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Cloning and expression of the gene encoding solanesyl diphosphate synthase from *Hevea brasiliensis*

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Summary

Plastoquinones play important roles as electron carriers in the light-dependent reactions of photosynthesis and also as a cofactor of phytoene desaturation in the synthesis of carotenoid. A plastoquinone-9 (PQ-9) was identified in Frey-Wyssling organelles of fresh rubber latex from *Hevea brasiliensis*. This indicates that a *Hevea* solanesyl diphosphate synthase (HbSDS) must be present for the synthesis of the C_{45} prenyl side chain of the PQ-9 found in the rubber latex. Based on the sequence information of other *trans*-prenyl diphosphate synthases, the cDNA encoding HbSDS was cloned from a *Hevea* rubber latex cDNA library. Sequence analysis revealed the presence of an ORF consisting of 1254 bp capable of encoding a 46,095 Da polypeptide. The deduced amino acid sequence of the clone contained all conserved regions found in typical *trans*-prenyl chain elongating enzymes. This cDNA, expressed in *Escherichia coli* cells as an insoluble but Trx-His-tagged fusion protein, showed a distinct solanesyl diphosphate synthase activity in the presence of Triton X-100. The expression level of HbSDS mRNA in latex was found to be higher than those in leaves, stems and roots. The expressed HbSDS is suggested to be a specific enzyme in the Frey-Wyssling particles of rubber latex.
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Keywords: Solanesyl diphosphate synthase; Plastoquinone; Frey-Wyssling particle; Prenyl diphosphate synthase; *Hevea brasiliensis*; Rubber latex

1. Introduction

Prenylquinones present in photosynthetic organisms belong to a large multifunctional family of lipid soluble compounds that include plastoquinones, phylloquinones, tocopherols and tocotrienols. All prenylquinones are composed of hydrophobic prenyl tails of various lengths attached to aromatic head groups that are capable of donating and accepting electrons. The aromatic compound homogenistic acid is used for condensation with phytyl diphosphate (PDP), geranylgeranyl DP (GGDP), or solanesyl DP (SDP) in tocopherol, tocotrienol and plastoquinone synthesis, respectively [1–4]. The length of the prenyl side chain is controlled by specific *trans*-prenyl diphosphate synthase. In general, a long-chain *trans*-prenyl diphosphate product is derived from its short-chain prenyl diphosphate precursor produced by short-chain *trans*-prenyl diphosphate synthases which includes farnesyl diphosphate synthase (FDS) or geranylgeranyl diphosphate synthase (GGDS). Several genes encoding for short-chain *trans*-prenyl diphosphate synthases have been cloned and characterized including *Arabidopsis thaliana* FDS [5] and GGDS [6–8], *Hevea* FDS [9] and GGDS [10], *Citrus sinensis* and *Capsicum annuum* geranyl diphosphate synthases (GDS) [11–13]. At present, there have been only two plant genes encoding for a long-chain *trans*-prenyl diphosphate synthase, solanesyl diphosphate synthase (SDS) reported from a small cruciferous plant, *A. thaliana* [14–16].

To gain further insight into the structure, function and evolution of the long-chain *trans*-prenyl diphosphate synthases among various plants, we became interested in isolating the SDS gene from the latex of the rubber tree (*Hevea brasiliensis*). The incentive for this isolation arises from a preliminary detection of plastoquinone with a C_{45} prenyl side chain moiety

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(plastoquinone-9) in specialized rubber latex organelles called the Frey-Wyssling (FW) particles [17]. The FW particles are surrounded by a double membrane and had been shown to contain plastochromanol and carotenoids that give characteristic yellow to orange colors [18,19]. So far, the detailed biological function of these particles in latex, besides their possible involvement in carotenoid biosynthesis, has not been established [20].

2. Materials and methods

2.1. Materials

Escherichia 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]IDP was from Amersham. Non-labelled IDP, GDP, FDP and GGDP were synthesized according to the procedure of Davisson et al. [21]. 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. Ubiquinone-10 (UQ-10) was a product of Sigma.

2.2. Plant materials and RNA isolation

Fresh *Hevea* latex and other tissue samples were obtained from regularly tapped rubber trees (*Hevea*) 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, roots and whole young seedling tissues were isolated by using the RNeasy Plant Mini Kit (Qiagen). Mitochondria and chloroplasts were prepared from *Hevea* leaves by the previously described method [22].

2.3. Frey-Wyssling particle isolation

Fresh latex containing most numerous rubber particles, in addition to other lutoid and FW particles [23], was subjected to a series of centrifugations to isolate the FW particles. First a low speed centrifugation ($1700 \times g$ at 10 °C for 30 min) with swing-out bucket rotors to allow all the particles to travel through the viscous latex media, according to their densities gradients, down the length of the centrifuge tube without being forced against the sides of the tube. This aimed to minimize inter-particle collisions, especially between FW and lutoid particles. The upper half of the centrifuged latex, containing the majority of the less density rubber particles, was removed whereas the lower half was further separated, by using a fixed angle rotor centrifuge ($7000 \times g$ 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 ($59{,}000 \times g$ at 4 °C for 20 min). The pellet was washed twice by suspending in 3 vol. of washing buffer (50 mM Tris–HCl, pH 7.4), and kept at −20 °C until used.

2.4. Prenylquinone analysis

H. brasiliensis leaves (50 g) were cut into small pieces and homogenized in a Waring blender with 300 ml of ice-cold medium (0.3 ml 1 M $MgCl_2$, 0.3 ml 1 M $MnCl_2$, 0.3 ml 1 M glycine, 0.6 ml 0.5 M Na_2 EDTA, 15 ml 1 M Tris–HCl, pH 7.5, 30.81 g sucrose, 0.48 g BSA, 211 g Na-ascorbate, added immediately before used, H_2O added to 300 ml). After filtration through four layers of cheesecloth, the filtrate was centrifuged at $2500 \times g$ at 4 °C for 5 min. The pellet contained the chloroplasts and the supernatant contained the mitochondria. The mitochondria were isolated from the supernatant by centrifugation at $15{,}000 \times g$ at 4 °C for 15 min [22]. Prenyllipids were separately extracted from the pellets (2 g, wet weight, each) of mitochondria, chloroplasts and FW particles under total reflux for 3 h with chloroform/methanol (2:1, v/v). The extracts were evaporated to dryness, dissolved in chloroform/methanol (1:1), and analyzed by normal-phase TLC (Kieselgel 60 F_{254}, Merck) with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) [24]. The UV-visualized areas contained the prenylquinones (R_f 0.27–0.59) and these areas were scraped from the TLC plates, extracted with chloroform/methanol (1:1, v/v) and evaporated to dryness. The dry sample was dissolved in solvent A (methanol/propan-2-ol; 4:1, v/v) and subjected to HPLC (Waters) on a reverse-phase C_{18} Symmetry 5 μm (3.9 mm × 150 mm) column, preequilibrated with a solvent mixture containing an equal volume of solvent A and solvent B (methanol/propan-2-ol; 1:1, v/v), at a flow rate of 1.0 ml/min. After sample injection, the column was eluted with a linear gradient from 50 to 100% solvent B for 10 min, followed by isocratic elution with solvent B for another 10 min. A standard solution of UQ-10 was prepared in solvent A and used to confirm identification of purified ubiquinone ($\lambda_{max} = 275$ nm). Plastoquinone ($\lambda_{max} = 256$ nm) was identified by a combination of spectroscopy and mass spectroscopy according to the method of Hirooka et al. [14].

2.5. PCR amplification of Hevea trans-prenyl diphosphate synthase cDNA fragment

The cDNA fragment containing the region corresponding to the *trans*-prenyl diphosphate synthases was amplified from the *Hevea* latex cDNA library that had been constructed using the ZAP-cDNA Synthesis Kit (STRATAGENE). Seven degenerate oligonucleotide primers were designed to amplify the *Hevea trans*-prenyl diphosphate synthase cDNA fragment after identifying highly conserved regions of other *trans*-prenyl diphosphate synthases; sense primers, TransF1 (GGKRVRP, region I) 5′-GGNGGHAARMGKRTNMGDCC-3′, TransF2 (VXLVSRA, region I) 5′-GTRYTSCTRRTATCACRWGC-3′, TransF3 (EMIHTAS, region II) 5′-GARATGATHCAYACNGCHAG-3′, and TransF4 (HDDXXDE, region II) 5′-CAHGAYGAYGTGKTAGAYGA-3′ and antisense primers, TransR1

(KTASLVA, region V) 5′-GCHACYAAWGANGCNGTYTT-3′, TransR2 (VDDILDF, region VI) 5′-AMRTCVAR-AATRTCRTCMAC-3′, and TransR3 (GKPAGXD, region VI) 5′-ACMBWMSCKRCHGGYAAHCC-3′, where R is G or A, K is G or T, S is G or C, W is A or T, M is A or C, Y is T or C, D is G or A or T, V is G or A or C, B is G or T or C, H is A or T or C, and N is G or A or T or C. PCR was performed in a total volume of 20 μl containing 1 μM of amplification primer pair for 30 cycles of 15 s at 94 °C, 30 s at 50 °C and 1 min at 68 °C. The resulting band of PCR products were extracted from agarose gel and subcloned into pGEMT-easy vector for sequencing by the ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software. One of the resulting clones, that showed homology to *trans*-prenyl diphosphate synthases, was termed LS300.

2.6. 3′- and 5′-RACE reaction and cloning of Hevea trans-prenyl diphosphate synthase cDNA

Cloning of the full length cDNA of *Hevea trans*-prenyl diphosphate synthase was performed by using total RNA extracted from young *Hevea* seedlings as the template for both 3′- and 5′-RACE reactions based on the cDNA sequence of the LS300. The procedures applied for the 3′- and 5′-RACE reactions were according to the manufacturer's instructions (GeneRacer Kit, Invitrogen). The primers used for 3′-RACE were 5′-CGGCCAGCTTAATCCTGATGATGT-3′ (HesF1), 5′-ACTCTATGGCACGAGGGTGGCAGT-3′ (HesF2) and 5′-CTGCGATGTTGAACTCGAGGAGTA-3′ (HesF3), respectively, and those used for 5′-RACE were 5′-ACTCCTC-GAGTTCAACATCGCAGT-3′ (HesR1), 5′-GTACTGCCAC-CCTCGTGCCATAGA-3′ (HesR2) and 5′-CATCATCATG-GATTAAGCTGGCGGTAT-3′ (HesR3), respectively. According to the sequence information obtained by the 3′- or 5′-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 HbSDS.

2.7. Construction of an expression vector system in E. coli and purification of Hevea trans-prenyl diphosphate synthase

An expression vector system for the HbSDS was constructed by choosing the pET-32(a+) vector (Novagen) designed to express the gene product as a thioredoxin- and His-tagged fusion protein, suitable for production of soluble protein in the *E. coli* cytoplasm and for rapid purification. The restriction enzyme recognition site for *Sac*I and *Sal*I was introduced by PCR at the 5′- and 3′-end of the coding regions of the HbSDS cDNA, respectively. The resulting fragments were sequenced, digested with *Sac*I and *Sal*I, and ligated into the *Sac*I–*Sal*I vector of pET-32(a+), yielding the expression plasmid pET-HbSDS. The expressed plasmid was used for transformation of *E. coli* BL21(DE3), and 1 ml of an overnight culture of the transformant in Luria–Bertani medium containing 100 μg/ml ampicilin was inoculated into 200 ml of the same medium. The cells were grown at 37 °C to reach an A_{600} value of 0.8. Isopropyl thio-β-D-galactoside was added to a final concentration of 0.1 mM, and the culture medium was further incubated at 30 °C for 5 h. Overproduction of the proteins was confirmed by SDS/PAGE according to the standard method of Laemmli [25]. The cells were harvested by centrifugation (5000 × *g*, 10 min) and then disrupted by sonication. The cell homogenates were separated into soluble and insoluble protein (inclusion body) fractions 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 Ni^{2+} nitrilotriacetic acid-agarose column. The insoluble proteins were extracted with buffer A (20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 8 M urea) at 37 °C for 1 h. The protein extract was collected by centrifugation at 8000 × *g* for 10 min, filtered through a filter membrane, and applied to a Ni^{2+} resin column. The column was washed with buffer A and the tagged protein was eluted with buffer B (20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.3 M imidazole). The purified protein was renatured by dialysis against buffer C (10 mM Tris–HCl, pH 7.4, containing 1% Triton X-100).

2.8. Solanesyl diphosphate synthase assay and product analysis

The incubation mixture, in a total volume of 100 μl, contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 20 mM β-mercaptoethanol, 50 μM FDP, 50 μM [1-^{14}C]IDP, 0.5% Triton X-100 and 200 μg of crude enzyme from the cell-free homogenate of HbSDS-overexpressed *E. coli* cells. After incubation at 30 °C for 1 h, the reaction was stopped by chilling on ice and followed by addition of 100 μl saturated NaCl solution. The products were extracted with 1 ml of 1-butanol saturated with water and the amount of [1-^{14}C]IDP incorporated into the 1-butanol-extractable polyprenyl diphosphate was measured in a liquid scintillation counter. The enzyme activity was expressed in terms of the radioactivity in the 1-butanol extract. The 1-butanol extracts were hydrolyzed with potato acid phosphatase according to Fujii et al. [26] The hydrolyzed products were extracted with pentane and analyzed by TLC on a reversed LKC-18 plate (Whatman) with acetone/water (19:1, v/v). The distribution of radioactive products on the TLC plate was analyzed with a Fuji BAS-100 Mac Bioimage analyzer and spots of marker prenols were visualized by exposing the plate to iodine vapor.

2.9. Expression analysis of HbSDS mRNAs

RT-PCR was performed for the analysis of *HbSDS* mRNA expression. Total RNAs (1 g) from various *Hevea* tissues was amplified with HbSDS specific primers, S1 (5′-CCTAGTGT-CAAGAGCCACAGCAGAA-3′) and C2 (5′-GTTTATACCCC-TGTAAAGCTTACCTA-3′). The reactions were carried out with 35 cycles of 15 s at 94 °C, 30 s at 54 °C and 1 min at 68 °C with a 2 min preheat at 94 °C. The products obtained were analyzed by agarose gel electrophoresis with ethidium bromide staining.

3. Results

3.1. Latex Frey-Wyssling prenylquinone identification

Frey–Wyslling particles are known to be very specialized chromoplasts containing β-carotene and found in lacticiferous vessels including rubber latex collected by tapping [23,27]. The prenylquinones in the lipids extracted from FW particles were identified, through a combination of TLC and HPLC, and compared to those of leaf chloroplast and mitochondria. A similar retention time (9.5 min), monitored by A_{256} in an HPLC chromatogram was obtained for the main chromatographic peaks of the prenylquinones from FW particles and chloroplasts (Fig. 1). Their chemical structures were confirmed as plastoquinone-9 (PQ-9) by mass spectroscopy (data not shown). This is different from the mitochondrial prenylquinone which was identified as UQ-10 (Fig. 1), by comparing the retention time of the chromatographic peak, monitored by A_{275}, with an authentic reference UQ-10 standard. These findings indicate a possible involvement of latex FW particles in the synthesis of PQ-9. Hence, further studies to isolate and characterize the *Hevea* solanesyl diphosphate synthase (HbSDS), involved in the synthesis of the C_{45} prenyl side chain of the PQ-9 present in rubber latex are thus warranted.

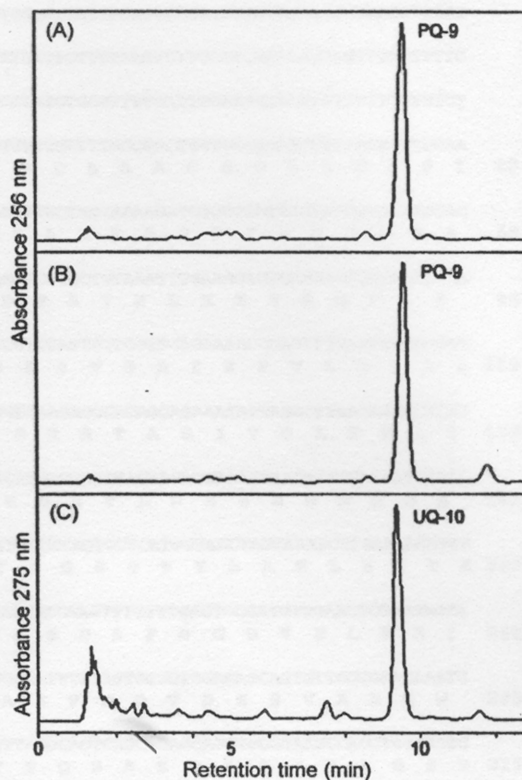

Fig. 1. HPLC chromatogram of *Hevea* prenylquinones. (A) Chloroplast plastoquinone-9 extracted from leaves; (B) Frey-Wyssling particle plastoquinone-9 extracted from latex; (C) mitochondrial ubiquinone-10 extracted from leaves.

3.2. Isolation of the gene encoding solanesyl diphosphate synthase

In order to obtain a gene fragment containing conserved amino acid regions of HbSDS, seven degenerated oligonucleotide primers were designed to identify highly conserved regions of various *trans*-prenyl diphosphate synthases such as octaprenyl diphosphate synthase (ODS) from *E. coli* [28], SDP synthase from *Anabaena* [29], SDP synthase 1 from *A. thaliana* [14], decaprenyl diphosphate (DDP) synthases from *Gluconobacter suboxydans* [30] and *Schizosaccharomyces pombe* [31]. Three kinds of products were obtained from PCR using a *Hevea* latex cDNA library as a template with primer pairs of TransF1 and TransR2. Then nested PCR was performed using primers TransF3 and TransR1. The resulting 0.3 kb cDNA fragment was cloned into the pGEMT-easy vector, and its nucleotide sequence was determined. The deduced amino acid sequence of this cDNA contained regions common to the conserved regions II, III, IV and V of *trans*-prenyl diphosphate synthases with 39, 65 and 89% identities to the corresponding regions of *E. coli* ODS, *Anabaena* SDP synthase and, *A. thaliana* SDP synthase 1, respectively. Specific primers, designed from the 0.3 kb cDNA fragment, were used to amplify 5′- and 3′-UTRs by 5′- and 3′-RACE, respectively. Two cDNA fragments that contained the same ORF were obtained from the 5′-end amplification and only a 762 bp cDNA fragment was obtained from the 3′-RACE. These sequences were used to design primers for the amplification of full length cDNA. The full length sequence revealed the presence of an ORF consisting of 1254 bp capable of encoding a 418 amino acid protein (Fig. 2). The protein encoded by this ORF contains the seven conserved regions found in typical prenyl diphosphate synthases (Fig. 3). This gene was thus designated as *HbSDS* (*Hevea* solanesyl diphosphate synthase, GenBank accession no. DQ437520). The amino acid identities of the protein encoded by this ORF compared to those of the SDP synthase 1 from *A. thaliana*, ODP synthase from *E. coli*, GDP synthase from *Citrus sinensis*, GGDP and FDP synthases from *Hevea* were 70, 46, 41, 33 and 23%, respectively.

3.3. Overproduction of recombinant HbSDS protein in E. coli

In order to obtain a HbSDS gene product, its cDNA was expressed in *E. coli* cells by means of a pET-32a(+) expression system that contains a thioredoxin fusion sequence suitable for production of soluble proteins in the *E. coli* cytoplasm. By induction with IPTG, the *E. coli* cells harboring HbSDS cDNA produced a recombinant protein of 60 kDa in the insoluble pellet fraction (Fig. 4). The urea extracted HbSDS protein was subjected to a metal affinity column for purification of the His-tagged fusion protein, under denaturing conditions. As shown in Fig. 4, the affinity-purified HbSDS protein was detected as a single band of 60 kDa corresponding to the His-tagged fusion protein.

3.4. Enzymatic activity of HbSDS protein

The activity of HbSDS was examined in the crude enzyme obtained from a cell-free homogenate of IPTG-induced *E. coli*

```
   1  [illegible]
 101  [illegible]
 201  [illegible]
 301  [illegible]
                  M M S N T C Y S L D F G R T V F D L A A C G C S S N A S I      29
 401  [illegible]
      D R C S V R N Y A R S V Y R T C N R D Y A A R R S P Y C R R D S A      62
 501  [illegible]
      W C R V S S T K A P E T L L N G V S Q D P A V N L K E S R G P I S      95
 601  [illegible]
      L I N V F E A V A G D L Q T L N Q N L R S I V G A E N P V L N S A A   129
 701  [illegible]
      D Q I F G A G G K R M R F A L V F L V S R A T A E I V G L K E L T     162
 801  [illegible]
      T K E R R L A S I I E M I E T A S L I H D D V L D E S N M R R G K     195
 901  [illegible]
      Q T V H Q L Y G T R V A V L A G D F M F A Q S S W Y L A N L E N I E   229
1001  [illegible]
      V I K L I S Q V I K D F A S G E I K Q A S S L F D C D V E L E E Y     262
1101  [illegible]
      L I K S Y Y K T A S L I A A S T K G A A I F S G V D S S V A E Q M     295
1201  [illegible]
      Y E Y G K N L G L S F Q V V D D V L D F T Q S A E Q L G K P A G S D   329
1301  [illegible]
      L A K G N L T A P V I F A L E K E P K L R E I I E S E F C E T G S     362
1401  [illegible]
      L D E A V E L V K Q C G G I E R A Q E L A K E K A D L A I Q N L N     395
1501  [illegible]
      C L P R G V F Q S H L K E M V L Y N L E R I D                         418
1601  [illegible]
1701  [illegible]
1801  [illegible]
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Fig. 2. Nucleotide and deduced amino acid sequences of HbSDS. The number of nucleotide sequence and amino acid sequence are indicated on the left and right, respectively.

harboring pET-HbSDS. The prenyl alcohol product clearly indicated that the cloned gene catalyzed the synthesis of a C_{45} prenyl moiety (Fig. 5). This confirmed that the expressed enzyme is solanesyl diphosphate synthase. When the primer substrate specificity was examined by using four allylic diphosphates namely dimethylallyl diphosphate (DMADP), GDP, FDP and GGDP (Table 1), the HbSDS was found to utilize only allylic primers that contain at least two C_5 isoprenyl units and its allylic substrate specificity is directly proportional to the isoprene unit contents of the primers, GGDP > FDP > GDP, respectively.

3.5. Expression analysis of HbSDS mRNAs

To examine the HbSDS mRNA expression in various *Hevea* tissues, RT-PCRs using total RNA extracted from young stems, roots, leaves and latex were performed. As shown in Fig. 6, the PCR products representing the HbSDS mRNA expressions were detected in all tissues but at a different level. The level of transcript accumulation for HbSDS was found to be highest in the latex and lowest in the root. This is opposite to the expression level of *Hevea* GGDP synthase, a short-chain prenyl

Table 1
Allylic substrate specificity of HbSDS

Substrate	Relative activity
DMADP	0
GDP	44
FDP	100
GGDP	257

Enzyme reactions were performed as described in Section 2. Each allylic substrate was used at 50 with 50 μM [1-^{14}C]IDP. Relative activities were determined based on the activity with FDP.

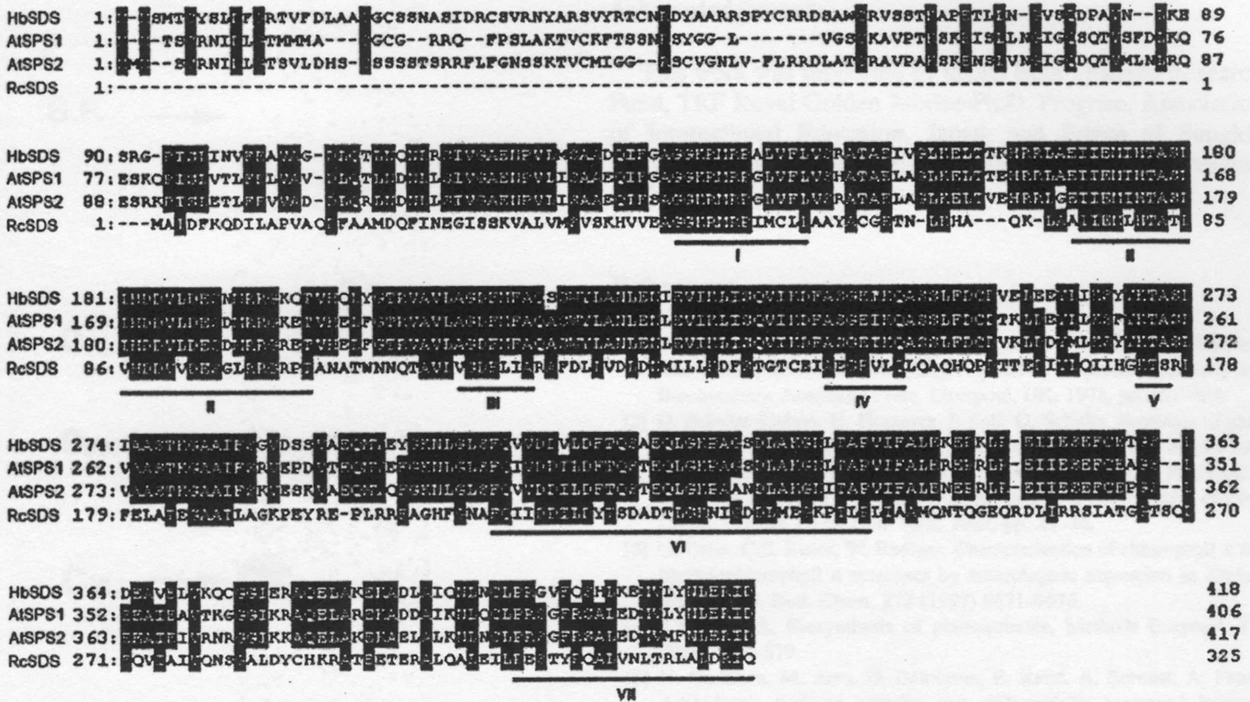

Fig. 3. Comparison of the deduced amino acid sequences of HbSDS. The deduced amino acid sequences of HbSDS are compared with those of solanesyl diphosphate synthases from *A. thaliana* (SPS1 GenBank accession no. BAD88533 and SPS2 GenBank accession no. BAD88534) and *Rhodobacter capsulatus* (GenBank accession no. BAA22867). The filled boxes indicate the positional identity of the sequences. The seven highly conserved regions are underlined.

diphosphate synthase, which had been shown to be higher in the leaves than in the latex [10].

4. Discussion

Our identification study on prenylquinones from *Hevea* tissues showed that the prenylquinone in leaf mitochondria possesses a C_{50} prenyl side chain which is longer than those C_{45} prenyl side chains found in leaf chloroplast and rubber latex FW particles. This finding indicates the need for at least two *Hevea trans*-long chain prenyl diphosphate synthases including a latex solanesyl diphosphate synthase. This is different from the prenylquinones found in *A. thaliana* where only a single species of side chain with the C_{45} prenyl moiety was found [14].

A *Hevea* rubber latex gene that encodes for a solanesyl diphosphate synthase was successfully cloned and expressed in *E. coli* cells. This gene contains a putative ORF encoding a protein consisting of 418 amino acid residues with a predicted molecular mass of 46 kDa. The expressed protein was found in the pellet fraction in spite of being fused with a His- and thioredoxin-tag suitable for production of soluble protein in the *E. coli* cytoplasm. This may result from a much higher proportion of hydrophobic amino acids (48%) as compared to the hydrophilic ones (28%). As a consequence, a non-ionic detergent (Triton X-100) was required to stimulate the enzymic activity of the HbSDS, and this is similar to reports for other long-chain prenyl diphosphate synthases found in microorganisms [32,33]. From the sequence analysis of HbSDS the Predotar program [34] has predicted the presence of a plastid targeting sequence. The plant plastids had previously been shown to accumulate isoprenoids, including carotenoids, prenyl chains of chlorophylls and plastoquinone, synthesized via the MEP pathway [35–37]. The latex FW particles have also been assumed to be modified plastids [38] due to the presence of

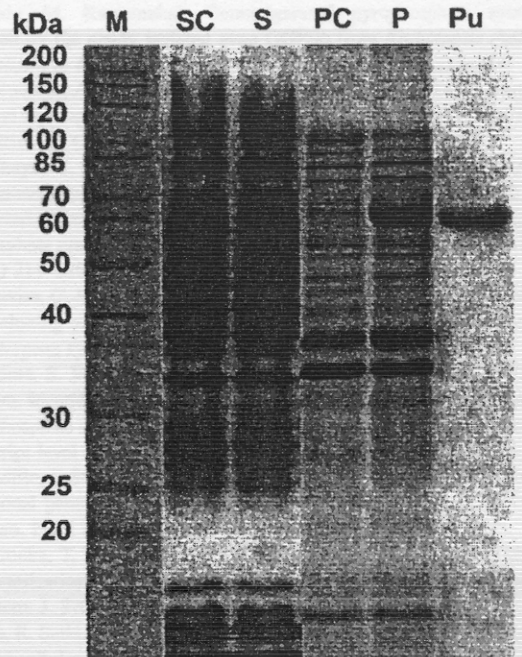

Fig. 4. SDS-PAGE gel of purified HbSDS. Lanes SC and S, soluble protein from non-induced and induced *E. coli* cells, respectively; lanes PC and P, insoluble proteins from non-induced and induced *E. coli* cells, respectively; lane Pu, purified HbSDS; lane M, molecular-mass markers.

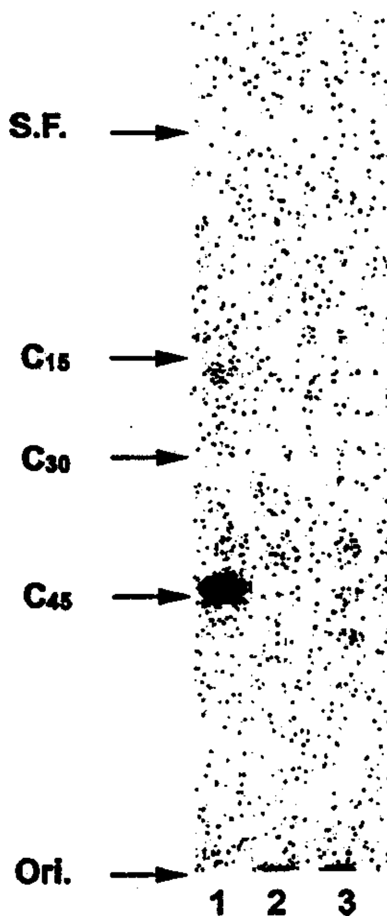

Fig. 5. TLC autoradiogram of the prenyl alcohols obtained by enzymatic hydrolysis of the products formed by the reaction with cell-free homogenate of IPTG-induced *E. coli* harboring pET-HbSDS (lane 1), non-induced *E. coli* harboring pET-HbSDS (lane 2), and *E. coli* harboring pET-32 (lane 3). S.F.: solvent front; C_{15}: farnesol; C_{30}: hexaprenol; C_{45}: solanesol; Ori.: origin.

plastoquinone, plastochromanol and isoprenoids. Hence, the expressed HbSDS may be specific for the synthesis of PQ-9 prenyl side chains in the latex FW particles. Accordingly, the level of HbSDS mRNA transcript was also found to be higher in the latex. We therefore suggest that it has an important function in the lacticiferous vessels where rubber biosynthesis is highly active. The PQ-9 in the latex FW particles may function as a cofactor in the sequential desaturation reactions, catalyzed by phytoene desaturase, required for carotenoid synthesis and play an antioxidative role similar to the function of ubiquinone in mitochondria and/or be involved in disease resistance as had been reported in other plants [39–41].

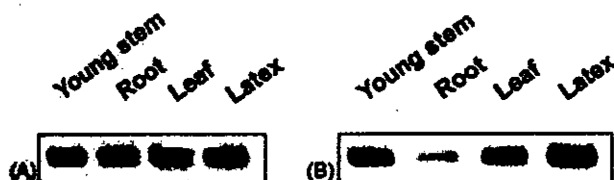

Fig. 6. Analysis of mRNA expression patterns by RT-PCR. (A) Control (18S rRNA); (B) mRNA expression of HbSDS (35 cycles).

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