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A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway

(deoxyxylulose-phosphate synthase/glyceraldehyde 3-phosphate/isopentenyl diphosphate biosynthesis/pyruvate/thiamin biosynthesis)

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Contributed by Rodney B. Croteau, December 19, 1997

ABSTRACT **Isopentenyl diphosphate, the common precursor of all isoprenoids, has been widely assumed to be synthesized by the acetate/mevalonate pathway in all organisms. However, based on *in vivo* feeding experiments, isopentenyl diphosphate formation in several eubacteria, a green alga, and plant chloroplasts has been demonstrated very recently to originate via a mevalonate-independent route from pyruvate and glyceraldehyde 3-phosphate as precursors. Here we describe the cloning from peppermint (*Mentha* × *piperita*) and heterologous expression in *Escherichia coli* of 1-deoxy-D-xylulose-5-phosphate synthase, the enzyme that catalyzes the first reaction of this pyruvate/glyceraldehyde 3-phosphate pathway. This synthase gene contains an ORF of 2,172 base pairs. When the proposed plastid targeting sequence is excluded, the deduced amino acid sequence indicates the peppermint synthase to be about 650 residues in length, corresponding to a native size of roughly 71 kDa. The enzyme appears to represent a novel class of highly conserved transketolases and likely plays a key role in the biosynthesis of plastid-derived isoprenoids essential for growth, development, and defense in plants.**

The isoprenoids comprise the largest family of natural products, with over 20,000 individual compounds described to date (1). They play numerous functional roles in plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (side chain of phytol, carotenoids), electron carriers (side chain of plastoquinone), and structural components of membranes (phytosterols). Isoprenoids also serve in communication and defense, for example as attractants for pollinators and seed dispersers and as competitive phytotoxins, antibiotics, and herbivore repellents and toxins (2). Until recently, it was generally assumed that all isoprenoids were synthesized from acetyl-CoA via the classical mevalonate pathway to the central precursor isopentenyl diphosphate (IPP) (3). However, in 1993, Rohmer and coworkers demonstrated that a nonmevalonate pathway, originating from pyruvate and glyceraldehyde 3-phosphate (GAP) (4, 5), operated in several eubacteria, including *Escherichia coli* and a green alga (6). Evidence subsequently emerged that the plastid-derived isoprenoids of plants, including carotenoids and the prenyl side chains of chlorophyll and plastoquinone (7), as well as isoprene (8), monoterpenes (9), and diterpenes (10, 11), are synthesized via the pyruvate/GAP route to IPP. This enzymatic pathway had been completely overlooked in the past. The first dedicated reaction of this mevalonate-independent pathway to IPP is considered to involve a transketolase-type condensation involving pyruvate and GAP to form 1-deoxy-D-xylulose 5-phosphate (5, 8, 12, 13) (Fig. 1). A recent abstract has described the cloning of a gene encoding 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) from *E. coli*, but no sequence information was provided (14). The cloning and characterization of this gene is now reported in a companion paper in this issue (15).

As part of an ongoing effort to isolate isoprenoid biosynthetic genes, we have employed a cDNA library derived from peppermint (*Mentha* × *piperita*) oil gland secretory cells, a plant cell type highly specialized for isoprenoid (monoterpene essential oil) formation (16) and, thus, a highly enriched source of the target mRNA species. Here we describe the cloning, heterologous expression, and transcriptional regulation of a new gene encoding DXPS from peppermint that marks the entry to the new mevalonate-independent pathway for the synthesis of isoprenoids. This gene defines a unique family of transketolases that are highly conserved between bacteria and plants but absent in animals, which rely entirely on the classical mevalonate pathway for isoprenoid biosynthesis.

MATERIALS AND METHODS

cDNA Library Construction and Screening. Secretory cells were isolated from 5-day-old peppermint leaves (16), and from these total RNA was extracted (17). Poly(A)$^+$ RNA was purified by chromatography on oligo(dT)-cellulose (Pharmacia), and 5 μg of the resulting mRNA was utilized to construct a λZAP cDNA library according to the manufacturer's instructions (Stratagene). Randomly picked and purified clones (150) were excised *in vivo*, and the resulting phagemids were sequenced by using T3 and T7 primers. A set of 3,000 plaques was then screened with a probe derived from one of the clones (designated pDS1) that was "transketolase-like" in sequence. This procedure afforded 47 positive signals under high stringency hybridization conditions. After one additional cycle of hybridization, the positive clones were excised *in vivo*, the insert sizes were determined by PCR, and the 20 largest clones were partially sequenced. Three of these clones (designated pDS16, pDS29, and pDS39) appeared to be of full-length and were entirely sequenced on both strands.

cDNA Expression, Enzyme Assay, and Product Identification. *E. coli* SOLR cells harboring pDS16, pDS29, or pDS39 were grown at 37°C in 5 ml of Luria–Bertani medium supplemented with appropriate antibiotics to an OD_{600} of 0.7, transferred to 50 ml of the same medium, and incubated at 20°C for 2 h. After induction with 200 μmol of isopropyl-1-thio-β-D-galactopyranoside (IPTG), the cells were maintained for another 14 h at 20°C. Bacteria were harvested by centrifugation (1,800 × *g*, 10 min), washed with 5 ml of assay buffer [100 mM sodium phosphate (pH 6.5) containing 3 mM $MgCl_2$,

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Abbreviations: IPP, isopentenyl diphosphate; GAP, glyceraldehyde 3-phosphate; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TPP, thiamin pyrophosphate.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF019383).

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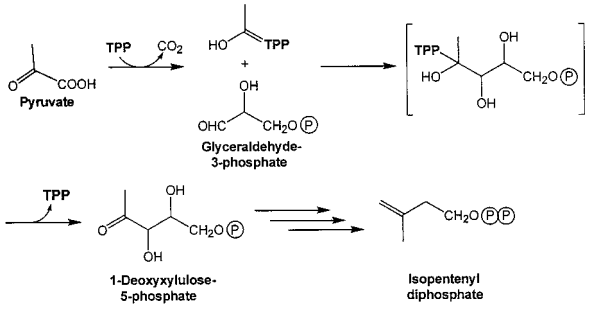

FIG. 1. Proposed mechanism of DXPS. The addition of hydroxyethyl TPP, formed by decarboxylation of pyruvate, to C1 of GAP and subsequent loss of TPP yields 1-deoxy-D-xylulose 5-phosphate, which ultimately gives rise to IPP. The circled P denotes the phosphate moiety.

0.1 mM EDTA, 5 mM NaF, 20 μM phenylmethanesulfonyl fluoride, and 100 μM thiamin diphosphate], and then resuspended in 1 ml of assay buffer. Cells were disrupted by brief sonication at 0–4°C, and the resulting homogenate was centrifuged as above to pellet debris.

An aliquot (50 μl) of the supernatant was transferred to a 600-μl Eppendorf tube to which 30 μM [2-^{14}C]pyruvate (18.5 kBq) and 0.4 μM DL-GAP (or 0.4 μM D-GAP) were added, and the mixture was incubated at 23°C for 30 min. The reaction was terminated by the addition of 70 μl of acetone and freezing at −20°C for 20 min. Following centrifugation (14,000 rpm, 5 min, bench-top centrifuge) to remove denatured protein, the supernatant was transferred to a new vial and evaporated to dryness. The residue was dissolved in 40 μl of H_2O and analyzed by reversed-phase (C_{18}) ion-pair radio-HPLC by using a procedure previously described with minor modifications (18). Enzyme assays performed with extracts of IPTG-induced cells harboring plasmid pDS29 or pDS39 evidenced the GAP-dependent appearance of a labeled product with R_t of 35.5 min that was formed in significantly higher amounts than in control assays (extracts from cells containing vector without insert). Protein content of samples was determined by using the Bio-Rad protein assay.

Fractions containing the radiolabeled product (32–37 min) were collected from semipreparative HPLC runs as above, and the solvent was removed by lyophilization. The remaining solid was suspended in 50 ml of H_2O, 1.0 g of Dowex 50X-200 cation exchange resin (H^+ form) was added, and the suspension was incubated with shaking at 23°C for 2 h. The resin was removed by filtration and washed with 2 × 20 ml of H_2O. To the combined filtrates, 50 units of wheat germ acid phosphatase (Sigma) were added, and the mixture was incubated at 23°C for 4 h. Protein was denatured by the addition of 90 ml of acetone, the suspension was frozen at −20°C for 20 min, and the protein was then precipitated by centrifugation (3,500 × *g*, 10 min). The supernatant was transferred to a new tube and lyophilized, and a portion of the dried sample was silylated [100 μl of bis(trimethylsilyl)trifluoroacetamide (Fluka), 10 μl of pyridine, and 100 μg of Na_2SO_4; 80°C for 1 h]. GC–MS analysis (of the dephosphorylated and then silylated biosynthetic product and of silylated authentic 1-deoxy-D-xylulose) was performed by using a Hewlett-Packard 6890 GC-MSD system equipped with a 30-m × 0.25-mm diameter fused silica column coated with a 0.25-μm film of HP 5MS (Hewlett-Packard). The oven was programmed from 90°C (2-min hold) at 20°C/min to 250°C (2-min hold) and then at 20°C/min to 300°C at 10 psi (1 psi = 6.89 kPa) He, and electron impact spectra were recorded at 70 eV with an electron multiplier voltage of 2,200 V. Full spectra were acquired, and selected diagnostic ions were monitored: m/z 307 [$M^+ - 43$ (CH_3CO)]; m/z 277 [$M^+ - 73$ ((CH_3)$_3$Si)]; m/z 218 [$M^+ - 43$ (CH_3CO) − 89 ((CH_3)$_3$SiO)]; m/z 205 [$M^+ - 145$ ($CH_3COCHOSi(CH_3)_3$)]; m/z 204 [((CH_3)$_3$SiOCHCH$_2$OSi(CH$_3$)$_3$)$^+$]; m/z 147 [((CH_3)$_2$SiOSi(CH$_3$)$_3$)$^+$]; m/z 132 [(Si(CH$_3$)$_3$)OCH$_2$CHO)$^+$]; m/z 117 [((CH_3)$_3$SiOCH$_2$CH$_2$)$^+$]; m/z 103 [((CH_3)$_3$SiOCH$_2$)$^+$]; m/z 89 [((CH_3)$_3$SiO)$^+$]; m/z 73 [((CH_3)$_3$Si)$^+$]. The silylated derivative of the biosynthetic product eluted at an R_t of 6.71 min; the silylated derivative of authentic 1-deoxy-D-xylulose eluted at an R_t of 6.70 min.

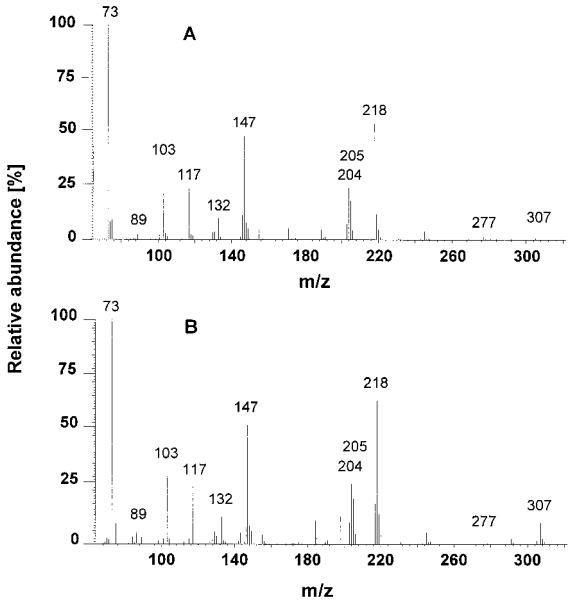

FIG. 2. GC–MS analysis of the biosynthetic product formed by the recombinant peppermint enzyme. (*A*) Mass spectrum of the biosynthetic product after dephosphorylation and trimethylsilylation (R_t = 6.71 min). (*B*) Mass spectrum of the silylated derivative of authentic 1-deoxy-D-xylulose (R_t = 6.70 min).

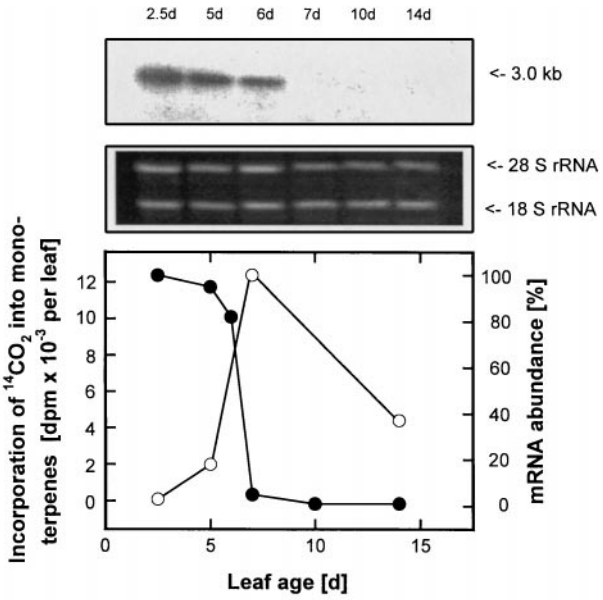

FIG. 3. Time course of relative steady-state *DXPS* mRNA levels (●) and rate of monoterpene biosynthesis as measured by $^{14}CO_2$ incorporation (○) during leaf development in peppermint. Total RNA was isolated from oil gland secretory cells of leaves of different developmental stages in days (d) from emergence. A ^{32}P-labeled probe derived from *DXPS* clone pDS29 detected a transcript of about 3 kbp. Ethidium bromide-stained bands of 18S and 28S rRNA are shown as the internal controls. Leaves are fully expanded by 2 weeks, and 2.5-day leaves are the smallest from which oil gland secretory cells can be isolated (16).

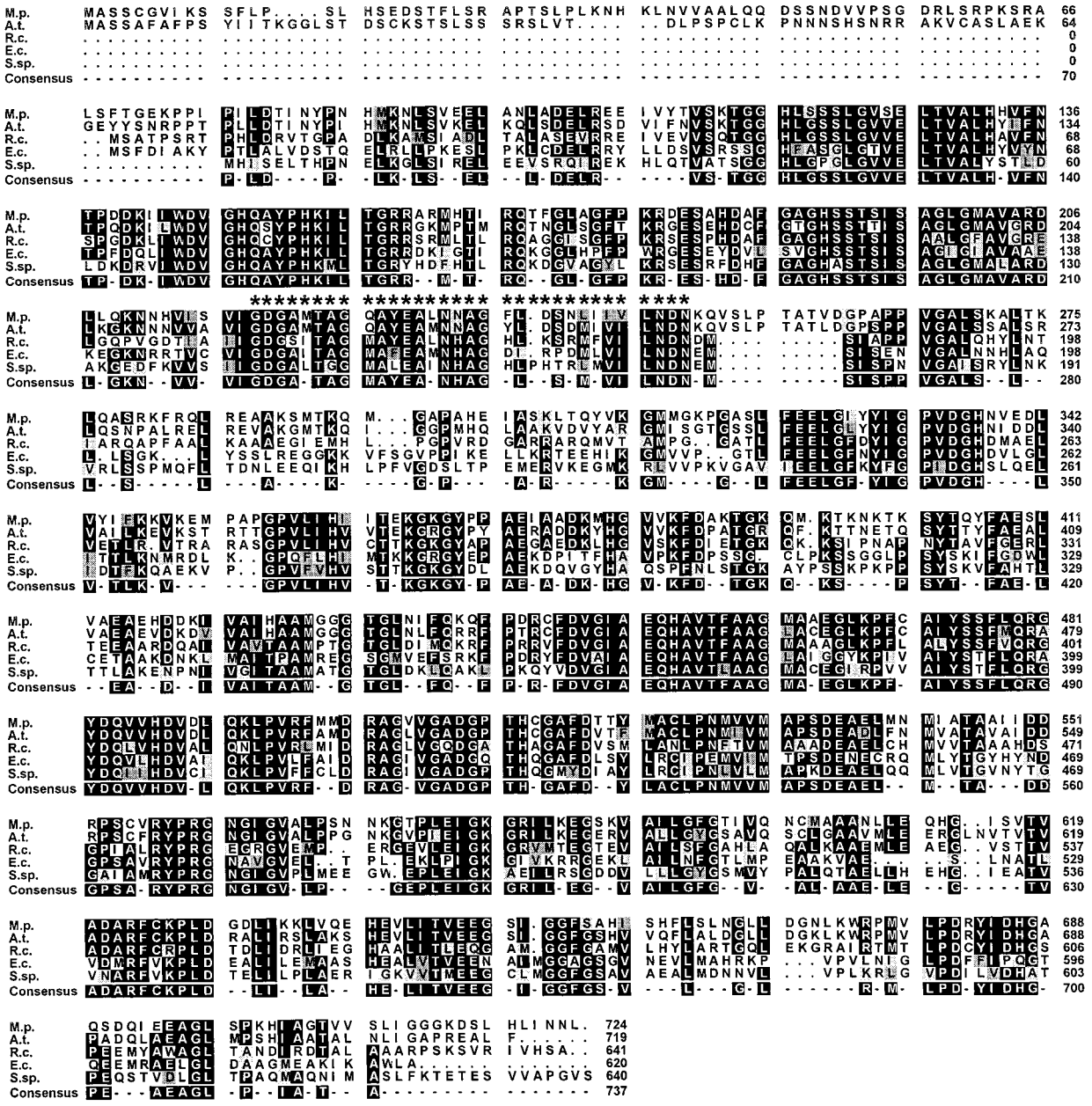

FIG. 4. Deduced amino acid sequence comparison (upper panel) of *DXPS* from peppermint (M.p.), *CLA1* from *A. thaliana* (A.t.), ORF *2814* from *R. capsulata* (R.c.), ORF *f620* from *E. coli* (E.c.), and a protein of unknown function from *Synechocystis* sp. strain PC6803 (S.sp.). Identical

RNA Blot Analysis and Monoterpene Biosynthetic Rate. Peppermint oil gland secretory cell RNA was isolated as before from leaves of different ages, separated on a 1.5% formaldehyde-agarose gel (5 μg each lane), and blotted onto nylon membranes (Amersham). The membranes were hybridized with a ^{32}P-labeled probe prepared from cDNA clone pDS29 in a hybridization solution containing 35% formamide (19) for 15 h at 42°C after prehybridization for 3 h at the same temperature. Blots were washed for 20 min at 60°C with 4× SSC (0.2% SDS) (19) and twice for 20 min at 60°C with 1× SSC (0.2% SDS). Administration of $^{14}CO_2$ to peppermint plants and the isolation and quantification of the leaf monoterpenes produced were performed as described previously (20).

RESULTS AND DISCUSSION

Cloning and Heterologous Expression of a cDNA Encoding a Novel Transketolase. For the cloning of genes involved in isoprenoid biosynthesis in peppermint (*M.* × *piperita*), a cDNA library was constructed from mRNA isolated from the epidermal oil glands, a tissue highly specialized for isoprenoid (monoterpene) biosynthesis. During a random cDNA library screen we obtained, in addition to several genes of known function in isoprenoid biosynthesis, two clones (designated pDS1 and pDS2), which exhibited very high sequence similarity to a recently described *Arabidopsis thaliana* transketolase gene (*CLA1*) of unknown function but which were distinguishable from the transketolases involved in the pentose phosphate pathway (21). To investigate the possible function of this transketolase gene in isoprenoid formation, we next screened 3,000 plaques from the peppermint oil gland cDNA library with a probe derived from clone pDS1. From 47 isolates, three clones appeared to be of full length (designated pDS16, pDS29, and pDS39), and these were evaluated by heterologous expression in *E. coli* of an enzyme capable of catalyzing the condensation reaction of pyruvate and GAP to a deoxypentulose phosphate (Fig. 1). *E. coli* cultures transformed with phagemids derived from pDS16, pDS29, and pDS39 were each induced with IPTG, the corresponding bacterial cells were harvested and homogenized, and the extracts were assayed by using [2-^{14}C]pyruvate and DL-GAP as cosubstrates. Preparations from *E. coli* cells harboring either pDS29 or pDS39 yielded a prominent new radioactive component in the reaction mixture that, on reversed-phase ion-pair radio-HPLC, exhibited a retention time (R_t = 35.5 min) consistent with that of a sugar (pentulose) phosphate (18). The same enzymatic product was generated with D-GAP as cosubstrate, indicating that the D-antipode is the likely natural substrate of the functionally expressed transketolase.

Characterization of the Enzyme Product. The presumptive pentulose phosphate obtained from preparative enzyme incubations (*E. coli* transformed with pDS29) was purified by HPLC and hydrolyzed with acid phosphatase, and the resulting sugar was silylated. This derivatized product of the recombinant enzyme was then analyzed by combined capillary GC–MS and shown to possess the identical retention time (6.71 ± 0.03 min) and mass spectrum as that of an authentic sample of silylated 1-deoxy-D-xylulose (Fig. 2). The combined evidence thus indicated that a cDNA encoding DXPS had been acquired. DXPS activity was significantly higher in extracts of the IPTG-induced *E. coli* cells expressing pDS29 than in extracts of identically treated cells containing the same plasmid devoid of cDNA insert (i.e., 7-fold higher than the endogenous activity of *E. coli*; $n = 7$, $P < 0.01$).

Time Course of Steady-State DXPS mRNA Levels and Monoterpene Biosynthesis. To examine the possible function of the *DXPS* gene in greater detail, total RNA was isolated from peppermint oil gland secretory cells obtained from leaves of different developmental stages following emergence. RNA blot analyses (with a probe derived from clone pDS29) showed the highest levels of steady-state *DXPS* message (at about 3 kbp) during the first 2.5–6 days of leaf development (Fig. 3). The rate of monoterpene biosynthesis, as determined by $^{14}CO_2$ incorporation, peaked at about day 7, thus suggesting activation of the pyruvate/GAP pathway to supply the IPP precursor for subsequent monoterpene biosynthesis in peppermint oil glands.

Sequence Analysis. *DXPS* clone pDS29, which yielded the highest expressed level of synthase activity, contains an ORF of 2,172 nucleotides (Fig. 4). The first 70 deduced amino acid residues reveal the general characteristics of plastidial targeting sequences (22), consistent with the proposed subcellular location of the enzyme in plant cells. By excluding the putative transit peptide residues, the sequence corresponds to a mature protein of about 650 amino acids, with a predicted size of roughly 71 kDa. This compares to a deduced protein of 621 residues with a predicted size of 67.6 kDa described by Lois *et al.* (15) in the companion report on a *DXPS* clone from *E. coli*. An alignment of translated transketolase sequences (devoid of plastid-targeting peptides where appropriate) shows very high similarity/identity scores between the peppermint *DXPS* and *CLA1* from *Arabidopsis* (21) (85/77%), ORF *2814* from the purple nonsulfur photosynthetic bacterium *Rhodobacter capsulata* (23) (part of the *puf* operon in the photosynthetic gene cluster; 72/56%), *DXPS* of *E. coli* (15) (map position 9.43 min, described in the accompanying paper; 69/48%), and a deduced protein from the cyanobacterium *Synechocystis* sp. strain PCC6803 (24) (65/45%) (Fig. 4, upper panel). These conserved sequences appear to form a new class of transketolases that is distinct from the well characterized transketolases involved in the pentose phosphate pathway, and the extensive sequence similarity among the members of this group suggests that they all encode DXPS or a very closely related synthase (Fig. 4, lower panel). In addition, the general transketolase consensus thiamin pyrophosphate (TPP) binding motif [GDG(X)$_{7-8}$E(X)$_{3-4}$A(X)$_{11-13}$NN] determined by Hawkins *et al.* (25) was observed in this new transketolase type as GDG(A/SXT(A/G)G(Q/M)AXEAXN(N/H)AG(X)$_{7-8}$(I/V)(V/I)LNDN (residues 219–250 of the peppermint sequence).

DXPS as a Branch-Point Enzyme. Mandel and associates (21) described an *Arabidopsis* transketolase gene (*CLA1*, almost certainly a DXPS based on sequence), the disruption of which results in an albino phenotype. The lack of chlorophyll and carotenoid pigments, resulting in very early arrest of chloroplast development in the mutant plants, indicates the central role of DXPS in the biosynthesis of plastidial isoprenoids essential for chloroplast function. In addition to the utilization of TPP as a cofactor, DXPS also provides a substrate for thiamin biosynthesis in plants (26). This newly established relationship between the plastidial, mevalonate-independent pathway to isoprenoids and cofactor biosynthesis (and utilization) now allows reconsideration of earlier studies with a thiamin-deficient tobacco mutant, in which McHale *et*

residues are shaded in black, residues of high similarity are indicated by gray shading, and residues of lower similarity are displayed by a pale shade. Asterisks indicate the position of the putative TPP-binding motif. Sequence comparison and clustering relationship analysis (lower panel) were carried out by using GCG version 9.0 of the University of Wisconsin Genetics Computer Group Package (1997). The following transketolase sequences are included: *DXPS* (*M.* × *piperita*, accession number AF019383); *CLA1* (*A. thaliana*, U27099); ORF *2814* (*R. capsulata*, P26242); ORF *f620* (*E. coli*, U82664); a protein of unknown function (*Synechocystis* sp. PC6803, D90903); transketolase 1 (human, A45050; yeast, P23254; and *E. coli*, P27302); transketolase 2 (human, P51854; yeast, P33315; *E. coli*, P33570); and a plastidial transketolase from potato (Z50099).

al. (27) reported the associated inability to produce chlorophyll and carotenoids to be fully reversible by the addition of exogenous thiamin. The authors (27) postulated that the thiamin deficiency prevented the production of acetyl-CoA from pyruvate by the thiamin-dependent pyruvate dehydrogenase complex and thus abolished plastidial isoprenoid formation via the acetate/mevalonate pathway. The utilization of TPP by DXPS now suggests an alternate direct metabolic linkage in the mutant, in which deprivation of the cofactor for DXPS eliminates chlorophyll and carotenoid production by incapacitating the plastidial, mevalonate-independent pathway for IPP biosynthesis.

The cloning of *DXPS* from peppermint provides direct evidence for the presence in plants of the plastidial mevalonate-independent pathway, which operates in parallel with the classical, cytosolic mevalonate pathway to IPP to produce a very broad range of isoprenoid compounds (7–11). The mevalonate-independent pathway offers a novel approach to transgenic manipulation of plant isoprenoid biosynthesis, and because this new pathway is present in bacteria and plants but not animals, it provides a unique target for the design of highly specific antibiotics and herbicides. Finally, an unusual experimental tool developed in this work (a cDNA library constructed from mRNA isolated from oil gland cells, a cell type highly specialized for isoprenoid biosynthesis) provides an ideal system for further investigation of the mevalonate-independent pathway and for examination of flux control within (and the communication between) the plastidial and cytosolic routes to IPP.

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