Isopentenyl diphosphate biosynthesis via a mevalonate-independent pathway: Isopentenyl monophosphate kinase catalyzes the terminal enzymatic step

B. Markus Lange and Rodney Croteau*

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6430

Contributed by Rodney Croteau, September 13, 1999

In plants, the biosynthesis of isopentenyl diphosphate, the central precursor of all isoprenoids, proceeds via two separate pathways. The cytosolic compartment harbors the mevalonate pathway, whereas the newly discovered deoxyxylulose 5-phosphate pathway, which also operates in certain eubacteria, including *Escherichia coli*, is localized to plastids. Only the first two steps of the plastidial pathway, which involve the condensation of pyruvate and glyceraldehyde 3-phosphate to deoxyxylulose 5-phosphate followed by intramolecular rearrangement and reduction to 2-*C*-methylerythritol 4-phosphate, have been established. Here we report the cloning from peppermint (*Mentha* × *piperita*) and *E. coli*, and expression, of a kinase that catalyzes the phosphorylation of isopentenyl monophosphate as the last step of this biosynthetic sequence to isopentenyl diphosphate. The plant gene defines an ORF of 1,218 bp that, when the proposed plastidial targeting sequence is excluded, corresponds to ≈308 aa with a mature size of ≈33 kDa. The *E. coli* gene (*ychB*), which is located at 27.2 min of the chromosomal map, consists of 852 nt, encoding a deduced enzyme of 283 aa with a size of 31 kDa. These enzymes represent a conserved class of the GHMP family of kinases, which includes *g*alactokinase, *h*omoserine kinase, *m*evalonate kinase, and *p*hosphomevalonate kinase, with homologues in plants and several eubacteria. Besides the preferred substrate isopentenyl monophosphate, the recombinant peppermint and *E. coli* kinases also phosphorylate isopentenol, and, much less efficiently, dimethylallyl alcohol, but dimethylallyl monophosphate does not serve as a substrate. Incubation of secretory cells isolated from peppermint glandular trichomes with isopentenyl monophosphate resulted in the rapid production of monoterpenes and sesquiterpenes, confirming that isopentenyl monophosphate is the physiologically relevant, terminal intermediate of the deoxyxylulose 5-phosphate pathway.

deoxyxylulose 5-phosphate | isoprenoid biosynthesis

Isopentenyl diphosphate (IPP) is the central intermediate in the biosynthesis of isoprenoids in all organisms. In higher plants, the formation of IPP is compartmentalized. The mevalonate (MVA) pathway, the enzymes of which are localized to the cytosolic compartment, produces the precursor of triterpenes (sterols) and certain sesquiterpenes (1); in plastids, the deoxyxylulose 5-phosphate (DXP) pathway operates to supply IPP for the synthesis of monoterpenes and diterpenes (2, 3), several sesquiterpenes (4), tetraterpenes (carotenoids), and the prenyl side chains of chlorophyll and plastoquinone (5). In addition, there are examples of cooperation between the cytosolic and plastidial pathways in the biosynthesis of stress-induced and constitutively emitted volatile terpenoids from a variety of plants (6) and of constitutive sesquiterpenes of chamomile (7). In mammals, where the DXP pathway does not operate (B.M.L., W. Martin, and R.C., unpublished results), and in plants, the individual biosynthetic steps of the MVA pathway have been well characterized (8, 9). However, for the recently discovered DXP pathway, which also occurs in many eubacteria (10), the biosynthetic sequence leading to the formation of IPP is still incompletely defined (Fig. 1). The initial step of the pathway involves a condensation of pyruvate (C2 and C3) with D-glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose 5-phosphate (11–14). The enzyme that catalyzes this reaction belongs to a family of transketolases, and the corresponding gene has been isolated from *Escherichia coli* (15, 16), peppermint (17), and pepper (18). In the second step of this pathway, rearrangement and reduction of DXP yield 2-*C*-methylerythritol 4-phosphate (MEP) (19–21) (Fig. 1). Recently, genes encoding this DXP reductoisomerase have been cloned from *E. coli* (22), peppermint (23), and *Arabidopsis thaliana* (23, 24). To date, no other intermediates on the route to IPP, the terminal product of the DXP pathway (25, 26), have been identified.

Sequencing of 1,300 anonymous clones (expressed sequence tags) from a cDNA library constructed from mRNA isolated from the oil-gland secretory cells of peppermint (*Mentha* × *piperita*) (4), afforded, after extensive database comparisons, two clones having homologues of unknown function in plants and several eubacteria, the sequences of which contained a motif with homology to the putative ATP-binding domain of the GHMP (*g*alactokinase, *h*omoserine kinase, *m*evalonate kinase, and *p*hosphomevalonate kinase) family of metabolite kinases. This putative kinase gene from peppermint and its *E. coli* orthologue, when overexpressed in *E. coli*, yielded a recombinant enzyme that catalyzes the ATP-dependent phosphorylation of isopentenyl monophosphate (IP) to IPP. Feeding experiments with IP and several other isoprenoid precursors, by using isolated peppermint secretory cells, confirmed the phosphorylation of IP to IPP to be the last step in the DXP pathway.

Materials and Methods

Substrates. [1-^{3}H]Dimethylallyl diphosphate (555 GBq·mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis) and was adjusted to a specific activity of 2.13 GBq·mmol^{-1} by dilution with the unlabeled compound (Sigma). [4-^{14}C]Isopentenyl diphosphate (2.13 GBq·mmol^{-1}) was obtained from DuPont/NEN. [1-^{3}H]Dimethylallyl monophosphate and [4-^{14}C]isopentenyl monophosphate were generated enzymatically by apyrase (Sigma, A6410) treatment (10 units in

Abbreviations: DMA, dimethylallyl alcohol; DMAP, dimethylallyl monophosphate; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose 5-phosphate; IP, isopentenyl monophosphate; IPK, isopentenyl monophosphate kinase; IPP, isopentenyl diphosphate; ISO, isopentenol; MEP, 2-*C*-methylerythritol 4-phosphate; MVA, mevalonate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [AF179283 (peppermint) and AF179284 (*E. coli*)].

*To whom reprint requests should be addressed. E-mail: croteau@mail.wsu.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

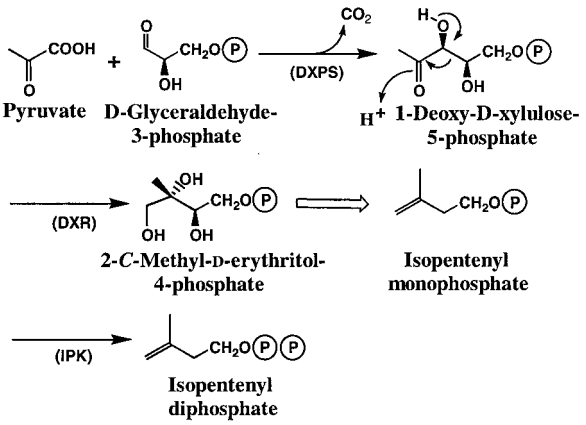

Fig. 1. Outline of the DXP pathway for the biosynthesis of IPP, and the proposed role of IPK. The circled P denotes the phosphate moiety. The large open arrow indicates several as-yet-unidentified steps.

200 μl of 0.2 M succinate buffer, pH 6.0; 5 min at 23°C) of the parent diphosphates (0.17 μmol each). Extended incubation (1 h) under the same conditions with a mixture of apyrase and acid phosphatase (Sigma, A6535) yielded [1-^{3}H]dimethylallyl alcohol (DMA) and [4-^{14}C]isopentenol (ISO). Each phosphorylated product was purified by semipreparative ion-pair HPLC, and identity was established by dephosphorylation and subsequent GC/MS analysis of the derived alcohol by using methods modified from a published protocol (23). 1-Deoxy-D-[1-^{14}C]xylulose 5-phosphate and 2-*C*-methyl-D-[2-^{14}C]erythritol were prepared enzymatically from [2-^{14}C]pyruvic acid (DuPont/NEN, 0.59 GBq·mmol^{-1}) as described (17, 23). *R,S*-[2-^{14}C]Mevalonic acid (2.12 GBq·mmol^{-1}) was purchased from DuPont/NEN.

Bacterial Strains and Plasmid Constructs. A λ ZAP cDNA library was constructed from mRNA obtained (27) from isolated peppermint oil-gland secretory cells (4) according to the manufacturer's instructions (Stratagene). Randomly picked and purified clones were excised *in vivo*, and inserts of the resulting pBluescript SK− phagemids were partially sequenced from both ends by using T3 and T7 primers. An apparently full-length clone (designated ml100) of the putative peppermint IP kinase (IPK) gene was acquired by this means and was used as a template to amplify by PCR a 1,218-bp fragment, by using the primers 5′-ATGGCTTCCTCCTCCCATTTCCTC-3′ (forward) and 5′-TTCAGCATCCTGAGGAAAAGACGG-3′ (reverse), which was subsequently cloned into the expression vector pBAD TOPO TA (Invitrogen). *E. coli* strain BL21-CodonPlus-RIL [F$^-$ *ompT*, *hsdS*($r_B^- m_B^-$), *dcm*$^+$, Tet^R, *gal*, *endA*, Hte, (*argU*, *ileY*, *leuW*, Cam^R); Invitrogen] served as host in the transformation. The putative *E. coli* IPK gene was amplified by PCR using the primers 5′-ATGCGGACACAGTGGCCCTC-3′ (forward) and 5′-AAGCATGGCTCTGTGCAATG-3′ (reverse), and genomic DNA from the strain K-12 MG1655 (wild-type) as a template. For expression, the amplicon was cloned into pBAD TOPO TA (Invitrogen) and transformed into *E. coli* strain TOP10 One Shot [F$^-$, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15, Δ*lac*X74, *recA1*, *deoR*, *araD139* Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*, (Str^R), *endA1*, *nupG*; Invitrogen].

Kinase Assays and Product Identification. Bacterial cells were grown in LB medium supplemented with appropriate antibiotics (ampicillin and chloramphenicol for the expression of the putative peppermint kinase, ampicillin for the expression of the putative *E. coli* kinase) to an OD_{600} of 0.2 and then treated with either 0.02% arabinose (induction of transgene expression) or 0.02% glucose (repression of transgene expression) and incubated at 20°C for 20 h. After harvest by centrifugation (1,800 × *g*, 5 min), the cells were resuspended in 2 ml of assay buffer (100 mM Tris·HCl, pH 7.5, containing 20 mM $MgCl_2$, 20 mM ATP, and 1 mM DTT), and disrupted by sonication at 0–4°C; the protein content of the resulting homogenate was determined by using the Bio-Rad protein assay. To aliquots containing 400 μg of total protein, 0.79 nmol of the appropriate substrate was added, and the mixture was incubated at 30°C for 5–60 min. Cell debris was pelleted by centrifugation (10,000 × *g*), protein was removed by filtration through a Nanosep cartridge (10-kDa cutoff; Pall Filtron), and the filtrate was analyzed by reversed-phase ion-pair radio-HPLC by using a modification of a previously published method (28); column: Adsorbosphere HS C18 (Alltech Associates, 5-μm particle size, 4.6-mm i.d., 250-mm length); solvents: A, 10 mM tetrabutylammonium acetate (pH 6); B, 10 mM tetrabutylammonium acetate in 70% aqueous methanol (pH 6); C, 70% aqueous methanol; gradient: 100% A (hold for 10 min), gradient to 80% B/20% A (65 min), hold for 10 min, gradient to 100% C (10 min), hold for 10 min; flow rate: 1 ml·min^{-1}; retention times: IPP, 72.6 min; dimethylallyl diphosphate (DMAPP), 70.4 min; IP, 64.6 min; dimethylallyl monophosphate (DMAP), 63.7 min; ISO, 32.9 min; DMA, 34.6 min; DXP, 49.4 min; deoxyxylulose, 21.2 min; MEP, 46.8 min; MVA, 32.6 min.

Partial Purification of Recombinant *E. coli* IPK. Bacteria were grown as described above. After centrifugation (1,800 × *g*, 5 min), cells were resuspended in 1.5 ml of lysis buffer [20 mM sodium phosphate (pH 7.8) containing 500 mM NaCl], incubated with 100 μg of egg white lysozyme (15 min on ice), and sonicated with three 10-s bursts at medium intensity. The crude lysate was treated with RNase (5 μg, 10 min, 30°C) and DNase (5 μg, 10 min, 30°C), insoluble debris was removed by centrifugation (15,000 × *g*, 10 min), and the supernatant was transferred to a tube containing 4 ml of ProBond resin (Invitrogen) preequilibrated with lysis buffer. To bind the recombinant enzyme, which was expressed as a fusion protein containing a 6-His affinity tag, the suspension was gently agitated for 20 min at 4°C. After brief centrifugation (700 × *g*, 2 min), the supernatant was carefully decanted, and the remaining resin was washed twice with 40 ml of lysis buffer. The resin was transferred to a gravity flow column and washed with an additional 5 ml of lysis buffer, and the recombinant protein was then eluted with 3 ml of elution buffer [20 mM potassium phosphate (pH 6.4) containing 500 mM NaCl and 300 mM imidazole]. The eluent was transferred to Econo-Pac 10-DG cloumns (Bio-Rad), and the IPK was desalted by elution with 100 mM Tris·HCl buffer (pH 7.5). Aliquots of this partially purified preparation (15 μg of total protein) were assayed for kinase activity as described above.

Isolation of and Feeding Studies with Peppermint Oil-Gland Secretory Cells. Leaves (15–20 g; <10 mm in length) were excised from peppermint (*Mentha* × *piperita* L. cv. Black Mitcham) plants, and the oil gland secretory cells were isolated by the glass bead abrasion method (4). After isolation, the secretory cells were washed with 25 mM Tris·HCl buffer (pH 7.3) containing 200 mM sorbitol, 10 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, 1 mM ethyleneglycol bis(β-aminoethyl ether), 8.5 mM Na_2HPO_4, and 0.1 mM $Na_4P_2O_7$ and then suspended in the same buffer supplemented with 2 mM ATP, 0.1 mM NADPH, 0.1 mM NAD^+, 5 mM phospho*enol*pyruvate, and 5 mM glucose 6-phosphate. Cell density was determined by using a hemocytometer and was adjusted to 1–2 × 10^5 cellular disks (each containing eight secretory cells) per milliliter of suspension. Aliquots (1–1.5 ml) were transferred to 15-ml screw-cap glass vials, and the suspended cells were aerated and incubated at 23°C for 2 h. At the end of the incubation period, the suspension was extracted

three times with 1 ml of diethyl ether. The combined organic extract was washed with 1 ml of 1 M Na_2CO_3 and dried over Na_2SO_4. An aliquot was removed for liquid scintillation counting and, to the remainder, authentic standards (10–50 μg each) of ISO, DMA, geraniol, farnesol, limonene, menthone, menthol, pulegone, humulene, and caryophyllene were added. These extracts were then slowly concentrated on ice under a gentle stream of N_2 to $\approx$200 μl and were then transferred to conical glass vials and further concentrated to 5–10 μl at -20°C in preparation for chromatographic analysis.

Radio-GLC Analysis of Peppermint Isoprenoids. Radio-GC was performed according to a published method (29) with several modifications; 3.2 mm o.d. $\times$ 5 m stainless steel, column with 15% AT 1000 on 100/120 mesh Gas Chrom Q (Alltech Associates); helium carrier gas at 30–60 ml·min^{-1}; temperature program from 70 to 200°C at 5°C·min^{-1}; injector at 200°C; thermal conductivity detector at 220°C and 140 mA; propane quench gas at 10–15 ml·min^{-1}. The relative peak area corresponding to each biosynthetic product was converted to dpm as a fraction of the total radioactivity determined by liquid scintillation counting before concentration. The absolute amount of each component formed was then calculated based on the specific activity of the corresponding radiolabeled substrate.

Results and Discussion

Cloning and Heterologous Expression of a Putative Kinase From Peppermint. The oil glands (glandular trichomes) of mint species are highly specialized for the production of monoterpenes and sesquiterpenes, and the secretory cells of these structures are thus highly enriched in the machinery for terpenoid biosynthesis (30). As part of an ongoing effort to isolate and define genes involved in isoprenoid metabolism, we analyzed the sequences of 1,300 random clones obtained from an enriched cDNA library constructed specifically from mRNA isolated from peppermint glandular trichome secretory cells. Because the most advanced, defined intermediate of the plastidial DXP pathway to isoprenoids is MEP (19–21), and the end product of the pathway is IPP (25, 26), a phosphorylation step must occur at some point during this reaction sequence. Accordingly, metabolite kinases were sought, but only two clones with similarity to adenylate kinases were noted by searching the common databases. However, a more detailed search of the Prosite database (http://www.expasy.ch/prosite) revealed another more promising clone (designated ml100) as well as a truncated version (ml164) that was also sequenced, which shared a region of high sequence similarity to the putative ATP-binding domain of the GHMP family of kinases (31). The deduced amino acid sequence of this peppermint clone additionally showed significant homology to a chromoplast-directed protein of unknown function from ripening tomato fruits (32) and to a number of hypothetical proteins from several eubacteria.

To examine the possible function of the peppermint clone ml100, the coding region was amplified by PCR, transferred into the expression vector pBAD TOPO TA (to yield pBAD-MPK), and, for convenient purification, expressed as a 6-His fusion protein in *E. coli* BL21-CodonPlus-RIL cells (which contain an extra plasmid encoding rare tRNAs that specify Arg, Leu, and Ile). However, the expressed enzyme strongly tended to form inclusion bodies, and, even after several attempts to increase the soluble activity by decreasing the amount of arabinose added for induction (0.001–0.01%) and by prolonging the incubation at lower temperatures (8–15°C), soluble protein could not be obtained in sufficient quantity for affinity-based (Ni^{2+}-binding) purification. Thus, crude extracts of sonicated transformed cells served as the enzyme source for kinase assays. As a control, *E. coli* harboring pBAD-MPK were treated with 0.02% glucose, which leads to repression of transgene expression (Fig. 2).

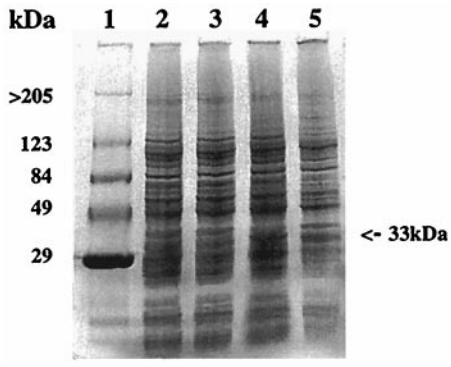

Fig. 2. Expression analysis of recombinant peppermint and *E. coli* IPK, and partial purification of the *E. coli* IPK. SDS/PAGE lanes are: 1, molecular mass markers; 2, peppermint IPK expressed from pBAD-MPK in *E. coli* BL21-CodonPlus-RIL cells (induction with 0.02% arabinose); 3, *E. coli* background control (pBAD-MPK in *E. coli* BL21-CodonPlus-RIL cells (repression with 0.02% glucose); 4, *E. coli* IPK expressed from pBAD-ECK in *E. coli* Top 10 One Shot cells (induction with 0.02% arabinose); and 5, partially purified *E. coli* IPK (from 4 above) after affinity chromatography.

Consistently, the expressed recombinant peppermint kinase gave detectably elevated levels of activity with IP [1.43 pmol·(s·g of protein)$^{-1}$] and ISO [0.10 pmol·(s·g of protein)$^{-1}$] as substrates when compared with the *E. coli* background (repressed) controls [IP, 0–1.0 pmol·(s·g of protein)$^{-1}$; ISO, 0.08 pmol·(s·g of protein)$^{-1}$] (Table 1). No kinase activity was detected with DMAP or MVA as substrate. Kinase activity with DMA as a substrate, under the standard assay conditions, was always detectable, but never exceeded 0.01 pmol·(s·g of protein)$^{-1}$. With DXP, deoxyxylulose, and MEP as substrates, kinase activity of $<$0.2 pmol·(s·g of protein)$^{-1}$ occasionally was detected but, in most experiments, no activity was observed. Because these assays with crude extracts were severely compromised by the presence of competing phosphatases (as evidenced by the production of the corresponding dephosphorylated products on HPLC analysis), the conversion rates observed must be regarded as minimum values.

Cloning, Expression, and Purification of an IPK from *E. coli*. As an alternative to further expression studies with the recombinant peppermint enzyme [e.g., by truncation of the putative plas-

Table 1. Substrate specificity of recombinant IPKs

	Rate,* pmol·(s·g protein)$^{-1}$			
Substrate	Peppermint†	*E. coli*† (repressed)	*E. coli*† (induced)	*E. coli* (partially purified)
IP	1.43	$<$1.0‡	2.41	178 $\pm$ 81
ISO	0.10	0.08	0.10	79 $\pm$ 24
DMAP	ND	ND	ND	ND
DMA	$<$0.01	$<$0.01	$<$0.01	11 $\pm$ 5
DXP	$<$0.1§	$<$0.1§	$<$0.1§	ND
Deoxyxylulose	$<$0.1§	$<$0.1§	$<$0.1§	ND
MEP	$<$0.2§	$<$0.2§	$<$0.2§	ND
MVA	ND	ND	ND	ND

ND, not detectable.

*Rates are given as averages of two to five separate experiments, with SD where appropriate.

†These crude extracts contained high phosphatase activity; the rates given are thus minimums.

‡The kinase activity in this assay varied but was always below the listed rate.

§In most of these assays, no kinase activity was observed; in some cases, minor levels of the apparent product were detected.

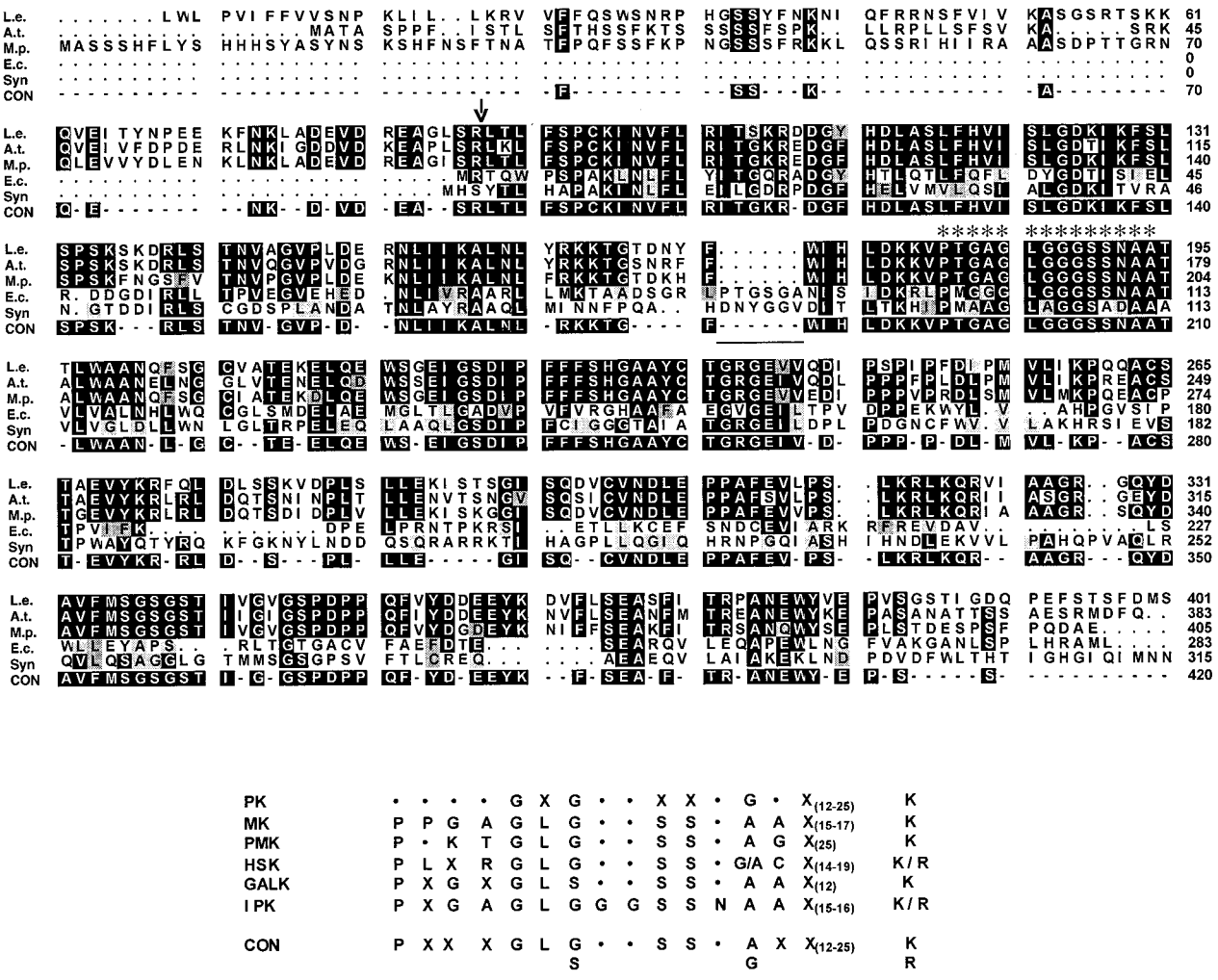

Fig. 3. (*Upper*) Deduced amino acid sequence comparison of IPK from peppermint (M.p.; AF179283), pTOM41 from tomato (L.e.; U62773), a hypothetical protein from *A. thaliana* (A.t.; AC005168, PID g3426035), IPK from *E. coli* (E.c.; AF179284), and a hypothetical protein from *Synechocystis* sp. strain PC6803 (Syn; D90899, PID g16665). CON indicates the consensus sequence. Identical residues are black with white lettering, residues of high similarity are indicated in gray, and residues of lower similarity are in light gray. Asterisks indicate the position of the putative ATP-binding motif, the putative cleavage site of the plastidial targeting sequence is shown by an arrow, and a conserved insert in bacterial sequences is underlined. (*Lower*) Comparison of sequences of an ATP-binding site found in representatives of protein kinases (PK), mevalonate kinases (MK), phosphomevalonate kinases (PMK), homoserine kinases (HSK), galactokinases (GALK), and isopentenyl monophosphate kinases (IPK).

tidial targeting sequence as an attempt to minimize protein misfolding (33)], the *E. coli* orthologue of the peppermint kinase was evaluated. This *E. coli* gene (*ychB*), which was found by database homology searching and which is located at 27.2 min of the chromosomal map, was amplified by PCR and transferred into pBAD TOPO TA (to yield pBAD-ECK), for similar expression as a 6-His fusion protein. Assay of crude extracts gave results similar to those obtained with the recombinant peppermint enzyme (Table 1). However, in this instance, the expressed, soluble enzyme, produced on induction with 0.02% arabinose, allowed a useful one-step affinity-based purification with the Ni^{2+}-binding column (Fig. 2). This partially purified protein readily catalyzed the conversion of IP to IPP [178 ± 81 pmol·(s·g of protein)$^{-1}$], ISO to IP [79 ± 24 pmol·(s·g of protein)$^{-1}$], and DMA to DMAP [11 ± 5 pmol·(s·g of protein)$^{-1}$]; however, no phosphorylating activity was detectable with DMAP, DXP, deoxyxylulose, MEP, or MVA as substrate (Table 1). This kinase activity (with IP as a substrate) depended on the presence of $MgCl_2$ as divalent cation and ATP as phosphate donor, whereas CTP, UTP, and GTP did not serve as alternate phosphate donors. As a control, pBAD-ECK expression in *E. coli* was repressed by addition of 0.02% glucose, and the extracted proteins were subjected to the same purification step as above; kinase assays with these enzyme preparations yielded no detectable activity with any of the above substrates. These results with the enzyme products of the peppermint ml100 clone and the *E. coli ychB* clone suggest that this gene encodes an IPK that is involved in the DXP pathway to isoprenoids.

Sequence Analysis. The peppermint IPK gene contains an ORF of 1,218 nt (GenBank accession no. AF179283). The first 98 deduced aa display the general characteristics of plastidial targeting sequences (34), and, when this putative leader peptide is excluded, a mature protein of 308 aa with a predicted size of ≈33 kDa is obtained. The gene encoding *E. coli* IPK (GenBank accession no. AF17924) consists of 852 nt, which corresponds to an enzyme of 283 aa with a size of 31 kDa. Database sequence comparison of translated putative IPK genes from several different organisms revealed very high

similarity/identity scores within the plant kingdom (>81.6/74.8% for presumptive orthologues found in tomato and *A. thaliana*) and a high degree of sequence variation among eubacteria (39.0–70.2/25.6–62.5%) and between plants and eubacteria (38.3–48.8/28.5–38.6%) (Fig. 3 *Upper*). The IPP kinases appear to share a conserved glycine-rich sequence motif [PXGAGLGGGSSNAAX$_{15–16}$(K/R)] similar to the conserved sequence PXXXGL(G/S)SS(A/G)XX$_{12–25}$(K/R) found in the GHMP family of kinases, including galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (31) (Fig. 3 *Lower*). A related motif is also present in protein kinases (35). The gene for the *A. thaliana* IPK orthologue is located on chromosome 2 (AC005168; BAC F12C20; PID g3426035), near the marker B68, and contains 10 introns. Neither the intron/exon organization nor a phylogenetic analysis reveals a direct evolutionary relationship among different classes of the GHMP kinase family (data not shown). A detailed survey of the available microbial genome project databases did not indicate the IPK gene to be part of a cluster with other (potential) genes of the DXP pathway.

Incorporation of IP into Isoprenoids of Peppermint Secretory Cells. Although IP was shown to be the preferred substrate of both peppermint and *E. coli* IPK, it remained to be directly demonstrated that IP was an intermediate of the DXP pathway. Previous experiments with isolated peppermint oil gland secretory cells had demonstrated that the MVA pathway in these cells is blocked at an early stage and that IPP used for both monoterpene and sesquiterpene biosynthesis is synthesized exclusively in the plastids from pyruvate (4), almost certainly via the DXP pathway (2). The high degree of metabolic specialization and the ability to synthesize monoterpenes and sesquiterpenes *de novo* from basic precursors, including phosphorylated intermediates (4), made the isolated secretory cells an ideal model system to establish the intermediacy of IP, and the activity of IP kinase, *in vivo*. Thus, isolated secretory cells were incubated under comparable conditions with several radiolabeled substrates (ISO, IP, IPP, DMA, DMAP, and DMAPP), and, after extraction of products with diethyl ether, the incorporation into monoterpenes and sesquiterpenes was quantified by liquid scintillation counting and radio-GC analysis of these volatile metabolites (Table 2). Because the IPK is plastidial, as is monoterpene biosynthesis, whereas enzymes specific for sesquiterpene biosynthesis are cytosolic (4), uptake and partitioning differences between the C_5 precursors influence the distribution between monoterpene and sesquiterpene biosynthetic pathways. In a similar fashion, endogenous phosphatases of both plastidial and cytosolic origin can complicate the partitioning of precursors into the pathways of these compartments. Thus, as a measure of the conversion efficiency of each C_5 precursor, total monoterpenoids (C_{10}) and sesquiterpenoids (C_{15}), including geraniol and farnesol released by phosphatases from the corresponding diphosphate ester intermediates, were recorded. By this measure, IPP was most readily converted to terpenoid end products, as expected [561 pmol·(h·10^5 cell clusters)$^{-1}$], followed by ISO [304 pmol·(h·10^5 cell clusters)$^{-1}$], most likely reflecting efficient plastidial uptake of this low molecular weight alcohol, and then IP [43 pmol·(h·10^5 cell clusters)$^{-1}$]. DMAPP and DMAP were not very efficient precursors of terpenoids in secretory cells [<6 pmol·(h·10^5 cell clusters)$^{-1}$], and the incorporation of DMA was negligible. Although ISO, likely because of uptake rates, and the more advanced precursor IPP were transformed to terpenoids *in vivo* at higher rates than was IP, the latter was incorporated at a rate [43 pmol·(h·10^5 cell clusters)$^{-1}$] comparable to that observed previously with pyruvate [67 pmol·(h·10^5 cell clusters)$^{-1}$] (4), an efficient, established precursor of the DXP pathway.

Table 2. Conversion of potential C_5 precursors into terpenoids by isolated peppermint oil-gland secretory cells

Precursor	Rate of incorporation into total terpenoids* (C_{10} + C_{15})
IPP	561
IP	43
ISO	304
DMAPP	4
DMAP	6
DMA	ND

ND, not detectable.

*Rates of incorporation into total monoterpenoids and sesquiterpenoids are given in pmol·(h·10^5 cell clusters)$^{-1}$ (average of two experiments) and include geraniol and farnesol released by phosphatases from the intermediates geranyl diphosphate (C_{10}) and farnesyl diphosphate (C_{15}).

Although IP and ISO were shown to be efficient *in vitro* substrates for this newly defined kinase, and the role of this kinase was demonstrated by *in vivo* feeding studies, several steps of the mevalonate-independent pathway still remain to be elucidated, and it cannot be ruled out that the required phosphorylation step also may occur with intermediates other than IP. Future studies to evaluate the effects of inactivating the *E. coli* IPK gene on isoprenoid metabolism and to explore potential inhibitors of IPK will not only help to define the role of IPK in IPP biosynthesis but also should assist in the development of a specific and novel class of herbicides and antibiotics that target a central metabolic pathway that is absent in mammals. Thus, for example, inhibitors of an early step of the mevalonate-independent pathway have been shown recently to be effective antimalarial drugs (36).

We thank C. Sanchez, E. Stauber, G. Munske, and D. Pouchnik for nucleotide sequencing and primer synthesis. This investigation was supported by grants from the U.S. Department of Energy and Pioneer Hi-Bred International.

1. Newman, J. D. & Chappell, J. (1999) *Crit. Rev. Biochem. Mol. Biol.* **34,** 95–106.
2. Eisenreich, W., Sagner, S., Zenk, M. H. & Bacher, A. (1997) *Tetrahedron Lett.* **38,** 3889–3892.
3. Eisenreich, W., Menhard, B., Hylands, P. J., Zenk, M. H. & Bacher, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6431–6436.
4. McCaskill, D. & Croteau, R. (1995) *Planta* **197,** 49–56.
5. Lichtenthaler, H. K., Schwender, J., Disch, A. & Rohmer, M. (1997) *FEBS Lett.* **400,** 271–274.
6. Piel, J., Donath, J., Bandemer, K. & Boland, W. (1998) *Angew. Chem. Int. Ed. Engl.* **37,** 2478–2481.
7. Adam, K.-P. & Zapp, J. (1998) *Phytochemistry* **48,** 953–959.
8. Goldstein, J. L. & Brown, M. S. (1990) *Nature (London)* **343,** 425–430.
9. Bach, T. J., Boronat, A., Campos, N., Ferrer, A. & Vollack, K.-U. (1999) *Crit. Rev. Biochem. Mol. Biol.* **34,** 107–122.
10. Rohmer, M. (1998) *Prog. Drug Res.* **50,** 135–154.
11. Rohmer, M., Knani, M., Simonin, P., Sutter, B. & Sahm, H. (1993) *Biochem. J.* **295,** 517–524.
12. Broers, S. T. J. (1994) Ph.D. thesis (Eidgenössische Technische Hochschule, Zürich, Switzerland).
13. Schwarz, M. K. (1994) Ph.D. thesis (Eidgenössische Technische Hochschule, Zürich, Switzerland).
14. Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S. & Sahm, H. (1996) *J. Am. Chem. Soc.* **118,** 2564–2566.
15. Sprenger, G. A., Schörken, U., Wiegert, T., Grolle, S., De Graaf, A. A., Taylor, S. V., Begley, T. P., Bringer-Meyer, S. & Sahm, H. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 12857–12862.
16. Lois, L. M., Campos, N., Putra, S. R., Danielsen, K., Rohmer, M. & Boronat, A. (1997) *Proc. Natl. Acad. Sci. USA* **95,** 2105–2110.

17. Lange, B. M., Wildung, M., McCaskill, D. & Croteau R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 2100–2104.
18. Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R. A. & Camara, B. (1998) *Plant Physiol.* **117,** 1423–1431.
19. Duvold, T., Bravo, J. M., Pale-Grosdemange, C. & Rohmer, M. (1997) *Tetrahedron Lett.* **38,** 4769–4772.
20. Duvold, T., Calí, P., Bravo, J. M. & Rohmer M. (1997) *Tetrahedron Lett.* **38,** 6181–6184.
21. Sagner, S., Eisenreich, W., Fellermeier, M., Latzel, C., Bacher, A. & Zenk, M. H. (1998) *Tetrahedron Lett.* **39,** 2091–2094.
22. Takahashi, S., Kuzuyama, T., Watanabe, H. & Seto, H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9879–9884.
23. Lange, B. M. & Croteau R. (1999) *Arch. Biochem. Biophys.* **365,** 170–174.
24. Schwender, J., Müller, C., Zeidler, J & Lichtenthaler, H. K. (1999) *FEBS Lett.* **455,** 140–144.
25. McCaskill, D. & Croteau R. (1999) *Tetrahedron Lett.* **40,** 653–656.
26. Arigoni, D., Eisenreich, W., Latzel, C., Sagner, S., Radykewicz, T., Zenk, M. H. & Bacher, A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 1309–1314.
27. Logemann, J., Schell, J. & Willmitzer, L. (1987) *Anal. Biochem.* **163,** 16–20.
28. McCaskill, D. & Croteau, R. (1993) *Anal. Biochem.* **215,** 142–149.
29. Croteau, R. & Satterwhite, D. M. (1990) *J. Chromatogr.* **500,** 349–354.
30. Lange, B. M. & Croteau, R. (1999) *Curr. Opin. Plant Biol.* **2,** 139–144.
31. Tsay, Y. H. & Robinson, G. W. (1991) *Mol. Cell. Biol.* **11,** 620–631.
32. Lawrence, S. D. Cline, K. & Moore, G. A. (1997) *Plant Mol. Biol.* **33,** 483–492.
33. Williams, D. C., McGarvey, D. J., Katahira, E. J. & Croteau, R. (1998) *Biochemistry* **37,** 12213–12220.
34. von Heijne, G., Steppuhn, J. & Herrmann, R. G. (1989) *Eur. J. Biochem.* **180,** 535–545.
35. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
36. Jomaa, H. Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Türbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, K., *et al.* (1999) *Science* **285,** 1573–1576.