Plant Physiology, January 2000, Vol. 122, pp. 215–223, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Developmental Regulation of Monoterpene Biosynthesis in the Glandular Trichomes of Peppermint[1]

Marie E. McConkey, Jonathan Gershenzon[2], and Rodney B. Croteau*

Institute of Biological Chemistry, and Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164–6340

Monoterpene production in peppermint (*Mentha* × *piperita* L.) glandular trichomes is determined by the rate of biosynthesis, as determined by $^{14}CO_2$ incorporation, and is restricted to leaves 12 to 20 d of age. Using oil glands isolated from peppermint leaves of different ages, in vitro assay of the eight sequential enzymes responsible for the biosynthesis of the principal monoterpene (−)-menthol indicated that all but one biosynthetic enzyme had a very similar developmental profile. Activities were highest in leaves 12 to 20 d of age, with a sharp peak centered at 15 d. The exception, (−)-menthone reductase, the last enzyme of the pathway, exhibited a later peak of activity, which was centered at approximately 21 d. The correlation between in vitro enzyme activity and the rate of biosynthesis measured in vivo suggests that monoterpene formation is controlled mainly by the coordinately regulated activity of the relevant biosynthetic enzymes. Developmental immunoblotting of limonene synthase, which catalyzes the committed step of the pathway, demonstrated a direct correlation between enzyme activity and enzyme protein, suggesting that the dynamic time course for the remaining pathway enzyme activities also reflects the corresponding protein levels. RNA-blot analyses indicated that the genes encoding enzymes of the early pathway steps are transcriptionally activated in a coordinated fashion, with a time course superimposible with activity measurements and immunoblot data. These results demonstrating coincidental temporal changes in enzyme activities, enzyme protein level, and steady-state transcript abundances indicate that most of the monoterpene biosynthetic enzymes in peppermint are developmentally regulated at the level of gene expression.

Monoterpenes (C_{10}) comprise the major components of the essential oils of the mint (Lamiaceae) family, including peppermint (*Mentha* × *piperita*) and spearmint (*Mentha spicata*) (Lawrence, 1981). Peppermint has been developed as a model system for the study of monoterpene metabolism because of the commercial value of the essential oil, the fact that the plant is clonal and easily propagated vegetatively, and because the oil is chemically complex and the biosynthetic pathway involves essentially all of the representative reaction types of terpenoid metabolism (Croteau and Gershenzon, 1994). Monoterpene biosynthesis and accumulation in mint is specifically localized to the glandular trichomes (Gershenzon et al., 1989; McCaskill et al., 1992), and the pathway originates in the plastids (leucoplasts) of the secretory cells of these highly specialized, nonphotosynthetic glandular structures (Turner et al., 1999). The monoterpene family of natural products therefore is derived from the plastidial, mevalonate-independent pathway for isoprenoid metabolism (Eisenreich et al., 1997; Sagner et al., 1998), which provides isopentenyl diphosphate (and, by isomerization, dimethylallyl diphosphate) as the universal precursors of the terpenoids (Lichtenthaler et al., 1997; Eisenreich et al., 1998; McCaskill and Croteau, 1999).

The monoterpenes diverge from primary metabolism by conversion of isopentenyl diphosphate and dimethylallyl diphosphate, via the action of the prenyltransferase geranyl diphosphate synthase, to geranyl diphosphate (Burke et al., 1999), which undergoes subsequent cyclization by limonene synthase to (4*S*)-(−)-limonene (Alonso et al., 1992). (−)-Limonene serves as the common olefinic precursor of the essential oil terpenes of both peppermint and spearmint (Kjonaas and Croteau, 1983) by way of a series of secondary, largely redox, transformations (Croteau and Gershenzon, 1994) (Fig. 1). In peppermint, a microsomal cytochrome (Cyt) P450 limonene-3-hydroxylase introduces an oxygen atom in an allylic position to produce (−)-trans-isopiperitenol and thereby establishes the oxygenation pattern of all subsequent derivatives.

A soluble NADP-dependent dehydrogenase oxidizes the alcohol to a ketone, (−)-isopiperitenone, thereby activating the adjacent double bond for reduction by a soluble, NADPH-dependent, regiospecific reductase to afford (+)-cis-isopulegone. An isomerase next moves the remaining double bond into conjugation with the carbonyl group, yielding (+)-pulegone. A NADPH-dependent reductase then converts (+)-pulegone to (+)-isomenthone and (−)-menthone, which predominates. Finally, two stereoselective NADPH-dependent reductases convert (−)-menthone and (+)-isomenthone to (−)-menthol and (+)-neoisomenthol, respectively, and (−)-menthone and (+)-isomenthone to (+)-neomenthol and (+)-isomenthol, respectively. (−)-Menthol greatly predominates among the menthol isomers (often exceeding 50% of the essential oil) and is primarily responsible for the characteristic flavor and cooling sensation of peppermint.

[1] This work was supported in part by the U.S. Department of Energy Division of Energy Biosciences, the Mint Industry Research Council, and project no. 0268 from the Agricultural Research Center, Washington State University.

[2] Present address: Max Planck Institut für Chemische Ökologie, Tatzendpromenade 1a, D–07745 Jena, Germany.

* Corresponding author; e-mail croteau@mail.wsu.edu; fax 509–335–7643.

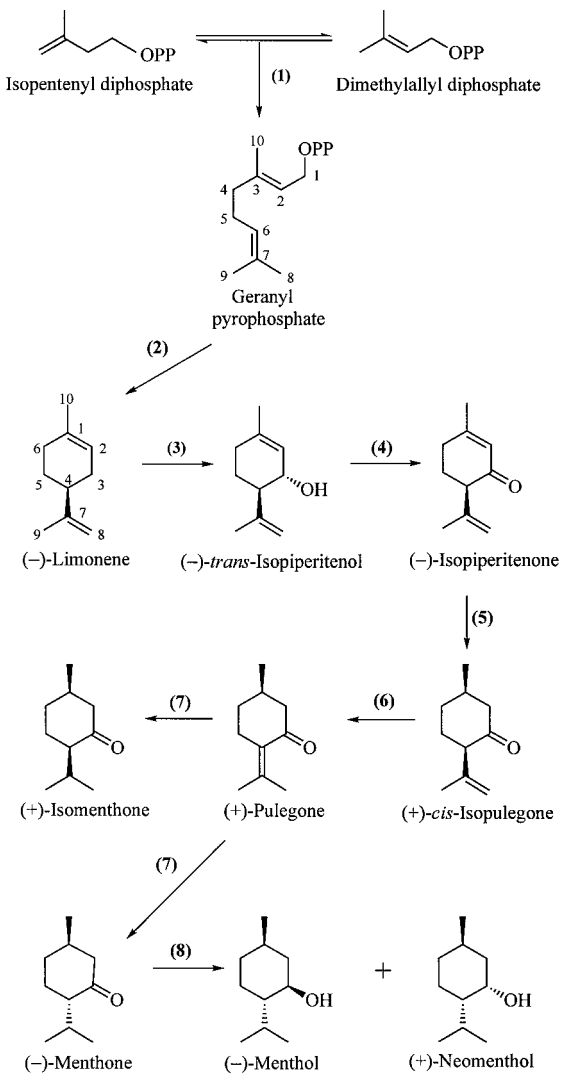

Figure 1. The principal pathway for monoterpene biosynthesis in peppermint. The responsible enzymes are: (1) geranyl diphosphate synthase, (2) (4*S*)-(−)-limonene synthase, (3) Cyt P450 (−)-limonene-3-hydroxylase, (4) (−)-trans-isopiperitenol dehydrogenase, (5) (−)-isopiperitenone reductase, (6) (+)-cis-isopulegone isomerase, (7) (+)-pulegone reductase, and (8) (−)-menthone reductase.

Although the pathway for the biosynthesis of peppermint monoterpenes is now well defined, the regulation of monoterpene metabolism in this species is only poorly understood (Gershenzon and Croteau, 1990, 1993). Both developmental and environmental factors are known to markedly influence the yield and composition of peppermint oil, with obvious consequences for the commercial production of this commodity (Burbott and Loomis, 1967; Clark and Menary, 1980); however, the means by which these variables exert regulatory control over the pathway flux to isopentenyl diphosphate and the specific steps of monoterpene metabolism are not known. Recent studies at the level of the intact plant indicate that monoterpene production (measured by incorporation of $^{14}CO_2$) is restricted to leaves 12 to 20 d of age, prior to full expansion, and that metabolic turnover of oil components (Mihaliak et al., 1991) and evaporative losses of oil from the storage compartment play only minor roles in determining oil yield and composition (Gershenzon et al., 2000). These results, coupled to the lack of evidence thus far for the control of pathway enzyme activity by allosteric modulation or covalent modification (Croteau, 1987; Wise and Croteau, 1999), suggest that oil composition and yield may reflect the simple kinetic consequences of the levels of biosynthetic enzymes present, as determined by transcriptional and translational production of these pathway catalysts and their subsequent proteolytic turnover.

More detailed study of the regulation of monoterpene metabolism has been seriously hampered by the strict localization of essential oil formation in the specialized oil glands (glandular trichomes), which constitute only a small fraction of the total leaf mass. This feature limits information that can be gained through experiments at the level of the intact tissue, and limits the utility of enzyme preparations and nucleic acid extracts from whole leaves in the development of refined tools for molecular level analysis. An early improvement over the use of whole leaf extracts was the development of methods for preparing surface cell extracts enriched in gland contents from the leaves of essential-oil-producing plants (Gershenzon et al., 1987).

Subsequently, this method was improved to allow the isolation of intact secretory cell clusters derived from the glandular trichomes of a number of plants (Gershenzon et al., 1992). This approach applied to mint species has allowed the examination of monoterpene biosynthesis in situ (McCaskill et al., 1992; McCaskill and Croteau, 1995) and the detailed characterization, including amino acid microsequencing, of biosynthetic enzymes isolated from this highly enriched source (Alonso et al., 1992; Ponnamperuma and Croteau, 1996; Lupien et al., 1999). More recently, isolated gland cells have been used as a source of mRNA for construction of highly enriched cDNA libraries, from which cDNA clones encoding enzymes of both early steps of isoprenoid biosynthesis (Lange et al., 1998; Lange and Croteau, 1999a) and committed steps of monoterpene metabolism have been isolated (Colby et al., 1993; Burke et al., 1999; Lupien et al., 1999). The availability of these DNA probes, and polyclonal antibodies directed to the corresponding proteins, has for the first time provided tools for examining transcriptional and translational control of monoterpene metabolism. In this paper, we describe the correlation of relevant monoterpene biosynthetic enzyme activities with developmental western and northern analyses to provide evidence that regulation of monoterpene metabolism in peppermint oil glands resides at the level of gene expression.

MATERIALS AND METHODS

Plant Material

Peppermint (*Mentha* × *piperita* L. cv Black Mitcham) plants were propagated from rhizomes in peat moss: pumice:sand (55:35:10, v/v) and grown in a controlled environment chamber with a mixture of fluorescent and incandescent bulbs (yielding 500 μmol m^{-2} s^{-1} photosyn-

thetically active radiation at plant height), a 16-h photoperiod, and a 22°C/10°C (day/night) temperature cycle. Plants were watered and fertilized daily with a complete fertilizer (N:P:K, 20:20:20) plus iron chelate and micronutrients. For the developmental survey, a single cohort of leaves was followed from initiation to senescence. This cohort was initiated on 3-week-old stems (as determined by microscopic dissection of the apical bud) that were 10 to 15 cm high. For biochemical analysis, initial samples were taken when leaves were 8 d old (average length = 0.2 cm, average fresh weight = 5 mg), because it was impractical to isolate large quantities of leaves at younger stages. Additional samples were harvested at 10, 12, 15, 18, 21, 25, 30, 40, and 55 d. Leaves grew rapidly, reaching full expansion (average length about 3.5 cm, average weight about 155 mg) at 21 d. At 30 to 35 d, flower buds first appeared on the apices of the stems, which began to open when leaves were 45 to 50 d old. By 55 d, nearly half of the leaves had senesced.

Preparation of Enzyme Extracts

Since monoterpene biosynthesis in peppermint is confined to the secretory cells of glandular trichomes (Gershenzon et al., 1989; McCaskill et al., 1992), to obtain extracts enriched in monoterpene biosynthetic enzymes, purified preparations of secretory cells were isolated by modification of a previously described surface abrasion protocol (Gershenzon et al., 1992). Freshly harvested leaves were soaked in deionized water for 1 h at 4°C, and their surfaces abraded using a cell disrupter (Bead-Beater, Biospec Products, Bartlesville, OK) filled with 20 g of plant material, 120 g of 0.5-mm-diameter glass beads, 20 g of XAD-4 polystyrene resin beads, and a buffer comprised of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, containing 200 mM sorbitol, 2 mM Suc, 10 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM KH_2PO_4, 0.1 mM $Na_2P_2O_7$, 1% (w/v) polyvinylpyrrolidone (M_r = 40,000), and 0.6% (w/v) methyl cellulose. Leaves longer than 2.0 cm were cut into smaller pieces prior to soaking. When the isolated secretory cells were to be used for RNA extraction, 1 mM aurintricarboxylic acid was added to the buffer (Gonzalez et al., 1980).

Abrasion was carried out for three 1-min periods at 4°C using a rotor speed controlled with a rheostat set at 85 V. Following abrasion, the contents of the disruption chamber were filtered through a series of nylon filters of successively smaller mesh size (350, 150, and 20 μm) to separate the secretory cell clusters from the glass beads, XAD-4 resin, and residual plant material. Secretory cell clusters (approximately 60 μm in diameter) were collected on the 20-μm mesh filter and then resuspended and refiltered several times in the 25 mM HEPES buffer described above (without methyl cellulose or polyvinylpyrrolidone) to remove impurities. To quantify the number of clusters obtained, a sample was removed for observation with a hemocytometer.

For enzyme assays, secretory cells were disrupted by grinding with liquid nitrogen in a prechilled mortar. The ground material was suspended in a 50 mM KH_2PO_4 buffer, pH 6.0, containing 10% (v/v) glycerol, 10 mM $Na_2S_2O_5$, 1 mM ascorbic acid, 1 mM EDTA, 1 mM dithiothreitol, 5 μM FAD, 5 μM FMN, 1% (w/v) polyvinylpyrrolidone (M_r = 10,000), and about 100 mg of XAD-4 resin. After passage through a 20-μm nylon filter, the filtrate was centrifuged at 18,000*g* for 20 min and the resulting supernatant recentrifuged at 150,000*g* for 90 min. The microsomal pellet was stored under argon at −80°C and used for limonene-3-hydroxylase assays. The remaining enzymes were assayed using the 150,000*g* supernatant, which was adjusted to the various assay conditions by desalting through a series of polyacrylamide columns (6-kD exclusion limit, Bio-Rad, Richmond, CA). No limonene-3-hydroxylase activity was detected in the 150,000*g* supernatant, and no activity of the remaining enzymes was found in the pellet.

Enzyme Assays

Geranyl diphosphate synthase (Burke et al., 1999) was assayed with a 1-mL aliquot of diluted supernatant in 25 mM piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 6.75, containing 10% (w/v) glycerol, 10 mM $MgCl_2$, and 1 mM dithiothreitol, with 100 μM dimethylallyl diphosphate and 100 μM [1-^{3}H]isopentenyl diphosphate (22.5 Ci/mol), synthesized by diphosphorylation of [1-^{3}H]isopentenol according to a previously published procedure (Croteau and Purkett, 1989). After addition of a 1-mL pentane overlay to trap volatile products, assays were incubated at 30°C for 30 min. The diphosphate ester products and remaining substrates were hydrolyzed by treatment with 1 unit each of wheat germ alkaline phosphatase and potato apyrase, added to each assay in a volume of 1 mL of 250 mM Tris buffer, pH 9.5, and allowed to incubate for at least 8 h at 30°C. The pentane overlay and a diethyl ether extract of the aqueous layer were combined and passed over anhydrous sodium sulfate to remove residual water.

After the addition of a mixture of unlabeled isopentenol, dimethylallyl alcohol, geraniol, *E*,*E*-farnesol, and isomeric geranylgeraniols as carriers, the extract was concentrated under a stream of nitrogen and an aliquot counted by liquid scintillation spectrometry. Analysis of the remainder by radio-GLC was carried out with a gas chromatograph (model 550P, Gow-Mac, Bridgewater, NJ; He carrier gas flow at 65 mL/min, injector at 220°C), equipped with a thermal conductivity detector (240°C, 150 mA) and attached to a gas proportional counter (model 7357, Nuclear Chicago, Des Plaines, IL). Separations were accomplished on a 3.6-m × 2.15-mm i.d. stainless steel column of 15% SE-30 (polydimethylsiloxane) coated on 80/100-mesh Chromosorb WHP (Alltech Associates, Deerfield, IL), and programmed from 80°C (5 min hold) to 250°C at 8°C/min. Thermal conductivity and radioactivity signals were monitored with a dual-channel data system, and all radioactivity measurements were externally calibrated with [^{3}H]toluene. The identity of the biosynthetic product was confirmed by co-chromatography of the alkaline phosphatase cleavage product with an authentic standard of geraniol on several different polarity phases, and by analysis

of a pooled sample by gas chromatography-mass spectrometry (GC-MS) (described below). Non-enzymatic activity was negligible.

(4*S*)-(−)-Limonene synthase assays were performed with 1-mL aliquots of diluted supernatant using [1-^{3}H]geranyl diphosphate as a substrate, as previously described (Alonso et al., 1992). (−)-Limonene-3-hydroxyalse assays were performed using the microsomal pellet as the enzyme source, as previously described (Karp et al., 1990).

(−)-trans-Isopiperitenol dehydrogenase was assayed with 1-mL aliquots of supernatant diluted with 50 mM Gly buffer, pH 10.5, containing 10% (w/v) sorbitol and 1 mM dithiothreitol, and incubated with 1 mM NADP and 200 μM (−)-trans-isopiperitenol for 30 min at 30°C (Kjonaas et al., 1985). The reaction was stopped by the addition of 1 mL of diethyl ether followed by vigorous shaking. After the addition of 25 nmol of (+)-camphor as an internal standard, the ether layer was removed and the reaction mixture re-extracted twice with additional 1-mL portions of ether. The combined ether extracts were decolorized with charcoal, washed with 1 mL of water, passed through a short column of silica gel (type 60A, Mallinckrodt, Chesterfield, MO) overlaid with anhydrous sodium sulfate in a Pasteur pipette, and concentrated to 40 μL under a stream of nitrogen.

Enzymatic products and unreacted substrate were separated by GC (model 5890 gas chromatograph with 3396 integrator, Hewlett-Packard, Palo Alto, CA) using a 30-m × 0.25-mm i.d. fused silica capillary column coated with a 0.25-μm film of AT-1000 (polyethyleneglycol ester, Alltech Associates) operated with H_2 as carrier (1.5 mL min^{-1}), with cool on-column injection (ambient temperature) and temperature programming (45°C for 5 min hold, then 10°C min^{-1} to 220°C, and 10 min hold) with a flame ionization detector (230°C). The product, (−)-isopiperitenone, was identified by comparison of retention time and mass spectrum with those of an authentic standard from our own collection (Kjonaas et al., 1985), and was quantified by comparison of the detector response to that of the internal standard. GC-MS was performed on a Hewlett-Packard 5840A-5985B system at 70 eV with column and separation conditions as described above.

The remaining enzymes were assayed similarly, by assays described in detail elsewhere (Croteau et al., 1991), using 1-mL aliquots of supernatant diluted in 50 mM KH_2PO_4 buffer, pH 7.0, containing 10% (w/v) sorbitol and 1 mM dithiothreitol. Substrates used were 180 μM (−)-isopiperitenone (for isopiperitenone reductase) (Kjonaas et al., 1985), 200 μM (+)-cis-isopulegone (for cis-isopulegone isomerase) (Croteau and Venkatachalam, 1986), 200 μM (+)-pulegone (for pulegone reductase) (Croteau et al., 1991), and 500 μM (−)-menthone (for menthone reductase) (Kjonaas et al., 1982). All of the reductase assays also included 1 mM NADPH and an NADPH-regenerating system consisting of 1 unit of Glc-6-P dehydrogenase and 6 mM Glc-6-P. Remaining assay conditions, extractive isolation and separation protocols, and GC and GC-MS analyses were the same as those described above for (−)-trans-isopiperitenol dehydrogenase. Two of these activities yielded more than one product. Pulegone reductase produced menthone and isomenthone in an average ratio of 2.5:1, while menthone reductase produced menthol and neomenthol in a ratio that declined from 7:1 in the early stages of leaf development to about 3:1 in the oldest leaves.

The assay of each enzyme activity at each developmental stage was run in duplicate on at least three independent enzyme preparations. Preliminary trials were conducted for all assays to determine the dilutions giving linearity for enzyme extracts at each developmental stage. Boiled control assays, assays extracted immediately after substrate addition (zero time controls), and controls without substrate or cofactor were used to measure the extent of non-enzymatic conversion and to determine the background of endogenous monoterpenes in each extract. The only substrate prone to substantial non-enzymatic conversion was isopiperitenol, which was oxidized to isopiperitenone at a rate as high as 5% of the total added. The only enzyme product present endogenously in the extract at detectable levels was menthone, which was found at up to 2% of the amount of product formed from added pulegone in the assays for pulegone reductase.

As an additional control, to ensure that developmental changes in enzyme activities were not caused by ontogenetic alterations in levels of interfering substances, extracts from several different ages were mixed together and assayed for activity. In all cases, the activity obtained was equal to the sum of the activities when measured separately. Unless specified, all substrates and standards were from our own collection. All of the enzyme assays were standardized based on oil gland numbers and were converted to a per leaf basis using the average number of glandular trichomes per developmental stage (G. Turner, J. Gershenzon, and R. Croteau, unpublished data). To determine whether there were significant differences between time points, statistical analyses were performed using Excel (Microsoft, Redman, WA). Each data point represents the mean of three to six independent measurements from which the SD was calculated.

Immunoblots

For immunoblotting, secretory cells were disrupted by sonication (Sonic 2000, Braun, Allentown, PA) with the microprobe operated at maximum power for four 30-s bursts at 0°C to 4°C in a buffer similar to that used for liquid nitrogen grinding (but without FAD and FMN), and the extract was filtered and centrifuged as described above. Proteins (150,000*g* supernatant) from the oil gland extracts of leaves of different ages were resolved on a 10% (w/v) SDS-polyacrylamide gel (Mini-Electrophoresis System, Bio-Rad) and transferred to nitrocellulose membranes using an Electro Transblot apparatus (Bio-Rad) according to the manufacturer's instructions. Immunodetection was performed according to the method of Ausubel et al. (1991) using rabbit anti-(4*S*)-(−)-limonene synthase polyclonal antibodies (Alonso et al., 1993) with alkaline phosphatase-conjugated goat anti-rabbit polyclonals as secondary antibody. Antigen bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride according to the manufacturer's specifications (Bio-Rad)

and were quantified by densitometry. Preimmune serum was used as a control. Multiple immunoblots for each time point were analyzed and a representative time course profile is presented.

RNA Isolation and Blot Analysis

Total RNA was prepared from the isolated secretory cells according to the method of Logemann et al. (1987). RNA electrophoresis and northern-blot analysis were performed according to the method of Sambrook et al. (1989). Total RNA was denatured and separated by 1.2% (w/v) formaldehyde-agarose gel electrophoresis. Following electrophoresis, the RNA was transferred with 20× SSC onto nitrocellulose or nylon membranes and cross-linked by UV irradiation (1.2×10^6 μJ). ^{32}P-Labeled DNA probes prepared by random priming of the cDNAs encoding geranyl diphosphate synthase (Burke et al., 1999), limonene synthase (Colby et al., 1993), Cyt P450 limonene-3-hydroxylase (Lupien et al., 1999), and NADPH-dependent Cyt P450 reductase (Ponnamperuma and Croteau, 1996) were used to detect the corresponding mRNAs.

Prehybridizaiton was conducted at 65°C for 1 h in 5× Denhardt's solution, 5× SSC, 0.5% (w/v) SDS, and 20 μg/mL sheared salmon sperm DNA, followed by hybridization with the ^{32}P-labeled probe ($3–6 \times 10^6$ cpm) under the same conditions overnight. Post-hybridization washes were done twice for 5 min in 1× SSC with 0.1% (w/v) SDS (10 mL) at room temperature, twice for 15 min in 1× SSC with 0.1% (w/v) SDS (50 mL) at room temperature, and twice for 15 min in 0.2× SSC with 0.1% (w/v) SDS (50 mL) at 68°C. Equal loading of total RNA was verified by comparing the ribosomal bands visualized on the ethidium bromide stained gel before transfer to membrane, and a phosphor imager (Bio-Rad) was used to quantify the signals from blots prepared from extracts of the different leaf ages. Multiple, independent northern blots were prepared and analyzed for each time point, and a representative time course profile is presented. All northern blots were exposed both to film for autoradiography and to the phosphor imager screen for quantification.

RESULTS AND DISCUSSION

In spite of the economic importance of the essential oils, relatively little is known about the regulation of the biosynthesis of essential oil terpenoids. This lack of information on metabolic controls is a serious impediment to the development of strategies for bioengineering of essential oil composition and yield (McCaskill and Croteau, 1997; Haudenschild and Croteau, 1998; Lange and Croteau, 1999b). The development of methods for the selective isolation from mint species of oil gland cells, in which the biosynthesis of essential oil terpenes specifically occurs (Gershenzon et al., 1989; McCaskill et al., 1992), has provided the means for verifying the target biosynthetic pathways, for isolating and purifying the corresponding enzymes, and for constructing highly enriched cDNA libraries from this very specialized tissue. This advance has led to the cloning of several monoterpene biosynthetic genes and has provided, for the first time to our knowledge, DNA probes and polyclonal antibodies as tools for examining the regulation of monoterpene biosynthesis at the molecular level. Using peppermint as a model system, and based on extensive knowledge of the pathway and enzymes of monoterpene biosynthesis in this species (Croteau and Gershenzon, 1994), the developmental regulation of metabolism was examined.

Developmental Regulation of Monoterpene Biosynthetic Activity

The rate of monoterpene biosynthesis determined by $^{14}CO_2$ pulse labeling (Gershenzon et al., 1999) has been shown to rise rapidly in newly emerging leaves, reaching a sharp peak between 12 and 20 d, and then rapidly declining as full leaf expansion is reached (Fig. 2A). Similarly, the monoterpene content of peppermint leaves rises rapidly between 12 and 20 d of age, levels off as full expansion is reached, and then remains stable for the remainder of leaf life (Fig. 2A) (Gershenzonet al., 1999). This pattern of development in monoterpene biosynthetic capability and accumulation is entirely consistent with the population dynamics of the peltate oil glands, which pass through the one-, two-, four-, and eight-celled stages and complete oil secretion prior to full leaf expansion (G. Turner, J. Gershenzon, and R. Croteau, unpublished data). Since neither monoterpene catabolism (Mihaliak et al., 1991) nor monoterpene volatilization contribute significantly to alterations in the level of stored product (Gershenzon et al., 1999), the pattern of monoterpene accumulation in peppermint glandular trichomes appears to chiefly reflect the rate of biosynthesis.

To examine the dynamics of monoterpene production in greater detail, changes in the in vitro activities of the eight enzymes involved in the conversion of the primary metabolites isopentenyl diphosphate and dimethylallyl diphosphate to the principal end product, (−)-menthol (see Fig. 1), were measured in cell-free extracts of oil gland cells obtained from leaves of different ages. The first seven enzymes responsible for the diversion of primary metabolism to (−)-menthone showed very similar and coordinated developmental profiles (Fig. 2, B–D). Activity for each enzyme was highest during early development in 12- to 20-d-old leaves, with a sharp peak centered at d 15. These enzyme activity profiles correlate very closely with changes in the rate of monoterpene biosynthesis measured by $^{14}CO_2$ incorporation (Fig. 2A), and correspond to leaves with high proportions of filling glands (G. Turner, J. Gershenzon, and R. Croteau, unpublished data).

The relative levels of the different enzyme activities readily rationalize the mature oil composition observed (Gershenzon et al., 1999). Thus, relatively low levels of the early pathway steps (geranyl diphosphate synthase, (−)-limonene synthase, Cyt P450, (−)-limonene-3-hydroxylase), coupled to much higher levels of the central pathway steps [(−)-trans-isopiperitenol dehydrogenase, (−)-isopiperitenone reductase, (+)-cis-isopulegone isomerase] and somewhat lower levels of the final steps (pulegone reductase, menthone reductase) combine to yield only very low levels

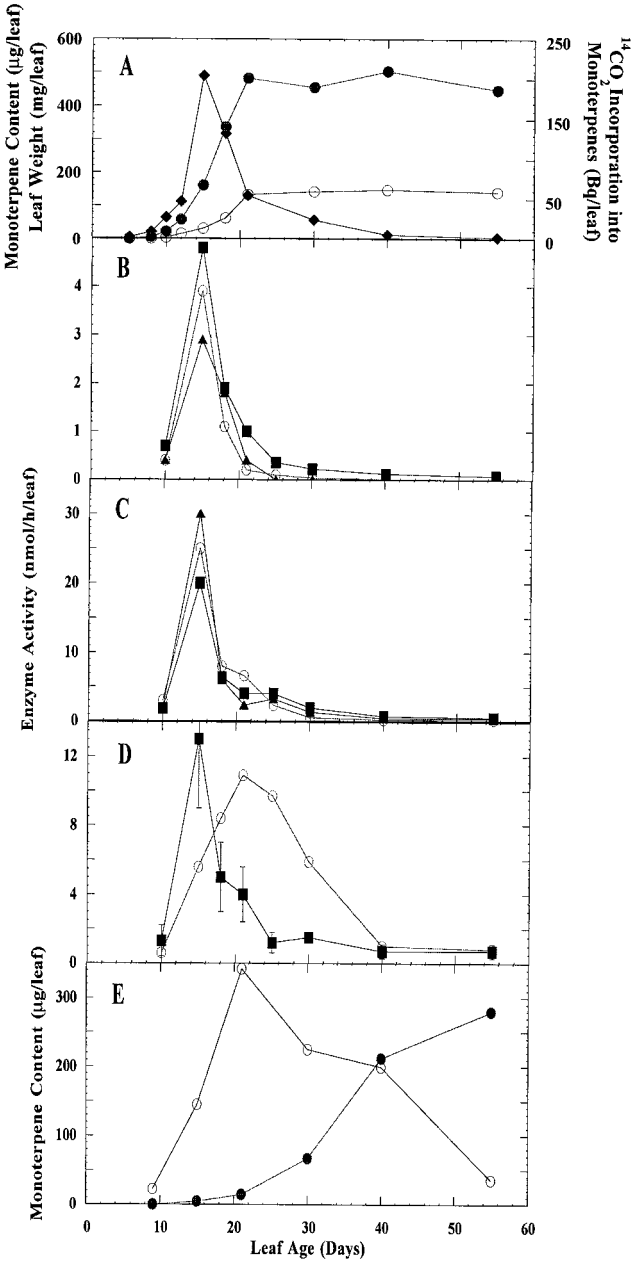

Figure 2. Changes in overall biosynthetic rate, biosynthetic enzyme activities, and monoterpene content of peppermint as a function of leaf development. A, Changes in monoterpene biosynthetic rate measured by $^{14}CO_2$ incorporation (♦), total leaf monoterpene content (●), and leaf weight (○) as a function of development (see Gershenzon et al., 1999). Peppermint leaves are fully expanded at 21 d. B, Changes in the activities of geranyl diphosphate synthase (■), (−)-limonene synthase (○), and (−)-limonene-3-hydroxylase ($\times 10^2$) (▲). C, Changes in the activities of (−)-trans-isopiperitenol dehydrogenase ($\times 10^{-1}$) (■), (−)-isopiperitenone reductase (○), and (+)-cis-isopulegone isomerase (▲). D, Changes in the activities of (+)-pulegone reductase (■) and (−)-menthone reductase ($\times 10^2$) (○). E, Changes in the essential oil content of (−)-menthone (○) and (−)-menthol (●) as a function of leaf development (see Gershenzon et al., 1999). (−)-Menthone is also converted to small amounts of (+)-neomenthol and menthyl esters; these data are not shown. The SDs are indicated for (+)-pulegone reductase activity data points (D); these error limits are typical for the other enzyme assays but are not plotted for the sake of clarity of the presentation. The complete data set, with error analysis, can be accessed at website www.wsu.edu/~ibc/faculty/rc.html. Enzyme assays were standardized based on the number of oil glands and, by using normalized gland count data, were converted to a per-leaf basis.

of pathway intermediates [a few percent each of (−)-limonene and (+)-pulegone, with all other intermediates at trace levels] and result in the accumulation of (−)-menthone and (−)-menthol as the principal products. The close correspondence between in vitro enzyme activity and the rate of monoterpene biosynthesis measured in vivo suggests that monoterpene formation in peppermint oil gland cells is tightly controlled by the levels of biosynthetic enzymes. Similar correlations have been noted for the formation of a variety of isoprenoids during flower (Pichersky et al., 1994), leaf (Croteau et al., 1981), and seedling (Green and Baisted, 1972) development, and after induction by pathogens (Dudley et al., 1986; Hanley et al., 1992), but this is the first time that a related array of isoprenoid biosynthetic enzymes has been investigated in a single specialized cell type.

The only enzyme for which anomalous time course kinetics were observed was (−)-menthone reductase, which converts (−)-menthone to (−)-menthol [lesser quantities of (+)-neomenthol are also formed in the assay]. In this instance, the activity level peaks 1 week later than all of the enzymes of the earlier pathway steps and the decline in activity is slower, giving rise to a significantly broader time course curve. This unusual developmental pattern is nevertheless consistent with the changes in monoterpene composition that accompany leaf development. (−)-Menthone is the major end product of monoterpene biosynthesis in young peppermint leaves, but this ketone declines in abundance during leaf development as conversion to (−)-menthol by the reductase occurs (Fig. 2E).

The apparent lag in the production of menthol from menthone following the developmental increase in the capacity for menthone reduction (Fig. 2., D and E) is explained by the observation that during early leaf development, when overall oil content is relatively low, some of the menthone produced by the glands present at this time is stored in the subcuticular compartment and thus is inaccessible for further metabolism. The cytosolic conversion of menthone to menthol is principally a function of those glands initiated and developed during the mid to late stages of leaf development, when the enzyme is present (i.e. because of temporal differences between the rate of gland development and the kinetics of menthone reductase appearance, a portion of the menthone "pool" produced is not accessible for reduction to menthol; this proportion of the total oil decreases with time as oil yield increases).

Since no evidence has been obtained as yet for the control of activity of these monoterpene biosynthetic enzymes by allosterism or covalent modification (Croteau, 1987; Wise and Croteau, 1999), it seemed likely that changes in enzyme activity as a function of leaf development instead reflect changes in the level of enzyme protein. To examine

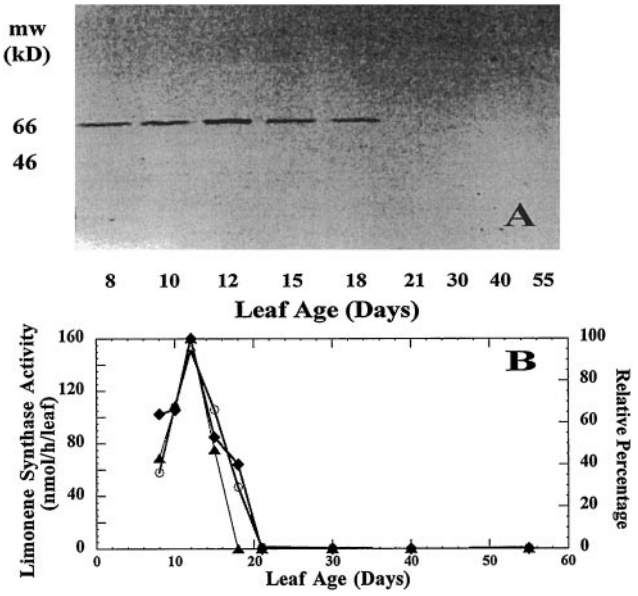

Figure 3. Temporal regulation of (−)-limonene synthase in the glandular trichomes of peppermint leaves as a function of development. A, The absolute amounts of limonene synthase protein determined by immunoblotting of extracts from the secretory cells of peppermint leaves of different ages. B, In vitro limonene synthase activity measured in soluble protein extracts from the glandular trichomes of peppermint leaves of different ages (○), the corresponding relative amounts of limonene synthase protein (♦) (from the immunoblot data of A), and the corresponding relative steady-state levels of limonene synthase transcripts (▲) (from the northern-blot data of Fig. 4). Peppermint leaves are fully expanded at 21 d.

this assumption in greater detail, (−)-limonene synthase was selected for further study. This enzyme catalyzes the committed step of monoterpene biosynthesis in mint and may be rate limiting in peppermint (Croteau and Gershenzon, 1994), and polyclonal antibodies directed against this synthase are available (Alonso et al., 1993). The absolute amounts of limonene synthase protein extracted from the secretory cells of leaves of different ages were determined by immunoblotting using the antibodies specific for the denatured enzyme. The time course of appearance and disappearance of enzyme protein compared with the developmental change in enzyme activity showed very close correlation (Fig. 3), which paralleled the overall rate of monoterpene biosynthesis (Fig. 2A), and gave no indication for the presence of inactive form(s) of limonene synthase at any stage of development. If it is assumed that the behavior of limonene synthase, for which enzyme activity is a direct measure of enzyme protein, is representative of the other enzymes of the menthol biosynthetic pathway, then it is apparent that these catalysts of monoterpene metabolism are present only for a brief period of overall leaf development.

Transcriptional and Translational Regulation of Monoterpene Biosynthesis

The regulation of gene expression for a subset of the above monoterpene biosynthetic enzymes was examined by RNA-blot analysis of total RNA isolated from oil glands of leaves at the same developmental stages employed in the above time course experiments. Since DNA probes were available only for geranyl diphosphate synthase (Burke et al., 1999), limonene synthase (Colby et al., 1993), Cyt P450 limonene-3-hydroxylase (Lupien et al., 1999), and NADPH-dependent Cyt P450 reductase (Ponnamperuma and Croteau, 1996), mRNA transcript accumulation for the respective enzymes of these early pathway steps (Fig. 1) was evaluated. This time course of steady-state message levels (Fig. 4), all peaking near d 12, clearly indicates the coordinated regulation of these initial biosynthetic steps that parallels the appearance of biosynthetic activity measured in vitro (Fig. 2) and by the developmental western blot (Fig. 3). These results suggest that the developmental regulation of monoterpene biosynthesis in peppermint oil glands resides at the level of gene expression, and indicate that transcriptional and immediate translational activity for these early pathway genes occurs during a relatively short period of leaf development that is followed by turn-

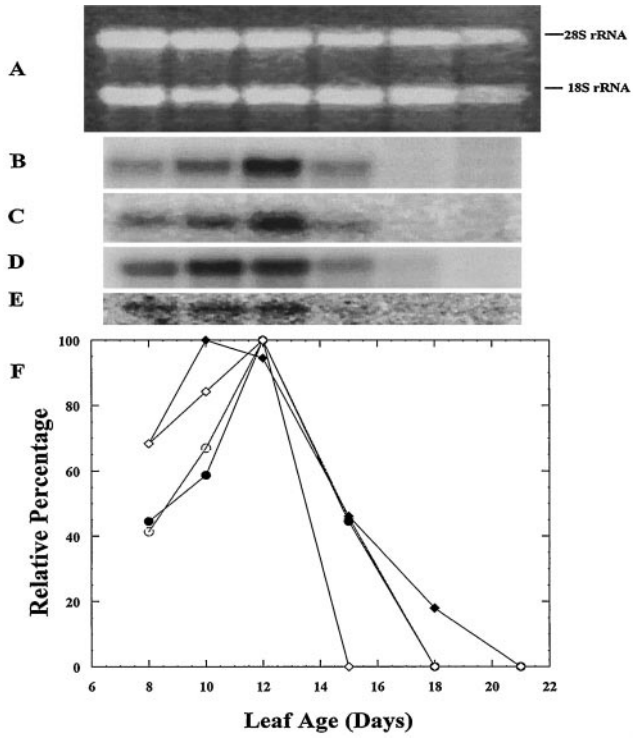

Figure 4. Temporal changes in steady-state mRNA levels for monoterpene biosynthetic enzymes in the glandular trichomes of peppermint leaves as a function of development. For each time point, total glandular RNA was isolated, separated on a denaturing agarose gel (5 μg/lane), blotted, and hybridized to radiolabeled DNA probes directed toward geranyl diphosphate synthase (B), (−)-limonene synthase (C), Cyt P450 limonene-3-hydroxylase (D), and NADPH-dependent Cyt P450 reductase (E). A, Ribosomal bands visualized with ethidium bromide that were used to verify loading of equal amounts of total RNA. F, Plot of the relative amounts of message determined by northern-blot analysis for geranyl diphosphate synthase (●), limonene synthase (○), limonene-3-hydroxylase (♦), and Cyt P450 reductase (◇).

over of the corresponding enzymes accompanied by the cessation of monoterpene production.

Previous studies have demonstrated that inducible monoterpene (Steele et al., 1998), sesquiterpene (Vögeli and Chappell, 1990; Facchini and Chappell, 1992) and diterpene (Lois and West, 1990) biosynthesis are regulated at the level of transcription. The present studies are the first to indicate that the developmental regulation of monoterpene biosynthesis in secretory trichomes also resides largely at the level of gene expression. The role of gene expression in the control of the early, non-specialized pathway steps leading to the universal terpenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate (Lange et al., 1998; Lange and Croteau, 1999a), and of menthone reductase and menthol acetyltransferase (Croteau and Hooper, 1978), which also appears late in leaf development, has not yet been evaluated. The time course of appearance of the latter two enzyme activities compared with the kinetics for earlier pathway steps suggests a subsequent period of transcriptional and translational activity late in leaf (oil gland) development. These regulatory questions are being addressed as DNA and antibody probes for these target enzymes become available.

ACKNOWLEDGMENTS

We thank Dr. Daniel Bergey for helpful discussions, Colette Gibbons for technical assistance, Thom Koehler for raising the plants, and Joyce Tamura for typing the manuscript.

Received July 27, 1999; accepted September 23, 1999.

LITERATURE CITED

Alonso WR, Crock JE, Croteau R (1993) Production and characterization of polyclonal antibodies in rabbits to 4*S*-limonene synthase from spearmint (*Mentha spicata*). Arch Biochem Biophys **301:** 58–63

Alonso WR, Rajaonarivony JIM, Gershenzon J, Croteau R (1992) Purification of 4*S*-limonene synthase, a monoterpene cyclase from the glandular trichomes of peppermint (*Mentha* × *piperita*) and spearmint (*M. spicata*). J Biol Chem **267:** 7582–7587

Ausubel FM, Brent R, Kingston RE, Moore DD, Siedman JG, Smith JA, Struhl K (1991) Current Protocols in Molecular Biology. John Wiley & Sons, New York

Burbott AJ, Loomis WD (1967) Effects of light and temperature on the monoterpenes of peppermint. Plant Physiol **42:** 20–28

Burke CC, Wildung MR, Croteau R (1999) Geranyl diphosphate synthase: cloning expression and characterization of this prenyltransferase as a heterodimer. Proc Natl Acad Sci USA **96:** 13062–13067

Clark RJ, Menary RC (1980) Environmental effects on peppermint (*Mentha piperita* L.). I. Effect of daylength, photon flux density, night temperature and day temperature on the yield and composition of peppermint oil. Aust J Plant Physiol **7:** 685–692

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4*S*-Limonene synthase from the oil glands of spearmint (*Mentha spicata*): cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. J Biol Chem **268:** 23016–23024

Croteau R (1987) Biosynthesis and catabolism of monoterpenoids. Chem Rev **87:** 929–954

Croteau R, Felton M, Karp F, Kjonaas R (1981) Relationship of camphor biosynthesis to leaf development in sage (*Salvia officinalis*). Plant Physiol **67:** 820–824

Croteau R, Gershenzon J (1994) Genetic control of monoterpene biosynthesis in mints (*Mentha*:Lamiaceae). Rec Adv Phytochem **28:** 193–229

Croteau R, Hooper CL (1978) Metabolism of monoterpenes: acetylation of (−)-menthol by a soluble enzyme preparation from peppermint (*Mentha piperita*) leaves. Plant Physiol **61:** 737–742

Croteau R, Karp F, Wagschal KC, Satterwhite DM, Hyatt DC, Skotland CB (1991) Biochemical characterization of a spearmint mutant that resembles peppermint in monoterpene content. Plant Physiol **96:** 744–752

Croteau R, Purkett PT (1989) Geranyl pyrophosphate synthase: characterization of the enzyme and evidence that this chain-length specific prenyltransferase is associated with monoterpene biosynthesis in sage (*Salvia officinalis*). Arch Biochem Biophys **271:** 524–535

Croteau R, Venkatachalam KV (1986) Metabolism of monoterpenes: demonstration that (+)-*cis*-isopulegone, not piperitenone, is the key intermediate in the conversion of (−)-isopiperitenone to (+)-pulegone in peppermint (*Mentha piperita*). Arch Biochem Biophys **249:** 306–315

Dudley MW, Dueber MT, West CA (1986) Biosynthesis of the macrocyclic diterpene casbene in castor bean (*Ricinus communis* L.) seedlings: changes in enzyme levels induced by fungal infection and intracellular localization of the pathway. Plant Physiol **81:** 335–342

Eisenreich W, Sagner S, Zenk MH, Bacher A (1997) Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Lett **38:** 3889–3892

Eisenreich W, Schwarz M, Cartayrade A, Arigoni D, Zenk NH, Bacher A (1998) The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. Chem Biol **5:** R221–R233

Facchini PJ, Chappell J (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. Proc Natl Acad Sci USA **89:** 11088–11092

Gershenzon J, Croteau R (1990) Regulation of monoterpene biosynthesis in higher plants. Rec Adv Phytochem **24:** 99–160

Gershenzon J, Croteau R (1993) Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes. *In* TS Moore, Jr, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, pp 339–388

Gershenzon J, Duffy MA, Karp F, Croteau R (1987) Mechanized techniques for the selective extraction of enzymes from plant epidermal glands. Anal Biochem **163:** 159–164

Gershenzon J, Maffei M, Croteau R (1989) Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). Plant Physiol **89:** 1351–1357

Gershenzon J, McCaskill D, Rajaonarivony JIM, Mihaliak C, Karp F, Croteau R (1992) Isolation of secretory cells from plant glandular trichomes and their use in biosynthetic studies of monoterpenes and other gland products. Anal Biochem **200:** 130–138

Gershenzon J, McConkey M, Croteau R (2000) Regulation of monoterpene accumulation in leaves of peppermint (*Mentha* × *piperita* L). Plant Physiol **122:** 205-213

Gonzalez RG, Haxo RS, Kirschner MW, Laemmli UK (1980) Mechanism of action of polymeric aurintricarboxylic acid, a potent inhibitor of protein-nucleic acid interactions. Biochemistry **19:** 4299–4303

Green TR, Baisted DJ (1972) Development of the activities of enzymes of the isoprenoid pathway during early stages of pea-seed germination. Biochem J **130:** 983–995

Hanley KM, Vögeli U, Chappell J (1992) A study of the isoprenoid pathway in elicitor-treated tobacco cell suspension cultures. *In* RJ Petroski, SP McCormick, eds, Secondary-Metabolite Biosynthesis and Metabolism. Plenum Press, New York, pp 329–336

Haudenschild CD, Croteau RB (1998) Molecular engineering of monoterpene production. *In* JK Setlow, ed, Genetic Engineering:

Principles and Methods. Vol 20. Plenum Press, New York, pp 267–280

Karp F, Mihaliak CA, Harris JL, Croteau R (1990) Monoterpene biosynthesis: specificity of the hydroxylations of (−)-limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and perilla (*Perilla frutescens*) leaves. Arch Biochem Biophys **276:** 219–226

Kjonaas R, Croteau R (1983) Demonstration that limonene is the first cyclic intermediate in the biosynthesis of oxygenated *p*-menthane monoterpenes in *Mentha piperita* and other *Mentha* species. Arch Biochem Biophys **220:** 79–89

Kjonaas R, Martinkus-Taylor C, Croteau R (1982) Metabolism of monoterpenes: conversion of *l*-menthone to *l*-menthol and *d*-neomenthol by stereospecific dehydrogenases from peppermint (*Mentha piperita*) leaves. Plant Physiol **69:** 1013–1017

Kjonaas RB, Venkatachalam KV, Croteau R (1985) Metabolism of monoterpenes: oxidation of isopiperitenol to isopiperitenone, and subsequent isomerization to piperitenone, by soluble enzyme preparations from peppermint (*Mentha piperita*) leaves. Arch Biochem Biophys **238:** 49–60

Lange BM, Croteau R (1999a) Isoprenoid biosynthesis via a mevalonate-independent pathway in plants: cloning and heterologous expression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase from peppermint. Arch Biochem Biophys **365:** 170–174

Lange BM, Croteau R (1999b) Genetic engineering of essential oil production in mint. Curr Opin Plant Biol **2:** 139–144

Lange BM, Wildung MR, McCaskill DG, Croteau R (1998) A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc Natl Acad Sci USA **95:** 2100–2104

Lawrence BM (1981) Monoterpene interrelationships in the *Mentha* genus: a biosynthetic discussion. *In* BD Mookherjee, CJ Mussinan, eds, Essential Oils. Allured Publishing, Wheaton, IL, pp 1–81

Lichtenthaler HK, Schwender J, Disch A, Rohmer M (1997) Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett **400:** 271–274

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Lois AF, West CA (1990) Regulation of expression of the casbene synthetase gene during elicitation of castor bean seedlings with pectic fragments. Arch Biochem Biophys **276:** 270–277

Lupien S, Karp F, Wildung M, Croteau R (1999) Regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha*) species: cDNA isolation, characterization, and functional expression of (−)-4*S*-limonene-3-hydroxylase and (−)-4*S*-limonene-6-hydroxylase. Arch Biochem Biophys **368:** 181–192

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha* × *piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta **197:** 49–56

McCaskill D, Croteau R (1997) Prospects for the bioengineering of isoprenoid biosynthesis. Advances Biochem Engin Biotechnol **55:** 102–146

McCaskill DG, Croteau R (1999) Isopentenyl diphosphate is the terminal product of the deoxyxylulose-5-phosphate pathway for terpenoid biosynthesis in plants. Tetrahedron Lett **40:** 653–656

McCaskill DG, Gershenzon J, Croteau R (1992) Morphology and monoterpene biosynthetic capabilities of secretory cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.). Planta **187:** 445–454

Mihaliak CA, Gershenzon J, Croteau R (1991) Lack of rapid monoterpene turnover in rooted plants: implications for theories of plant chemical defense. Oecologia **87:** 373–376

Pichersky E, Raguso RA, Lewinsohn E, Croteau R (1994) Floral scent production in *Clarkia* (Onagraceae). I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. Plant Physiol **106:** 1533–1540

Ponnamperuma K, Croteau R (1996) Purification and characterization of an NADPH-cytochrome P450 (cytochrome c) reductase from spearmint (*Mentha spicata*) glandular trichomes. Arch Biochem Biophys **329:** 9–16

Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH (1998) Differential incorporation of 1-deoxy-D-xylulose into monoterpenes and carotenoids in higher plants. J Chem Soc Chem Commun 221–222

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 7.20–7.43

Steele CL, Katoh S, Bohlmann J, Croteau R (1998) Regulation of oleoresinosis in grand fir (*Abies grandis*): differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding. Plant Physiol **116:** 1497–1504

Turner G, Gershenzon J, Nielson EE, Froehlich JE, Croteau R (1999) Limonene synthase responsible for monoterpene biosynthesis in peppermint is localized to leucoplasts of oil gland secretory cells. Plant Physiol **120:** 879–886

Vögeli U, Chappell J (1990) Regulation of a sesquiterpene cyclase in cellulase-treated tobacco cell suspension cultures. Plant Physiol **94:** 1860–1866

Wise ML, Croteau R (1999) Monoterpene biosynthesis. *In* DE Cane, ed, Comprehensive Natural Products Chemistry: Isoprenoids, Vol 2. Elsevier Science, Oxford, pp 97–153