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RESEARCH REPORT

Isoprenoid Biosynthesis via a Mevalonate-Independent Pathway in Plants: Cloning and Heterologous Expression of 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase from Peppermint[1]

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Two distinct pathways are utilized by plants for the biosynthesis of isopentenyl diphosphate, the universal precursor of isoprenoids. The classical acetate/mevalonate pathway operates in the cytosol, whereas plastidial isoprenoids originate via a novel mevalonate-independent route that involves a transketolase-catalyzed condensation of pyruvate and D-glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate as the first intermediate. Based on *in vivo* feeding experiments, rearrangement and reduction of deoxyxylulose phosphate have been proposed to give rise to 2-*C*-methyl-D-erythritol-4-phosphate as the second intermediate of this pyruvate/glyceraldehyde-3-phosphate pathway (1–3). The cloning of an *Escherichia coli* gene encoding an enzyme capable of converting 1-deoxy-D-xylulose-5-phosphate to 2-*C*-erythritol-4-phosphate was recently reported (4). A cloning strategy was developed for isolating the gene encoding a plant homolog of this enzyme from peppermint (*Mentha* × *piperita*), and the identity of the resulting cDNA was confirmed by heterologous expression in *E. coli*. Unlike the microbial reductoisomerase, the plant ortholog encodes a preprotein bearing an N-terminal plastidial transit peptide that directs the enzyme to plastids where the mevalonate-independent pathway operates in plants. The peppermint gene comprises an open reading frame of 1425 nucleotides which, when the plastidial targeting sequence is excluded, encodes a deduced enzyme of approximately 400 amino acid residues with a mature size of about 43.5 kDa. © 1999 Academic Press

Key Words: **1-Deoxy-D-xylulose-5-phosphate; 2-*C*-methyl-D-erythritol-4-phosphate; reductoisomerase; isoprenoid biosynthesis; peppermint; *Mentha* × *piperita*.**

Isoprenoids are a large and structurally diverse group of compounds that play essential roles in plants as hormones, photosynthetic pigments, electron carriers, and components of membranes and that also serve in communication and defense (5). Until recently, it was widely accepted that all isoprenoids were synthesized via the acetate/mevalonate pathway (6). However, evidence has emerged over the last few years that isopentenyl diphosphate, the central intermediate of isoprenoid biosynthesis, originates from pyruvate and D-glyceraldehyde-3-phosphate via a new mevalonate-independent pathway in several eubacteria (7–9), algae (10), and plant plastids (11, 12). The first step in this novel pathway involves a transketolase-type condensation reaction of pyruvate and glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (Scheme 1). Genes encoding the enzyme which catalyzes this reaction, deoxyxylulose phosphate synthase, have been cloned from *Escherichia coli* (13, 14), peppermint (*Mentha* × *piperita*) (15), and pepper (16). The second step of the mevalonate-independent pathway is considered to involve an intramolecular rearrangement and subsequent reduction of deoxyxylulose phosphate to yield 2-*C*-methyl-D-erythritol-4-phosphate (1–3) (Scheme 1), and Seto and co-workers (4) have recently reported the isolation and characterization of such a reductoisomerase gene from *E. coli*. Here we present evidence for the existence of plant

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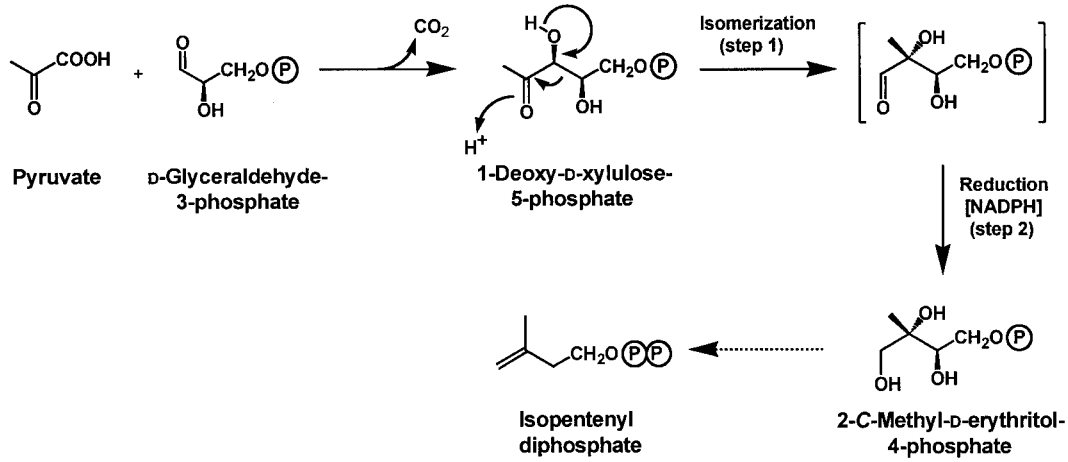

SCHEME 1. Outline of the pyruvate/glyceraldehyde 3-phosphate pathway for the biosynthesis of isopentenyl diphosphate and the proposed reaction mechanism of the deoxyxylulose reductoisomerase in the conversion of 1-deoxy-D-xylulose-5-phosphate to 2-*C*-methyl-D-erythritol-4-phosphate. The circled P denotes the phosphate moiety. The broken arrow indicates several as yet unidentified steps.

orthologs of this deoxyxylulose phosphate reductoisomerase in *Arabidopsis thaliana* and peppermint.

As a tool for the isolation of genes involved in terpenoid biosynthesis in plants, we have prepared a cDNA library constructed from mRNA of isolated peppermint oil gland secretory cells, a cell type highly specialized for monoterpene (essential oil) biosynthesis (17). Based on likely conserved regions of the reductoisomerase gene, PCR primers were designed (P1, 5′-CGAGATTATGCCAGGAGAGC-3′; P2, 5′-GGCTTCAGGCAAACCCTTG-3′) and employed with peppermint oil gland library cDNA as template to amplify a fragment (223 bp,[3] designated pMPDXR1) with significant homology to the *E. coli* reductoisomerase gene. By screening the peppermint oil gland cDNA library (2.5×10^4 plaques) with a labeled probe derived from pMPDXR1, five full-length clones were obtained. Screening an *A. thaliana* flower bud cDNA library (CD4-6 from the *Arabidopsis* Biological Resource Center (http://aims.cps.msu.edu/aims/); 2×10^5 plaques) with the same probe afforded 20 positive clones, all of which were slightly 5′-truncated. An additional primer set (P3, 5′-GTCTCAACTCTGGAAGCTTTATGAAGCAACTC-TCAC-3′; P4, 5′-CTCTGTAGCCGGACCTAGGTCAGCTT-GCGAGAC-3′) was employed to amplify the full-length *E. coli* reductoisomerase gene, and the resulting amplicon was inserted into pBluescript KS(−) for use as a positive control in the functional expression of the enzyme.

The peppermint deoxyxylulose phosphate reductoisomerase homolog contains an open reading frame of 1425 bp encoding a protein of 475 deduced amino acid residues (Fig. 1). The first 73 amino acids display typical characteristics of plastidial targeting sequences (18), consistent with the subcellular localization of this enzyme in plant plastids where the mevalonate-independent pathway operates (11, 12). When the residues defining the putative transit peptide are excluded, the size of the mature enzyme is estimated at about 43.5 kDa. Alignment of translated sequences (Fig. 1; excluding comparison of plastidial targeting peptides where appropriate) reveals significant homology between the peppermint reductoisomerase and the putative reductoisomerase fragment from *A. thaliana* (88.0% similarity/84.2% identity), as well as with SLL0019 from the cyanobacterium *Synechocystis* sp. PCC6803 (72.3/63.7%), BG13409 from *Bacillus subtilis* (56.9/45.5%), the reductoisomerase of *E. coli* (53.4/43.0%), HI0807 from *Haemophilus influenzae* (55.5/41.8%), Rv2870c from *Mycobacterium tuberculosis* (52.8/43.6%), and HP0216 from *Helicobacter pylori* (50.7/38.1%). Although the reaction mechanism of deoxyxylulose phosphate reductoisomerase and that of ketol acid reductoisomerase, which catalyzes the rearrangement and reduction of 2-acetolactate to 2,3-dihydroxyisovalerate and of 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate in the biosynthesis of branched-chain amino acids (19), share some similarity, the deduced amino acid sequences of these enzymes are quite distinct (~35% similarity). However, the N-termini of the deoxyxylulose phosphate reductoisomerase sequences contain a conserved motif (GSTGSIG) with some homology to the signature sequence of the proposed NADPH binding site of ketol acid reductoisomerase (GXGXXGXXXG) (20).

A full-length peppermint reductoisomerase cDNA (designated pMPDXR18) and the *E. coli* reductoisomerase clone (pECDXR20) were evaluated by expression in *E. coli* of an enzyme capable of catalyzing the rearrangement and pyridine nucleotide-dependent reduction of 1-deoxy-D-xylulose-5-phosphate to 2-*C*-methyl-D-erythritol-4-phosphate. *E. coli* cells transformed with phagemids derived from pMPDXR18 and pECDXR20 were grown and induced with IPTG, and the resulting enzyme preparations were assayed in the presence of NADPH and Mn^{2+} using biosynthetically prepared [1-^{14}C]deoxyxylulose phosphate (15) as substrate. Each preparation yielded a single new product upon radio-HPLC analysis by modification of a published reversed-phase ion-pairing chromatography method (21), with a retention time (34.0 min) considerably different from that of deoxyxylulose phos-

[3] Abbreviations used: bp, base pair(s); GC, gas chromatography; HPLC, high-performance liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MS, mass spectrometry; Tris, tris(hydroxymethyl)aminomethane.

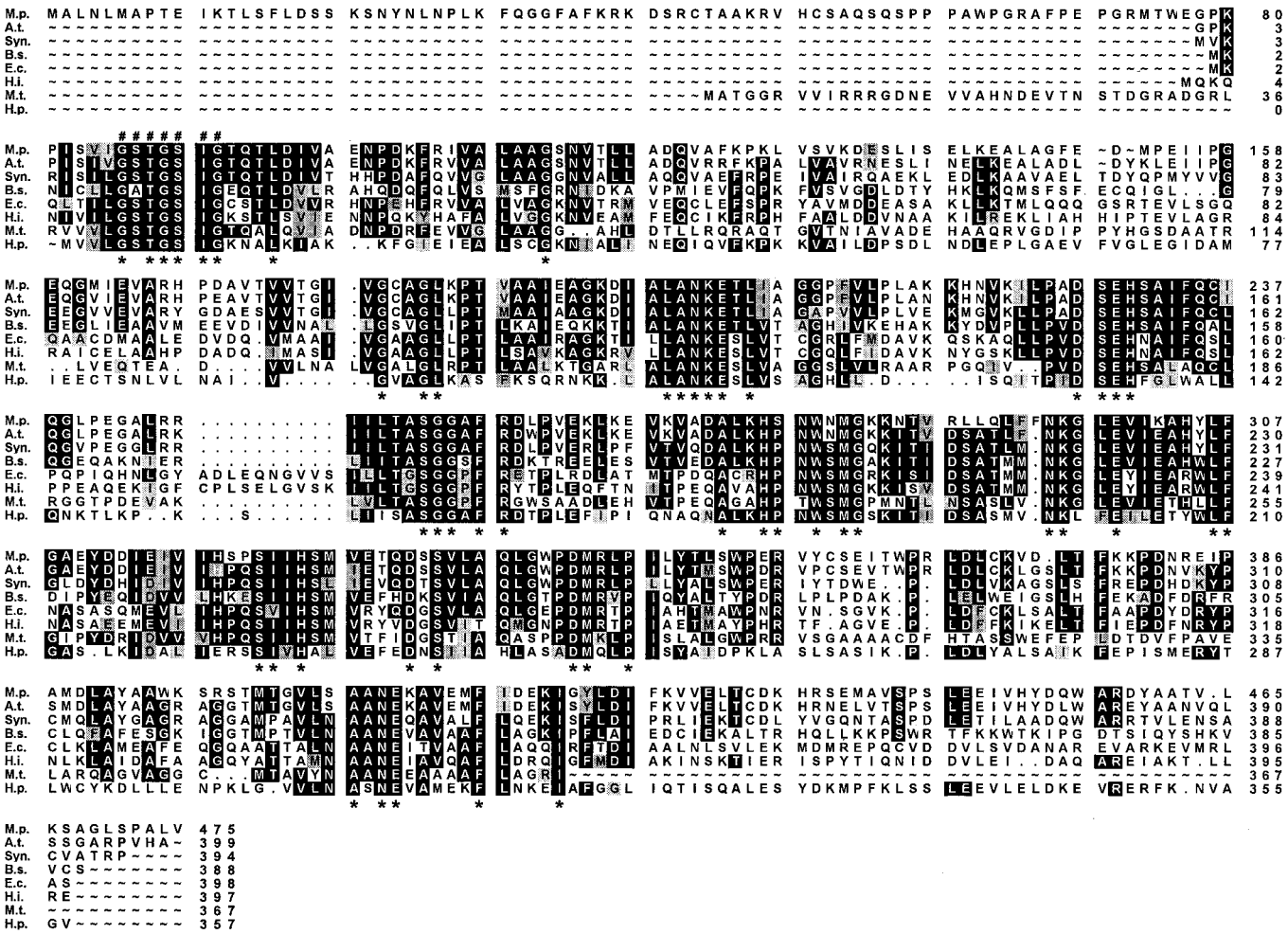

FIG. 1. Deduced amino acid sequence comparison of the reductoisomerase from peppermint (M.p., Accession No. AF116825), a putative reductoisomerase (longest available clone) from *A. thaliana* (A.t.), SLL0019 from *Synechocystis* sp. PCC6803 (Syn., Q55663), BG13409 from *Bacillus subtilis* (B.s., B69881), reductoisomerase from *E. coli* (E.c., AB013300), HI0807 from *Haemophilus influenzae* (H.i., P44055), Rv2870c from *Mycobacterium tuberculosis* (M.t., Q10798), and HP0216 from *Helicobacter pylori* (H.p., P56139). Residues boxed in black indicate positional identity for at least four of the eight compared sequences; similar amino acids are indicated by gray shading. Asterisks (*) indicate conserved residues in all reductoisomerase sequences; the pound signs (#) indicate a putative NADPH binding motif. The alignment was created with the PILEUP program (Wisconsin Package Version 9.0; Genetics Computer Group, Madison, WI). A search of genome project databases (incomplete for mammals) revealed the absence of reductoisomerase homologs in humans, mice and rats, and several archaebacteria (*Methanobacterium thermoautotrophicum*, *Methanococcus jannaschii*, and *Archaeoglobus fulgidus*).

phate (55.7 min). Control preparations from identically treated cells containing the same plasmids (pBluescript SK(⁻) for pMPDXR18 and pBluescript KS(⁻) for pECDXR20) but devoid of cDNA inserts did not yield measurable levels of the new metabolite derived from deoxyxylulose phosphate. Preparative incubations of the recombinant enzyme from both sources were employed to generate the new product at larger scale for HPLC-based purification. The resulting product (R_t = 34.0 min) was hydrolyzed with acid phosphatase, converted to the corresponding trimethylsilyl ether derivative, and analyzed by GC-MS. The derivatized product from both sources exhibited the same retention time (7.1 ± 0.1 min) and mass spectrum as an authentic sample of 2-*C*-methyl-D,L-erythritol identically derivatized (Fig. 2), thereby confirming the identity of the reductoisomerase and indicating that the plant enzyme is active in the preprotein form.

The reaction catalyzed by ketol acid reductoisomerase obeys an ordered mechanism in which NADPH and the metal ion cofactor bind first, followed by the acetohydroxy acid substrate (22). Since NADPH and manganese (or magnesium) are also required for the enzymatic conversion of deoxyxylulose phosphate to methylerythritol phosphate (no intermediates, such as methylerythrose phosphate, were observed in the presence or absence of these cofactors), a similar reaction mechanism may be postulated for deoxyxylulose phosphate reductoisomerase.

The isolation of cDNAs encoding both deoxyxylulose phosphate synthase (15) and deoxyxylulose phosphate reductoisomerase from peppermint provides substantial evidence for the operation of similar catalytic machinery in the pyruvate/glyceraldehyde-3-phosphate pathway in plant plastids and several eubacteria. Since this essential pathway is present in

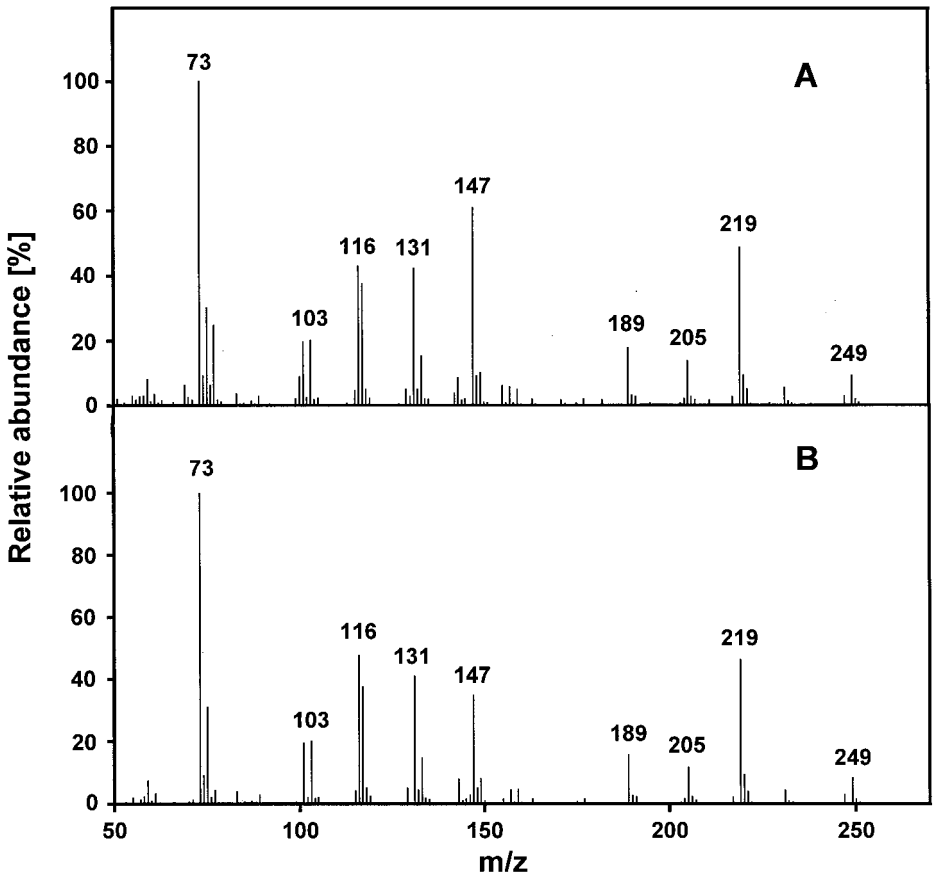

FIG. 2. GC-MS analysis of (A) the trimethylsilyl ether derivative of the dephosphorylated biosynthetic product (R_t = 7.1 ± 0.1 min) generated by recombinant peppermint reductoisomerase and (B) the trimethylsilyl ether derivative of authentic 2-*C*-methyl-D,L-erythritol (R_t = 7.1 ± 0.1 min) identically prepared. The slight difference in relative intensity of ions in the *m*/*z* 116, 131, and 147 clusters in spectrum A is due to background subtraction of contaminants in the case of the biosynthetic product for which the total ion abundance was 10-fold less than for the standard (B). For enzyme preparation, transformed *E. coli* cells were grown to A_{600} of 0.5 at 37°C in 50 ml of Luria–Bertani medium supplemented with appropriate antibiotics. Cells harboring pMPDXR18 were then incubated at 20°C for 2 h, induced with 0.1 mM IPTG, and maintained at 20°C for 15 h. Cells harboring pECDXR20 were similarly induced, but with 1 mM IPTG, and maintained at 37°C for 5 h. Bacteria were harvested by centrifugation, washed with 1 ml of assay buffer (0.1 M Tris–HCl (pH 7.5) containing 2 mM $MnCl_2$ and 0.5 mM NADPH), resuspended in 1 ml of assay buffer, and then disrupted by brief sonication at 0–4°C. The resulting homogenates were centrifuged to pellet debris, and an aliquot (15 μl) of each preparation was incubated with 0.1 mmol [1-^{14}C]deoxyxylulose phosphate (18.5 kBq) for 10 min at 23°C. To the reaction mixtures, 50 μl of 10 mM $NaHCO_3$ was added, the suspensions were filtered through Nanosep columns (Pall Filtron, 30,000-kDa cutoff), and the filtrates were analyzed by modification of an established reversed-phase ion-pair radio-HPLC method (21) using 10 mM tetrabutylammonium acetate as ion-pairing reagent. Enzyme assays from both sources revealed the presence of a new radiolabeled product at R_t = 34.0 min, which was isolated by semipreparative HPLC as above. Following solvent removal under vacuum, the residual material was dissolved in 50 μl of 0.1 M potassium phosphate buffer (pH 5.0) to which 10 units of wheat germ acid phosphatase were added (Sigma) followed by incubation at 23°C for 2 h. The reaction was terminated by addition of 50 μl of acetone, followed by centrifugation, transfer of the supernatant, and removal of solvent under vacuum. The residual material was dissolved in 20 μl of anhydrous diethyl ether and converted to the trimethylsilyl ether derivative for GC-MS analysis as previously described (15). The mass spectra of the products derived from the recombinant peppermint reductoisomerase and *E. coli* reductoisomerase (data not shown) were identical.

plants and bacteria but not in animals (B. M. Lange, W. Martin, and R. Croteau, unpublished results), both the synthase and reductoisomerase are obvious targets for the development of novel classes of highly specific herbicides and antibiotics (23). Whereas deoxyxylulose phosphate serves as the precursor for the biosynthesis of thiamin (24) and probably pyridoxol (25) in higher plants, as well as isopentenyl diphosphate (26), the reductoisomerase catalyzes the first committed step in the conversion of this common intermediate to plastidial isoprenoids, including carotenoids and the prenyl side chains of chlorophyll and plastoquinone (12). This specific transformation may be expected to be a regulated (and potentially rate-limiting) step of isoprenoid biosynthesis in plastids. Overexpression of this gene might lead to increased flux through the pathway with consequent increase in the rate of formation of plastidial monoterpene (C_{10}), diterpene (C_{20}), and tetraterpene (C_{40}) products, as well as related prenylated metabolites.

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