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Enzymes Encoded by the Farnesyl Diphosphate Synthase Gene Family in the Big Sagebrush *Artemisia tridentata* ssp. spiciformis*

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Farnesyl diphosphate synthase catalyzes the sequential head-to-tail condensation of two molecules of isopentenyl diphosphate with dimethylallyl diphosphate. In plants the presence of farnesyl diphosphate synthase isozymes offers the possibility of differential regulation. Three full-length cDNAs encoding putative isoprenoid synthases, FDS-1, FDS-2, and FDS-5, with greater than 89% similarity were isolated from a Big Sagebrush *Artemisia tridentata* cDNA library using a three-step polymerase chain reaction protocol. One of the open reading frames, FDS-5, encoded a protein with an N-terminal amino acid extension that was identified as a plastidial targeting peptide. Recombinant histidine-tagged versions of three proteins were purified, and their enzymatic properties were characterized. FDS-1 and FDS-2 synthesized farnesyl diphosphate as the final chain elongation product, but their kinetic behavior varied. FDS-1 prefers geranyl diphosphate over dimethylallyl diphosphate as an allylic substrate and is active at acidic pH values compared with FDS-2. In contrast, FDS-5 synthesized two irregular monoterpenoids, chrysanthemyl diphosphate and lavandulyl diphosphate, when incubated with dimethylallyl diphosphate and an additional product, the regular monoterpene geranyl diphosphate, when incubated with isopentenyl diphosphate and dimethylallyl diphosphate. Specific cellular functions are proposed for each of the three enzymes, and a scenario for evolution of isoprenyl synthases in plants is presented.

Plants synthesize a vast array of structurally diverse isoprenoid molecules (1). These compounds have important functional roles in plants, and many are useful in industry, agriculture, and medicine. As a result there are intense research efforts under way to characterize the isoprenoid pathway, its enzymes, and its regulation *in vivo*. Some isoprenoid compounds in plants, such as phytosterols, growth hormones, and plastidic pigments, are essential for development. Others, such as mono-, sesqui-, and some diterpenes, are not necessary for cell viability but frequently act as important mediators in plant ecology (2). Plants are able to alter isoprenoid production in response to their environment, and elucidation of the isoprenoid pathway and its regulation is important if one wishes to alter the flux of isoprenoid metabolites for production of industrially useful compounds and pharmaceuticals. Among the complexities associated with deciphering isoprenoid regulatory mechanisms is the presence of gene families or multiple copies of the same genes, the selective synthesis of isoprenoid compounds in specific organelles, and the operation of two independent pathways for synthesis of the C_5 building blocks isopentenyl diphosphate (IPP)[1] and dimethylallyl diphosphate (DMAPP) (3). The mevalonate pathway for the synthesis of IPP and DMAPP is located in the cytosol where the triterpenoids, including phytosterols, sesquiterpenoids, and prenylated proteins, are synthesized, whereas the 2-*C*-methyl-D-erythritol 4-phosphate pathway operates in plant plastids, where isoprenoids involved in photosynthesis (carotenoids, chlorophyll, and plastoquinone) and many secondary metabolites are synthesized (4). Mitochondrial isoprenoids, such as ubiquinones, are synthesized directly in the mitochondria from IPPs imported from the cytosol (5). The metabolic flux through different pathways and in different compartments of the plant cell can vary substantially depending upon its particular requirements. Understanding how common isoprenoid intermediates, such as IPP and farnesyl diphosphate (FPP), are managed is an important step in understanding the regulation of isoprenoid metabolism within a plant cell.

Several isoprenoid enzymes are encoded by gene families. A prominent example is farnesyl diphosphate synthase (FDS), (EC 2.5.1.10). FDS catalyzes the sequential 1′-4 condensations of IPP with DMAPP to produce geranyl diphosphate (GPP) and with GPP to give FPP. At least two different FDS isoforms are found in rice (*Oryza sativa*) (6), guayule (7) and white lupin (8). The presence of isogenes coupled with the fact that FPP is located at a multiple branch point in the isoprenoid biosynthetic pathway suggests that FPP biosynthesis is tightly regulated. Evidence for this hypothesis was obtained by Cunillera *et al.*, who demonstrated that *Arabidopsis thaliana* contains at least two differentially regulated genes coding for FDS (9) and later showed that one of these genes is controlled at the transcriptional level. It can be transcribed and translated into an FDS isoform that bears a mitochondrial targeting peptide or an

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY308477 (FDS-1), AY308476 (FDS-2), and AY308478 (ArtCDS).

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[1] The abbreviations used are: IPP, isopentenyl diphosphate; CPP, chrysanthemyl diphosphate; CDS, chrysanthemyl diphosphate synthase; DMAPP, dimethylallyl diphosphate; FDS, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; GDS, geranyl diphosphate synthase; GFP, green fluorescent protein; GPP, geranyl diphosphate; GGDS, geranylgeranyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; ORF, open reading frame; PIPES, 1,4-piperazinediethanesulfonic acid; GC, gas chromatography; MS, mass spectroscopy; LPP, lavandulyl diphosphate.

TABLE I
Oligonucleotides used in this study

Name	Sequence	Use
$(dT)_{18}$*Not*I	5′-pGACTAGTTCTAGATCGCGAGCGGCCGCCC(T)$_{15}$-3′	cDNA preparation
*Sal*I adapter	5′-TCGA(C/G)(C/G)(G/C)(A/T)(C/G)(G/C)(C/G)(G/C)(T/A)(C/G)(C/G)(G/Cp)-3′	cDNA preparation
PTSIIa	5′-(C/T)TT(C/T)(C/T)TIGTII(C/T)IGA(T/C)GA(C/T)ATIATGGA-3′	First-step PCR
PTSIIb	5′-GA(T/C)GA(T/C)ATIATGGA(T/C)-3′	First-step PCR
PTSVa	5′-TA(A/G)TC(A/G)TC(T/C)TGIAT(C/T)TG(A/G)AA-3′	First-step PCR
PTSVb	5′-(G/A)TCIA(G/A)(G/A)TA(A/G)TC(A/G)TC(T/C)TG-3′	First-step PCR
B29	5′-GGTATTGCACGCGTCGACCCACGCGTCCG-3′	Third-step PCR
B31	5′-TAGTTCTAGATCGCGAGCGGCCGCCCTTTTT-3′	Second-step PCR
F1FDS1	5′-TGTCTATTCACCGCCGGATTG-3′	P3 for FDS-1 cloning
R1FDS1	5′-GGAGAATGCGAATGATTTTCC-3′	P4 for FDS-1 cloning
F1FDS2	5′-GGTGAGAATTTGGACGATCATG-3′	P3 for FDS-2 cloning
R1FDS2	5′-GACTAGGAGACCTACTTTTGCCTCTTA-3′	P4 for FDS-2 cloning
F1FDS5	5′-GATACGATCTCTAGACTAGCTGG-3′	P3 for FDS-5 cloning
R1FDS5	5′-GACATCTTATTGTTCCGTTAC-3′	P4 for FDS-5 cloning
PT5GFPF	5′-CATGCCATGGCATCCTTTATTAGTCTTTCTTCC-3′	GFP fusion protein
PT5GFPR	5′-GTCCACCATGGTGAGCGGAGAATAGCTGGG-3′	GFP fusion protein
*Nde*I-H_6-Xa-F1	5′-GGGAATTCCATATGCACCATCATCATCATCATATAGAGGGGCGGATGAGTAGTAGTAAAAGTATC-3′	Enzyme expression
*Bam*HI-F1	5′-CGCGGATCCGCGGGTACGGTACATCTACTTTTGCCT-3′	Enzyme expression
*Nde*I-H_6-Xa-F2	5′-GGGAATTCCATATGCACCATCATCATCATCATATAGAGGGGCGGATGAGCATTGATCTCAAGT CTAGG-3′	Enzyme expression
*Bam*HI-F2	5′-CGCGGATCCGCGCTAGGAGACCTACTTTTGCCTC-3′	Enzyme expression
*Nde*I-H_6-Xa-F5B	5′-GGGAATTCCATATGCACCATCATCATCATCATATAGAGGGGCGGACTACAACATTGAGCAGCA ATCTG-3′	Enzyme expression
*Bam*HI-F5	5′-CGCGGATCCGCGCAAGCTAAGTTACTTATGTCC-3′	Enzyme expression

FDS protein without the signaling sequence (10). Thus, synthesis of FPP is tightly controlled and is catalyzed by enzymes localized to different compartments.

Recently, chrysanthemyl diphosphate synthase (CDS), an enzyme with ~80% sequence similarity to FDS in *Artemisia annua*, was isolated from *Chrysanthemum cinerariaefolium* (11). Instead of catalyzing chain elongation to produce FPP, CDS condenses two molecules of DMAPP to produce an irregular monoterpene, chrysanthemyl diphosphate (CPP). This reaction is the committed step for biosynthesis of the monoterpene component of the industrially important pyrethrin insecticides (12). The cyclopropane structure of CPP is similar to those of presqualene diphosphate and prephytoene diphosphate, the transient intermediates formed by squalene synthase and phytoene synthase during the committed steps of sterol and carotenoid biosynthesis, respectively (13). Interestingly, CDS has very low sequence homology with either squalene synthase or phytoene synthase. The high sequence homology between FDS and CDS from closely related plants suggests that the mechanisms for chain elongation catalyzed by FDS and the cyclopropanation reactions are similar (11).

We thought that identification and characterization of genes for FDS and CDS from the same organism might provide an insight into how biosynthesis of FPP and CPP is regulated in plants. *Artemisia tridentata* ssp. spiciformis, a plant found in the Great Basin region of the Western United States, was selected as an ideal candidate for this investigation for several reasons. Natural products derived from CPP, GPP, and FPP have been isolated from members of the *Artemisia* genus (14, 15). One of these materials, artemisinin, is a sesquiterpenoid derived from FPP with antimalarial properties (16, 17). Also, *Artemisia* plants produce a great variety of irregular or non-head-to-tail structures, including chrysanthemol (18, 19) and lavandulol (20, 21). We now describe the isolation of genes for two isoforms of FDS and a gene for CDS from an *A. tridentata* ssp. spiciformis cDNA library. The genes were cloned into expression vectors, and the recombinant enzymes were characterized. A model for the evolution of isoprenoid synthases in plants is described.

EXPERIMENTAL PROCEDURES

Materials—*A. tridentata* ssp. spiciformis shoots were collected from the Wasatch Mountains, Utah in late June 1998, from a stand of genetically pure plants. They were immediately frozen and stored in liquid nitrogen until use. *Escherichia coli* XA-90 and pKEN2 were provided by Gregory Verdine (Harvard University, Boston, MA). [4-^{14}C]IPP (58 mCi/mmol) and [1,2,4-^{14}C]FPP (50 mCi/mmol) was purchased from PerkinElmer Life Sciences and Invitrogen, respectively, and [1-^{14}C]DMAPP (55 mCi/mmol) was from American Radiolabeled Chemicals, Saint Louis, MI. Restriction enzymes were purchased from Invitrogen, Stratagene, or Amersham Biosciences. Unlabeled DMAPP, GPP, and IPP were synthesized by the procedure of Davisson *et al.* (22), and concentrations were determined by phosphate analysis (23). Synthesis of oligonucleotide primers (Table I) and DNA sequencing were performed at the Huntsman Cancer Institute Core Facility at the University of Utah.

General Methods—General molecular techniques were adapted from Sambrook *et al* (24). Plasmid DNA was purified with the Qiagen Tip-100 Plasmid Midi Kit (Qiagen, Chatsworth, CA). PCR amplifications were performed in a PerkinElmer Gene-Amp PCR System 2400 DNA thermal cycler and the polymerase mix provided in the Advantage PCR kit (Clontech). DNA electrophoresis was conducted with 0.8% agarose gels and visualized by staining with ethidium bromide. Protein samples were analyzed by SDS-PAGE and stained with Coomassie Blue R according to the procedure of Laemmli (25). Protein concentrations were determined by the method of Bradford (26) (Bio-Rad protein assay) using bovine serum albumin as a standard.

Cloning of FDS Isogenes—A guanidinium thiocyanate, phenol-chloroform procedure (27) was used to isolate total RNA from *A. tridentata* shoots. Poly(A)$^+$ RNA was purified by using the Dynabeads mRNA purification kit (Dynal, Great Neck, NY). Double-strand cDNA synthesis was carried out using 2 μg of mRNA, 1 μg of the oligonucleotide $(dT)_{18}$*Not*I, and the SuperScript™ system for cDNA synthesis (Invitrogen) according to the manufacturer's instructions. A *Sal*I adapter was used to extend the 5′ blunt end of the double-stranded cDNA. Template cDNA was PCR-amplified with two sets of degenerated primers designed from conserved motifs (domain II and domain V) in FDS (28). A 50-μl reaction mixture containing 0.4 μM each sense (PTSIIa or PTSIIb) and antisense (PTSVa or PTSVb) primer, 1 μl of cDNA, 240 μM dNTP and 50× Advantage Klentaq polymerase mix with the provided buffer (Clontech, San Diego, CA) was performed using a step cycle (30 cycles) program of 94 °C for 1 min, 42 °C for 1 min, and 68 °C for 2 min. The ~450-bp products were cloned into pGEMT-easy (Promega, Madison, WI) and sequenced from the 5′ end of the cDNA. The PCR fragments were classified in groups depending on the determined sequence, and for each group a specific forward primer P3 was designed. This primer in combination with the reverse B31 primer specific to the poly(A)$^+$ region of the cDNAs was used to amplify the 3′ region of the gene. Finally a fourth reverse and specific primer, P4, was designed in the noncoding region of each gene, and a third PCR step allowed the amplification of the full-length cDNA using the forward B29 primer, which is specific to the *Sal*I adapter. This three-step PCR was per-

formed at least twice to avoid mistakes introduced by the use of *Taq* polymerase.

Heterologous Expression in E. coli and Purification of Recombinant Poly-His-FDS Isoenzymes—PCR was performed using the sense *Nde*I-H_6-Xa-FY and the antisense *BamHI*-FY primers under the standard conditions described above (Y indicates the numbering of each gene, and Xa corresponds to nucleotide fragment coding for the recognition sequence of the Xa factor, which can be used to remove the N-terminal His_6 tag from the enzymes). The products were subcloned into pMPM3B, a derivative of pKEN2, which was digested with *Nde*I and *Bam*HI to generate pKEN-His_6-FDS-1, pKEN-His_6-FDS-2, and pKEN-His_6-FDS-5. *E. coli* XA-90 was transformed with pKEN-His_6-FDS-1, pKEN-His_6-FDS-2, or pKEN-His_6-FDS-5, and overnight cultures were diluted 100-fold in Luria Bertani (LB) medium containing 100 μg/ml ampicillin. Cells were incubated at 37 °C and induced with isopropyl-β-D-thiogalactoside to a final concentration of 0.5 mM when the A_{600} reached 0.4. Cells were incubated at 23 °C for 12–16 h after induction and were harvested by centrifugation. Proteins were purified using either Ni^{2+} spin columns or Ni^{2+}-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer's protocol. Proteins were dialyzed against 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM β-mercaptoethanol and stored at −80 °C in the presence of 20% of glycerol. Activities of the enzymes were checked immediately and after 1 month.

Construction and Expression of FDS-5 with an N-terminal GFP Fusion—The ORF encoding the targeting sequence corresponding to the first 52 amino acids of *AtsFDS-5* was modified by PCR using the respective primer sets PT5GFPF (sense) and PT5GFPR (antisense). Amplified DNA fragments were digested with *Nco*I and cloned in-frame into the *Nco*I site of the GFP expression vector pGFP-MRC (29). This resulted in a fusion of the transit peptide to the N terminus of GFP (pGFP-PT-FDS-5). The plasmids pGFP-MRC and pGFP-PT-FDS-5 under the control of the cauliflower virus 35 S promoter, were transiently transformed into tobacco *Nicotiana tabacum* cv. xanthi suspension cells by tungsten particle shooting with an inflow gun. After bombarding, the cells were incubated for 16 h at 23 °C, and transformed cells were selected using a Leica MZ12 epifluorescence loupe equipped with an HBO 50 lamp and examined using a Zeiss (Jena, Germany) LSM510 confocal laser-scanning microscope equipped with an inverted Zeiss Axiovert 10 M microscope and a 63×, 1.2 numerical aperture water immersion objective. GFP was excited at 488 nm using an argon laser, and emission spectra were recorded from 505 to 530 nm. Chlorophyll was excited at 543 nm using a helium laser, and emission was recorded at wavelengths higher then 585 nm. Images were handled with the Zeiss LSM Image Browser Version 2.50.0929 software and exported as TIFF files before being processed for printing using Photoshop 5.0 (Adobe Systems, Mountains View, CA).

Enzyme Assays—FDS activity was assayed by the acid lability method (30) in 100 μl (total volume) of 30 mM HEPES-NaOH buffer, pH 7.0, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.5% (v/v) bovine serum albumin, and an appropriate amount of enzyme (between 30 ng and 1 μg). After preincubation for 5 min at 30 °C, reactions were initiated by the addition of substrates (allylic diphosphate and radiolabeled IPP). For routine assays 50 μM [4-^{14}C]IPP (5 μCi/μmol) and 150 μM DMAPP or GPP were used. After 15 min, reactions were quenched by the addition of 200 μl of methanol-HCl (3:1 v/v) and incubated for another 15 min at 37 °C. Acid-labile material was extracted three times with *n*-hexane and analyzed for radioactivity. CDS activity was assayed as previously described (11) with one change; the silica plate was developed in 30:70:16:10 (v/v/v/v) chloroform:pyridine:formic acid:water (88% solution).

pH Dependence—Reactions were incubated as described above, except that a poly buffer with overlapping pK_a values consisting of 60 mM PIPES, HEPES, and TRIS was used. The pH range was from 6 to 9.

Product Analysis by TLC—Reactions were carried out in 20 μl of 35 mM HEPES (pH 7.5) containing 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and the appropriate substrate (50 μM [4-^{14}C]IPP (10 μCi/μmol), 150 μM DMAPP, 150 μM GPP, or 200 μM [1-^{14}C]DMAPP (1.0 μCi/μmol). The reactions were initiated by the addition of 5–10 μg of enzyme and incubated for 1 h at 30 °C before a 5-μl portion was removed and spotted on a 20 × 20-cm 60-Å silica plate (Merck). The plates were developed in 25:15:4:2 (v/v/v/v) $CHCl_3$:methanol:water:acetic acid and dried for 10 min with a gentle stream of air. The TLC plates were imaged (Molecular Dynamics Storm 840 PhosphorImager), and the resulting autoradiogram was analyzed to determine the R_f of the radiolabeled products.

Product Analysis by GC and GC-MS—Purified CDS (100 μg) was added to 35 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 4.0 mM dithiothreitol, 50 μM IPP, and 200 μM DMAPP to a final volume of 200 μl. The mixture was incubated for 2 h at 30 °C. The isoprenoid diphosphate products were hydrolyzed to the corresponding alcohols by the addition of 40 μl of 500 mM glycine, pH 10.5, containing 5 mM $ZnCl_2$ and 40 units of calf alkaline phosphatase (Sigma) followed by incubation at 37 °C for 1 h. Approximately 0.2 g of NaCl was added, and the aqueous phase was extracted three times with 100 μl of *tert*-butyl methyl ether. The volume of the extract was reduced to ~5 μl with a stream of dry nitrogen, and 50 μl of fresh methyl *t*-butyl ether were added. A 1-μl portion of the extract was analyzed by gas chromatography on a 30-m × 0.252-mm × 0.25-μm DB-5 capillary column (J&W Scientific) with a temperature gradient from 70 to 150 °C at 2 °C/min. The mass spectra of eluted peaks were compared with authentic samples of lavandulol, geraniol, and *trans*-chrysanthemol.

RESULTS

Cloning and Sequence Analysis of cDNA Members of the Farnesyl Diphosphate Synthase Gene Family in A. tridentata—Initial screening of more than 300 sequenced 450-bp PCR products gave 5 different groups with strong FDS sequence homologies (FDS-1, FDS-2, FDS-3, FDS-4, and FDS-5). Group 1 showed high identity with group 4 and group 3 with group 5. A Southern blot analysis did not show the presence of additional genes. Consequently, full-length cDNAs coding for the isozymes FDS-1, FDS-2, and FDS-5 were isolated in a ratio of 40:2:1 for FDS-1:FDS-2:FDS-5. Assuming that the primers recognized each isogene equally, FDS-1 was highly expressed in *A. tridentata* shoots. Full-length cDNAs were cloned using the three-step PCR reaction as described under "Experimental Procedures," and the nucleotide sequence of each gene was determined. cFDS-1 contained 1378 bp with an ORF of 1038 bp, flanked by a 70-bp 5′-untranslated region and a 270-bp 3′-untranslated region including a poly(A) tail of 16 bp. A polyadenylation signal was identified at 1265 bp (AATAAA). The gene codes for a 346-amino acid protein with a theoretical pI of 5.82 and a theoretical molecular mass 39,705.6 Da. *cFDS-2* contained 1221 bp, with an ORF of 1026 bp, flanked by a 177-bp 5′-untranslated region containing a TATA box and a 18-bp 3′-untranslated region. The gene encodes a 342-amino acid protein with a theoretical pI of 5.57 and a theoretical molecular mass of 39,570.45 Da. Finally, *cFDS-5* contained 1360 bp, with an ORF of 1182 bp, flanked by a 39-bp 5′-untranslated region and a 139-bp 3′ untranslated region. A polyadenylation signal was identified at 1339 bp (AATAAA). The gene codes for a 394-amino acid protein with a theoretical pI of 5.97 and a theoretical molecular mass of 45,022.41 Da. Despite several attempts, we were not able to isolate full-length cDNA corresponding to *cFDS-3* or *cFDS-4*. At this point we don't know if the partial sequences represent poorly expressed genes or perhaps PCR mistakes.

The three full-length cDNAs showed greater than 69% overall identity. Pairwise, *FDS-1* had 83% identity with *FDS-2* and 75% with *FDS-5*. *FDS-2* had 78% identity with *FDS-5*. *FDS-1* had 97% identity to the *FDS-1* orthologue in *A. annua* (31). A second *A. annua* gene encoding a putative FDS, called *FDS-2*, is reported in databases (accession number AAD32648). Because of its high identity to *FDS-1*, the *A. annua* gene may correspond to *FDS-4* cDNA in *A. tridentata*. The full-length *FDS-2* cDNA from *A. tridentata* did not match the sequences available from *A. annua*.

Sequence Alignments of Prenyltransferases and Construction of a Phylogenetic Tree—A comparison of the deduced amino acid sequences for FDS-1, FDS-2, and FDS-5 with those of other prenyltransferases (farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, chrysanthemyl diphosphate synthase, and geranyl diphosphate synthase) was performed using ClustalX (32) (downloaded at ftp-igbmc.u-strasbg.fr). With one exception, the three isozymes had the five conserved regions identified by Chen *et al.* (28) that are characteristic of

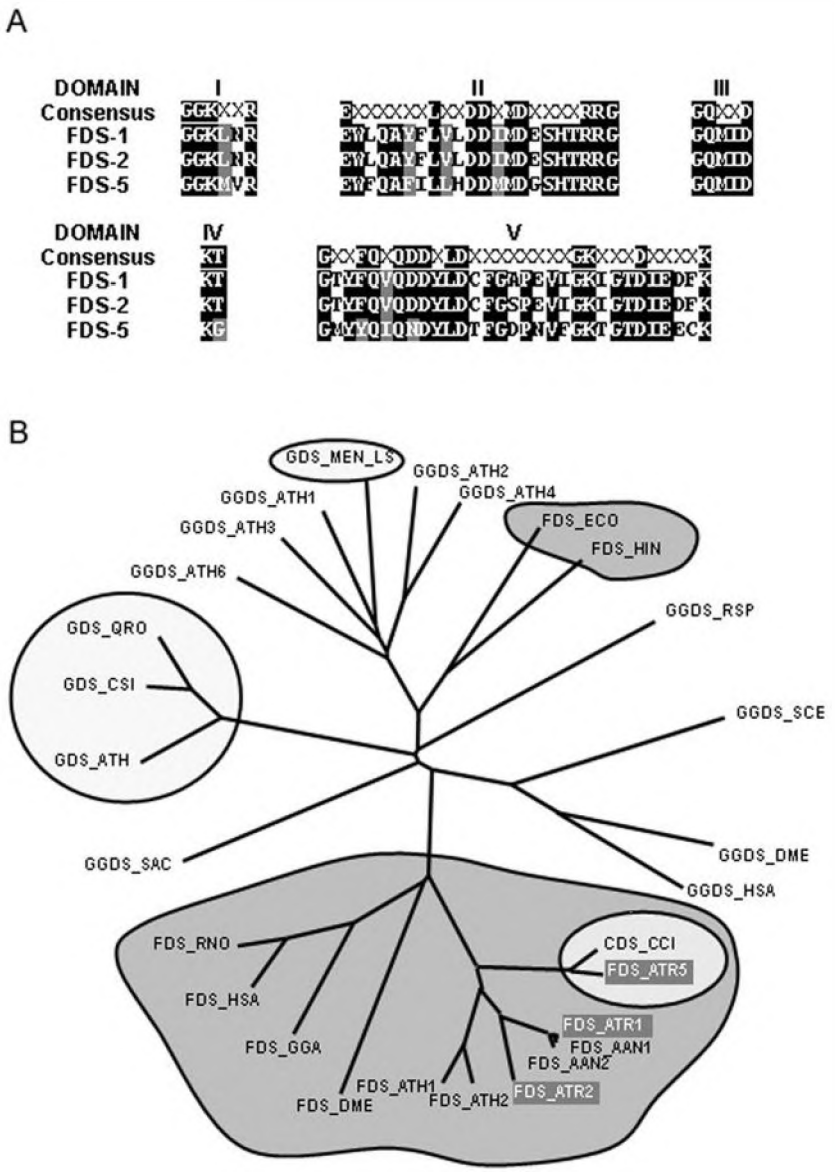


FIG. 1. **Amino acid sequences of *A. tridentata* FDS-1, FDS-2, and FDS-5 within the five conserved domains of prenyltransferases and a phylogenetic tree for prenyltransferases.** *A*, comparisons of amino acid sequences in FDS-1, FDS-2, and FDS-5 with the five conserved regions in chain elongation prenyltransferases. Identical residues are shown in *black*, and conserved residues are shown in *gray*. *B*, a phylogenetic tree comparing FDS isoforms from *A. tridentata* with CDS, FDS, GDS, and GGDS from other organisms. Alignments were performed with ClustalX using the full-length amino acid sequences (with transit peptides). The tree was calculated using the bootstrap neighbor-joining method by making 1000 random samples of sites or trials from the alignment and is represented in an unrooted form. GGDS_RSP, *Rhodobacter sphaeroides* (CAB38744); GGDS_ATH6, *A. thaliana* (BAA23157); GGDS_ATH3, *A. thaliana* (BAB02385); GGDS_ATH1, *A. thaliana* (AAA32797); GDS_MEN_LS, *Mentha x piperita* large subunit (AAF08793); GGDS_ATH2, *A. thaliana* (AAB67730); GGDS_ATH4, *A. thaliana* (AAM15136); FDS_ECO, *E. coli* (JQ0665); FDS_HIN, *Haemophilus influenzae* (C64123); GDS_ATH, *A. thaliana* (CAC16849); GDS_CSI, *Citrus sinensis* (CAC16851); GDS_Qro, *Quercus robur* (CAC20852); GGDS_SAC, *Sulfolobus acidocaldarius* (P39464); GGDS_SCEm *Saccharomyces cerevisiae* (AAA83262); GGDS_DME, *Drosophila melanogaster* (AAC05273); GGDS_HSA, *Homo sapiens* (BAA75909); FDS_ATH1, *A. thaliana* (AAF44787); FDS_ATH2, *A. thaliana* (AAB07248); FDS_ATR2, *A. tridentata* (AY308476); FDS_AAN2, *A. annua* (AAD32648); FDS_AAN1, *A. annua* (JC4846); FDS_ATR1, *A. tridentata* (AY308477); FDS_ATR5, *A. tridentata* (AY308478); CDS_CCI, *C. cinerariaefolium* (I13995, nucleotidic sequence); FDS_DME, *D. melanogaster* (AAD27853); FDS_GGA, *Gallus gallus* (PO8836); FDS_HSA, *H. sapiens* (NP_001995); FDS_RNO, *Rattus norveginus* (PO5369). Prenyltransferases leading to the formation of a precursor of monoterpenes are indicated in *light gray*, and the principal farnesyl diphosphate synthase family is indicated in *dark gray*.

the chain elongation prenyltransferase that synthesizes isoprenoid diphosphates with *E*-double bonds. FDS-5 had a T → G substitution in region IV and a D → N substitution in the first aspartate in region V (Fig. 1*A*). Other amino acids known to be important for FDS activity or substrate binding are conserved in FDS-1, FDS-2, and FDS-5 (33). A phylogenetic tree (Fig. 1*B*) indicates that FDS-5 is closely related to CDS isolated from *C. cinerariaefolium*. The striking similarity between plant FDS and CDS indicates that the proteins evolved recently from the same FDS ancestor. Interestingly, plant GPP synthase (GDS), which synthesizes GPP from IPP and DMAPP, is more closely related to plant geranylgeranyl diphosphate synthases. FDS-1 and FDS-2 cluster with other plant FDS proteins.

Pairwise comparison of the amino acid sequences in FDS-1, FDS-2, and FDS-5 showed >69% identity and >89% similarity among the three proteins, suggesting that the three genes are very closely related. One notable difference between the DNA sequences was a 5′ extension in the ORF of *FDS-5*. This sequence encodes 52 amino acids not found in FDS-1 or FDS-2 and was identified as a potential chloroplastidial targeting

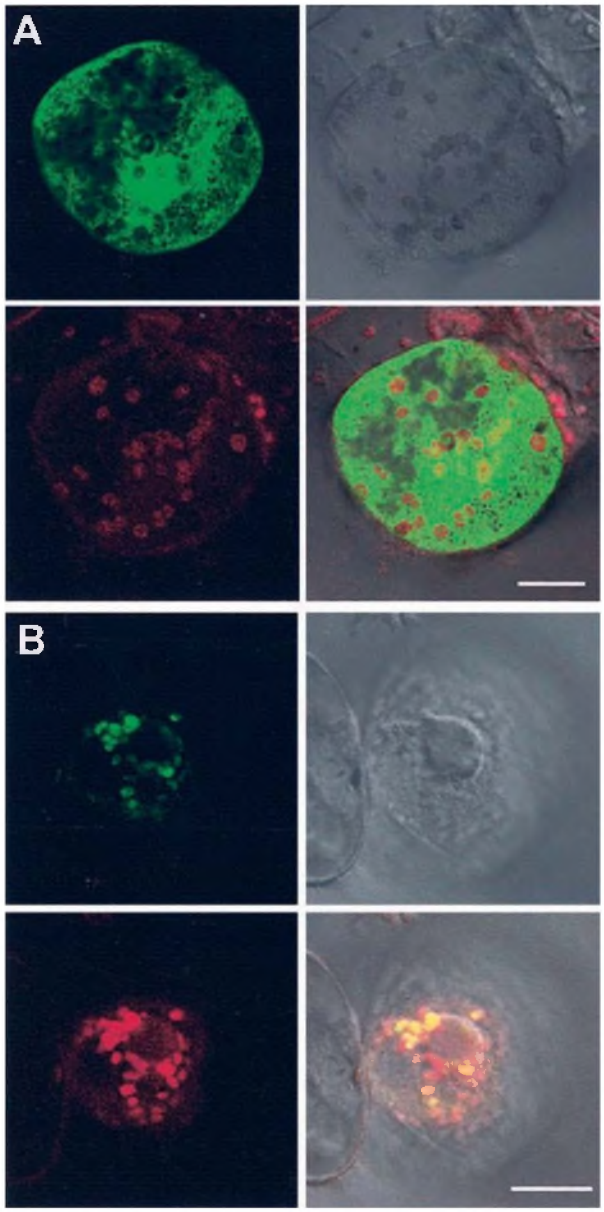


FIG. 2. **Chloroplastic localization of the fusion FDS-5 transit peptide-GFP protein in *N. tabacum* cv. xanthi cells.** Pictures depicting 16-h-old cell transformations obtained 1) with excitation at 488 nm and emission between 505 and 530 nm (*upper left*) to detect green fluorescent protein, 2) with excitation at 543 nm and emission of the natural red fluorescence of chlorophyll (*lower left*), and 3) in bright field (*upper right*). The *lower right* picture is a superposition of all three channels, which shows colocalization of the fusion protein with chloroplasts (*panel B*). In contrast, GFP fluorescence is not colocalized with the red fluorescence from chlorophyll in control cells (*panel A*). The *white scale bars* represent 20 μm.

peptide by the TargetP Version 1.01 program for prediction of subcellular location, with a probability of 93% (www.cbs.dtu.dk/services/TargetP) (34). Despite multiple attempts, a 3′ sequence encoding a putative mitochondrial-targeting peptide was not found in any of the FDS proteins.

Identification of the Function of the 52-Amino Acid N-terminal Extension in FDS-5—To confirm the function of the putative plastidial transit peptide, the DNA sequence coding for the N-terminal of the ORF was fused to the 5′ end of a mutated version of the gene for jellyfish *Aequorea victoria* GFP (29). Transient expression of the fused GFP in tobacco *N. tabacum* cv. xanthi cells verified that the N-terminal propeptide of *A. tridentata* FDS-5 was sufficient to target the reporter protein

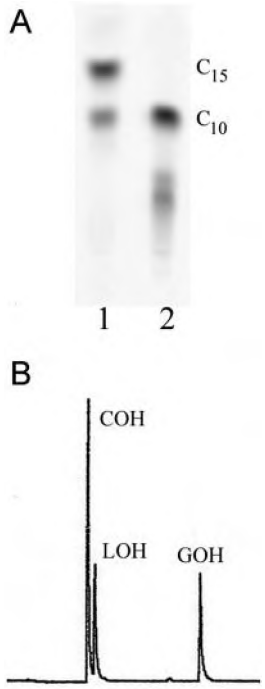


FIG. 3. **Product Analysis (see "Experimental Procedures" for details).** *A*, TLC analysis of products from incubations of FDS-1 (*lane 1*) and FDS-5/CDS (*lane 2*) with 50 μM [^{14}C]IPP and 200 μM DMAPP. Products were visualized by autoradiography. *B*, GC analysis from incubation of CDS with unlabeled IPP and DMAPP under similar conditions after removal of the diphosphate moieties from the products by treatment with alkaline phosphatase. The identities of the products were established by co-injection with authentic samples and by GCMS. *COH*, chrysanthemol; *LOH*, lavandulol; *GOH*, geraniol.

GFP to organelles (Fig. 2*B*). The green fluorescence co-localized with the natural emitted red fluorescence of chlorophyll-excited green light. Control cells expressing only GFP showed its localization to the cytosol surrounding the red fluorescence emitting chloroplasts (Fig. 2*A*). Thus, FDS-5 appears to be targeted to the plastidial compartment.

Expression and Purification of the Farnesyl Diphosphate Homologue Enzymes—Recombinant N terminal His_6-tagged FDS-1, FDS-2, and FDS-5 without the 52-amino acid transit peptide were purified from cell-free *E. coli* homogenates using Ni^{2+} affinity chromatography. The recombinant proteins were greater than 95% pure as judged by SDS-PAGE, with molecular masses corresponding to the calculated values.

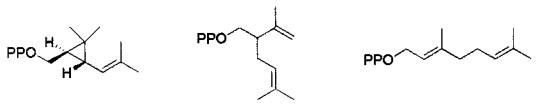
Products Analysis—The products for each enzyme were first determined using a radioactive TLC analysis of the diphosphates and later verified by liquid chromatography-MS or GC-MS of the alcohols produced by treatment with alkaline phosphatase. GPP and FPP were detected by TLC when [^{14}C]IPP and DMAPP were incubated with FDS-1 and FDS-2 for 1 h at 30 °C (Fig. 3*A*). TLC analysis of products from incubation of [^{14}C]IPP and DMAPP with FDS-5 indicated that a C10 product was formed (Fig. 3*A*). When FDS-5 was incubated with [^{14}C]DMAPP, a spot characteristic of a C_{10} product, which co-migrated with both GPP and CPP (11), was seen. The structures of the diphosphates produced by FDS-5 were verified by GC-MS of the corresponding alcohols. In these experiments, unlabeled substrates were incubated with FDS-5, and the alcohols produced by hydrolysis with alkaline phosphatase were analyzed. When the enzyme was incubated with IPP and DMAPP, three monoterpene alcohols, chrysanthemol, lavandulol, and geraniol, were formed in a 4:1:1 ratio (Fig. 3*B*). For incubations with DMAPP, only two of the alcohols, chrysanthemol and lavandulol, were seen. Thus, in the absence of IPP,

FDS-5 behaves like a CDS, with properties similar to the enzyme from *C. cinerariaefolium*. Thus, FDS-5, or more appropriately CDS, catalyzes three competing isoprenoid condensation reactions when incubated with IPP and DMAPP, a chain elongation to give GPP, a cyclopropanation to give CPP, and a branching to give LPP (Scheme I).

Metal Ion, pH, and Temperature Dependence of FDS-1 and FDS-2 Activities—Like other farnesyl diphosphate synthases, FDS-1 and FDS-2 require a divalent metal ion for activity. Maximal rates for FDS-1 were observed when the concentration of Mg^{2+} was between 1 and 4 mM. The optimum Mg^{2+} concentration for FDS-2 was between 0.5 and 2 mM. Mg^{2+} could be replaced by Mn^{2+}, but only one-third of the maximum activity was observed. Neither Zn^{2+}, which is a cofactor for protein prenyltransferases, nor Ca^{2+} could be used to restore FDS activity. In combination with Mg^{2+}, Ca^{2+} even inhibited FDS. The pH-rate profiles were different for the two enzymes (Fig. 4). FDS-1 had a pH optimum of 6.5. The profile for FDS-2 had a broad plateau between pH 7.0 and 8.0, and activity dropped rapidly on either side. Because both FDS-1 and FDS-2 display nearly their full activity at pH 7.0, HEPES was selected as the buffer for all enzymatic assays.

Both enzymes show the same temperature dependence profile with a maximum of activity at 40 °C. Activation energies of 43 and 52 J/mol were calculated from Arrhenius plots for FDS-1 and FDS-2, respectively.

Steady-state Kinetic Constants for FDS-1, FDS-2 and FDS-5—The steady-state kinetic constants are given in Table II. The K_m for FDS-1 suggests a greater affinity (4.5 times) for GPP *versus* DMAPP, whereas the K_m for FDS-2 suggests a preference for DMAPP. K_m values for IPP are similar for both enzymes. In general, FDS-1 has a higher specific activity than FDS-2. The enzyme efficiency (k_{cat}/K_m) was 3-fold higher in the presence of DMAPP and almost 7-fold higher in the presence of GPP. For IPP, the difference is only 2-fold. These differences were not due to the storage, since both enzymes lost less than half of their activity over the period of 1 month at −20 °C. To confirm that FDS-1 and FDS-2 have different affinities for DMAPP and GPP, a competition experiment was performed using increasing concentrations of GPP and decreasing concentrations of DMAPP at a total combined concentration of 50 μM for the allylic substrates (Fig. 5). As GPP concentrations increase, FDS-1 activity increases, whereas the activity of FDS-2 decreases. The catalytic constants for synthesis of CPP and LPP from DMAPP by CDS (FDS-5) were similar to those reported for the *C. cinerariaefolium* enzyme (11). K_m for the *A. tridentata* enzyme was slightly larger, and k_{cat} was 4-fold higher. The corresponding catalytic constants for synthesis of GPP by CDS were not evaluated because of inhibition by both substrates. However, relative values for k_{cat}^{IPP} and K_m^{IPP} were obtained at a fixed concentration of DMAPP that was slightly above K_m^{DMAPP} for the formation of CPP and LPP. K_m^{IPP} for CDS was ~200–240-fold higher than for the related values for FDS-1 and FDS-2. Thus, CDS appears to bind IPP substantially less tightly than the closely related FDS enzymes.



SCHEME I

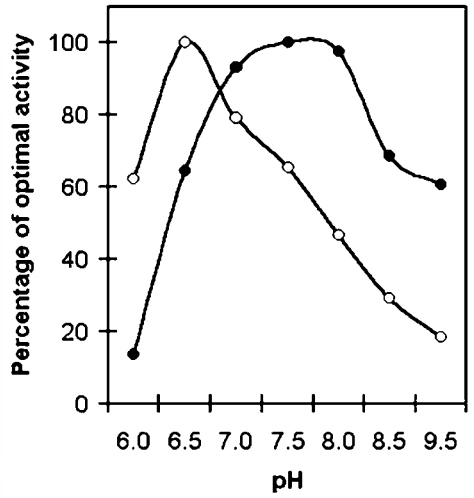


FIG. 4. **Effect of pH on the enzyme activities of FDS-1 and FDS-2.** The activities of purified His_6-tagged FDS proteins were measured between pH 6.0 and 9.5 in a poly buffer consisting of 60 mM PIPES (6.0–6.5), HEPES (7.0–8.0), and Tris (8.0–9.5) containing 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mg of bovine serum albumin, 50 μM GPP, and 20 μM IPP in a total volume of 100 μl. Activities were normalized to the maximal values for FDS-1 (*open circles*) and FDS-2 (*closed circles*).

DISCUSSION

Recent evidence indicates that isoprenoid biosynthesis is highly regulated in plants, often in response to environmental changes. For example, floral scents composed of volatile monoterpenes (C_{10}) and sesquiterpenes (C_{15}) serve to attract a variety of animal pollinators via largely unknown mechanisms. Once the flower is pollinated, the scent profile changes drastically (35). Plants also alter their isoprenoid production in response to herbivore or pathogen attack (36, 37). Upon elicitation, the production of sesquiterpenoid defense compounds has been shown to be coupled to a decrease in sterol biosynthesis (38). Because FPP is the starting material for many of these biosynthetic pathways, it is likely that the biosynthesis of this

TABLE II
Steady-state kinetic constants for farnesyl diphosphate synthases and chrysanthemyl diphosphate synthase

Enzyme	FDS-1	FDS-2	FDS-5	CCI-CDS
K_m^{DMAPP} (μM)	7.1 ± 0.6	6.0 ± 0.2	1430 ± 150	600 ± 150
k_{cat}^{DMAPP} (min^{-1})	397[a]	113[a]		
k_{cat}^{DMAPP} (min^{-1}) for CPP + LPP			3.2	0.5
$k_{cat}^{DMAPP}/K_m^{DMAPP}$	55.9	18.8	0.0022	0.0008 (CPP)
K_m^{GPP} (μM)	1.6 ± 0.5	13.1 ± 1.0		
k_{cat}^{GPP} (min^{-1})	159[a]	198[a]		
k_{cat}^{GPP}/K_m^{GPP}	99.4	15.1		
K_m^{IPP} (μM)	5.5 ± 1.5	5.8 ± 2.3	1334 ± 130[b]	ND[c]
k_{cat}^{IPP} (min^{-1})	265[a]	132[a]	0.75 ± 0.05[b]	ND[c]
k_{cat}^{IPP}/K_m^{IPP}	48.53	22.72		ND[c]
Reference	This study	This study	This study	Rivera *et al.* (11)

[a] Values for k_{cat} were calculated for the dimeric form of the enzyme using preparations with highest specific activity.
[b] A relative value measured at 2 mM DMAPP.
[c] ND, not determined.

branch point metabolite is highly regulated. Newman and Chappell (39) suggest that isoprenoid biosynthesis in plants might be organized in metabolic channels. This concept was tested by Brodelius and co-workers (40) in *A. annua*. They used the gene corresponding to FDS-1 to generate a chimeric fusion protein with epi-aristolochene synthase, the sesquiterpene cyclase required for biosynthesis of capsidiol in *N. tabacum*. The resulting recombinant enzyme produced epi-aristolochene from IPP and DMAPP with a higher efficiency than was seen for the individual enzymes. Thus, a close interaction of both enzymes in space facilitated synthesis of the downstream product. The presence of differentially regulated FDS genes and the occurrence of multiple FDS isoforms suggests that the isozymes are produced for specific functions. For example, one FDS isozyme may play a housekeeping role, whereas another is synthesized or up-regulated when larger amounts of FPP are required.

The primary amino acid sequences of members in the FDS gene family of chain-elongation prenyltransferases contain five highly conserved motifs (28). Four amino acids immediately preceding the first conserved DD*XX*D motif are located on an α-helix that forms part of the wall of a hydrophobic pocket that binds the hydrocarbon moiety of the allylic diphosphate substrate. These amino acids are important for regulating the length of the growing polyisoprenoid chain (41, 42). Two of the three isogenes isolated in this study, FDS-1 and FDS-2, synthesized FPP and retained the highly conserved amino acids characteristic of the FDS family of prenyltransferases. The steady-state kinetic constants for FDS-1 and FDS-2 from *A. tridentata* indicate that the two enzymes have different the catalytic efficiencies and affinities for their allylic substrates. Also, FDS-1 is a more efficient catalyst than FDS-2. The third isogene, which encodes CDS, had an asparagine residue in place of the first conserved aspartate residue in the second DD*XX*D motif. In contrast to the FDSs, CDS synthesized three monoterpenoid diphosphates.

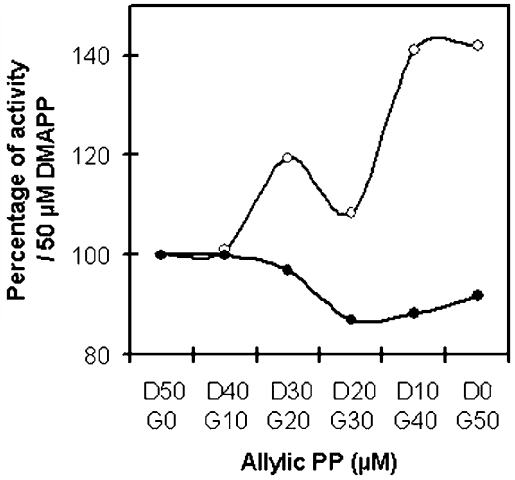


FIG. 5. **A plot of competition for DMAPP and GPP by FDS-1 and FDS-2.** FDS assays were performed as described under "Experimental Procedures" with 50 μM total concentration of allylic diphosphate and 20 μM [^{14}C]IPP. Activities were measured at allylic substrate concentrations from 50 μM DMAPP (*D*) to 50 μM GPP (*G*). The activities for FDS-1 (*open circles*) and FDS-2 (*closed circles*) were normalized to the value for 50 μM DMAPP and plotted against the proportion of allylic substrate.

The level of *FDS-1* transcripts was 20-fold higher than *FDS-2* in our cDNA library established from fresh shoots. Thus, the two FDSs may function optimally under growth conditions. For example, FDS-1 might provide high levels of FPP for biosynthesis of isoprenoid compounds needed in rapidly growing shoots; for example, sesquiterpenes required for defense. In contrast, FDS-2 might play a housekeeping function for biosynthesis of other metabolites such as phytosterols or prenylated proteins. pH-rate profiles indicate that FDS-1 functions optimally in an acidic environment commonly found in the plant cytosol during elicitation (43), whereas FDS-2 prefers the pH of the cytosol in non-elicited cells. The different preferences that FDS-1 and FDS-2 have for DMAPP and GPP have an interesting parallel in FDS isozymes in camomile. Adam *et al.* (44) show that the carbon skeletons of sesquiterpenoids produced by the plant are synthesized in the cytosol using GPP produced in plastids as the allylic substrate. Consequently, the farnesyl diphosphate synthase responsible for synthesizing the sesquiterpenoids preferred GPP over DMAPP as an allylic substrate. FDS-1 has a lower K_m for GPP than DMAPP, indicating that GPP is the preferred allylic substrate, whereas FDS-2 prefers DMAPP. Perhaps FDS-1 has a major role in sesquiterpenoid biosynthesis, whereas FDS-2 maintains a pool of FPP for other branches of the pathway.

Members of another family of enzymes that catalyze isoprenoid chain elongation, the geranylgeranyl diphosphate synthase, are localized in three different subcellular compartments in *A. thaliana* (45). Multiple organelles and compartments are also utilized to regulate FPP biosynthesis. An FDS isoform is directed to mitochondria in *A. thaliana* (9, 10). Thus far an FDS gene bearing a plastidial targeting encoding sequence has not been reported. Based on immunolocal-

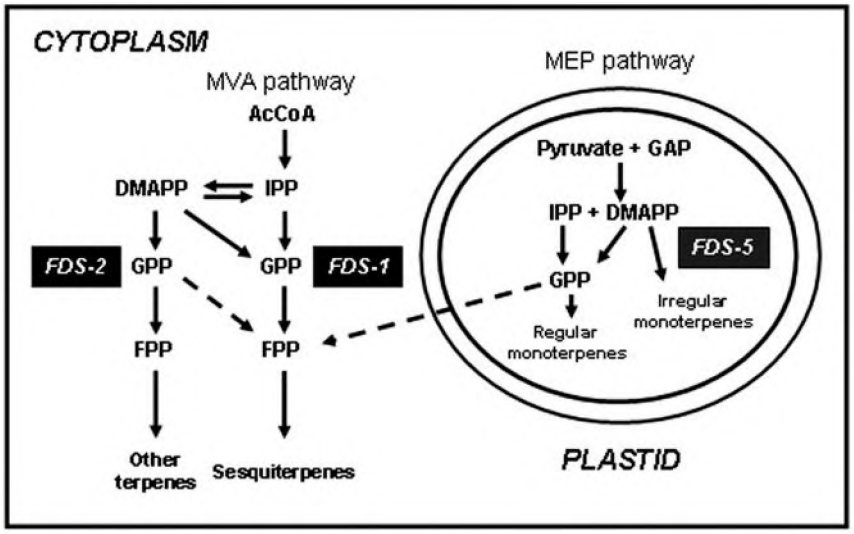


FIG. 6. **A function/compartmentation model for the FDS isoforms isolated from *A. tridentata* ssp. spiciformis.** The mevalonate (*MVA*) pathway is localized in the cytosol, and the methylerythritol phosphate (*MEP*) pathway is localized in plastids. FDS-1, with a higher affinity for GPP, may be induced under stress conditions for production of FPP required for biosynthesis of phytoalexin. FDS-2 may be a housekeeping enzyme for biosynthesis of FPP. FDS-5 is targeted to plastids for the biosynthesis of irregular monoterpenes.

izations, Sanmiya *et al.* (6) suggest that a chloroplastic form of FDS occurs in mono- and dicotyledons. However, this seems unlikely since no plastidial targeting sequences were clearly identified in the ORFs for FDS in the rice and tobacco genes used in their analysis. Likewise, we did not find N-terminal targeting sequences in FDS-1 and FDS-2 from *A. tridentata*. The isoprenoid metabolites in chloroplasts are derived from GPP or GGPP. Typically, GPP is synthesized by a dedicated GDS. The biosynthesis of di- and tetraterpenes in chloroplasts probably does not depend upon FDS activity. Plant and bacterial geranylgeranyl diphosphate synthases (GGDS) catalyze the successive condensation of three molecules of IPP beginning with a DMAPP primer (46). Consequently, the biosynthesis of carotenoids can proceed independently of the production of FPP by an FDS. Thus, FDS activity is not required in plant plastids. A model for compartmentation of the three FDS isoforms is sagebrush is presented in Fig. 6.

Most naturally occurring monoterpenes are derived from GPP and have "regular" structures where the isoprenoid units are joined by 1′-4 linkages (47). In plants these biosynthetic pathways are localized in plastids (48). GDS and the downstream enzymes for biosynthesis of regular monoterpenes are directed to the plastids by N-terminal targeting sequences (49–51). In contrast, CPP and LPP are examples of irregular monoterpenes. These compounds are much less common and are typically found in plants that belong to the *Anthemidiae* tribe of the *Compositae* (20). There are five skeletal classes of irregular isoprenoids: lavandulyl (1′-2), artemisyl (1′-3), santolinyl (2–1′-3), 1′-2–2′-3, and chrysanthemyl (c1′-2–3) (13, 20). Epstein and Poulter (20) and Poulter and co-workers (52) propose that members of the artemisyl and santolinyl families were synthesized from a chrysanthemyl precursor by carbocationic rearrangements of the cyclopropyl-carbinyl skeleton. The origin of the lavandulyl skeleton was less clear, but can now be explained by a 1′-2 condensation of two molecules of DMAPP. It now appears likely that the lavandulyl and chrysanthemyl structures are both synthesized from DMAPP, presumably by similar chemical mechanisms, by CDS in *A. tridentata*. CDS also has GDS activity.

Two different plant GDSs have been reported. The enzyme from peppermint is a heterodimer (49), whereas GDSs from *A. thaliana* (50), *Abies grandis* (53), and *Marchantia polymorpha* (54) are homodimers. Interestingly, these enzymes do not cluster with plant FDS in multiple sequence alignments but instead show high homology to plant GGDS. A phylogenic analysis of chain elongation prenyltransferases (Fig. 1) indicates that plant GGDSs are more closely related to bacterial rather than eukaryotic FDSs. Kuntz *et al.* (55) show that GGPP in plants is biosynthesized directly from DMAPP and IPP, whereas fungal and animal GGDSs prefer FPP as their allylic substrate (56, 57). Thus, both genetic and biochemical properties of plant GGDSs suggest that the enzymes evolved from bacterial FDS. It now appears that GDS and CDS, the two branch point enzymes for monoterpene biosynthesis, evolved independently (Fig. 1). CDS, which lies at the branch point to irregular monoterpenes, originated from FDS in a eukaryotic host, whereas GDS, which is the branch point enzyme for regular monoterpenes appears to have originated from GGDS in a prokaryotic host. We did not isolate clones for GDS from our cDNA library using probes that were optimized for FDS. At this point, we do not know if CDS is also the source of GPP for biosynthesis of regular monoterpenes in *A. tridentata* or if the plant has a dedicated GDS. Further work will be needed to unravel the isoprenoid biosynthetic pathways and their regulation in plants. The chain elongation family provides an interesting platform for these studies.

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