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Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha × piperita*) rely exclusively on plastid-derived isopentenyl diphosphate

David McCaskill, Rodney Croteau

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA

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Abstract. The subcellular compartmentation of isopentenyl diphosphate (IPP) synthesis was examined in secretory cells isolated from glandular trichomes of peppermint (*Mentha × piperita* L. cv. Black Mitcham). As a consequence of their anatomy and the conditions of their isolation, the isolated secretory cells are non-specifically permeable to low-molecular-weight water-soluble metabolites. Thus, the cytoplasm is readily accessible to the exogenous buffer whereas the selective permeability of subcellular organelles is maintained. With the appropriate choice of exogenous substrates, this feature allows the assessment of cytoplasmic and organellar (e.g. plastidic) metabolism in situ. Glycolytic substrates such as [^{14}C]glucose-6-phosphate and [^{14}C]pyruvic acid are incorporated into both monoterpenes and sesquiterpenes with a monoterpene:sesquiterpene ratio that closely mimics that observed in vivo, indicating that the correct subcellular partitioning of these substrates is maintained in this model system. Additionally, exogenous [^{14}C]mevalonic acid and [^{14}C]IPP, which are both intitially metabolized in the cytoplasm, produce an abnormally high proportion of sesquiterpenes. In contrast, incubation with either [^{14}C]citrate or [^{14}C]acetyl-CoA results in the accumulation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) with no detectable isoprenoids formed. Taken together, these results indicate that the cytoplasmic mevalonic acid pathway is blocked at HMG-CoA reductase and that the IPP utilized for both monoterpene and sesquiterpene biosynthesis is synthesized exclusively in the plastids.

Key words: Isopentenyl diphosphate – Isoprenoid biosynthesis – Leucoplast – *Mentha* – Mevalonic acid pathway

Abbreviations: DMAPP = dimethylallyl diphosphate; FPP = farnesyl diphosphate; GLC = gas liquid chromatography; GPP = geranyl diphosphate; HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA; IPP = isopentenyl diphosphate; PEP = phosphoenolpyruvic acid

Correspondence to: R. Croteau; FAX. 1 (509) 335 7643; Tel.: 1 (509) 335 1790; E-mail: croteau@wsuvm1.csc.wsu.edu

Introduction

Isopentenyl diphosphate (IPP) is the immediate, essential precursor leading to the formation of over 22 000 known isoprenoids (Connolly and Hill 1992). The subcellular location(s) of the synthesis of IPP in plants, and of the allylic prenyl diphosphates utilized for isoprenoid biosynthesis, has been the subject of considerable study (reviewed in Liedvogel 1986; Gray 1987; Kleinig 1989). The two subcellular compartments responsible for the bulk of isoprenoid biosynthesis in plants are the cytoplasm/endoplasmic reticulum involved in the synthesis of farnesyl diphosphate (FPP)-derived isoprenoids such as sterols, and the plastids involved in the formation of geranylgeranyl diphosphate (GGPP)-derived isoprenoids such as phytol and carotenoids (the mitochondrial synthesis of ubiquinones will not be considered here). However, in addition to ubiquitous isoprenoids such as sterols and carotenoids, plants often accumulate large amounts of a wide variety of defense-related compounds including mono-, sesqui-, di- and triterpenes (see e.g. Gershenzon and Croteau 1991). The subcellular compartmentation of IPP utilization is well established. Geranyl diphosphate (GPP) and GGPP are synthesized from IPP, and subsequently converted into monoterpenes and diterpenes in the plastid, whereas FPP is synthesized and subseqently converted into sesquiterpenes in the cytoplasm/endoplasmic reticulum (reviewed in Gershenzon and Croteau 1993).

Despite this general agreement regarding the localization of steps utilizing IPP, the subcellular locations of IPP synthesis is not completely resolved. In addition, the regulation of partitioning of IPP between the different families of isoprenoids in the plant cell is currently only poorly understood. There are two competing models of subcellular compartmentation of IPP synthesis: the first involves the parallel synthesis of IPP in each compartment in which it is utilized; the second involves the exclusive synthesis of IPP in the cytoplasm, followed by distribution of the IPP between the different locations at which it is utilized (reviewed in Gray 1987). However, more recent studies with developing chloroplasts in barley leaves have

indicated that the subcellular localization of IPP synthesis can change during development (Heintze et al. 1990). In very young barley leaf tissue, plastidic isoprenoids are synthesized from plastid-derived IPP, whereas chloroplasts in mature leaf tissue utilize IPP imported from the cytoplasm for isoprenoid biosynthesis. Studies demonstrating developmental changes in plastid envelope permeability towards acetate, citrate and mevalonate (Wellburn and Hampp 1976; Schneider et al. 1977) and IPP (Heintze et al. 1990) reinforce the view that the subcellular localization of IPP synthesis depends on the type of tissue examined and may change during development of the tissue in response to different metabolic requirements.

Glandular trichomes on peppermint (*Mentha* × *piperita*) leaves synthesize and store an essential oil composed primarily of monoterpenes with smaller amounts of sesquiterpenes (Amelunxen et al. 1969). The secretory cells of these glandular trichomes are not photosynthetic and contain only leucoplasts (Werker et al. 1985), thus these cells must rely on the import of carbon from the underlying tissue to support isoprenoid synthesis that leads to oil accumulation. Secretory cells of these glandular trichomes can be mechanically isolated in high yield as a disc of eight cells each (Gershenzon et al. 1992), reflecting the original anatomy of the gland. Several features of these isolated cells make them an ideal system for examining the subcellular compartmentation of isoprenoid biosynthesis. The glandular trichomes from which the secretory cells are isolated are at a developmental stage where cell growth and division have ceased (Amelunxen 1965) and rapid accumulation of essential oil occurs (Croteau and Martinkus 1979). For this reason, the rates of sterol and carotenoid formation should be insignificant relative to the massive levels of monoterpene and sesquiterpene biosynthesis leading to essential-oil accumulation, simplifying analysis and interpretation of the results. The isolated cells are capable of the de-novo synthesis of monoterpenes from basic precursors such as [^{14}C]sucrose (McCaskill et al. 1992), indicating that they contain all of the enzymes necessary for the synthesis of the essential-oil terpenes characteristic of peppermint. In addition, the isolated cells are non-specifically permeable towards low-molecular-weight water-soluble metabolites as a consequence of the anatomy of the trichome and the conditions of their isolation. The molecular-weight exclusion limit for this permeability is less than 1800 based on the exclusion of fluorescein-dextran (McCaskill et al. 1992) and is believed to be due to the exposure of a large number of plasmodesmata between the secretory cells and the underlying stalk cell to the external buffer as the secretory cells are isolated. As a result, endogenous low-molecular-weight constituents are washed out of the cytoplasm during isolation of the cells. This feature allows exogenous manipulation of the cytoplasmic composition of cofactors and substrates.

The judicious choice of radiolabeled substrates and cofactors added to the isolated cells, followed by quantification of the radiolabeled monoterpenes and sesquiterpenes produced, permits delineation of the subcellular partitioning of the different substrates in the cells. This in-situ approach avoids the difficulties associated with attempting to quantify the relative contributions of parallel pathways for IPP synthesis in different subcellular compartments using cell-free preparations. This report describes the use of different radiolabeled substrates to assess the relative contributions of cytoplasmic and plastidic IPP synthesis for both monoterpene and sesquiterpene biosynthesis.

Materials and methods

Plant material, chemicals and substrates. Peppermint (*Mentha* × *piperita* L. cv. Black Mitcham) plants were propagated and grown under greenhouse conditions as previously described (Gershenzon et al. 1992). Unless otherwise indicated, all chemicals and enzymes were purchased from either Sigma Chemical Co. (St. Louis, Mo., USA) or Research Organics (Cleveland, Ohio, USA). Flo-Scint III scintillation fluid for radio-high performance liquid chromatography (HPLC) was from Radiomatic Instruments and Chemical Co. (Meriden, Conn., USA). Mevinolin was a generous gift of Merck, Sharpe and Dohme Research Laboratories (Rahway, N.J., USA). Monoterpene and sesquiterpene standards were from our own collection.

[2-^{14}C]Pyruvic acid (0.59 GBq · mmol^{-1}), [1,5-^{14}C]citric acid (3.0 GBq · mmol^{-1}), *RS*-[2-^{14}C]mevalonic acid (2.12 GBq · mmol^{-1}), [1, 2-^{14}C]acetic acid (sodium salt, 2.08 GBq · mmol^{-1}), and *n*-[1-^{14}C]butyryl-CoA (0.15 GBq · mmol^{-1}) were all purchased from DuPont Co. (Boston, Mass, USA). [1-^{14}C]Acetyl-coA (2.18 GBq · mmol^{-1}), phosphoenol-[1-^{14}C]pyruvate (PEP, cyclohexylammonium salt, 0.96 GBq · mmol^{-1}) and [1-^{4}C]isopentenyl diphosphate (1.93 GBq · mmol^{-1}) were purchased from Amersham Corp., (Arlington Heights, Il., USA). D-[U-^{14}C]glucose-6-phosphate (11.0 GBq · mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, Mo., USA). [1-^{3}H]Farnesyl diphosphate (3.71 GBq · mmol^{-1}; Munck and Croteau 1990) and [8-^{3}H]geranyl diphosphate (2.30 GBq · mmol^{-1}; Coates et al. 1987) were synthesized as previously described.

Isolation and incubation of secretory cells. Apical leaves (10–15 g, < 10 mm length) were harvested from vegetative stems of peppermint. Secretory cells were isolated from the leaves by mechanical abrasion with glass beads and washed essentially as described in McCaskill et al. (1992). The buffer used for washing the isolated cells consisted of 200 mM sorbitol, 10 mM sucrose, 50 mM KCl, 5 mM succinic acid, 5 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 0.5 mM Na_2HPO_4, 0.1 mM $Na_4P_2O_7$ and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), adjusted to pH 7.3 with KOH.

Incubation of the isolated cells with radiolabeled substrates was carried out essentially as described previously by McCaskill et al. (1992). After isolation, the cells were washed into a cofactor-rich buffer consisting of the above wash buffer supplemented with 5 mM ATP and 0.1 mM each of NAD^+, NADPH, CoA, FAD and thiamine pyrophosphate. The cell density was then adjusted to give approx. $2.5 \cdot 10^6$ cells ($3.1 \cdot 10^5$ cell disks) per 1 ml assay. Aliquots of the cells were distributed into tubes and the cells were aerated (approx. 3 ml air/min) at room temperature for 15 min using a fused silica capillary inserted into each tube. Any inhibitors or other additions to the assays were also added at this time. At the end of this preincubation period, the cells were pelleted by brief centrifugation (approx. 30 s at 100 · *g*) and then resuspended in 1 ml fresh cofactor-rich buffer per assay. At this point, the radiolabeled substrates were added along with any inhibitors or unlabeled substrates used, the cells were overlaid with 2 ml of pentane to trap the volatile isoprenoids formed, and the cells were aerated as before for 1 h at room temperature. When indicated, $^{14}CO_2$ produced from ^{14}C-labeled substrates was trapped by passing the air from the assay tubes into KOH traps as previously described (McCaskill et al. 1992).

At the end of the incubation period, the cells were extracted with the pentane overlay, followed by 3 × 1 ml of diethyl ether. After combining, the organic extracts were backwashed twice with 1 ml of