequence of this DNA fragment, two sets of gene specific primers were designed to generate 5′- and 3′- ends using RNA ligase-mediated rapid amplification cDNA 5′ and 3′

cDNA ends (RLM-RACE) PCR. The GSP1 and GSP2, in combination with a 5′-oligo primer and 5′-nested oligo primer, were used to amplify the 5′-end of cDNA. A 511 bp product was obtained by nested PCR. For the 3′- RACE, the first PCR was performed with GSP3 and oligodT primer. After 3′-nested PCR with GSP4 and the 3′- nested primer, a 572 bp fragment was obtained. Three contiguous regions were assembled, and the resulting full-length cDNA contained an open reading frame (ORF) of 1,413 bp capable of encoding a protein of 471 amino acids. We termed this clone *Hbdxr*. The GenBank accession number of this cDNA had been assigned as **DQ437520**. The amino acid sequence deduced from the nucleotide sequence was compared with the NCBI databases. It had a very high homology throughout the entire ORF with the plant *dxrs*: *L. esculentum* (86%), *A. thaliana* (82%), *S. rebaudiana* (82%), and *O. sativa* (80%) (Fig. 2).The highly conserved GSTGSIG motif, proposed to constitute the NADPH binding site of a ketol acid reductoisomerase [40], was present near the N-terminal of HbDXR (positions 81-87).

To characterize the sequence of the N-terminal region, two prokaryotic DXRs; *E.coli* [40] and *Synechococcus leopoliensis* [41] DXRs were aligned with the plant DXRs. The plant enzyme contains an extension of about 80 residues that is not present in the prokaryotic sequence. The extended region at the N-terminal of the plant DXRs indicated that it was likely to be a transit peptide for a plastid protein and contained a conserved cleavage site [37]. A putative conserved processing site, Cys-Ser-Ala motif, was present at positions 49-51 of the HbDXR sequence. The extended region at the C-terminal side of the putative cleavage site contains a consensus motif PPPPAWPGRA (positions 53-62 of HbDXR), defined as the Pro-rich region at the N-terminus of the mature plant DXR [40].

Phylogenetic analysis, based on the alignment of plant *dxrs*, revealed a relationship between the phylogeny of *dxr* sequences and phylogenic distances (Fig. 3). There appear to be three major lineages of these genes; gymnosperms, monocots, and eudicots. The eudicot genes formed two major clades, rosids and asterids. The *Hevea* gene was most closely

related to the one from *Croton stellatopilosus* (GenBank accession no EF451544) which is in the same Euphorbiaceae family as the rubber tree.

3.3 Expression analysis of Hevea mRNAs

To examine the *Hbdxr* mRNA expression in various *Hevea* tissues,
RT-PCRs using total RNA extracted from inflorescences, seedling stems, leaves and latex were performed. As shown in Fig. 4, the levels of PCR products in the inflorescences and young stems were higher than that of the latex tapped from the tree. The PCR product was, however, barely detected in the mature leaves.

3.4 Subcellular localization of Hevea DXR

HbDXR was predicted by the ChloroP algorithm (http://www.cbs.dtu.dk/services/ChloroP/) to have a putative plastid transit peptide in the 80 amino acid region at the N-terminus thus indicating the function of HbDXR in the plastid. As expected, a fluorescence of HbDXR fused with GFP at the C-terminal region was detected in chloroplasts of *Arabidopsis* cultured cells, and this was confirmed by an overlapping of the autofluorescence of chloroplasts (Fig. 5).

4. Discussion

The presence of [^{14}C] C_{45}- prenyl DP products, derived from [^{14}C] glucose, on the autoradiogram from the membrane free preparation derived from sedimented FW particles indicated a successful preparation able to initiate synthesis of prenyl DP products. Previously, C_{45} prenyl side chains of plastoquinone-9 had been detected in the FW particles isolated from fresh *Hevea* latex [12] and C_{15}-, C_{20}- and C_{45}–prenyl DP products had been detected among the prenyl DP extraction products from a freeze-dried bottom fraction, containing both lutoid and FW particles, after incubation with [^{14}C] IDP [19]. The allylic

diphosphate, especially FDP, had been identified as an initiating species for rubber formation [20]. Hence, it is interesting to see whether the FW particles make a major contribution for providing both the initiating primer and the IDP, from the MEP pathway, for *in vivo* rubber formation in the proposed *Hevea* latex thread-like reticulum [13]. Moreover, the involvement of an endoplasmic reticulum had previously been reported in the ontogeny of rubber formation in guayule, *Parthenium argentatum* [42].

A *Hevea* rubber latex gene (*Hbdxr*) encoding for the rate limiting enzyme in the MEP pathway was successfully cloned from rubber latex cDNA. This gene contains a putative ORF encoding a protein consisting of 471 amino acid residues with a predicted molecular mass of 50.97 kDa. Accordingly, the study on the localization of HbDXR in the chloroplast of *Arabidopsis* cells indicated that HbDXR has a distinct transit peptide function in translocation of the HbDXR to a plastid. Latex FW particles have been assumed to be modified plastids due to the presence of plastoquinone, plastochromanol and isoprenoids [11, 12]. Hence, the *Hbdxr* may be expressed and localized in the latex FW plastid-like particles. Accordingly, the levels of *Hbdxr* mRNA transcripts in young latex containing tissues, inflorescences and seedling stems, were shown to be higher than that of the latex tapped from mature latex vessels localized in the bark of the rubber tree. This result is opposite to the barely detectable transcript found in the leaves rich in light gathering plastids. Thus, this *Hbdxr* homolog may be specific to the putative FW plastids specialized in the biosynthesis of rubber. It remains to be seen whether the IDP and FDP required for the biosynthesis of rubber polymer, in the proposed thread-like reticulum, is also mainly contributed by the FW particles.

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References

1. S. Hamzah, J. B. Gomez, Electron microscopy of ultracentrifugation fractions of *Hevea* latex. I. Top fractions, J. Rubb. Res. Inst. Malaysia. 30 (1982) 161-173.
2. S. Hamzah, J. B. Gomez, Electron microscopy of ultracentrifugation fractions of *Hevea* latex. II. Bottom fractions, J. Rubb. Res. Inst. Malaysia, 31 (1983) 117-128.
3. D. Wititsuwannakul, R. Wititsuwannakul, Biochemistry of natural rubber and structure of latex. In: Steinbuchel, A. (Ed.), Biopolymers, vol. 2, WILEY-VCH, Germany, 2001, pp. 151-202.
4. K. Cornish, D. F. Wood, J. J. Windle, Rubber particles from four different species, examined by transmission electron microscopy and electron-paramagnetic-resonance spin labeling, are found to consist of a homogeneous rubber core enclosed by a contiguous, monolayer biomembrane, Planta 210 (1999) 85-96.
5. J. Dupont, F. Moreau, C. Lance, J. L. Jacob, Phospholipid composition of the membrane of lutoids from *Hevea brasiliensis* latex, Phytochemistry 15 (1976) 1215-1217.
6. S. Pujarniscle, Caractère lysosomal des lutoïdes du latex d'*Hevea brasiliensis*. Müll Arg, Physiol. Veg. 6 (1968) 27-46.
7. P. B. Dickenson, The ultrastructure of the vessel of *Hevea brasiliensis*. In: Mullins, L. (Ed), Proc. Natl. Rubber Prod. Res. Assoc., Jubillee Conf. Mc Laren and Sons, London, 1965, pp. 52-56.
8. P. B. Dickenson, Electron microscopical studies of latex vessel system of *Hevea brasiliensis*, J. Rubb. Res. Inst. Malaya. 21 (1969) 543-559.
9. G. F. J. Moir, Latex metabolism, J. Rubb. Res. Inst. Malaya 21 (1969) 401-416.
10. B. L. Archer, B. G. Audley, G. P. Mc Sweeney, T. C. Hong, Studies on the composition of latex serum and bottom fraction, J. Rubb. Res. Inst. Malaya 21 (1969) 560-569.

11. N. Ohya, T. Koyama,Biosynthesis of natural rubber and other natural polyisoprenoids. In: Steinbuchel, A. (Ed.), Biopolymers, vol. 2, Wiley-VCH, Germany, 2001, pp. 73-109.

12. Phatthiya, S. Takahashi, N. Chareonthiphakorn, T. Koyama, D. Wititsuwannakul, R. Wititsuwannakul, Cloning and expression of the gene encoding solanesyl diphosphate synthase from *Hevea brasiliensis*, Plant Sci. 172 (2007) 824- 831.

13. W. A. Southorn, Microscope studies on fresh *Hevea* latex, Rubber Development 14 (1961) 2-6.

14. J. Tangpakdee, Y. Tanaka, K. Ogura, T. Koyama, R. Wititsuwannakul, D. Wititsuwannakul, Rubber formation by fresh bottom fraction of *Hevea* latex, Phytochemistry 45 (1997) 269-274.

15. D. Wititsuwannakul, A. Rattapittayaporn, R. Wititsuwannakul, Rubber biosynthesis by a *Hevea latex* bottom-fraction membrane, J. Appl. Polym. Sci. 87 (2003) 90-96.

16. B. L.Archer, B. G. Audley, New aspects of rubber biosynthesis, Bot. J Linnean Soc. 94 (1987) 181-196.

17. S. Madhavan, G. A. Greenblatt, M. A. Foster, C. R. Benedict, Stimulation of isopentenyl pyrophosphate incorporation into polyisoprene in extracts from guayule plants (*Parthenium argentatum*) by low temperature and 2-(3,4-dichlorophenoxy) triethanoamine, Plant Physiol. 89 (1989) 506-511.

18. K. Cornish, R. A. Backhaus, Rubber transferase activity in rubber particles of guayule, Phytochemistry 29 (1990) 3809- 3813.

19. J. Tangpakdee, Y. Tanaka, K. Ogura, T. Koyama, R. Wititsuwannakul, D. Wititsuwannakul, K. Aswatreeratanakul, Isopentenyl diphosphate isomerase and prenyl transferase activities on bottom fraction and C-serum from *Hevea* latex, Phytochemistry 45 (1997) 261-267.

20. Y. Tanaka, A. H. Eng, N. Ohya, N. Nishiyama, J. Tangpakdee, S. Kawahara, R. Wititsuwannakul, Initiation of rubber biosynthesis in *Hevea brasiliensis*:

characterization of initiating species by structural analysis, Phytochemistry 41 (1996) 1501-1505.

21. Takaya, Y. Zhang, K. Asawatreratanakul, D. Wititsuwannakul, R. Wititsuwannakul, S. Takahashi, T. Koyama, Cloning, expression and characterization of a functional cDNA clone encoding geranylgeranyl diphosphate synthase of *Hevea brasiliensis*, Biochim. Biophys. Acta 1625 (2003) 214-220.
22. D. R. Light, M. S. Dennis, Purification of a prenyltransferase that elongates *cis*-polyisoprene rubber from the latex of *Hevea brasiliensis*, J. Biol. Chem. 264 (1989) 18589-18597.
23. K. Cornish, The separate roles of plant *cis* and *trans* prenyl transferase in *cis*- 1, 4-polyisoprene biosynthesis, Eur. J. Biochem. 218 (1993) 267-271.
24. K. Asawatreratanakul, Y-W. Zhang, D. Wititsuwannakul, R. Wititsuwannakul, S. Takahashi, A. Rattanapittayaporn, T. Koyama, Molecular cloning, expression and characterization of cDNA encoding *cis*-prenyltransferase from *Hevea brasiliensis*: A key factor participating in natural rubber biosynthesis, Eur. J. Biochem. 270 (2003) 4671-4680.
25. K. Cornish, Similarities and differences in rubber biochemistry among plant species, Phytochemistry 57 (2001) 1123-1134.
26. W. Eisenreich, M. Schwarz, A. Cartayrade, D. Arigoni, M. Zenk, A. Bacher, The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorgasims, Chem Biol. 5 (1998) 221-223.
27. M. Rohmer, The discovery of a mevalonate-independent pathway for isoprenoid bipsynthesis in bacteria, algae and higher plants, Nat. Prod. Rep. 16 (1999) 565-574.
28. H. K. Lichtenthaler, Non-mevalonate isoprenoid biosynthesis: enzymes, genes and inhibitors, Biochem. Soc. Trans. 28 (2000) 785-789.
29. J. H. Julliard, R. Douce, Biosynthesis of the thiazole moiety of thiamin (vitamin B1) in higher plant chloroplasts, Proc Natl Acad Sci USA. 88 (1991) 2042-2045.

30. R. E. Hill, K. Himmeldirk, I. A. Kennedy, R. M. Pauloski, B. G. Sayer, E. I. Wolf, D. Spenser, The Biogenetic Anatomy of Vitamin B_6: A ^{13}C NMR Investigation of the biosynthesis of pyridoxol in *Escherichia coli*, J. Biol. Chem. 271 (1996) 30426-30435.

31. J. Ko, K. Chow, K. Han, Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree), Plant Mol. Biol. 53 (2003) 479-492.

32. H. Fujii, T. Koyama, K. Ogura, Efficient enzymatic hydrolysis of polyprenyl pyrophosphates, Biochim. Biophys. Acta. 712 (1982) 716-718.

33. M. Axelos,C. Curie, C. Bardet, B. Lescure, A protocol for transient expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures, Plant Physiol. Biochem. 30 (1992) 123-128.

34. M. Suzuki, T. Xiang, K. Ohyama, H. Seki, K. Saito, T. Muranaka, H. Hayashi, Y. Katsube, T. Kushiro, M. Shibuya, Y. Ebizuka, Lanosterol Synthase in Dicotyledonous Plants, Plant Cell Physiol. 47 (2006) 565 - 571.

35. J. P. Jouanneau, C. Péaud-Lenoël, Growth and synthesis of proteins in cell suspensions of kinetin dependent tobacco, Physiol Plant. 20 (1967) 834-850.

36. W. Ji, C. R. Benedict, M. A. Foster, Seasonal variations in rubber biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and rubber transferase activities in *Parthenium argentatum* in the Chihuahuan desert, Plant Physiol. 103 (1993) 535-542.

37. L. Carretero-Paulet, I. Ahumada, N. Cunillera, M. Rodríguez-Concepción, A. Ferrer, A. Boronat, N. Campos, Expression and molecular analysis of the Arabidopsis *DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-*C*-methyl-D-erythritol 4-phosphate pathway, Plant Physiol. 129 (2002) 1581-1591.

38. M. Rodríguez, I. Ahumada, E. Diez-Juez, S. Sauret-Güeto, L. María Lois, F. Gallego, L. Carretero-Paulet, N. Campos, A. Boronat, 1-Deoxy-D-xylulose 5-

phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening, The Plant Journal 27 (2001) 213-222.

39. N. M. L. C. Totte, W. Van den Ende, E. J. M. Van Damme, F. Compernolle, I. Baboeuf, J. M. C. Geuns, Cloning and heterologous expression of early genes in gibberellinand steviol biosynthesis via the methylerythritol phosphate pathway in *Stevia rebaudiana*, Plant Physiol. 81 (2003) 517-522.
40. T. Kuzuyama, S. Takahashi, M. Takagi, H. Seto, Characterization of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic aminoacid residues, J. Biol. Chem. 275 (2000) 19928-19932.
41. B. Miller, T. Heuser, W. Zimmer, Functional involvement of a deoxy-D-xylulose 5-phosphate reductoisomerase gene harboring locus of *Synechococcus leopoliensis* in isoprenoid biosynthesis, FEBS Lett. 481 (2000) 221-226.
42. R. A. Backhaus, S. Walsh, Ontogeny of rubber formation in guayule, *Parthenium argentatum* Gray, Bot. Gaz 144 (1983) 391-400.

Figure legends

Fig. 1. TLC autoradiogram of the prenyl alcohols obtained by enzymatic hydrolysis of products formed by the reaction mixture containing [^{14}C] glucose and FW particles. The products obtained from reaction in the absence (lane 1) and presence of mevinolin (lane 2) was analyzed by TLC on a reversed-phase LKC-18 plate with a solvent system of acetone/water (19:1, v/v). S.F.: solvent front; Ori: origin.

Fig. 2. Multiple sequence alignment of the deduced amino acids of HbDXR and DXRs from four other plants and two from bacteria. The residues boxed in black indicate the positional identity for at least four of six compared sequences. Dashes indicate gaps introduced in order to optimize the alignment. Asterisks indicate the NADPH-binding motif. The putative cleavage site is indicated with an arrowhead. The cDNA accession

numbers are *A. thaliana*; **AF148852**, *L. esculentum*; **AF331705_1**, *O. sativa*; **AF367205**, *S. rebaudiana*; **AJ429233**, *H. brasiliensis*; **DQ437520**, *S. leopoliensis*; **AJ250721** and *E. coli*; **AB013300**.

Fig. 3.A phylogenetic tree generated by MEGA3 program based on the degree of similarity between plants *dxrs*. Sequence analysis was performed using CLUSTAL W. The branches were validated by bootstrap analysis from 500 replications, which are represented by percentages in the branch nodes. The cDNA accession number are Salvia (*S. miltiorrhiza*); **DQ991431**, Mentha (*M.* x *piperita*); **AF116825**, Plectranthus (*P. barbatus*); **AY515699**, Antirrhinum (*A. majus*); **AY770406**, Picrohiza (*P. kurroa*); **DQ347963**, Catharanthus (*C. roseus*); **AF250235**, Nicotiana (*N. tabacum*); **DQ839130**, Lycopersicon (*L. esculentum*); **AF331705**, Stevia (*S. rebaudiana*); **AY834755**, Artemisia (*A. annua*); **AF182287**, Chrysanthemum (*C.* x *morifolium*); **AB205048**, Arabidopsis (*A. thaliana*); **AF148852**, Pueraria (*P. Montana var lobata*); **AY315651**, Cistus (*C. creticus*); **AY297794**, Populus (*P. tremula* x *P. alba*); **AJ574852**, Croton (*C. Stellatopilosus*); **EF451544**, Hordeum (*H. vulgare*); **AJ583446**, Oryza (O. sativa); **AF367205**, Ginkgo (*G. biloba*); **AY494186** and Taxus (*T. cuspidata*); **AY575140**.

Fig. 4. Analysis of mRNA expression patterns by RT-PCR. (A) mRNA expression of *Hbdxr* (28 cycles); (B) 18S rRNA expression as an internal control.

Fig. 5. Subcellular localization of *Hevea* DXR in Arabidopsis T87 cells. Cells expressing HbDXR, fused with GFP at the C-terminus and GFP, were observed by laser confocal microscopy. The panels indicated as BA510IF, BA585IF, and Merge show fluorescence images of GFP, the autofluorescence of chlorophylls, and the merged image of green and red fluorescence, respectively.

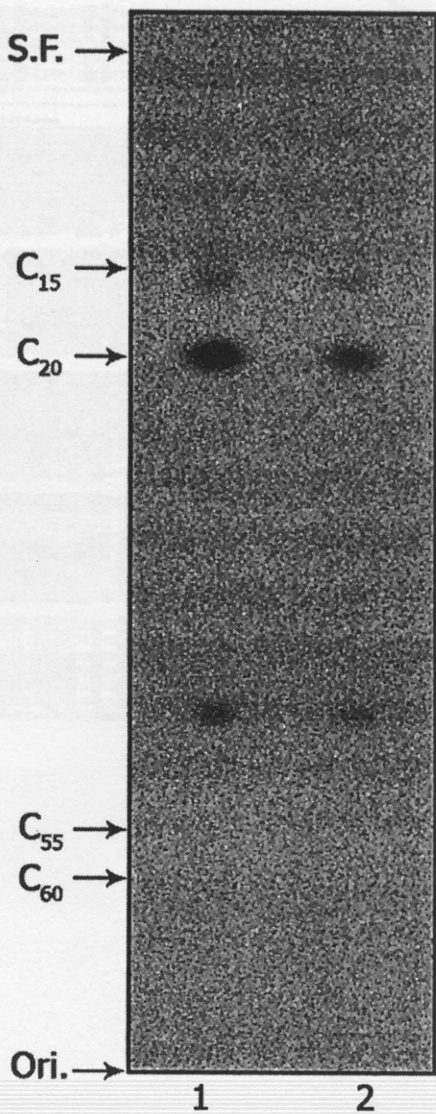

Fig. 1

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                      *
A. thaliana       1:MMTLNSLS-PAESKAISFLDTSRF---NPIPKLSGGFSLRRRNQGRGFGKGVKCSVKV---QQQQQPPPAWPGRA-VPEAPRQS-WDG 79
L. esculentum     1:-MALNLLS-PAEIKSISFLDNSKSSYNLSHLKFTGGLSIRRKECSGAFAKRVQCSA-------QLPPPPAWPGRAVAEPGRQS--WDG 77
O. sativa         1:-MALKVVSFPGDLAAVSFLDSNRGG-AFNQLKVDLPFQTRDRRAVSL--RRTCCSM-------QQAPPPAWPGRAVVEPGRRS--WDG 75
S. rebaudiana     1:-MSLSYLS-PTQTNLITPSSTCKSQ--THLLKLQGGFCFKRKDVKLAG-KGIRCSA-------QPPPPPAWPGTALVDPGTKN--WDG 74
H. brasiliensis   1:-MALNLLS-PAEIKAISFLDSTKS---SHLTKLPGGFSLKRKDFGAAFGKKVQCSA--------QPPPPAWPGRAFPDLGRKT--WDG 73
S. leopoliensis   1:--------------------------------------------------------------------------------------- 1
E. coli           1:--------------------------------------------------------------------------------------- 1

                           *******
A. thaliana      80:PKPISIVGSTGSIGTQTLDIVAENPDKFRVVALAAGSNVTLLADQVRRFKPALVAVRNESLINELKEALADLDYKLEIIPGEQGVIEV 167
L. esculentum    78:PKPISIVGSTGSIGTQTLDIVAENPDKFRVVALAAGSNVTLLADQVKTFRPKLVAVRNESLVEELKDALADMEDKPEIIPGEQGVIEV 165
O. sativa        76:PKPISIVGSTGSIGTQTLDIVAENPDKFRVVALAAGSNVTLLADQVKTFKPKLVAVRNESLVDELKEALADCDWKPEIIPGEQGVIEV 163
S. rebaudiana    75:PKPISIVGSTGSIGTQTLDIVAENPDKFRVVALAAGSNVTLLAEQIKAFKPQLVSICNESLVGELKEALADADYMPEIIPGDQGIIEV 162
H. brasiliensis  74:PKPISVVGSTGSIGTQTLDIVAENPDKFRVVALAAGSNVTLLADQVKTFKPQLVAVRNESLVHELREALADVEEKPEIIPGEQGVVEV 161
S. leopoliensis   1:MKAVTLLGSTGSIGTQTLDILEQYPDRFRLVGLAAGRNVALLSEQIRRHRPEIVAIQDAAQLSELCAAIADLDNPELLTGEAGVTEV 88
E. coli           1:MKQLTILGSTGSIGCSTLDVVRHNPEHFRVVALVAGKNVTRMVEQCLEFSPRYAVMDDEASAKLLKTMLQQQGSRTEVLSGQQAACDM 88

A. thaliana     168:ARHPEAVTVVTGIVGCAGLKPTVAAIEAGKDIALANKETLIAGGP-FVLPLANKHNVKILPADSEHSAIFQCIQGLPE---GA----- 246
L. esculentum   166:ARHPEAVTVVTGIVGCAGLKPTVAAIEAGKDIALANKETLIAGGP-FVLPFAHKHKVKILPADSEHSAIFQCIQGLPE---GA----- 244
O. sativa       164:ARHPEAVTVVTGIVGCAGLKPTVAAIEAGKDIALANKETLIAGGP-FVLPIAQKHKVKILPADSEHSAIFQCIQGLPE---GA----- 242
S. rebaudiana   163:ARHPECVTVVTGIVGCAGLKPTVAAIEAGKNIALANKETLIAGGP-FVLPLARKHNVKILPADSEHSAIFQCIQGFPE---GA----- 241
H. brasiliensis 162:ARHPEAVSVVTGIVGCAGLKPTVAAIEAGKDICLANKETLIAGGP-FVLPLAHKYNVKILPADSEHSAIFQCIQGLPD---GA----- 240
S. leopoliensis  89:ARYGEAEIVVTGIVGCAGLLPTIAAIEAGKDIALANKETLIAAGP-VVLPLLQKHGVTITPADSEHSAIFQCIQGLSTHADFRPACVV 175
E. coli          89:AALEDVDQVMAAIVGAAGLLPTLAAIRAGKTILLANKESLVTCGRLFMDAVKQSKAQLLPV-DSEHNAIFQSLPQPIQHNLGYADLEQ 175

A. thaliana     247:--LRKIILTASGGAFRDWPVEKLKEVKVADALKHPNWNMGKKITVDSATLFNKGLEVIEAHYLFGAEYDDIEIVIHPQSIIHSMIETQ 332
L. esculentum   245:--LRRIILTASGGAFRDWPVEKLKEVKVADALKHPNWNMGKKITVDSATLFNKGLEVIEAHYLFGAEYDNIEIVIHPQSIIHSMVETQ 330
O. sativa       243:--LRRIILTASGGAFRDWPVDKLKEVKVADALKHPNWNMGKKITVDSATLFNKGLEVIEAHYLFGAEYDDIEIVIHPQSIIHSMIETQ 328
S. rebaudiana   242:--LRRIILTASGGAFRDLPVEKLKDVKVADALKHPNWSMGKKITVDSATLFNKGLEVIEAHYLYGSDYDNIEIVIHPQSIIHSMVETQ 327
H. brasiliensis 241:--LRRIILTASGGAFRDWPVDKLKEVKVADALKHPNWNMGKKITVDSATLFNKGLEVIEAHYLFGAEYDNIEIVIHPQSIIHSMVETQ 326
S. leopoliensis 176:AGLRRILLTASGGAFRDWPVERLSQVTVADALKHPNWSMGRKITVDSATLMNKGLEVIEAHYLFGLDYDYIDIVIHPQSIIHSLIELE 263
E. coli         176:NGVVSILLTGSGGPFRETPLRDLATMTPDQACRHPNWSMGRKISVDSATMMNKGLEYIEARWLFNASASQMEVLIHPQSVIHSMVRYQ 263

A. thaliana     333:DSSVLAQLGWPDMRLPILYTMSWPDRVPCSEVTWPRLDLCKLGSLTFKKPDNVKYPSMDLAYAAGRAGGTMTGVLSAANEKAVEMFID 420
L. esculentum   331:DSSVLAQLGWPDMRLPILYTLSWPDRVYCSEITWPRLDLCKLGSLTFKAPDNVKYPSMDLAYSAGRAGGTMTGVLSAANEKAVELFIS 418
O. sativa       329:DSSVLAQLGWPDMRIPILYTMSWPDRIYCSEVTWPRLDLCKLGSLTFKAPDNVKYPSMDLAYAAGRAGGTMTGVLSAANEKAVELFID 416
S. rebaudiana   328:DSSVLAQLGWPDMRLPILYTLSWPDRISCSEITWPRLDLCKLGSLTFKAPDNVKYPSMDLAYAAGRAGGTMTGVLSAANEKAVEMFID 415
H. brasiliensis 327:DSSVLAQLGWPDMRLPILYTMSWPDRIYCSEITWPRLDLCKLGSLTFKAPDNVKYPSMDLAYAAGRAGGTMTGVLSAANEKAVEMFIN 414
S. leopoliensis 264:DTSVLAQLGWPDMRLPLLYALSWPDRLSTQ---WSALDLVKAGSLEFREPDHAKYPCMDLAYAAGRKGGTMPAVLNAANECAVALFLE 348
E. coli         264:DGSVLAQLGEPDMRTPIAHTMAWPNRVNSGVKPLDFCKLSALTFAAPDYDRYPCLKLAMEAFEQGQAATTA---LNAANEITVAAFLA 348

A. thaliana     421:EKISYLDIFKVVELTCDKHRNELVTSPSLEEIVHYDLWAREYAANVQLSSGA-RFVHA 477
L. esculentum   419:ERISYLDIFKIVELTCAKHREELVSSPSLEEIIHYDLWARDYAASLEPSSGL-SFALV 475
O. sativa       417:EKIGYLDIFKVVELTCDAHRNELVTRPSLEEIIHYDLWAREYAASLQPSTGL-SFVPV 473
S. rebaudiana   416:EKIQYLDIFKVVELTCAKHQSELVTAPSLEEIVHYDLWARDYAASLKSSSPGLTAVALV 473
H. brasiliensis 415:EKIGYLDIFKIVELTCDKHRSELVASPSLEEIIHYDLWARDYAASLQPTSGL-SFVLA 471
S. leopoliensis 349:EQIHFSDIPRLIEPACDRHQTEWQQQPSLDDILAYDAWARQFVCASYQSLESVV     402
E. coli         349:QQIRFTDIAAINLSVLEKMDMREPQCV-DDVLSVDANAREVARKEVMRLAS        398
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Fig. 2

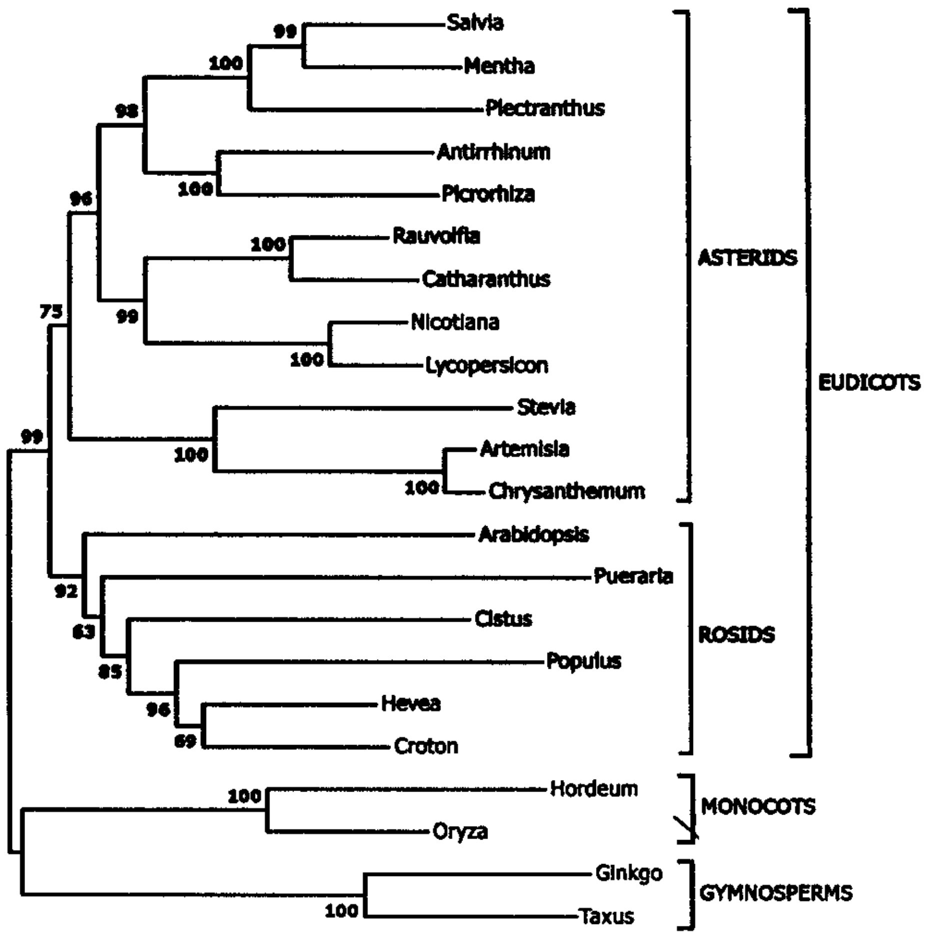
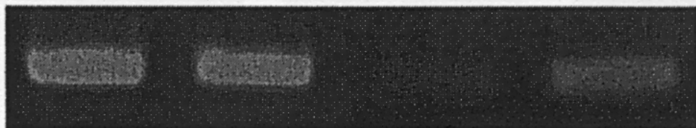


Fig. 3

Inflorescence Seedling stem Leaf Latex

A



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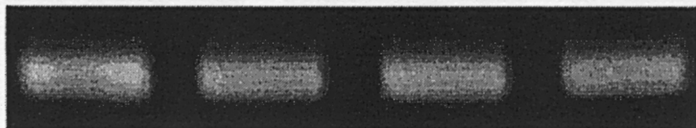

Fig. 4

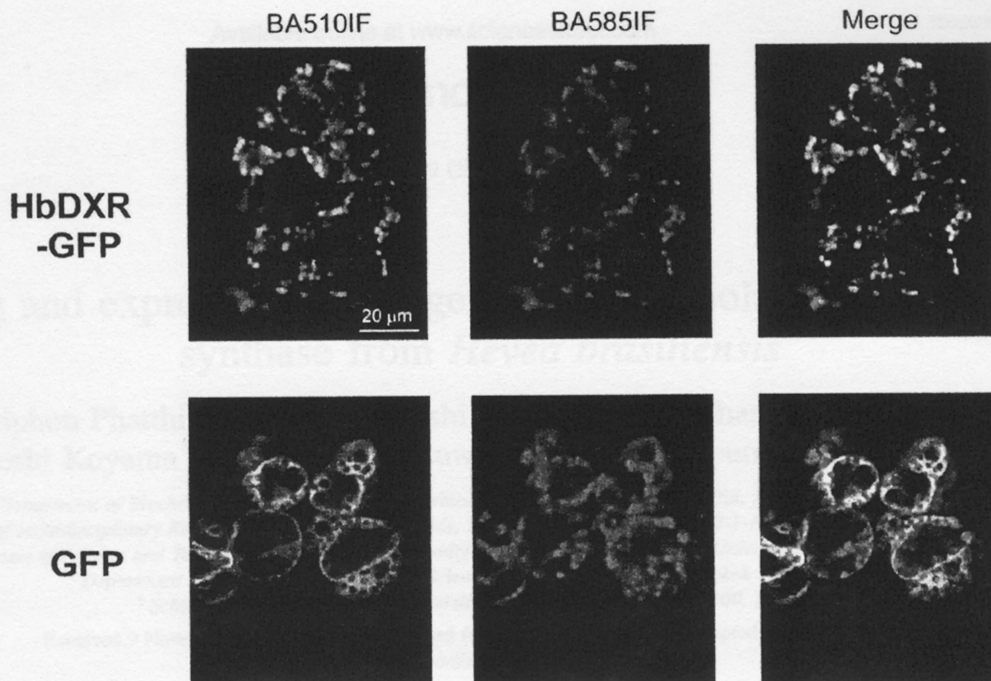

Fig. 5