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Morphology and monoterpene biosynthetic capabilities of secretory cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.)

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Abstract. Secretory cells were isolated from the monoterpene-producing glandular trichomes (peltate form) of peppermint as clusters of eight cells each. These isolated structures were shown to be non-specifically permeable to low-molecular-weight, water-soluble cofactors and substrates. Short incubation periods with the polar dye Lucifer yellow iodoacetamide ($M_r = 660$) resulted in a uniform staining of the cytoplasm, with exclusion of the dye from the vacuole. The molecular-weight exclusion limit for this permeability was shown to be less than approx. 1800, based on exclusion of fluorescein-conjugated dextran ($M_r \sim 1800$). Intact secretory cell clusters very efficiently incorporated [^{3}H]geranyl pyrophosphate into monoterpenes. The addition of exogenous cofactors and redox substrates affected the distribution of monoterpenes synthesized from [^{3}H]geranyl pyrophosphate, demonstrating that the cell clusters were permeable to these compounds and that the levels of endogenous cofactors and redox substrates were depleted in the isolated cells. When provided with the appropriate cofactors, such as NADPH, NAD^+, ATP, ADP and coenzyme A, the isolated secretory cell clusters incorporated [^{14}C]sucrose into monoterpenes, indicating that these structures are capable of the de-novo biosynthesis of monoterpenes from a primary carbon source, and that they maintain a high degree of metabolic competence in spite of their permeable nature.

Key words: Glandular trichome – Isoprenoid biosynthesis – *Mentha* – Monoterpene biosynthesis

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Abbreviations: GLC = gas liquid chromatography; LSCM = laser scanning confocal microscopy; LY-IA = Lucifer yellow iodoacetamide

Introduction

Modified epidermal hairs known as glandular trichomes are a widespread anatomical feature of higher plants. These glandular structures are responsible for the synthesis and secretion of a broad range of natural products such as terpenoids, cannabinoids, sucrose esters and phenolic compounds (reviewed in Kelsey et al. 1984). Ultrastructural evidence has long indicated that the secretory cells of glandular trichomes are responsible for the synthesis of the secondary metabolites stored in these structures (reviewed in Fahn 1979; Schnepf 1974). However, attempts to study the physiology and enzymology of secretory cells have been hampered by the lack of suitable isolation procedures.

Members of the mint family (Lamiaceae) bear non-photosynthetic glandular trichomes on both upper and lower leaf surfaces (Werker et al. 1985). Peppermint (*Mentha piperita* L.) contains peltate (Amelunxen 1965) and capitate (Amelunxen 1964) glandular trichomes, both of which are believed to be involved in monoterpene biosynthesis (Amelunxen et al. 1969). However, only the peltate glandular trichomes accumulate monoterpenes in an extracellular, subcuticular space. The peltate glands of peppermint consist of a single basal cell embedded in the epidermis, a single stalk cell, and eight radially distributed secretory cells surrounded by an extended cuticle that also encloses an oil droplet rich in monoterpenes (Amelunxen 1965). Before flowering, the major monoterpene accumulated by the glandular trichomes of peppermint is (−)-menthone, derived from the cyclization of the ubiquitous isoprenoid precursor geranyl pyrophosphate to (−)-limonene, by limonene cyclase, followed by a series of enzymatic redox transformations (reviewed in Croteau 1987) (see Fig. 1). Mechanized techniques for leaf surface abrasion that preferentially remove the contents of glandular trichomes have provided cell-free enzyme extracts enriched in these monoterpene biosynthetic enzymes (Gershenzon et al. 1987). In spearmint (*Mentha spicata*), it was shown that both (−)-limonene cyclase and (−)-limonene-6-hydroxylase were located

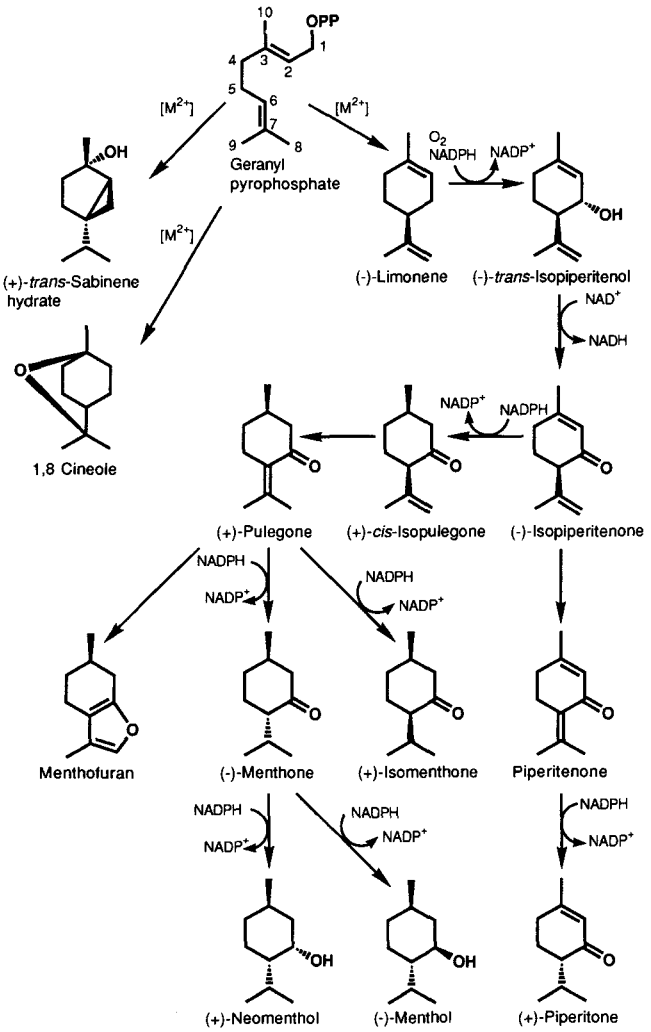

Fig. 1. Major pathways and cofactor requirements for monoterpene biosynthesis in peppermint. [M^{2+}] is the divalent metal ion cofactor (either Mg^{2+} or Mn^{2+}) required by monoterpene cyclases

exclusively in leaf-surface extracts, rather than the remainder of the leaf, indicating that many, it not all, of the enzymes involved in monoterpene biosynthesis occur in the cells of the glandular trichomes (Gershenzon et al. 1989).

Few techniques are available for isolating intact, viable cells from glandular trichomes. Intact glandular heads have been isolated from tobacco leaves (Keene and Wagner 1985; Kandra and Wagner 1988), and have been shown to incorporate both [^{14}C]acetate and [^{14}C]carbonate into diterpenoids and sucrose esters. The technique used with tobacco relies upon the sticky exudate from tobacco trichomes to detach the gland heads from the leaf surface. Thus, the application of this technique appears limited to species exuding sticky rather than oily materials. The secretory cells from glandular trichomes of sagebrush *(Artemisia tridentata)* have been isolated by macerating leaf and floral tissue in a blender followed by purification on a Percoll density gradient (Slone and Kelsey 1985), although these preparations have not yet been exploited for biochemical or physiological studies. Using a mechanized surface-abrasion technique, Gershenzon et al. (1992) developed a procedure for the isolation of large numbers of highly purified secretory cells derived from the peltate glands of peppermint. The cells are isolated as clusters of eight cells each, indicating that these structures are each derived from a single peltate glandular trichome. In this report we demonstrate that the isolated secretory cell clusters are non-specifically permeable to low-molecular weight, water-soluble substrates and cofactors. Such permeability results in depletion of cytoplasmic cofactors and redox substrates. If provided with the necessary cofactors, the cell clusters are capable of de-novo biosynthesis of monoterpenes from a basic metabolic precursor such as sucrose, indicating that these structures contain all of the enzymes necessary for the biosynthesis of the monoterpenes found in the essential oil of peppermint. These findings also demonstrate that the cell clusters maintain a high degree of metabolic competence in spite of their leaky nature.

Material and methods

Plant material, substrates and chemicals. Peppermint (*Mentha piperita* L. cv. Black Mitcham) plants were propagated vegetatively and grown under controlled conditions as previously described (Gershenzon et al. 1992). Lucifer yellow iodoacetamide (LY–IA) was purchased from Molecular Probes (Eugene, Ore. USA). All other reagents and chemicals were purchased from either Research Organics (Cleveland, Oh., USA), Sigma Chemical Co. (St. Louis, Mo., USA) or Aldrich Chemical Co. (Milwaukee, Wis., USA) unless otherwise indicated. Nylon mesh (350, 105 and 20 μm mesh size) was purchased from Small Parts, (Miami, Fla., USA).

[U-^{14}C]Sucrose (24.8 GBq/mmol), [U-^{14}C]palmitic acid (31.4 GBq/mmol) and [1,2-^{14}C]acetic acid (sodium salt, 2.04 GBq/mmol) obtained from DuPont Co. (Boston, Mass., USA) were diluted with unlabeled carrier as indicated in the text. [8-^{3}H]Geranyl pyrophosphate (2.30 GBq/mmol) was synthesized as described in Coates et al. (1987). Monoterpene standards and other substrates were from our own collection.

Isolation of secretory cell clusters. Isolated secretory cell clusters were prepared essentially according to Gershenzon et al. (1992) using approx. 15 g of apical leaves (< 10 mm in length) harvested from vegetative stems of three- to seven-week-old peppermint. The isolation buffer used consisted of 200 mM sorbitol, 2 mM sucrose, 10 mM KCl, 5 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.5 mM KH_2PO_4, 0.1 mM $Na_2P_2O_7$, 1% (w/v) polyvinylpyrrolidone (M_r = 40000), 0.6% (w/v) methyl cellulose (viscosity of 2% solution in water = 1500 cp), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), adjusted to pH 7.3 with KOH. The buffer used for subsequent washing of the glands was of the same composition, but without the polyvinylpyrrolidone or methyl cellulose. After washing, the secretory cell clusters were resuspended in buffer and counted with a hemocytometer before use in subsequent experiments.

Synthesis and purification of dyes. Fluorescein-conjugated dextran was synthesized by nucleophilic reaction of dextran under anhydrous, basic conditions with the triazine moiety of dichlorotriazinyl amino fluorescein (modified from de Belder and Granath 1973). Dextran (10 mg, approx. 8 μmol, $M_r \sim 1270$; Fluka Chemical Corp., Ronkonkoma, N.Y., USA) was dissolved in 1 ml of anhydrous dimethyl sulfoxide. A small amount of sodium hydride (approx. 5 mg of a 60% (w/w) suspension in mineral oil) was added and the mixture was held for 15 min at room temperature. Dichlorotriazinyl amino fluorescein (4 mg, 8 μmol; Research Organ-