Functional Analysis of Two Solanesyl Diphosphate Synthases from Arabidopsis thaliana

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Received November 4, 2004; Accepted December 16, 2004

Solanesyl diphosphate (SPP) is regarded as the precursor of the side-chains of both plastoquinone and ubiquinone in Arabidopsis thaliana. We previously analyzed A. thaliana SPP synthase (At-SPS1) (Hirooka et al., Biochem. J., 370, 679–686 (2003)). In this study, we cloned a second SPP synthase (At-SPS2) gene from A. thaliana and characterized the recombinant protein. Kinetic analysis indicated that At-SPS2 prefers geranylgeranyl diphosphate to farnesyl diphosphate as the allylic substrate. Several of its features, including the substrate preference, were similar to those of At-SPS1. These data indicate that At-SPS1 and At-SPS2 share their basic catalytic machinery. Moreover, analysis of the subcellular localization by the transient expression of green fluorescent protein-fusion proteins showed that At-SPS2 is transported into chloroplasts, whereas At-SPS1 is likely to be localized in the endoplasmic reticulum of the A. thaliana cells. It is known that the ubiquinone side-chain originates from isopentenyl diphosphate derived from the cytosolic mevalonate pathway, while the plastoquinone side-chain is synthesized from isopentenyl diphosphate derived from the plastidial methylerythritol phosphate pathway. Based on this information, we propose that At-SPS1 contributes to the biosynthesis of the ubiquinone side-chain and that At-SPS2 supplies the precursor of the plastoquinone side-chain in A. thaliana.

Key words: isoprenoid; prenyltransferase; nonaprenyl diphosphate; plastoquinone; ubiquinone

Plants have two major prenylquinones, plastoquinone and ubiquinone.⁴ Although both share the structural feature of a trans-polyrenyl tail attached to the benzoquinone skeleton and have common oxidation-reduction properties, their subcellular localization and biochemical roles are distinct. Plastoquinone exists in the thylakoid membrane of the chloroplast and acts as an electron carrier in the photosynthetic electron transfer reaction,² whereas ubiquinone exists in the inner membrane of the mitochondrion and transfers electrons in the respiratory chain reaction.³ The hydrophobic tails of prenylquinones are C₃₀–C₅₀ in length and serve as membrane anchors. The lengths of the polyrenyl side-chains differ between plastoquinone and ubiquinone, and even among plant species.⁶ It is thought that these polyrenyl chains are derived from C₃₀–C₅₀ prenyl diphosphates formed by the consecutive condensation of isopentenyl diphosphate (IPP; C₅) with allylic diphosphate in the trans-configuration.⁵ As for the enzymes giving these precursors, several genes isolated from microorganisms have been well characterized,⁶–⁸ but information about the plant-origin enzymes has been limited. Recently, we succeeded in the molecular cloning of Arabidopsis thaliana solanesyl diphosphate synthase (SPP; C₄₅) synthase (At-SPS1; formerly designated At-SPS), which catalyzes the trans-type condensation of IPP to yield the C₄₅ product.⁹ Enzymological analysis indicated that At-SPS1 utilizes both farnesyl diphosphate (FPP; C₁₅) and geranylgeranyl diphosphate (GGPP; C₂₀), but prefers GGPP to FPP as a starter substrate. We also performed an analysis of the

Abbreviations: At-SPS1, Arabidopsis thaliana solanesyl diphosphate synthase 1; At-SPS2, Arabidopsis thaliana solanesyl diphosphate synthase 2; IPP, isopentenyl diphosphate; SPP, solanesyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; MEP, methylerythritol phosphate; DMAAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; UTR, untranslated region; RACE, rapid amplification of cDNA ends; ORF, open reading frame; sGFP(S65T), engineered green fluorescent protein; DiOC₅, 3,3′-dihexyloxacarbocyanine iodide; FARM, first aspartate-rich motif
side-chains of plastoquinone and ubiquinone extracted from A. thaliana plant and found that both prenylquinones contain the C_{55} prenyl moiety. This suggests that At-SPS1 might be devoted to the biosynthesis of either or both of the prenylquinone side-chains.

IPP is the common precursor for all isoprenoid biosyntheses, and there are two different IPP biosynthetic pathways in plants.\(^\text{[10,11]}\) One is the mevalonate pathway and the other is the methylerythritol phosphate (MEP) pathway. By labelling experiments, it has been shown that, in plastids, IPP is synthesized by the MEP pathway, whereas cytosolic and mitochondrial IPPs are derived from the mevalonate pathway.\(^\text{[12,13]}\) These studies also indicated that the plastoquinone prenyl-chain is synthesized from the MEP-derived IPP in a few plants,\(^\text{[12]}\) while the ubiquinone prenyl-chain is synthesized from the mevalonate-derived IPP in tobacco BY2 cells,\(^\text{[13]}\) which suggests that the SPP for the plastoquinone side-chain should be synthesized in plastids and that SPP for the ubiquinone side-chain should be synthesized in the cytosol or mitochondria in A. thaliana. Hence, we speculated that At-SPS1 might be transported into different organelles, viz., chloroplasts or cytosol/mitochondria, to yield the precursor of the side-chain of both prenylquinones. Alternatively, At-SPS1 might participate in only one prenylquinone biosynthesis, and an SPP synthase other than At-SPS1 might serve to produce the precursor of the other prenylquinone side-chain. Screening of the A. thaliana sequence database using the At-SPS1 amino acid sequence as a query resulted in the identification of a highly homologous sequence (At-SPS2). In this study, we isolated the corresponding cDNA and enzymologically characterized the recombinant protein. Moreover, we also investigated the transcription levels and intracellular localization of both At-SPS1 and At-SPS2. The results shown here should provide new insight into the intracellular compartmentalization of two prenylquinone biosyntheses.

Materials and Methods

Materials. A. thaliana (ecotype Columbia) seeds were grown on sterile soil at 23 °C with a light intensity of 50 μmol·m\(^{-2}\)·s\(^{-1}\) and a day length of 16 h. A pBluescript\(^\text{®}\) II KS (−) vector (Stratagene) and a pT7Blue T-vector (Novagen) were used for subcloning. Escherichia coli BL21(DE3) and a pET-15b vector (Novagen) were used for expression of the recombinant protein. [4-\(^{14}\)C]IPP was purchased from NEN\(^\text{™}\) Life Science Products. Non-labeled IPP, dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), (all-trans)-FPP, (all-trans)-GGPP, and acid phosphatase (potato) were from Sigma. A pre-coated reverse-phase TLC plate (RP-18) was from Merck.

cDNA cloning. Total RNA was isolated from the total tissue of 4-week-old A. thaliana plants and then first-strand cDNA was synthesized according to the method reported previously.\(^\text{[9]}\) To isolate the coding sequence of the At-SPS1 homolog (accession no. AC007651), PCR was performed using the first-strand cDNA as the template and specific primers, At-SPS2-F (5’-GATGTCATGCGAATAGACTTGGG-3’) and At-SPS2-R (5’-CCCTTTGAAACTAATCACTTTC-3’), which were designed from sequence information on the database. The PCR product was subcloned into the EcoR V site of a pBluescript\(^\text{®}\) II KS (−) vector to give a plasmid designated pBS-At-SPS2. To determine the 5’-untranslated region (UTR) and 3’-UTR of the entire cDNA corresponding to the above-mentioned coding sequence, 5’- and 3’-rapid amplification of cDNA ends (RACE) was conducted. For 5’-RACE, the 5’-Full RACE Core Set (TaKaRa) was used with A. thaliana total RNA as the template and a set of specific primers, At-SPS2-RT (5’-pATAGCTCGTGAACCG-3’) for reverse transcription, At-SPS2-1st-F (5’-TTGATACACGATGTGTTTAGAC-3’) and At-SPS2-1st-R (5’-TTCCGGTGTGAATCATCTCATC-3’) for the first PCR, and At-SPS2-2nd-F (5’-AAAGTGATATGCGAAAGGGAAGG-3’) and At-SPS2-2nd-R (5’-CACCTACCGGGATCTTTAC-3’) for the second PCR. For 3’-RACE, the 3’-Full RACE Core Set (TaKaRa) was used with A. thaliana total RNA as the template and a set of specific primers (At-SPS2-1st-F for the first PCR and At-SPS2-2nd-F for the second). These sequence data, combined with those of the coding sequence, were compared with the corresponding genome sequence, and it was confirmed that non-specific mutagenesis had not been introduced during PCR. The sequence of the full-length cDNA is registered in the DDBJ/GenBank/EMBL/GSDB DNA databases under accession no. AB104727. The open reading frame (ORF) of this cDNA was designated At-SPS2 (A. thaliana solanesyl diphosphate synthase 2).

Expression and purification. The E. coli expression system for the full length of the At-SPS2 protein (At-SPS2) was constructed as follows: Restriction endonuclease sites at both ends of At-SPS2 were introduced by PCR using pBS-At-SPS2 as the template and a pair of primers, At-SPS2-NdeI-F (5’-GCCCATATGATGTGTCACTGCGGAATTAG-3’) and At-SPS2-BamHI-R (5’-CCTGGATCCCTATCTCCCTTCTTAC-3’; the Nde I and BamH I sites are underlined). The At-SPS2 fragment was then digested with Nde I and BamH I and ligated into the same sites of a pET-15b vector, yielding the expression plasmid pET-15-At-SPS2, which encodes an N-terminal in-frame fusion of At-SPS2 with the His\(_6\) tag. We also constructed the E. coli expression system for the truncated form of At-SPS2 (At-SPS2Δ), in which the 30 N-terminal residues of a putative signal-peptide were removed, according to the procedure mentioned above, except for the use of a primer, At-SPS2Δ-NdeI-F (5’-ATTCCATATGGGAATTTTCTCAGGACC-3’; the Nde I site is underlined, instead of At-SPS2-NdeI-F.
The resultant expression plasmid pET-15-At-SPS2Δ encodes an N-terminal in-frame fusion of At-SPS2Δ with the His6 tag.

*E. coli* BL21(DE3) was transformed with either of these expression plasmids. The cells were grown at 37 °C in 1 liter of Luria–Bertani medium supplemented with 60 μg/ml of ampicillin until OD600 reached 1.0. Subsequently, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM, followed by further cultivation for 4 h at 25 °C. Cells were harvested by centrifugation (4000 g, 10 min) and then disrupted by the addition of BugBuster™ HT (Novagen) supplemented with 1 mM phenylmethylsulfonyl fluoride. All subsequent operations were carried out at 4 °C. After centrifugation (8000 g, 30 min) and filtration (0.45 μm), the supernatant was applied to a HiTrap Chelating HP column (Amersham Biosciences) charged with Ni2+ and equilibrated with buffer 1 (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) containing 10 mM imidazole. The column was washed with buffer 1 containing 50 mM imidazole and eluted with a stepwise gradient of 100–500 mM imidazole in buffer 1. The fractions of the targeted protein were concentrated and then subjected to gel filtration performed with a Superdex 200 HR 10/30 column (Amersham) at 0.3 ml/min with buffer 2 (50 mM Mops, 150 mM NaCl, pH 8.0).

After purification, the homogeneity of the protein was evaluated by SDS/PAGE with Coomassie Brilliant Blue staining. We estimated the purity of the protein by measuring the intensities of the stained bands on the gel with NIH Image (http://rsb.info.nih.gov/nih-image/). Protein concentrations were measured by the method of Bradford with bovine serum albumin as a standard.

**Measurement of enzymic activity and product analysis.** Enzymic activity was measured by determination of the amount of [4-14]CIPP incorporated into butanol-extractable polypropenyl diphosphates. The standard assay mixture contained, in a total volume of 200 μl, a 100 mM Mops buffer (pH 8.0), 8 mM MgCl2, 0.05% (w/v) Triton X-100, 50 μM [4-14]CIPP (37 GBq·mol−1), a 50 μM allylic substrate (DMAPP, GPP, FPP or GGPP), and 50–100 ng of the purified enzyme. Incubation was carried out at 30 °C for 10–20 min and stopped by adding 200 μl of NaCl-saturated water before 10% of the substrate had been consumed. We confirmed that the addition of NaCl-saturated water completely terminates this enzymic reaction by carrying out a control experiment (results not shown). The reaction products were then extracted with 1 ml of 1-butanol saturated with NaCl-saturated water, and the radioactivity in the 1-butanol extract was measured by the d.p.m. mode with an LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter) and a Clear-sol I scintillation cocktail (Nacalai Tesque).

For kinetic studies, the concentration of the allylic substrate (FPP/GGPP) or of [4-14]CIPP was varied, while the counter-substrate ([4-14]CIPP or FPP/GGPP respectively) was kept at a saturating concentration. Kinetic parameters and their standard errors were estimated by non-linear regression analysis using Enzyme-Kinetics software version 1.5 (Trinity Software).

To analyze the radioactive reaction products, the extracted prenyl diphosphates were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously. It was verified that prenyl diphosphates shorter than C50 can be hydrolyzed completely and that prenyl diphosphates longer than C45 can be hydrolyzed to some degree to the corresponding alcohols by this method. The alcohols were extracted with n-pentane and analyzed by TLC on a reversed phase RP-18 plate with a solvent system of acetone/water (19:1, v/v). The positions of authentic standards were visualized with iodine vapor, and the absolute radioactivities of the spots were detected with a Bio-image Analyzer BAS1500 (Fuji). The product distributions were determined on the basis of the molar ratios of the products that were obtained by division of the absolute radioactivity of each spot by the number of the IPPs incorporated into the corresponding prenyl alcohol.

**Real-time PCR analysis.** Total RNA was prepared and purified individually from leaves and roots of 3-week-old *A. thaliana* plants according to the above-mentioned method. Using each of these total RNAs, first-strand cDNA was synthesized by M-MLV Reverse Transcriptase RNase H− (Toyobo), treated with RNase H, and then purified with a SUPREC-02 ultrafilter (TaKaRa). The *At-SPS1* (the ORF of *At-SPS1*) and *At-SPS2* transcripts were quantified by real-time PCR on the GeneAmp® 5700 Sequence Detection System (Applied Biosystems), in which the first-strand cDNA as the template and a set of specific primers, *At-SPS1*-RT-F (5’-TGGATTTTCATTCAGTGCACA-3’) and *At-SPS1*-RT-R (5’-GCTGTTAAGTTACCTTAGCCA-ATC-3’) for *At-SPS1*, and *At-SPS2*-RT-F (5’-GGTTAGCCCTGCAATGCTAC-3’) and *At-SPS2*-RT-R (5’-GCTCATTCTCTTACGCGAAGA-3’) for *At-SPS2*, were used along with the SYBR® Green PCR Master Mix (Applied Biosystems). Thermal cycling conditions were 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. We confirmed that each pair of primers specifically amplified the targeted sequence but not its counterpart by performing a control experiment using pET-15-At-trans-PT, in which *At-SPS1* is included, or pET-15-At-SPS2 as the template (results not shown). The elongation factor EF1α (accession no. NM_125432) was used to normalize the first-strand cDNA concentrations. A pair of primers, At-EF1α-RT-F (5’-TGGATT-CTTCCACCTTCCAGGATGTC-3’) and At-EF1α-RT-R (5’-CAGGCTTGTACATACCAGTCTCAA-3’), was used to amplify the EF1α sequence fragment.

Data analysis was conducted according to the user bulletins for the ABI PRISM 7700 Sequencing Detec-
tion System provided by the manufacturer (http://docs.appliedbiosystems.com/search.taf). Because the ΔC_{T} values of At-SPS2 varied slightly from the amount of the template cDNA, we did not use the comparative C_{T} method. Alternatively, the relative standard curve method was adopted for relative quantitation.

**Transient expression of green fluorescent protein-fusion proteins.** The full length of At-SPS1 was amplified by PCR using pET-15-At-trans-PT as the template and a pair of primers, At-SPS1-BamHI-F (5'-GGATCCATGATGATGTCATGTCGGAATATAG-3') and At-SPS1-NcoI-R (5'-CCATGGGAATCCTTTCGAGGTATACAAC-3'); the BamH I and Nco I sites are underlined. To amplify the full length of At-SPS2, PCR was also performed with pET-15-At-SPS2 as the template and a pair of primers, At-SPS2-BamHI-F (5'-GGATCCATGATGATGTCATGTCGGAATATAG-3') and At-SPS2-NcoI-R (5'-CCATGGGAATCCTTTCGAGGTATACAAC-3'); the BamH I and Nco I sites are underlined. Each of the fragments was digested with BamH I and Nco I and ligated at the same sites into the 35S-C10-sGFP(S65T) vector, which encodes the engineered green fluorescent protein (sGFP(S65T)). Because the cauliflower mosaic virus 35S promoter region was removed from the 35S-C10-sGFP(S65T) vector by the BamH I digestion, this promoter region was reinserted at the BamH I site into the vector containing the sGFP(S65T) gene and At-SPS1 or At-SPS2 to construct the plasmids pAt-SPS1-sGFP(S65T) and pAt-SPS2-sGFP(S65T) respectively. These plasmids were designed to express the N-terminal in-frame fusion of sGFP(S65T) with the full-length At-SPS1 or the full-length At-SPS2 under the control of the cauliflower mosaic virus 35S promoter in plant cells.

For transient expression of the sGFP(S65T)-fused proteins, mesophyll protoplasts were prepared from 3-week-old *A. thaliana* plants and transfected with each of the above-mentioned plasmids according to the protocol described by Sheen (http://genetics.mgh.harvard.edu/sheenweb/). About 4 x 10^4 protoplasts were transfected with 20 μg of the plasmid DNA. The transfected protoplasts were then incubated under dark conditions at 22°C for 16 h in a W5 solution (154 mM NaCl, 125 mM CaCl_2, 5 mM KCl, 2 mM Mes, pH 5.7) to allow accumulation of the fusion proteins.

Fluorescence images of the protoplasts were taken using the Laser Scanning System Radiance 2100 (Bio-Rad). Wavelengths of 488 and 543 nm were used for fluorescence excitation of sGFP(S65T) and chlorophyll respectively. Fluorescence emission was collected from 515–530 nm for sGFP(S65T) and 570–700 nm for chlorophyll. An image of the mitochondria and the endoplasmic reticulum (ER) stained with 3,3'-dihexyl-oxacarbocyanine iodide (DiOC_6; Molecular Probes) was observed using the same excitation and collection wavelengths as for sGFP(S65T).

**Results**

**Isolation of cDNA for At-SPS2**

We searched the *A. thaliana* sequence database for homologs of At-SPS1 (formerly At-SPS; accession no. BAB86941) using the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/). We found one amino acid sequence (accession no. AAD50025) that showed 79% identity with the At-SPS1 amino acid sequence (Fig. 1). The coding sequence of AAD50025 (accession no. AC007651) was obtained by PCR with sequence-specific primers and the first-strand cDNA derived from *A. thaliana* total tissue as the template. Subsequently, 5'- and 3'-UTRs were determined by 5'-RACE and 3'-RACE respectively. We designated the ORF encoded by the full-length cDNA (accession no. AB104727) At-SPS2 (*A. thaliana* solanesyl diphosphate synthase 2). The At-SPS2 protein (At-SPS2) consists of 417 amino acids with a calculated M_r of 46,044 and contains several conserved regions through...
trans-prenyl diphosphate synthases, including the first aspartate-rich motif (FARM) and the second aspartate-rich motif, which are regarded as the substrate-binding domains. Like At-SPS1, At-SPS2 possesses small residues (A117 and S178) at the fifth and fourth positions before the FARM and contains no insertions within the FARM, characteristic of trans-long-chain prenyl diphosphate synthase.

At-SPS2 showed 23–24% identity with two A. thaliana FPP synthases (accession nos. AAF44787 and S71182) and 30–37% identity with five A. thaliana GGPP synthases (accession nos. AAA32797, AAB-67730, BAB02387, AAD12206, and BAA23157). Moreover, At-SPS2 has significant similarity (40% identity) with A. thaliana GPP (C10) synthase (accession no. CAC16849), which catalyzes only a single condensation of IPP with DMAPP. Although At-SPS2 was highly homologous to At-SPS1 within the regions implicated in the enzymic activity, the similarity was low in their N-terminal regions, which are assumed to be signal sequences to target-specific organelles.

Substrate and product specificities of At-SPS2

For functional analysis, At-SPS2 was heterologously expressed as the His6-tagged fusion protein in E. coli cells. Although most of the recombinant protein formed insoluble inclusion bodies, small amounts of the protein were obtained in the soluble fraction. We tried to purify the soluble protein in two chromatographic steps and succeeded in partially purifying it to about 10% purity. Williams et al. have reported that the solubility of (−)-4s-limonen synthase was improved by removal of its plastidial targeting sequence. Hence, in order to obtain sufficient amounts of soluble active enzyme, we constructed an expression system for the truncated form of At-SPS2 (At-SPS2Δ) in which the 30 N-terminal residues of the putative signal-peptide were removed and, instead, the His6 tag was attached to the N-terminus. At-SPS2Δ was heterologously expressed in E. coli cells, and most of the recombinant protein was acquired as a soluble protein. The soluble At-SPS2Δ was readily purified to about 90% purity by the same chromatographic procedure as for the full-length At-SPS2. Both the partially purified At-SPS2 and the sufficiently purified At-SPS2Δ were examined as to their condensation activity using 50 μM FPP and 50 μM [4,14]CIPP as the substrates. The result was that the maximum activity of both forms was achieved at pH 8.0 (100 mM Mops buffer), with 8 mM MgCl2 and 0.05% Triton X-100, consistently, with the case of At-SPS1.

Four kinds of allylic diphosphates at 50 μM were tested as a primer substrate with 50 μM [4,14]CIPP under optimum conditions, described above. Both At-SPS2 and At-SPS2Δ were determined to utilize FPP and GGPP as a primer substrate but not DMAPP or GPP. The reaction products with FPP or GGPP were dephosphorylated and then analyzed by reversed-phase TLC. Whether FPP or GGPP was used as the primer substrate, solanesol (C45) was predominantly detected by TLC analysis, indicating that At-SPS2 and At-SPS2Δ gave solanesyl diphosphate (SPP, C45) as the major product (Table 1). Hence, we have confirmed that At-SPS2 is an SPP synthase (EC 2.5.1.11), and that A. thaliana possesses at least two SPP synthases (At-SPS1 and At-SPS2). When GGPP was used as the allylic substrate, At-SPS2 and At-SPS2Δ produced a large amount of SPP. In contrast, when FPP was used as the primer substrate, considerable amounts of intermediates, such as GGPP, were formed, although the main product was still SPP in the both case of At-SPS2 and that of At-SPS2Δ (Table 1). The substrate and product specificities of both forms of the enzyme were almost the same as those of At-SPS1. Because At-SPS2Δ showed the same enzymic features as the full-length At-SPS2 and was more tractable than the full-length enzyme, we carried out the kinetic analysis using mainly At-SPS2Δ.

Table 1. Product Distributions of At-SPS2 and At-SPS2Δ

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Allylic substrate</th>
<th>Product distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At-SPS2</td>
<td>FPP</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>GGPP</td>
<td>11</td>
</tr>
<tr>
<td>At-SPS2Δ</td>
<td>FPP</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>GGPP</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Kinetic analysis

Kinetic parameters of At-SPS2Δ were determined with [4,14]CIPP and FPP or GGPP as substrates. The results are summarized in Table 2. At-SPS2Δ showed an 8.2-fold lower Km value for GGPP than for FPP and a slightly lower kcat value for GGPP than for FPP. Consequently, the kcat/Km value for GGPP was 5.1-fold higher than that of FPP. Moreover, when GGPP was used as the counter substrate, At-SPS2Δ showed a 6.3-fold lower Km value for IPP than that obtained with FPP as the counter substrate. An almost equal kcat value for IPP was observed in each case. Consequently, the kcat/Km value for IPP determined with GGPP was 3.8-fold higher than that for IPP with FPP. Using the partially purified enzyme, we also determined the Km values of full-length At-SPS2 and found that these Km values were similar to the corresponding values of At-SPS2Δ. These results indicate that both At-SPS2Δ and At-SPS2 prefer GGPP to FPP for the primer substrate and that the substrate specificity of At-SPS2 is not affected by removal of the putative signal-peptide. A preference for GGPP over FPP was also observed in the case of At-SPS1.
The kinetic parameters of At-SPS2 and At-SPS2Δ were determined at 30°C for 10–20 min, as described in “Materials and Methods”. The concentrations of the counter-substrates were set at saturating levels as indicated. The kcat value was defined as the nmolar amount of IPP converted into products per s by 1 nmol of one subunit of the enzyme. Because the purity of At-SPS2 was insufficient, its kcat values were not determined.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Counter-substrate</th>
<th>kcat (μmol·min⁻¹·g⁻¹)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/km (s⁻¹·μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At-SPS2Δ</td>
<td>FPP</td>
<td>500 μM IPP</td>
<td>6.89 ± 0.74</td>
<td>3.77 ± 0.15</td>
<td>0.547</td>
</tr>
<tr>
<td></td>
<td>GGPP</td>
<td>100 μM IPP</td>
<td>0.843 ± 0.157</td>
<td>2.33 ± 0.13</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>IPP</td>
<td>20 μM FPP</td>
<td>182 ± 30</td>
<td>2.83 ± 0.21</td>
<td>0.0155</td>
</tr>
<tr>
<td></td>
<td>IPP</td>
<td>4 μM GGPP</td>
<td>28.9 ± 4.2</td>
<td>1.72 ± 0.10</td>
<td>0.595</td>
</tr>
<tr>
<td>At-SPS2</td>
<td>FPP</td>
<td>500 μM IPP</td>
<td>5.12 ± 0.57</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>GGPP</td>
<td>100 μM IPP</td>
<td>0.565 ± 0.178</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>IPP</td>
<td>20 μM FPP</td>
<td>248 ± 40</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>IPP</td>
<td>4 μM GGPP</td>
<td>37.7 ± 6.9</td>
<td>—</td>
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</table>

Real-time PCR analysis of two SPP synthases

To examine the mRNA levels of At-SPS1 and At-SPS2, real-time PCR analysis was carried out with first-strand cDNAs prepared individually from leaves and roots of 3-week-old A. thaliana plants and used as the template along with the primer sets specific for each of the targeted sequences. The data were normalized against the level of elongation template along with the primer sets specific for each of the targeted sequences. The result was represented as relative values based on the mean value of At-SPS1 in roots (mean value ± S.D. (n = 9)).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene</th>
<th>Relative mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>At-SPS1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td></td>
<td>At-SPS2</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Root</td>
<td>At-SPS1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>At-SPS2</td>
<td>0.75 ± 0.09</td>
</tr>
</tbody>
</table>

Subcellular localization of two SPP synthases

Because both At-SPS1 and At-SPS2 appear to have putative signal-peptides at the N-terminal regions, we analyzed their subcellular localization using the transient expression of the N-terminal in-frame fusion of sGFP(S65T) with full-length At-SPS1 or full-length At-SPS2 (At-SPS1-sGFP(S65T) or At-SPS2-sGFP(S65T)) in A. thaliana mesophyll protoplasts. The transfected cells were observed with a fluorescence microscope.

In the protoplast cells expressing sGFP(S65T) alone, the green fluorescence of the protein was detected in the cytoplasm and the nucleus under blue light (Fig. 2A), as reported previously,18 and chloroplasts were seen as red dots due to the autofluorescence of chlorophylls under green light (Fig. 2B, E, H, and K). When At-SPS1-sGFP(S65T) was expressed, the green fluorescence of the fusion protein showed a blurred image mainly around the nucleus (Fig. 2D). A similar green fluorescence pattern was observed when the ER was stained with DiOC6 (Fig. 2J). Hence, we judged that At-SPS1-sGFP(S65T) was localized in the ER. In the case of At-
indicates that At-SPS2-sGFP(S65T) was translocated into chloroplasts (Fig. 2G, H, and I). This result clearly indicates that At-SPS2-sGFP(S65T) was translocated into chloroplasts.

**Discussion**

We isolated the cDNA for the At-SPS1 homolog from *A. thaliana* and performed enzymological characterization of the recombinant protein. Product analysis indicated that the isolated cDNA also encodes an SPP synthase (Table 1), and that *A. thaliana* possesses at least two SPP synthases. The full-length of At-SPS2 could not be purified to homogeneity, probably because its solubility was impaired by the putative signal-peptide. Hence, we constructed an expression system for the N-terminus-truncated form of At-SPS2 (At-SPS2Δ) and carried out purification to acquire the At-SPS2Δ protein in high purity. The full-length At-SPS2 and At-SPS2Δ showed similar features to those previously reported for the full-length At-SPS1,9) which indicates that the basic catalytic machinery of At-SPS1 and At-SPS2 is almost the same and that the putative signal-peptide attached to each of the N-terminal regions does not seriously affect the catalytic function itself. Indeed, the amino acid sequences of At-SPS1 and At-SPS2, except for their putative signal-peptides, showed very high similarity with each other, suggesting that they originated from a common ancestor by gene duplication (Fig. 1).

We also analyzed subcellular localization of At-SPS1 and At-SPS2 using green fluorescent protein-fusion proteins. It was found that At-SPS2-sGFP(S65T) was translocated into chloroplasts whereas the fluorescence image of At-SPS1-sGFP(S65T) was not clear, but it is likely that this fusion protein was localized in the ER. As for At-SPS2, the result shown here clearly indicates that At-SPS2 is transported into chloroplasts and serves to supply the precursor of the plastoquinone side-chain. Considering that At-SPS2 does not appear to have a transmembrane domain,27) and that it showed maximum activity under weakly basic conditions (pH 8.0), we predict that At-SPS2 is localized in the chloroplastic stroma. Previous studies have reported that the most abundantly expressed GGPP synthase of the five isoenzymes is transported into the chloroplastic stroma to give the allylic substrate for At-SPS2,24,28) and that the enzymes responsible for the final steps of plastoquinone biosynthesis, including transfer of the solanesyl group to homogentisate, are localized on the inner envelope membrane of chloroplasts.29) Hence, we assume that plastoquinone biosynthesis proceeds in the closed space of chloroplasts (Fig. 3).

Although it is not sufficient to reach a conclusion, the result for At-SPS1-sGFP(S65T) strongly suggests that At-SPS1 is localized in the ER. Like At-SPS2, At-SPS1 is unlikely to contain a transmembrane domain,27) which implies that At-SPS1 might be localized in the ER lumen or associated with an ER-membrane-bound protein. At-SPS1 does not have a well-known ER-retention signal, such as the H/KDEL or KKXX sequence, at its C-terminus.30) Thus this enzyme might be retained in the ER by another mechanism: it might be transferred to another compartment through the ER/Golgi protein sorting system. Given that At-SPS1 is localized in the ER, this enzyme probably utilizes IPP derived from the mevalonate pathway. Because the side-chain of ubiquinone is synthesized from the mevalonate-derived IPP,13) it is plausible that At-SPS1 participates in the biosynthesis of the ubiquinone side-chain. It is thought that plant FPP synthase is localized mainly in the cytosol.31) In addition, it has been reported that in *A. thaliana*, two GGPP synthases of five isoenzymes are localized in the ER, although their expressed tissues and levels are limited.24) Hence, we assume that At-SPS1 utilizes either or both of two kinds of allylic substrates, FPP and GGPP, which are synthesized from mevalonate-derived IPP (Fig. 3).

To date, many enzymes involved in ubiquinone biosynthesis have been cloned from yeasts, and it has been found that most of them are localized in the mitochondrion6,32–36) Among plant ubiquinone biosynthetic enzymes, *A. thaliana* 3,4-dihydroxy-5-trans-prenyl-benzoate methyltransferase and *A. thaliana* 4-hydroxy benzoate polyprenyltransferase have thus far been characterized, and both enzymes have been shown to be localized in mitochondria.37,38) 4-Hydroxy benzoate polyprenyltransferase catalyzes attachment of the polyprenyl group to 4-hydroxy benzoate to yield 4-hydroxy-3-polyenyl benzoate. Hence, we speculate that SPP synthesized by At-SPS1 in the ER is transported into the mitochondria by an unknown mechanism to synthesize ubiquinone in *A. thaliana* (Fig. 3). Recent studies have found that under the specific condition that one IPP biosynthetic pathway is inhibited, isoprenoid intermediates synthesized via the other IPP biosynthetic pathway were supplied into the IPP-depleted intracellular compartment.39,40) The unidentified SPP-transporting mechanism might be included in this metabolic cross-talk system between cytosolic and plastidial pathways of isoprenoid biosynthesis. On the other hand, other research groups have reported that SPP is synthesized by condensation of IPP to GPP or GGPP followed by transfer of the solanesyl group to 4-hydroxy benzoate and homogentisate to produce the precursors of ubiquinone and plastoquinone respectively in the Golgi/ER system of spinach leaves.41,42) The SPP synthase activity that they detected might correspond to that of At-SPS1. In any case, the localizations of two prenylquinone biosyntheses are still controversial in plants. Additional experiments, such as *in vitro* translation followed by incorporation into isolated organelles, are required to conclude that At-SPS1 is localized in the ER. Moreover, further detailed analysis, such as cloning and
clarification of the subcellular localizations of the other prenylquinone-biosynthetic enzymes, are also necessary to elucidate how and where biosynthesis of the two prenylquinones proceeds in plants, including *A. thaliana*. However, at the least, the results presented here strongly suggest that the precursors of the side-chains of plastoquinone and ubiquinone are synthesized by distinct enzymes in different intracellular compartments, and should provide some insight into the intracellular compartmentalization of prenylquinone biosynthesis in *A. thaliana*.

**Acknowledgment**

We thank Dr. Y. Niwa of the University of Shizuoka, Japan, for providing plasmid 35Ω-xGFP(S65T). This work was obtained from the Earth Program, Development of Transgenic Plants for Production of Industrial Materials Project from the New Energy and Industrial Technology Development Organization (NEDO), in coordination with the Ministry of Economy, Trade and Industry (METI).
References


