Journal of Plant Physiology 167 (2010) 292–300

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Cloning and expression of 1-deoxy-D-xylulose 5-phosphate synthase cDNA from *Croton stellatopilosus* and expression of 2*C*-methyl-D-erythritol 4-phosphate synthase and geranylgeranyl diphosphate synthase, key enzymes of plaunotol biosynthesis

Worapan Sitthithaworn [a,*], Juraithip Wungsintaweekul [b,**], Tanawan Sirisuntipong [b], Tossaton Charoonratana [b], Yutaka Ebizuka [c], Wanchai De-Eknamkul [d]

[a] *Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok 26120, Thailand*
[b] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[c] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[d] *Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

ARTICLE INFO

Article history:
Received 30 April 2009
Received in revised form
31 August 2009
Accepted 1 September 2009

Keywords:
1-Deoxy-D-xylulose 5-phosphate synthase
2*C*-methyl-D-erythritol 4-phosphate synthase
Croton stellatopilosus
Geranylgeranyl diphosphate synthase
Plaunotol biosynthesis

ABSTRACT

1-Deoxy-D-xylulose 5-phosphate synthase (DXS, EC: 4.1.3.37), the first enzyme in the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, is known to be responsible for the rate-limiting step of isoprenoid biosynthesis in *Escherichia coli* and *Arabidopsis thaliana*. In this study, the *dxs* gene from *Croton stellatopilosus*, designated *csdxs*, was cloned from leaf tissue using the rapid amplification of cDNA ends (RACE) technique. Leaves of *C. stellatopilosus* contain plaunotol, an acyclic diterpene alcohol. The *csdxs* cDNA containing the open reading frame of 2163 base pairs appeared to encode a polypeptide of 720 amino acids. Analysis of the deduced amino acid sequence revealed that the NH_2-terminus of CSDXS carried a chloroplast transit peptide, a thiamine diphosphate binding site, and a transketolase motif, which are the important characteristics of DXS enzymes in higher plants. Multiple alignments of CSDXS with other plant DXSs have indicated that CSDXS has identity ranging between 68% and 89%. Expression levels of *csdxs* and genes encoding key enzymes in the plaunotol biosynthetic pathway, namely 2*C*-methyl-D-erythritol 4-phosphate synthase (*meps*) and geranylgeranyl diphosphate synthase (*ggpps*), were analysed by measuring transcript levels in leaves of different developmental stages. The results showed that *dxs*, *meps,* and *ggpps* are all active in young leaves prior to full expansion when plaunotol is synthesised from the DXP precursor in chloroplasts. The dense presence of chloroplasts and oil globules in the palisade cells of these leaves support the view that these genes are involved in plaunotol biosynthesis in chloroplast-containing tissues.

Introduction

Croton stellatopilosus (Euphorbiaceae) is a Thai medicinal plant, formerly known as *C. sublyratus* (Esser and Chayamarit, 2001). Since 1978, it has been reported that plaunotol isolated from its leaves has an anti-peptic ulcer activity (Ogiso et al., 1978). Presently, the leaves of *C. stellatopilosus* are used as raw materials for manufacturing an anti-peptic ulcer drug, Kelnac®, which contains plaunotol as an active ingredient.

Plaunotol is an acyclic diterpene alcohol derived from four isoprene units. As shown in Fig. 1, one molecule of dimethylallyl diphosphate (DMAPP) and three molecules of isopentenyl diphosphate (IPP) are connected by head-to-tail condensation, which is catalysed by geranylgeranyl diphosphate synthase (GGPPS), yielding geranylgeranyl diphosphate (GGPP) (Sitthithaworn et al., 2001). Recently, membrane-bound GGPP phosphatases have been shown to catalyse the two-step monodephosphorylation of GGPP to form geranylgeraniol (GGOH) (Nualkaew et al., 2005). For the final step in plaunotol biosynthesis, GGOH 18-hydroxylase has been reported to

Abbreviations: DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; *dxs*, / DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGOH, geranylgeraniol; *ggpps*, / GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; IPP, isopentenyl diphosphate; IspD, 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase; IspG, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; *meps*, / MEPS, 2*C*-methyl-D-erythritol 4-phosphate synthase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulphate

* Corresponding author. Tel.: +66 74288887; fax: +66 74428220.
** Also to be corresponded to.
E-mail address: juraithip.w@psu.ac.th (J. Wungsintaweekul).

0176-1617/$ - see front matter
doi:10.1016/j.jplph.2009.09.001

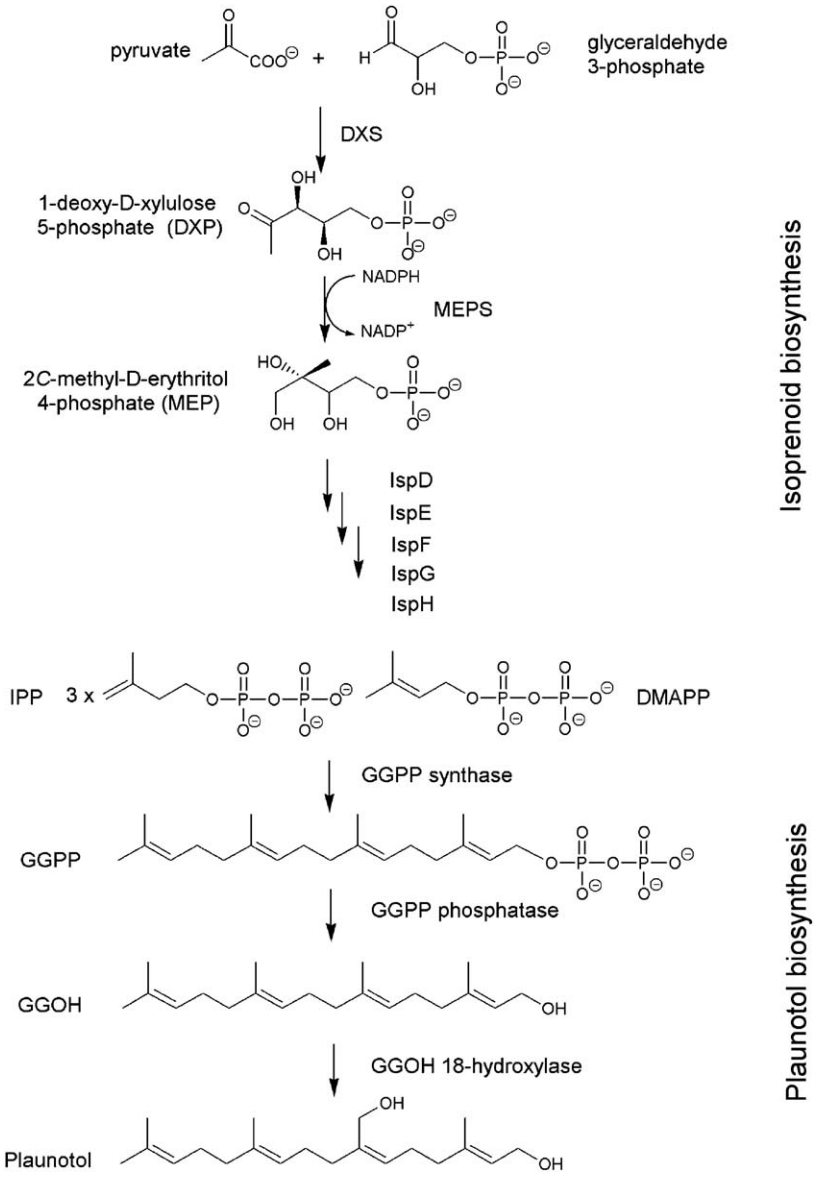

Fig. 1. Plaunotol biosynthesis via the methylerythritol phosphate pathway.

catalyse the addition of a hydroxyl group at the C-18 position of GGOH, yielding 18-hydroxygeranylgeraniol or plaunotol (Tansakul and De-Eknamkul, 1998).

Studies of the organelle localisation of plaunotol in *C. stellatopilosus* leaves have suggested that plaunotol accumulates as oily globules mainly in the palisade cells of the leaves (Sitthithaworn et al., 2006). These data are also in agreement with the localisation of the gene encoding the enzyme GGPPS (Sitthithaworn et al., 2001), and are supported by results showing a correlation between plaunotol production and chlorophyll accumulation in the chloroplast (Morimoto and Murai, 1989).

During the past few decades, information on isoprenoid biosynthesis via the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway has been uncovered (for a review, see Rohdich et al., 2003). The initial step begins with the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by the condensation of pyruvate and glyceraldehyde 3-phosphate, which is catalysed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Sprenger et al., 1997). In the second step, which is catalysed by 2*C*-methyl-D-erythritol 4-phosphate synthase (MEPS), DXP is further transformed into 2*C*-methyl-D-erythritol 4-phosphate (MEP). The formation of MEP from DXP is synthesised in a single step by the rearrangement of DXP to an intermediate with a branched carbon skeleton, 2*C*-methyl-D-erythrose 4-phosphate, followed by a reduction step using NADPH (Takahashi et al., 1998). The resulting MEP is then converted into its cyclic diphosphate by the sequential actions of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (IspE), and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF) (Rohdich et al., 1999; Lüttgen et al., 2000; Herz et al., 2000). 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate is transformed into IPP and DMAPP via 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate by the sequential actions of 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (IspG) and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (IspH) (Hecht et al., 2001; Altincicek et al., 2001) (Fig. 1). In *C. stellatopilosus*, the origin of IPP in the plaunotol pathway has been studied by feeding experiments using isotopic glucose. Feeding young *C. stellatopilosus* shoots with

either [U-^{13}C]glucose or [1-^{13}C]glucose revealed that the IPP moieties in plaunotol, all originate from the MEP pathway (Wungsintaweekul and De-Eknamkul, 2005).

In terpenoid biosynthesis, DXS has been reported to be a target of antimicrobials and herbicides in pathogenic bacteria and plants (Rohdich et al., 2005). Moreover, DXS has been suggested to be a regulatory enzyme in isoprenoid biosynthesis, as shown in *Coleus forskolii* (Engprasert et al., 2005) and *Elaeis guineensis* (Khemvong and Suvachittanont, 2005). In *Arabidopsis thaliana*, knocking out DXS causes albino phenotype. That study suggests that DXS plays a role in chloroplast development (Mandel et al., 1996). Homologous overexpression of both the *Escherichia coli dxs* and *meps* genes has led to increased lycopene production compared with cells that overexpressed *dxs* alone (Kim and Keasling, 2001). Thus, *dxs* and *meps* are probably good candidates for metabolic engineering to increase plaunotol synthesis in *C. stellatopilosus*.

Recently, we have reported a cDNA clone encoding 2*C*-methyl-D-erythritol 4-phosphate synthase (*csmeps*) from *C. stellatopilosus* leaves (Wungsintaweekul et al., 2008). Results obtained from such studies of the regulatory roles of *csdxs* and *csmeps* genes in leaf, twig, and root tissues of *C. stellatopilosus* have clearly suggested that *csdxs* and *csmeps* mRNAs are expressed in chloroplast-containing tissues, where plaunotol accumulates. In this study, *csdxs* cDNA was cloned and functionally analysed based on the deduced amino acid sequence of CSDXS. The expression profiles of *dxs*, *meps,* and *ggpps* in *C. stellatopilosus* leaves of different developmental stages were determined in parallel with plaunotol contents and cellular organisation, in order to understand the role of the chloroplast in plaunotol biosynthesis.

Materials and methods

Plant materials

The leaves, shoots, and roots of *Croton stellatopilosus* Ohba (Euphorbiaceae) used in this study were obtained from plants growing in an open field at the Faculty of Pharmacy, Srinakharinwirot University. A voucher specimen was collected and deposited at the Department of Pharmaceutical Chemistry and Pharmacognosy, the Faculty of Pharmacy, Srinakharinwirot University.

Cloning of C. stellatopilosus dxs cDNA

Total RNA was isolated from young leaves of 2-year-old *C. stellatopilosus* plants using a phenol–sodium dodecyl sulphate (SDS) procedure and lithium chloride precipitation (Sambrook et al., 1989). Total RNA (10 μg) was reverse-transcribed using the SuperScript™ III reverse transcriptase (Invitrogen, USA) with a RACE32 primer (Table 1), according to the manufacturer's protocol. After RNase H treatment, the resulting single-strand cDNA mixtures were used as templates for the following PCR steps.

For core fragment amplifications, sets of degenerate oligonucleotide primers, corresponding to the highly conserved amino acid sequences of known plant DXSs, were designed and used for the PCR amplifications (Table 1). The first PCR was performed with the external set of primers P126S and P434A using Ex-Taq™ DNA polymerase (Takara, Japan). The PCR program was 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 42 °C for 2 min, and 72 °C for 3 min, with a final extension at 72 °C for 10 min. The resulting PCR product was purified with a Suprec™ 02 column (Takara, Japan) to remove excess primers. Then, nested PCR was performed with the internal set of primers, P194S and P393A, and the first PCR product was then used as a template under the same PCR conditions.

Table 1
Primers used in this study.

Primer	Sequence (5′→3′)
	Reverse transcription
RACE32	GACTCGAGTCGACATCGATTTTTTTTTTTTTT
	Core fragment
P126S	CCICAYAARATHYTIACIGG (^{148}PHKILTG)
P194S	GTIATHGGIGAYGGIGCIATG (^{213}VIGDGAM)
P393A	GTIACIGCRTTYTGYTCIGCDAT (^{447}AEQHAVTF)
P434A	GCRAAIARIACIGGIARYTTYTGIA (^{487}LQKLPVRFA)
	3′ and 5′-RACE
3′-346S	AATCAACTGGGCCAGTCTTGATTCATGT
3′-521S	GCGTGCCTTCCTAACATGGTGGTG
5′-280A	ACCCTTGGCAATCTCTCTTAGTTCTCTT
AP1	CCATCCTAATACGACTCACTATAGGGC
	Transcription profile analysis (expected size, bp)
DXS-S	GAACTGAAGCAGCTAGCAGAAG (5 9 2)
DXS-A	AACACCCTTGGCAATCTCTC
MEPS-S	AATTGGGGTACCATGGCTCTTAATTTG (4 2 6)
MEPS-A	TGCAAGTGCCACCACTTTAAA
GGPPS-S	GCGTGTGAACTTGTTGGCGGAG (6 6 8)
GGPPS-A	CAATGGAGCCGCCTTCTCAGG
18SRNA-S	CAAAGCAAGCCTACGCTCTG (3 0 0)
18SRNA-A	CGCTCCACCAACTAAGAACG

The amino acid sequences corresponding to the core fragment primers are those of CSDXS.

The 5′ and 3′ cDNA ends were amplified using the rapid amplification of cDNA ends (RACE) method (Frohman et al., 1998) with the Marathon™ cDNA amplification kit (BD Bioscience, Clontech®). First-strand cDNA was synthesised from total RNA (10 μg) with the Marathon™ cDNA synthesis primer and avian myeloblastosis virus (AMV)-reverse transcriptase (Clontech®), according to the manufacturer's protocol. Second-strand cDNA was synthesised from the first-strand cDNA with a second-strand enzyme cocktail and T4 DNA polymerase. After treatment with solvent extraction and precipitation, the resultant cDNA was ligated to the Marathon cDNA adaptor to create adaptor primer binding sites for amplification of the 5′ and 3′ ends. Sets of specific primers were synthesised based on the core sequences obtained (Table 1). The 5′ end of cDNA was synthesised with 5′-280A and adaptor primer AP1, and the 3′ end was amplified using the AP1 and 3′-346S primers. Additionally, amplification with AP1 and 3′-521S was performed to complete the 3′ end of the cDNA. All PCR reactions were performed using Advantage 2 polymerase (Clontech®). The PCR program was 94 °C for 30 s and 30 cycles of 94 °C for 5 s and 68 °C for 3 min. PCR products were purified using Wizard® PCR Preps (Promega) and ligated into pT7Blue T-vector (Novagen). The resulting plasmids were chemically transformed into *Escherichia coli* strain DH5α competent cells (Invitrogen). Transformants were selected on ampicillin LB-agar plates. Recombinant plasmids were extracted using the GFX Micro plasmid Prep kit (Amersham Bioscience), cut with *Pst*I and *Eco*RI, and the insertion was analysed by agarose gel electrophoresis.

DNA sequence determination and sequence analysis

DNA fragments were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). After amplification, the samples were separated in an ABI PRISM® Applied Biosystems 3700 DNA analyser, equipped with computer workstation Model 3100, Version 3.7. The nucleotide sequence obtained in this study was deposited in the EMBL/GenBank/DDBJ databank with the accession number AB354578. Comparative analyses of nucleotide sequences and deduced amino acid sequences were performed using BLAST programs (http://www.

ncbi.nlm.nih.gov/BLAST/) and DNASIS v3.5 software. Multiple alignments were conducted using Clustal W (2.0) and BioEdit v7.0.5.

Semiquantitative RT-PCR

Total RNA was isolated from *C. stellatopilosus* tissue using a phenol–SDS procedure and lithium chloride precipitation. First-strand cDNA was synthesised using a clone AMV reverse transcription kit (Invitrogen). Reverse transcription was performed for 50 min at 42 °C in 20 μL of standard buffer containing 1 μL of 50 μM oligo(dT)$_{20}$ primer, 500 ng of total RNA isolated from each plant tissue, and 15 units of AMV reverse transcriptase.

The levels of the *dxs*, *meps*, and *ggpps* transcripts and that of a housekeeping gene (*18S rRNA*) were determined in parallel in each RNA sample, which was isolated from the plant tissue. The primer sets used to amplify *dxs*, *meps*, *ggpps*, and *18S rRNA* are shown in Table 1. The PCR reactions were performed under the optimal conditions for each gene. The PCR products were separated by 2.0% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The band intensities were measured using a Gel Doc Model 1000 (Bio-Rad, USA) equipped with Molecular Analyst® software (Windows Software for Bio-Rad's Image Analysis Systems, Version 1.4). Expression of the transcripts was calculated as the ratio of the band intensity of the gene in question and that of *18S rRNA*.

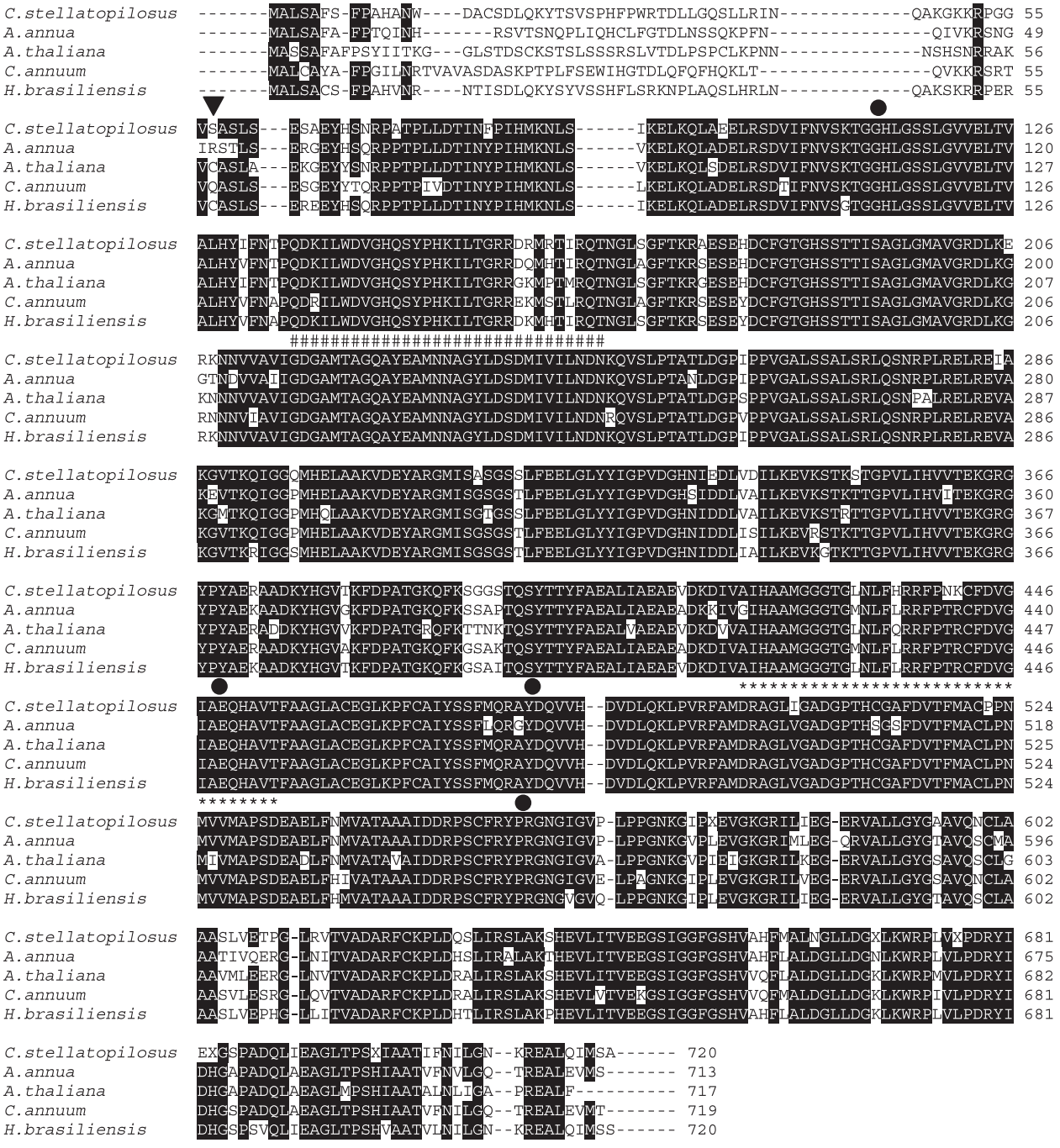

Fig. 2. Multiple alignments of the deduced amino acid sequences of DXSs obtained from *C. stellatopilosus* (this study), *Artemisia annua*, *Arabidopsis thaliana*, *Capsicum annuum*, and *Hevea brasiliensis*. The arrowhead denotes the cleavage site of chloroplast transit peptide. # and ∗ indicate the regions corresponding to the putative thiamine diphosphate-binding site and transketolase motif, respectively. Closed circles denote the catalytic amino acid residues H114, E449, D480, and R557 of the CSDXS protein.

Quantitative analysis of plaunotol by gas chromatography (GC)

Leaves of different developmental stages (harvested from the same stem), stems, and roots were collected and used for plaunotol content determination. The samples were prepared separately as previously described (Vongchareonsathit and De-Eknamkul, 1998). Dried samples (2 g) were extracted with 10 mL of absolute ethanol (Merck, Darmstadt, Germany) under reflux for 1 h and filtered. The filtrates were evaporated under reduced pressure. The residue was dissolved in 2 mL of 50% (v/v) ethanol in water in the presence of 1.6% (w/v) NaOH solution and gently heated for 30 min. The mixtures were partitioned with 3 mL of *n*-hexane three times. The *n*-hexane fractions were pooled and evaporated to dryness. The dried residue was re-dissolved in 0.5 mL of *n*-hexane, centrifuged, and subjected to a GC system (Agilent 6850 instrument equipped with an FID detector). The GC conditions were as follows: HP1 methylsiloxane column (GC-Hewlett Packard, CA, USA), 30 m × 0.32 mm internal diameter, 0.25-μm film thickness, 1.0 mL/min helium flow; splitless injection; temperature program from 235 °C (hold 1 min) to 280 °C (rate 15 °C/min) with a hold of 2.5 min; injector temperature 220 °C; detector temperature 280 °C; and sample size 1.0 μL. Plaunotol was eluted at 5.5 min. The peak areas were converted to the concentration by using a plaunotol calibration curve (linearity range 0.01–0.5 μg/μL, with R^2 of 0.999). Samples were analysed in triplicate.

Transmission electron microscopy

Shoot and young leaf (leaf no. 3) samples were cut into small pieces (1.0 × 1.0 mm^2), fixed in a solution of 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, kept at 4 °C for 2 h, and then washed thoroughly with distilled water. After staining with 2% (w/v) uranyl for 20 min, the tissue samples were transferred into dehydrating agents as follows: 70%, 80%, and 90% (v/v) ethanol for 5 min, absolute ethanol for 10 min, propylene oxide for 15 min, mixtures of propylene oxide and epoxy resin (1:1, 1:2) for 2 h each, and finally epoxy resin for 3 h. The resulting samples were embedded in liquid paraffin at 70 °C for 16 h. Blocks of specimens were sectioned at 80- to 90-nm thickness with an ultramicrotome (RMA, model MT-XL, AZ, USA). The specimens were viewed and photographed under a JEOL JEM-2010 CX transmission electron microscope (Tokyo, Japan) at 80 kV.

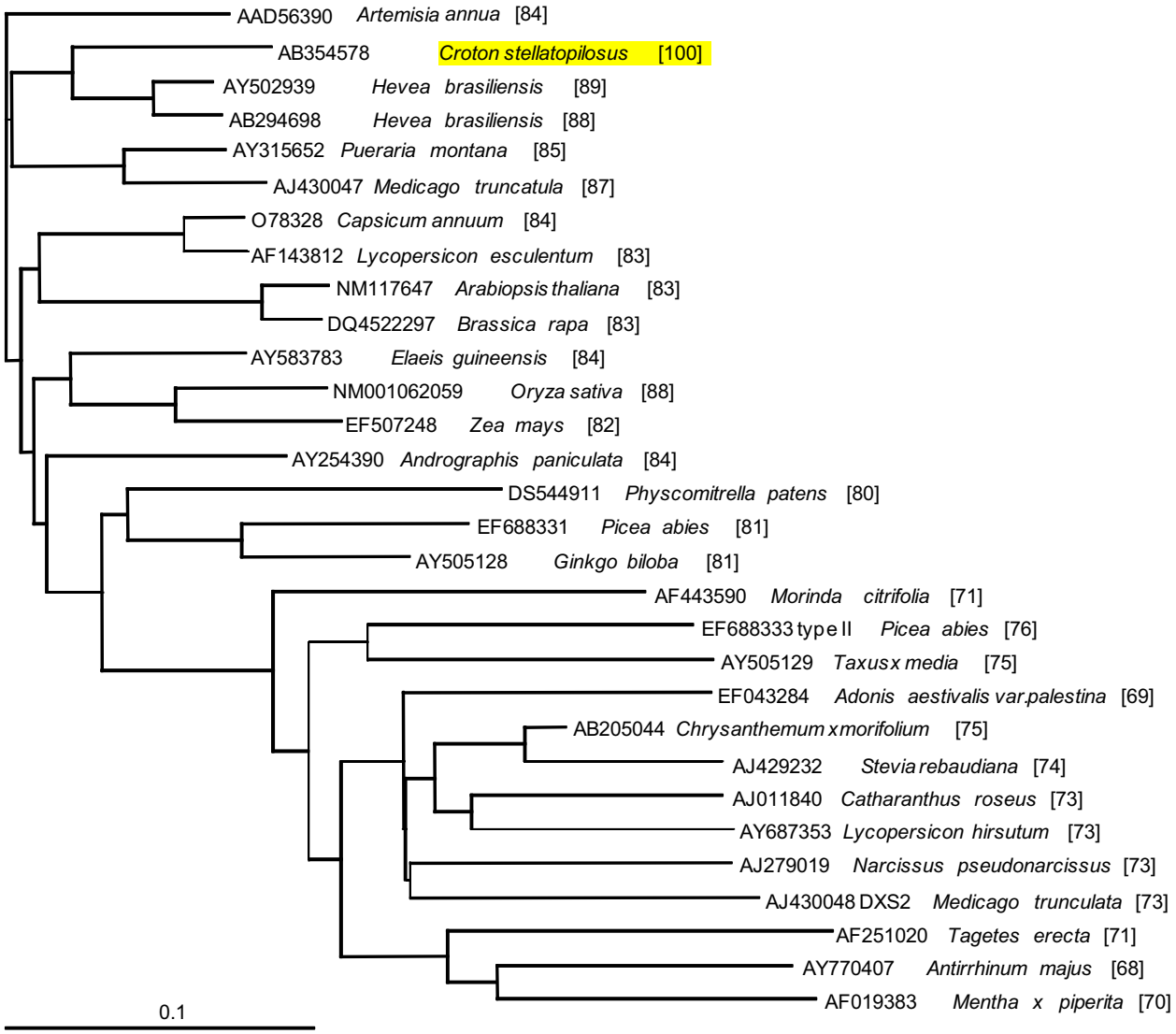

Fig. 3. Phylogenetic tree analysis of plant DXSs from ClustalW XXL alignments, viewed using TreeView v 1.6.6. Accession numbers are indicated in front of the plant names. Numbers in brackets indicate the % identity from the PSI-BLAST.

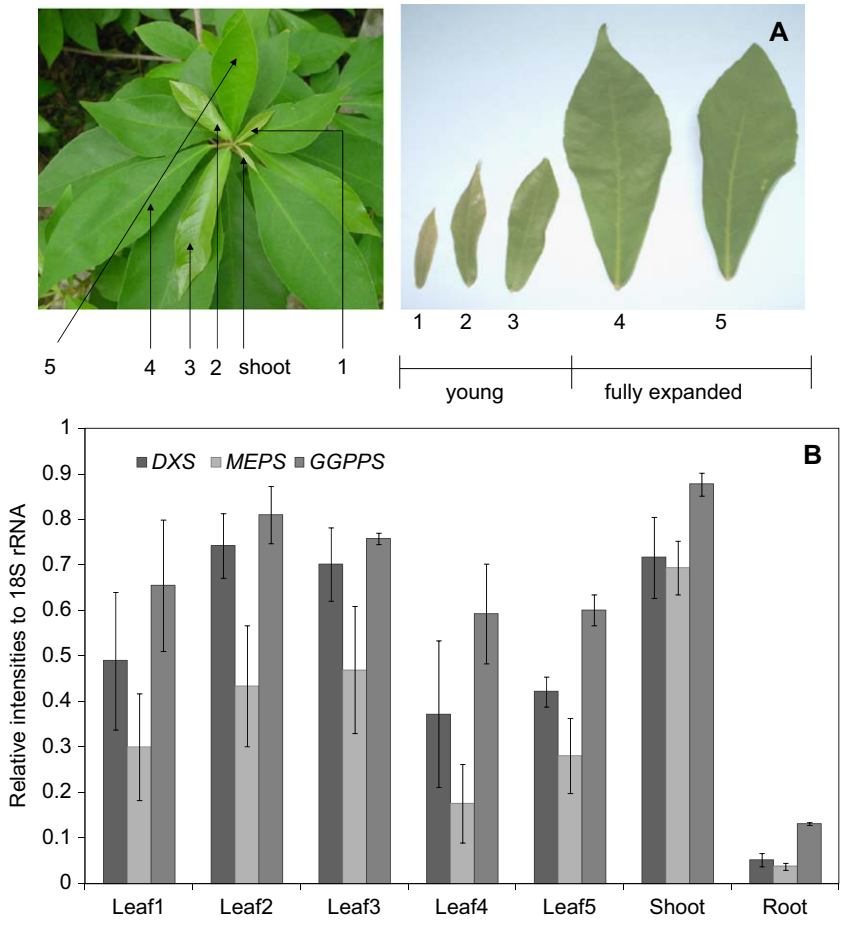

Fig. 4. The mRNA expression levels of *dxs*, *meps*, and *ggpps* from various organs of *C. stellatopilosus* using semiquantitative RT-PCR. (A) Indicates the position of selected leaves. (B) Illustrates the relationships of the relative intensities to *18S rRNA*.

Results and discussion

Cloning of C. stellatopilosus dxs cDNA

The open reading frame of *dxs* cDNA encodes a protein of 720 amino acid residues.

Sequence comparison with other plant *dxs* cDNAs revealed high levels (68–89%) of identity throughout the entire coding region (Fig. 2). As shown in Fig. 3, CSDXS has high homology (89% identity) with DXS from *Hevea brasiliensis*. Some of these enzymes, such as the one from *Capsicum annuum*, have been shown to belong to the DXS class II (Bouvier et al., 1998). However, sequence similarity with prokaryotic *dxs* cDNAs from *Mycobacterium marinum*, *Geobacter metallireducens*, *Rhodobacter sphaeroides*, and *E. coli* was low (30–50%). The computer program BLAST P (http://www.ncbi.nlm.nih.gov/BLAST/) suggested that this protein belonged to the transketolase and dehydrogenase E1 component family. It contained the consensus sequence for the thiamine diphosphate (TPP)-binding motif $(GDG(X)_8E(X)_4A(X)_{11}NDN$, where X denotes any amino acid) (Hawkins et al., 1989) at position 216–246. In addition, the consensus sequence for the transketolase motif (Schenk et al., 1997) has been designated as $DRAGX_{28}PXD$, where X denotes any amino acid. This motif is located at position 498–532. The catalytic amino acid residues include H114, E449, D480, and R557, which were previously identified in *E. coli* (Sprenger et al., 1997; Lois et al., 1998), *Mentha piperita* (Lange et al., 1998), and *C. annuum* (Bouvier et al., 1998). The protein has a predicted pI of 6.80 and a calculated molecular weight of 77,623 Da (http://au.expasy.org/cgi-bin/pi_tool).

The computer program TargetP (www.cbs.dtu.dk/services/TargetP/), which predicts the subcellular localisation and cleavage site of a given protein by a computational neural network-based method (Emanuelsson et al., 1999), predicted that CSDXS would be localised to chloroplasts. Earlier expression studies with *Taxus canadensis* GGPPS in yeast (Schmidt and Gershenzon, 2007) and spearmint limonene synthase in *E. coli* (William et al., 1998) have demonstrated that a high level of heterologous expression can be achieved upon removal of the plastidial transit sequence at the N-terminus. It has been suggested that a change in secondary structure from a beta sheet to a more helical structure marks a transit peptide cleavage site (von Heijne et al., 1989). Sequence alignment (Fig. 2) and predicted secondary structures based on ChloroP v1.1 (Emanuelsson et al., 1999) suggest that the putative cleavage site is located in the vicinity of Ser-57.

Transcription profile analyses of dxs, meps, and ggpps during plaunotol biosynthesis

The expression profiles of *dxs*, *meps*, and *ggpps* mRNAs were relatively similar (Fig. 4). High levels of mRNA transcripts were first observed in the shoot, and the levels of the transcripts increased greatly again during leaf development. The highest level of transcripts detected occurred in young leaves prior to full expansion, decreasing when the leaves reached full expansion. Although the mRNA levels in fully expanded leaves were less than those observed in young leaves, they were higher than the level found in the root, in which chloroplasts are rarely observed. Thus,

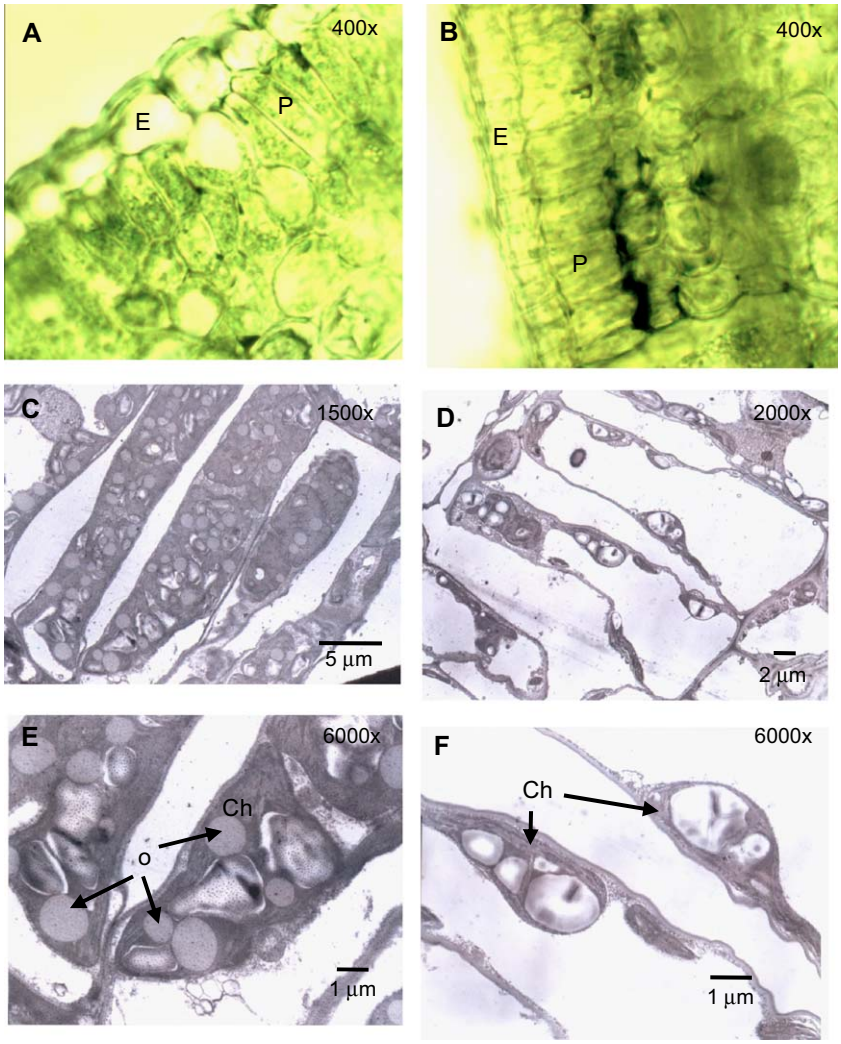

Fig. 5. Electron micrographs of young leaf (A, C, E) and shoot (B, D, F) samples from *C. stellatopilosus*. Ch, chloroplast; E, upper epidermis; O, oil globule; P, palisade cells.

it was found that the *dxs*, *meps,* and *ggpps* mRNAs are abundant in chloroplast-containing tissues. Very low levels of expression in root tissues were detected, suggesting that the activities of DXS, MEPS, and GGPPS are required, at least at a basal level, in non-photosynthetic tissues.

It has been reported that the biosynthesis of plaunotol is catalysed by the enzymes involved in the plastidial pathway (Wungsintaweekul and De-Eknamkul, 2005) and that plaunotol was found to accumulate in chloroplasts (Sitthithaworn et al., 2006). These reports suggested that plaunotol is actively biosynthesised in leaves during plastid differentiation or leaf greening process but not in shoot tissue. To confirm this hypothesis, shoot and young leaf (leaf no. 3) tissue samples were sectioned to capture their transmission electron micrographs. In the young leaf (Figs. 5A, C, E), the layers of palisade cells contained an enrichment of chloroplasts as well as oil globules. In contrast, few chloroplasts and no oil globules were observed in the shoot (Figs. 5B, D, F). These data are in agreement with the plaunotol contents in those tissues (Table 2). A small amount of plaunotol has been detected in shoots, where plastid development is incomplete, although high levels of gene expression have been observed. This suggests that DXP, MEP, and GGPP synthases in the shoot may provide DXP, MEP, and GGPP substrates, which are immediately channelled into the crucial compounds leading to the plastid development. A remarkably high amount of plaunotol was detected in young leaves (leaves no. 1–3), while its levels declined in fully expanded leaves (Table 2, Fig. 4). These results were consistent with *dxs*, *meps*, and *ggpps* gene-expression patterns in the leaf tissues and suggested strong induction of *dxs*, *meps*, and *ggpps* gene expression in leaves at the stage with the highest rate of plaunotol accumulation. The function of plaunotol in *C. stellatopilosus* is unknown, however. The large amount of plaunotol in young leaves before they reach full expansion may

Table 2
Plaunotol contents from various organs of *C. stellatopilosus*.

Sample	Plaunotol content[a] (%w/w) ± S.D.
1st leaf	1.48 ± 0.08
2nd leaf	1.68 ± 0.16
3rd leaf	0.92 ± 0.17
4th leaf	0.07 ± 0.01
5th leaf	0.51 ± 0.04
Shoot	n.d.[b]
Stem	0.24 ± 0.08
Root	n.d.

[a] Samples were analysed in triplicate.
[b] n.d.=not determined (concentration ≤0.002 µg).

be due to the maturity of the chloroplast, and it is presumably produced for as a defence mechanism, particularly for young leaves. These genes were slightly expressed in root tissues, where a small amount of plaunotol accumulated. The low level of gene expression in non-photosynthetic tissue suggested that these genes are responsible for plaunotol biosynthesis in the chloroplast.

A strong correlation between plaunotol accumulation and leaf development has been observed. Plaunotol is restricted to young leaves prior to full expansion. Thus, it has been suggested that biosynthesis of plaunotol is highly functional during plastid development in the leaf greening process. The results are in agreement with an earlier study of the production of diterpenes in *Scoparia dulcis* leaf organ cultures, which revealed that the concentration of diterpenes increased in parallel with the differentiation of green leaves (Hayashi et al., 1999). Moreover, the production of monoterpenes in the oil glands of peppermint leaves was limited only to young leaves prior to full expansion (Turner et al., 1998).

A high level of gene expression was also observed in other plants when a secondary metabolite was biosynthesised. It has been reported that *dxs* mRNA levels are highest during the first stages of peppermint leaf development, preceding the peak of monoterpene biosynthesis (Lange et al., 1998). In tomato fruit, *dxs* induction is associated with the activation of carotenoid biosynthesis at the onset of fruit ripening (Lois et al., 2000), while its expression in young to fully expanded leaves remained constant, consistent with the fact that no specific isoprenoid product accumulates at high levels during leaf development.

The results also indicate an unambiguous correlation between levels of accumulated *dxs*, *meps*, and *ggpps* mRNA in a given tissue and the plastid isoprenoid requirements of that tissue. Most plastid isoprenoid products are related to photosynthesis, therefore more active gene expression in shoot and leaf tissues compared with the root should be expected. The relationship between gene expression and plaunotol biosynthesis during plastid development indicates that overexpression of *dxs*, *meps*, and *ggpps* is an attractive goal for metabolic engineering to increase plaunotol synthesis.

Acknowledgements

This work was supported by grants from the National Centre for Genetic and Biotechnology (BIOTEC), the Thailand Research Fund (TRF, Grant no. MRG4880016), the International Foundation for Science (IFS, Grant no. F/4093-1), and the Joint Research Program of the NRCT-JSPS. The authors acknowledge Associate Professor Tanomjit Supavita for her suggestions on electron microscopy.

References

Altincicek B, Kollas AK, Sanderbrand S, Wiesner J, Hintz M, Beck E, et al. GcpE is involved in the 2-*C*-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*. J Bacteriol 2001;183:2411–16.

Bouvier F, d'Harlingue A, Suire C, Backhaus RA, Camara B. Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. Plant Physiol 1998;117:1423–31.

Emanelsson O, Nielsen H, von Heijne G. ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 1999;8:978–84.

Engprasert S, Taura F, Shoyama Y. Molecular cloning, expression and characterization of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *Coleus forskohlii* Briq. BMC Plant Biol 2005;169:287–94.

Esser HJ, Chayamarit K. Two new species and a new name in Thai *Croton* (Euphorbiaceae). Thai For Bull 2001;29:51–7.

Frohman MA, Dush MK, Martin GR. Rapid production of full-length cDNAs from rare transcripts by amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 1998;85:8998–9002.

Hawkins CF, Borges A, Perham RN. A common structural motif in thiamin pyrophosphate-binding enzymes. FEBS Lett 1989;255:77–82.

Hayashi H, Asai T, Sankawa U. Mevalonate-independent biosynthesis of bicyclic and tetracyclic diterpenes of *Scoparia dulcis* L. Tetrahedron Lett 1999;40:8239–8243.

Hecht S, Eisenreich W, Adam P, Amslinger S, Kis K, Bacher A, et al. Studies on the nonmevalonate pathway to terpenes: the role of the GcpE (ispG) protein. Proc Natl Acad Sci USA 2001;26:14837–42.

Herz S, Wungsintaweekul J, Schuhr CA, Hecht S, Löttgen H, Sagner S, et al. Biosynthesis of terpenoids: YgbB protein converts 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate to 2*C*-methyl-D-erythritol 2, 4-cyclodiphosphate. Proc Natl Acad Sci USA 2000;97:2486–90.

Khemvong S, Suvachittanont W. Molecular cloning and expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate synthase from oil palm Elaeis guineensis *Jacq*. Plant Sci 2005;*169*:*571*–8.

Kim SW, Keasling JD. Metabolic engineering of the nonmevalonate isopentenyldiphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. Biotechnol Bioeng 2001;72:408–15.

Lange BM, Wildung MK, McCaskill D, Croteau R. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc Natl Acad Sci USA 1998;95:2100–4.

Lois LM, Campos N, Putra SR, Danielsen K, Rohmer M, Boronat A. Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyze the synthesis of 1-deoxy-D-xylulose 5-phosphate, a common precursor for isoprenoid, thiamine, and pyridoxal biosynthesis. Proc Natl Acad Sci USA 1998;95:2105–10.

Lois LM, Rodríguez-Concepción M, Gallego F, Campos N, Boronat A. Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J 2000;22:503–13.

Lüttgen H, Rohdich F, Herz S, Wungsintaweekul J, Hecht S, Schuhr CA, et al. Biosynthesis of terpeniods: YchB protein of *Escherichia coli* phosphorylates the 2-hydroxy group of 4-diphosphocytidyl-2*C*-methyl-D-erythitol. Proc Natl Acad Sci USA 2000;97:1062–7.

Mandel MA, Feldmann KA, Herrera-Estrella L, Rocha-Sosa M, Leon P. CLA1, a novel gene required for chloroplast development, is highly conserved in evolution. Plant J 1996;9:649–58.

Morimoto H, Murai F. The effect of gelling agents on plaunotol accumulation in callus cultures of *Croton sublyratus*. Plant Cell Rep 1989;8:210–13.

Nualkaew N, De-Eknamkul W, Kutchan TM, Zenk MH. Geranylgeraniol formation in *Croton stellatopilosus* proceeds via successive monodephosphorylations of geranylgeranyl diphosphate. Tetrahedron Lett 2005;46:8727–31.

Ogiso A, Kitazawa K, Kurabayashi M, Sato A, Takahashi M, Noguchi H, et al. Isolation and structure of antipeptic ulcer diterpene from Thai medicinal plant. Chem Pharm Bull 1978;26:3117–23.

Rohdich F, Wungsintaweekul J, Fellermeier M, Sagner S, Herz S, Kis K, et al. Cytidine 5′-triphosphate-dependent biosynthesis of isoprenoids: YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2-*C*-methylerythritol. Proc Natl Acad Sci USA 1999;96:11758–63.

Rohdich F, Hecht S, Bacher A, Eisenreich W. Deoxyxylulose phosphate pathway of isoprenoid biosynthesis. Discovery and function of *ispDEFGH* genes and their cognate enzymes. Pure Appl Chem 2003;75:393–405.

Rohdich F, Bacher A, Eisenreich W. Isoprenoid biosynthetic pathways as anti-infective drug targets. Biochem Soc Trans 2005;33:785–91.

Sambrook J, Fritsch EF, Maniatis T. In: Molecular cloning: a laboratory manual, second ed. New York: Cold Spring Harbor Laboratory Press; 1989.

Schenk G, Layfield R, Candy JM, Duggleby RG, Nixon PF. Molecular evolutionary analysis of the thiamine-diphosphate-dependent enzyme, transketolase. J Mol Evol 1997;44:552–72.

Schmidt A, Gershenzon J. Cloning and characterization of isopentenyl diphosphate synthases with farnesyl diphosphate and geranylgeranyl diphosphate synthase activity from Norway spruce (*Picea abies*) and their relation to induce oleoresin formation. Phytochemistry 2007;68:2649–59.

Sitthithaworn W, Kojima N, Viroonchatapan E, Suh DY, Iwanami N, Hayashi T, et al. Geranylgeranyl diphosphate synthase from *Scoparia dulcis* and *Croton sublyratus* plastid localization and conversion to a farnesyl diphosphate synthase by mutagenesis. Chem Pharm Bull 2001;49:197–202.

Sitthithaworn W, Potduang B, De-Eknamkul W. Localization of plaunotol in the leaf of *Croton stellatopilosus* Ohba. ScienceAsia 2006;32:17–20.

Sprenger GA, Schörken N, Wiegert T, Grolle S, Graaf AA, Taylor S, et al. Identification of thiamine-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamine and pyridoxal. Proc Natl Acad Sci USA 1997;94:12857–62.

Takahashi S, Kuzuyama T, Watanabe H, Seto HA. 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2*C*-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpeniod biosynthesis. Proc Natl Acad Sci USA 1998;95:9879–84.

Tansakul P, De-Eknamkul W. Geranylgeraniol-18-hydroxylase: The last enzyme on the plaunotol biosynthetic pathway in *Croton sublyratus*. Phytochemistry 1998;47:1241–6.

Turner GW, Gerhenzon J, Croteau R. A family of transketolases that directs isoprenoid biosynthesis via a menalonate-independent pathway. Proc Natl Acad Sci USA 1998;96:2100–4.

von Heijne G, Steppuhn J, Hermann RG. Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem 1989;180:535–45.

Vongchareonsathit A, De-Eknamkul W. Rapid TLC-densitometric analysis of plaunotol from *Croton sublyratus* leaves. Planta Med 1998;64:279–80.

William DC, McGarvey DJ, Katahira EJ, Croteau R. Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase andreveals the function of the amino-terminal arginine pair. Biochemistry 1998;37:12213–20.

Wungsintaweekul J, De-Eknamkul W. Biosynthesis of plaunotol in *Croton stellatopilosus* proceeds via the deoxyxylulose phosphate pathway. Tetrahedron Lett 2005;46:2125–8.

Wungsintaweekul J, Sirisuntipong T, Kongduang D, Losuphanporn T, Ounaroon A, Tansakul P, et al. Transcription profiles analysis of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase and 2*C*-methyl-D-erythritol 4-phosphate synthase in plaunotol biosynthesis from *Croton stellatopilosus*. Biol Pharm Bull 2008;31:852–6.