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Biosynthesis and Biotechnology of High-Value *p*-Menthane Monoterpenes, Including Menthol, Carvone, and Limonene

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Abstract Monoterpenes of the *p*-menthane group are volatile secondary (or specialized) metabolites found across the plant kingdom. They are dominant constituents of commercially important essential oils obtained from members of the genera *Mentha* (Lamiaceae), *Carum* (Apiaceae), *Citrus* (Rutaceae), and *Eucalyptus* (Myrtaceae). *p*-Menthane monoterpenes have also attracted interest as chiral specialty chemicals, and the harvest from natural sources is therefore supplemented by chemical synthesis. More recently, microbial and plant-based platforms for the high-level accumulation of specific target monoterpenes have been developed. In this review chapter, I discuss the properties of the genes and enzymes involved in *p*-menthane biosynthesis and provide a critical assessment of biotechnological production approaches.

Keywords Carvone · Cineole · Essential oil · Limonene · Menthol · Metabolic engineering

List of Abbreviations

DXR	1-Deoxy-D-xylulose 5-phosphate reductoisomerase
ER	Endoplasmic reticulum
FPPS	(*E,E*)-farnesyl diphosphate synthase
ISPD	(−)-*trans*-isopiperitenol dehydrogenase
ISPR	(−)-*trans*-isopiperitenone reductase
MDR	Medium chain dehydrogenase/reductase
MEP	2*C*-methyl-D-eryhritol 4-phosphate
MFS	(+)-menthofuran synthase
MMR	(−)-menthone:(−)-menthol reductase

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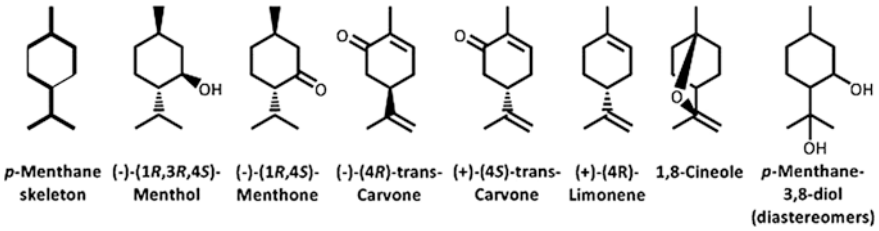
MNR	(−)-menthone:(+)-neomenthol reductase
MVA	Mevalonic acid
ORF	Open reading frame
PMD	*p*-menthane-3,8-diol
PR	(+)-pulegone reductase
SDR	Short chain dehydrogenase/reductase

Contents

1 Introduction

One of the most diverse and widely distributed classes of plant monoterpenes is the *p*-menthane (1-methyl-4-isopropylcyclohexane) type (Scheme 1). Larger amounts of these metabolites are often accumulated as essential oils and oleoresins. The current methods for the commercial production of specific high-value *p*-menthane monoterpenes are chemical synthesis or isolation from natural sources. The biosynthesis of *p*-menthane monoterpenes in the genus *Mentha* occurs exclusively in specialized anatomical structures called glandular trichomes [47]. Specifically, the pathway is expressed in secretory cells, which form an eight-celled disk within glandular trichomes. Secretory cells can be isolated and have been demonstrated to be a highly enriched source of transcripts that were characterized to be directly involved in *p*-menthane monoterpene biosynthesis [19, 45]. In *Citrus* species, the fruit peel contains secretory cavities that are filled with *p*-menthane monoterpene-containing essential oil. Recently, laser-microdissection has been demonstrated to allow the isolation of epithelial cells lining these cavities, which was followed by the extraction of RNA and subsequent transcriptome analysis [90]. An integration with physiological and metabolite data allowed a first glimpse at the metabolic specialization of this highly specialized oil biosynthetic cell type. A method for the isolation of oil-accumulating subdermal secretory cavities from *Eucalyptus* leaves was also developed recently [32]. Subsequently, RNA was isolated and a cDNA fragment with high homology to a monoterpene synthase amplified.

Building on cell type-specific transcriptome sequences, functional genomics efforts have led to the cloning and characterization of many of the genes involved in *p*-menthane monoterpene biosynthesis over the last 20 years. In this review chapter,



Scheme 1 Structures of selected *p*-menthane monoterpenes. The C5 units from which the parent skeleton is assembled are indicated in boldface on the left-hand side

I list the catalytic properties of the enzymes that are relevant for the discussion of *p*-menthane monoterpene formation but, for an in-depth coverage of the topic, the interested reader is referred to a comprehensive review article [19]. We also recently published an exhaustive review covering biotechnological efforts to generate volatile terpenoids in plants [48]. To avoid redundancy, this chapter focuses on attempts to produce high-value *p*-menthane monoterpenes (not biofuel components) through biotechnological methods using engineered plants and microorganisms.

2 Commercial Production and Applications for *p*-Menthane Monoterpenes

(−)-(1*R*,3*R*,4*S*)-Menthol (syn. *l*-menthol; Scheme 1) is the principal component [>60 % (w/v)] of the essential oil distilled from cornmint (*Mentha arvensis* L.). The oil of peppermint (*Mentha* x *piperita*) contains between 35 and 45 % *l*-menthol in a complex mixture with more than a dozen other volatiles [50]. *l*-Menthol has a characteristic cooling effect when ingested or applied to the skin. Its effects are mediated by TRPM8, a Na^+/Ca^{2+} ion channel that acts as a receptor for cold and cooling agents [69]. The physiological cooling effects and the minty taste underlie the widespread use of *l*-menthol in the flavor sector (e.g., chewing gum, beverages, candy, cigarettes, cosmetics), personal hygiene (e.g., toothpaste, mouthwash, skin and hair care products), and in nonprescription medical products (primarily topical analgesics, decongestants, and cough suppressants; [39]). Since the late 1980s, crystalline menthol has been registered as a pesticide for tracheal mite control in the United States. The annual production of *l*-menthol has been estimated to exceed 30,000 tons per year (t/year; [49]; Table 1), the majority of which (roughly 19,000 t/year in 2007) is obtained from cornmint essential oil by crystallization at low temperature [15]. India, the main supplier of natural *l*-menthol (roughly 80 % of the world market), has been experiencing weather-related fluctuations in production, and the compound is thus also produced synthetically on an increasingly large scale

(approximately 6,300 t/year in 2009; [27]) by (in alphabetical order) BASF (from *E*/*Z*-citral), Symrise/Lanxess (from *m*-cresol), and Takasago (from myrcene; [60]).

(−)-(1*R*,4*S*)-Menthone (syn. *l*-menthone; Scheme 1) is the second most abundant monoterpene [15–20 % (v/v)] of peppermint essential oil. The taste of *l*-menthone is refreshingly cool, but sharper than that of *l*-menthol and with a bitter afternote. It is added to adjust the taste and aroma of products containing mint oils (Table 1). If stored at room temperature, an epimerization of *l*-menthone will lead to the formation of (+)-(1*R*,4*R*)-isomenthone (syn. *d*-isomenthone), the aroma of which has a greener note. Commercial *l*-menthone is therefore always sold as a mixture with up to 29 % (v/v) *d*-isomenthone. There are several commercial ant and roach killer formulations that contain menthone as an active ingredient. Natural *l*-menthone is crystallized from dementholized peppermint or cornmint essential oil at about 1,300 t/year (Table 1). The compound is also prepared synthetically from different precursors, but these routes are only of minor commercial relevance [14].

(−)-(4*R*)-*trans*-Carvone (syn. *l*-carvone; Scheme 1) occurs at high levels [50–80 % (v/v)] in the essential oil of spearmint (*Mentha spicata* L.). Spearmint oil tastes very refreshing and cooling, but is milder than peppermint oil. *l*-Carvone is used commercially in the flavor and fragrance industries, mostly for cosmetic and personal hygiene products (applications that are similar to those listed for *l*-menthol). It is also an approved insect repellent in the United States. Roughly 1,800 t of spearmint oil are produced annually [49]. However, the majority of *l*-carvone (approximately 2,000 t/year) is not obtained by fractional crystallization of natural spearmint oil but by chemical synthesis from (+)-(4*R*)-limonene, which occurs at high levels in *Citrus* rind, a by-product of juice production (particularly orange fruit). Most commercial synthesis routes are based on a strategy that involves the formation of a characteristic nitrosochloride as an intermediate and was originally developed by Reitsema [71].

(+)-(4*S*)-*trans*-Carvone (syn. *d*-carvone; Scheme 1) is the major constituent in the oils distilled from caraway fruit [*Carum carvi* L.; 50–70 % (v/v)] and dill seed [*Anethum graveolens* L.; 40–60 % (v/v)]. Its aroma is, in contrast to that of the optical antipode *l*-carvone, characteristically pungent and anise-like. Caraway fruits are used as a spice in numerous traditional dishes in India (e.g., biryani) and several European countries (e.g., sauerkraut). Caraway oil is added as a fragrance to various personal hygiene products. *d*-Carvone is also sold as a potato-sprouting inhibitor in the Netherlands. About 30–40 t/year of caraway fruit oil (from which *d*-carvone is obtained by fractional distillation) are traded on the world market (Table 1; [58]). Synthetic *d*-carvone is produced from (−)-(4*S*)-limonene, a major component of turpentine oil (a by-product of pulp and paper processing).

(+)-(4*R*)-Limonene (syn. *d*-limonene; Scheme 1) is an intermediate in the biosynthesis of *d*-carvone in caraway fruit (accumulates to 25–30 % (v/v) of the essential oil). The most characteristic source of *d*-limonene, however, is the oil obtained from the fruit peel of various *Citrus* species where it accumulates to 70–98 % (v/v) of the oil [83]. *d*-Limonene imparts the characteristic citrusy smell of orange and lemon fruit and is used widely in the flavor and fragrance industries. Orange oil is an excellent, environmentally friendly, solvent employed in adhesives,

Table 1 Commercial sources and applications of *p*-menthane monoterpenes

Monoterpene	Source		Global production	Commercial applications
	Scientific name	Common name		
(−)-(1*R*,3*R*,4*S*)-Menthol (syn. L-Menthol)	*Mentha arvensis* L. (Lamiaceae) *Mentha* x *piperita* L. (Lamiaceae)	Cornmint Japanese mint	>30,000 t/year natural from cornmint and peppermint oil (*l*-Menthol obtained by low-temperature crystallization) 6,300 t/year synthetic in 2009 Starting materials for synthetic production: Myrcene (Takasago process) m-Cresol (Symrise process) E/Z-Citral (BASF process) Price of synthetic product: $15–20/kg	Flavoring ingredient Cosmetics and personal hygiene additive Tobacco additive Pesticide (repellent of tracheal mites of honey bees) Main nonprescription medical products: Topical analgesics, decongestants, cough suppressants
(−)-(1R,4S)-Menthone (syn. L-Menthone)	*Mentha* x *piperita* L. (Lamiaceae) *Mentha arvensis* L. (Lamiaceae)	Peppermint Cornmint Japanese mint	~1,300 t/year natural from peppermint and cornmint oil (*l*-Menthone crystallized from dementholized oil) Synthetic from various precursors (only minor production)	Flavoring ingredient Pesticide ant and roach killer formulations
(−)-(4*R*)-*trans*-Carvone (syn. l-Carvone)	*Mentha spicata* L. (Lamiaceae)	Spearmint	1,800 t/year natural spearmint oil produced globally (*l*-Carvone obtained by fractional distillation) ~2,000 t/year synthetic; starting materials: *d*-Limonene from *Citrus* peel oil Price of synthetic product: $15–20/kg	Flavoring ingredient Insect repellent (approved as pesticide in United States)

(continued)

Table 1 (continued)

Monoterpene	Source		Global production	Commercial applications
	Scientific name	Common name		
(+)-(4*S*)-trans-Carvone (syn. d-Carvone)	*Carum carvi* L. (Apiaceae)	Caraway Meridian fennel Persian cumin Shahi Jeera	30–40 t/year natural caraway oil produced globally (*d*-Carvone obtained by fractional distillation) Starting materials for synthetic production: *l*-Limonene from turpentine (biproduct of pulp/paper prod.) Price of synthetic product: $40–50/kg	Flavoring ingredient Potato sprouting inhibitor (marketed under trade name Talent® in the Netherlands)
(+)-(*R*)-Limonene (syn. d-Limonene)	*Citrus* sp. (Rutaceae)	Fruit peel oil of *Citrus* species (mostly orange and lemon)	>60,000 t/year natural orange and lemon oil produced globally (*d*-Limonene obtained by cold pressing or steam distillation)	Flavoring ingredient Cosmetics and personal hygiene additive Solvent for cleaning purposes and paints Pesticide (organic weedkiller formulations)
1,8-Cineole (syn. Eucalyptol)	*Eucalyptus* sp. (Myrtaceae)	Distillates of *Eucalyptus* species	~4,000 t/year natural *Eucalyptus* oil produced globally (Eucalyptol obtained by fractional distillation)	Flavoring ingredient Cosmetics and personal hygiene additive Nonprescription medical products
p-Menthane-3,8-diols (PMD) (syn. Menthoglycols)	*Eucalyptus citriodora* (Myrtaceae)	Lemon-scented gum Lemon eucalyptus	~1,500 t/year from lemon eucalyptus oil (PMD obtained from refined oil) Starting materials for semisynthetic production: Citronellal (Takasago process)	Insect repellent (mixture of PMD isomers marketed under trade name Citriodiol®)

stain removers, household cleaners, and strippers. *d*-Limonene is also employed as an active ingredient in organic weed killer formulations. *Citrus* oils are obtained commercially by cold pressing or distillation from orange and lemon fruit peel at >60,000 t/year (Table 1; [49]). Because of the comparatively low price ($9–10/kg) of *Citrus* essential oil, which is a by-product of fruit juice production, synthetic *d*-limonene is not produced on a larger commercial scale.

1,8-Cineole (syn. eucalyptol; Scheme 1) is the principal component [50–90 % (v/v)] of the essential oil of *Eucalyptus* (various species in this genus are grown commercially; [9]). It has a camphor-like smell and a spicy cooling taste. The flavor, fragrance, and cosmetic industries employ *Eucalyptus* oil as a fragrant ingredient in numerous consumer products (e.g., baked goods, confectionery, and beverages). It is also a characteristic component of over-the-counter mouthwash formulations and cough suppressants. Technical-grade 1,8-cineole is obtained by fractional distillation from *Eucalyptus* essential oil, whereas fragrance-grade materials require chromatographic isolation from the oil (total production approximately 4,000 t/year; Table 1; [49]). Synthetic 1,8-cineole does not play a significant role in the commercial sector.

***p*-Menthane-3,8-diol** (syn. menthoglycol; Scheme 1). The essential oil of lemon eucalyptus (*Eucalyptus citriodora* Hook.; aka *Corymbia citriodora*) contains (+)-(*R*)-/(−)-(*S*)-citronellal [50–80 % (v/v)] and (+)-(*R*)-/(−)-(*S*)-citronellal [7–20 % (v/v)] as the main products (Scheme 1; [9, 49]). Commercial insect repellents derived from the oil contain diastereomeric *p*-menthane-3,8-diols (PMDs), which are obtained at about 1,500 t/year from (+)-(*R*)-/(−)-(*S*)-citronellal by acid-catalyzed cyclyzation and subsequent crystallization (Table 1; [97]).

3 Genes and Enzymes of *p*-Menthane Monoterpene Biosynthesis

Monoterpene Synthases. The cyclization of geranyl diphosphate to *l*-limonene, catalyzed by (−)-limonene synthase, constitutes the first committed step in the biosynthetic pathway specific for *p*-menthane monoterpenes in peppermint and spearmint (Fig. 1). The gene encoding (−)-limonene synthase was first cloned from spearmint and the corresponding recombinant enzyme characterized by heterologous expression in *Escherichia coli* [17]. The open reading frame (ORF) of 1,800 bp, which is 96 % identical to the peppermint orthologue [45], codes for a 600 amino acid polypeptide. After import into leucoplasts (nongreen plastids of secretory cells in glandular trichomes), the N-terminal plastidial targeting sequence of the preprotein is proteolytically cleaved to generate a mature protein of approximately 545 residues and a size of ~65 kDa [84, 91]. The catalytic cascade of the (−)-limonene synthase reaction is thought to involve (1) ionization by removal of the diphosphate from C1 of geranyl diphosphate, (2) syn-migration of diphosphate to C3 (thus forming 3*S*-linalyl diphosphate), (3) rotation of the C1–C2 ethenyl group around C3 (from a transoid to a

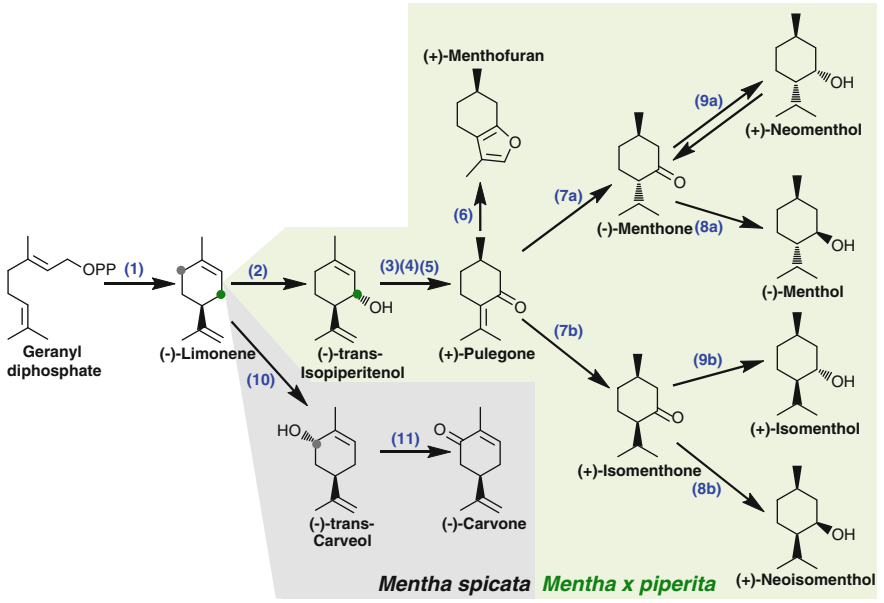


Fig. 1 *p*-Menthane monoterpene biosynthesis in peppermint (*Mentha* x *piperita* L.) and spearmint (*Mentha spicata* L.). The enzymes involved in this pathway are *1* (−)-limonene synthase; *2* (−)-limonene 3-hydroxylase; *3* (−)-*trans*-isopiperitenol dehydrogenase; *4* (−)-*trans*-isopiperitenone reductase; *5* (+)-*cis*-isopulegone isomerase; *6* (+)-menthofuran synthase; *7a* (+)-pulegone reductase ((−)-menthone-forming activity); *7b* (+)-pulegone reductase ((+)-isomenthone-forming activity); *8a* (−)-menthone:(−)-menthol reductase ((−)-menthol-forming activity); *8b* (−)-menthone:(−)-menthol reductase ((+)-neoisomenthol-forming activity); *9a* (−)-menthone:(+)-neomenthol reductase ((+)-neomenthol-forming activity); *9b* (−)-menthone:(+)-neomenthol reductase ((+)-isomenthol-forming activity); *10* (−)-limonene 6-hydroxylase; and *11* (−)-*trans*-carveol dehydrogenase

cisoid conformation), (4) a second ionization, (5) cyclization at C6–C1 to generate an α-terpinyl cation, and (6) deprotonation to yield primarily 4*S*-(−)-limonene (94 % of produced monoterpenes; [70]; Fig. 2, upper panel). Side reactions lead to the formation of myrcene (2 %), by premature deprotonation prior to step (5), and (−)-α-pinene/(−)-β-pinene (4 %) following a second ring closure between C2 and C8 of the geranyl diphosphate precursor and subsequent deprotonation. An analogous mechanism can be formulated for the (+)-limonene synthases of *Citrus* and caraway, but the reaction sequence in these enzymes proceeds via 3*R*-linalyl diphosphate (Fig. 2, lower panel, Fig. 3).

Limonene synthase sequences feature a conserved aspartate-rich motif (DDxxD) for binding the diphosphate moiety, which is released from geranyl diphosphate via divalent metal ions (Mg^{2+} or Mn^{2+}). A second metal cofactor binding region, termed the NSE/DTE motif [1], is also present in all limonene synthases. Despite a relatively low level of overall sequence conservation among monoterpene synthases, even when the nonconserved plastidial targeting sequence is excluded (45 % identity

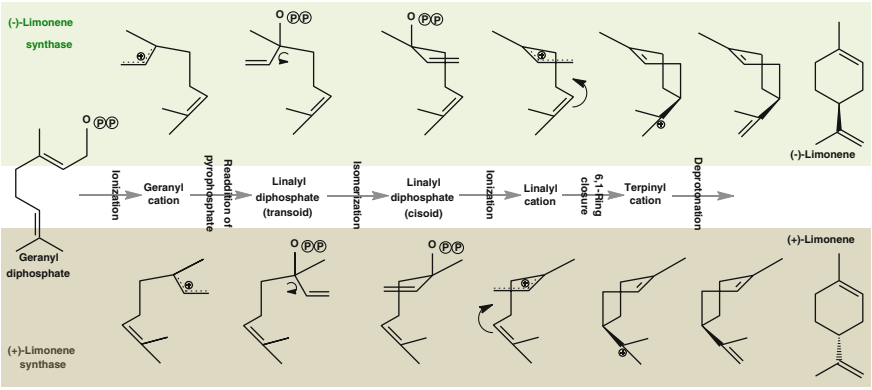


Fig. 2 Proposed reaction mechanism for (−)-limonene synthase in mint (*upper panel*) and (+)-limonene synthase in *Citrus* and caraway (*lower panel*). A helical folding of reaction intermediates is depicted. A mechanism involving extended conformations has also been proposed [36] but is not shown here

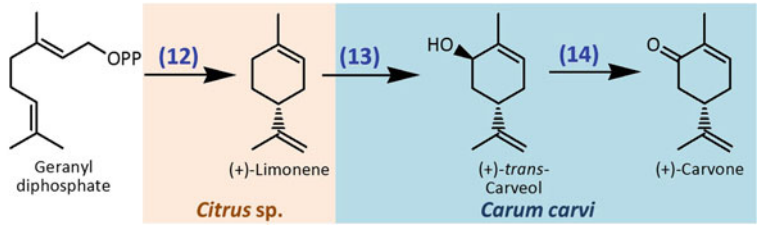


Fig. 3 *p*-Menthane monoterpene biosynthesis in *Citrus* and caraway (*Carum carvi* L.). The enzymes involved in this pathway are *12* (+)-limonene synthase; *13* (+)-limonene 6-hydroxylase; and *14* (+)-*trans*-carveol dehydrogenase

between (−)-limonene synthase (spearmint) and (+)-limonene synthase (orange); [52], the tertiary structures are remarkably similar. Spearmint (−)-limonene synthase shares a common fold, which consists mostly of α-helices with short connecting loops and turns, with all other members of the type I terpene synthases [36]. Limonene synthases have K_m values for geranyl diphosphate in the low micromolar range (12.6 μM for (−)-limonene synthase and 0.7 μM for (+)-limonene synthase) and a low catalytic rate constant (K_{cat} = 0.037 s^{-1} for (−)-limonene synthase; Table 2). The activity of (+)-limonene synthase in caraway has been detected [6] but the enzyme was not further characterized. All limonene synthases tested thus far generate only one enantiomer (either *d*- or *l*-limonene [22]).

Monoterpenoid Hydroxylases. *p*-Menthane monoterpenes are often accumulated as oxygenated metabolites. Hydroxylation reactions on monoterpenoid scaffolds are typically catalyzed by regiospecific cytochrome P450-dependent oxygenases localized to the endoplasmic reticulum (ER). In peppermint, the prevalent reaction is an oxygenation at C3 by (−)-limonene 3-hydroxylase, whereas in

Table 2 Genes and enzymes of *p*-menthane monoterpene biosynthesis

Annotation (Numbers used in figures given in parentheses)	Species	Gene information		Enzyme properties			
		ID	References	ID	Km (μM)	Kcat (s^{-1})	References
(−)-Limonene synthase (1)	*Mentha spicata* L. *Mentha* x *piperita* L. *Mentha arvensis* L.	L13459 EU108697 EF426463	Colby et al. [17] Not characterized Not characterized	EC 4.2.3.16 EC 4.2.3.16 EC 4.2.3.16	12.6 n.a. n.a.	0.037 n.a. n.a.	Williams et al. [91] n.a. n.a.
(−)-Limonene-3-hydroxylase (2)	*Mentha* x *piperita* L. *Mentha* x *piperita* L. *Mentha arvensis* L.	AF124817 (CYP71D13) AF124816 (CYP71D15) EF546776	Lupien et al. [55] Lupien et al. [55] Not characterized	EC 1.14.13.47 EC 1.14.13.47 EC 1.14.13.47	18 18 n.a.	n.a. n.a. n.a.	Karp et al. [40] Karp et al. [40] n.a.
(−)-*trans*-Isopiperitenol dehydrogenase (3)	*Mentha* x *piperita* L. *Mentha arvensis* L.	AY641428 AY641428	Ringer et al. [73] Not characterized	EC 1.1.1.223 EC 1.1.1.223	20 n.a.	n.a. n.a.	Ringer et al. [73] n.a.
(−)-*trans*-Isopiperitenone reductase (4)	*Mentha* x *piperita* L. *Mentha arvensis* L.	AY300162 AY300162	Ringer et al. [72] Not characterized	EC 1.3.1.82 EC 1.3.1.82	1.0 n.a.	1.3 n.a.	Ringer et al. [72] n.a.
(+)-*cis*-Isopulegone isomerase (5)	*Mentha* x *piperita* L.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
(+)-Menthofuran synthase (6)	*Mentha* x *piperita* L.	AF346833	Bertea et al. [5]	EC 1.14.13.104	n.a.	n.a.	n.a.
(+)-Pulegone reductase (7)	*Mentha* x *piperita* L. *Mentha arvensis* L.	AY300163 EF426467	Ringer et al. [72] Not characterized	EC 1.3.1.81 EC 1.3.1.81	2.3 n.a.	1.8 n.a.	Ringer et al. [72] n.a.
(−)-Menthone:(−)-menthol reductase (8)	*Mentha* x *piperita* L.	AY288138	Davis et al. [21]	EC 1.1.1.207	3.0[a] 41[b]	0.6 n.a.	Davis et al. [21] Davis et al. [21]
(−)-Menthone:(+)-neomenthol reductase (9)	*Mentha* x *piperita* L.	AY288137	Davis et al. [21]	EC 1.1.1.208	674[a] >1,000[b]	0.06 n.a.	Davis et al. [21] Davis et al. [21]
(−)-Limonene-6-hydroxylase (10)	*Mentha spicata* L.	AF124815 (CYP71D18)	Lupien et al. [55]	EC 1.14.13.48	20.0	n.a.	Karp et al. [40]
(−)-*trans*-Carveol dehydrogenase (11)	*Mentha spicata* L.	n.a.	Ringer et al. [73]	EC 1.1.1.243	n.a.	n.a.	n.a.

(continued)

Table 2 (continued)

Annotation (Numbers used in figures given in parentheses)	Species	Gene information		Enzyme properties			
		ID	References	ID	Km (μM)	Kcat (s^{-1})	References
(+)-Limonene synthase (12)	*Citrus limon* L. Burm. F. *Citrus limon* L. Burm. F. *Carum carvi* L.	AF514287 (LS1) AF514289 (LS2) n.a.	Lücker et al. [52] Lücker et al. [52] n.a.	EC 4.2.3.20 EC 4.2.3.20 EC 4.2.3.20	0.7 0.7 n.a.	n.a. n.a. n.a.	Lücker et al. [52] Lücker et al. [52] n.a.
(+)-Limonene 6-hydroxylase (13)	*Carum carvi* L. *(annual)* *Carum carvi* L. *(biennial)*	n.a. n.a.	n.a. n.a.	EC 1.14.13.48 EC 1.14.13.48	11.4 14.9	n.a. n.a.	Bouwmeester et al. [7] Bouwmeester et al. [7]
(+)-*trans*-Carveol dehydrogenase (14)	*Carum carvi* L.	n.a.	n.a.	EC 1.1.1.275	n.a.	n.a.	n.a.
1,8-Cineole synthase (15)	*Eucalyptus sideroxylon*	n.a.	Keszei et al. [41]	EC 4.2.3.108	n.a.	n.a.	n.a.
Geraniol synthase (16)	*Eucalyptus sideroxylon*	n.a.	n.a.	EC 3.1.7.11	n.a.	n.a.	n.a.
Geraniol reductase (16)	*Eucalyptus sideroxylon*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Citronellol dehydrogenase (17)	*Eucalyptus sideroxylon*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Note that this table does not provide a comprehensive listing of all gene orthologues. *n.a.* not available

[a] Values given for (−)-(1*R*,4*S*)-menthone as substrate. [b] Values given for (+)-(1*R*,4*R*)-isomenthone as substrate

spearmint the predominant oxygenation occurs at C6 and is catalyzed by (−)-limonene 6-hydroxylase ([85]; Fig. 2). Two genes encoding (−)-limonene 3-hydroxylases, designated as CYP71D13 (1,497 bp) and CYP71D15 (1,503 bp), were cloned from peppermint and characterized following heterologous expression in both yeast [33] and the baculovirus-*Spodoptera* system ([55]; Table 2). The (−)-limonene 6-hydroxylase gene was cloned from spearmint (CYP71D18) and also functionally characterized by heterologous expression [55]. The enzyme has roughly the same size (∼56 kDa) as the peppermint (−)-limonene 3-hydroxylase, but the sequences of these enzymes are only about 70 % identical. These hydroxylase sequences have the conserved features of typical cytochrome P450-dependent oxygenases, including an N-terminal membrane anchor for insertion into the ER, an oxygen-binding domain, a heme-binding motif, and a docking site for cytochrome P450 reductase (which is required for electron transfer from NADPH; [55]). The peppermint genome contains a C6 hydroxylase-like gene but the corresponding transcript is not expressed at appreciable levels. Spearmint expresses a (−)-limonene 3-hydroxylase-like gene but, compared to the 6-hydroxylase transcript, at fairly low levels (A. Ahkami, S. R. Johnson, N. Srividya, and B. M. Lange, unpublished results). The kinetic properties of the C3 and C6 hydroxylases (peppermint and spearmint, respectively) are very similar, with K_m values of 18–20 μM [40]. The gene encoding (+)-limonene 6-hydroxylase from caraway has not been cloned yet, but the kinetic properties (K_m of 11.4 μM for annual and 14.9 μM in biennial caraway) were very similar to those of (−)-limonene 6-hydroxylase from spearmint (Fig. 3; [7]). In contrast to limonene synthases characterized thus far, which generate either *d*- limonene or *l*-limonene stereospecifically [22], both the limonene C3 and C6 hydroxylases accept *d*-limonene as an unnatural substrate (thereby generating (+)-*cis*-carveol as a major product), albeit at lower affinity as natural *l*-limonene [40, 92].

The complex essential oil of peppermint contains, in addition to the major products *l*-menthone and *l*-menthol, (+)-(*R*)-menthofuran as a side product that is preferentially formed under stress conditions (Fig. 1; [10, 16, 74, 75, 89]). The enzyme responsible for the biosynthesis of this metabolite from the main pathway intermediate (+)-(1*R*)-pulegone is also a cytochrome P450-dependent oxygenase of the CYP71 family. The (+)-menthofuran synthase (MFS) gene (also known as (+)-pulegone 9-hydroxylase) was cloned from peppermint (1,479 nucleotides) and encodes a protein of 493 residues (∼55 kDa). The function of this gene was assessed by functional expression/characterization in both *E. coli* and yeast [5]. Its closest homologues are as yet uncharacterized CYP71A family members of the Lamiaceae and Solanaceae (55–58 % identity). Both (+)-(1*R*)-pulegone and (+)-(*R*)-menthofuran are toxic to humans [98] and low levels in essential oils are generally preferable. However, because both metabolites impart a desirable and characteristic camphor-like note if present in small amounts, but carry an off-note in higher amounts, adjustments in the concentrations of these metabolites are critical for the flavor and fragrance industries.

Monoterpenoid Dehydrogenases/Reductases. C3 and C6 hydroxylated monoterpenes are further oxidized in both mint and caraway (Figs. 1 and 3). A cDNA

clone with a 795 bp ORF (corresponding to a 265-residue protein) of the short-chain dehydrogenase/reductase (SDR) superfamily was cloned from peppermint and, following heterologous expression in *E. coli*, demonstrated to encode a 27 kDa protein under denaturing conditions (native protein likely a homodimer or homotetramer). The recombinant protein had relatively high affinity for (−)-(3*S*,4*R*)-*trans*-isopiperitenol and NAD^+ as cosubstrates (K_m values of 72 and 410 μM, respectively) but catalyzes a comparatively slow reaction (K_{cat} of 0.002 s^{-1}; Table 2; [73]). $NADP^+$ was a suitable alternative redox cofactor, but the activity of this dehydrogenase (for which the acronym ISPD was introduced) dropped to about $^1/_{10}$ of that with NAD^+. Interestingly, the affinity of peppermint ISPD for the spearmint pathway intermediate (−)-(4*R*,6*S*)-*trans*-carveol, when compared to the endogenous (−)-(3*S*,4*R*)-*trans*-isopiperitenol substrate, was significantly higher (K_m of 1.8). Furthermore, the reaction velocity with the unnatural substrate was also higher as with the endogenous pathway intermediate (K_{cat} of 0.02 s^{-1}). Various other alcohols occurring in members of the mint family were tested as substrates (NAD^+ as redox cofactor) but the ISPD activity was either very low or undetectable, indicating a notable specificity of this dehydrogenase. The reaction catalyzed by ISPD was irreversible (no reductase activity with either (−)-(4*R*)-*trans*-isopiperitenone or *l*-carvone as substrates).

The identity between putative ISPDs within the genus *Mentha* is >98 %, whereas the next closest SDR sequences in other plant families have approximately 60 % identity. Peppermint ISPD was localized to mitochondria of secretory cells within glandular trichomes using immunohistochemistry, with very little label found in other cell types [85]. An enzymatic activity for the NAD^+-dependent oxidation of various carveol isomers was detected in crude extracts of caraway fruit (highest specific activities for (+)-(4*R*,6*R*)-*trans*-carveol and (−)-(4*R*,6*R*)-*cis*-carveol), but the corresponding gene still awaits identification. The ISPD gene expression levels in secretory cells of both peppermint and spearmint glandular trichomes are relatively high, when compared to all other *p*-menthane pathway genes (A. Ahkami, S. R. Johnson, N. Srividya, and B. M. Lange, unpublished results), and the substrates of ISPD enzymes do not accumulate in the oil, indicating that this step is not rate-limiting for *p*-menthane monoterpene biosynthesis. The dehydrogenase reactions complete the pathway for the biosynthesis of the major spearmint and caraway monoterpenes, whereas further conversions, detailed in the following paragraphs, occur in peppermint.

The C3 oxidation of (−)-(3*S*,4*R*)-*trans*-isopiperitenol to (−)-(4*R*)-*trans*-isopiperitenone in peppermint is followed by a stereospecific reduction of the C_1–C_2 double bond to yield (+)-(1*R*,4*R*)-*cis*-isopulegone (Fig. 1). This reaction is catalyzed by (−)-*trans*-isopiperitenol reductase (ISPR), which is also a member of the SDR superfamily. The ISPR gene (942 bp ORF) codes for a cytosolic protein [86] with 314 residues and a size of approximately 34 kDa, which likely functions as a monomer [72]. ISPR shares fairly high sequence identity with (−)-menthone: (+)-neomenthol reductase (MNR; 64 % at the deduced amino acid level) and (−)-menthone:(−)-menthol reductase (MMR; 63 %) from peppermint (more details below), but is only distantly related to the ISPD gene (30 % identity). The native

ISPR protein, which was partially purified from peppermint glandular trichomes, is selective for NADPH as a redox cofactor (rather than NADH), and shows a high level of regioselectivity (reduction of C_1–C_2 double bond in *p*–menthadien-3-ones) as well as stereoselectivity (hydride attack from *si*-face of C_1–C_2 double bond to yield a product with 1*R* configuration; [18]). The reverse desaturation reaction (with $NADP^+$ as cofactor and (+)-(1*R*,4*R*)-*cis*-isopulegone as substrate) was not detectable. Recombinant ISPR (expressed in and purified from *E. coli*) has high affinity for (−)-(4*R*)-*trans*-isopiperitenone and NADPH (K_m values of 1.0 and 2.2 μM, respectively), with a fairly high turnover rate (*k*cat of 1.3 s^{-1}; [72]). (+)-(1*R*,4*R*)-*cis*-Isopulegone does not accumulate to measurable levels in peppermint essential oil, which is probably due to a combination of the relatively high abundance of ISPR transcript in secretory cells of peppermint glandular trichomes [45] and the comparatively high catalytic efficiency of the corresponding enzyme [72].

Pursuant to the ISPR reaction, an isomerization (not described in more detail here because the corresponding gene has not been identified yet) leads to the formation of (+)-(1*R*)-pulegone, which is then further reduced by (+)-pulegone reductase (PR; Fig. 1). The gene encoding PR consists of an ORF of 1,026 nucleotides, corresponding to a peptide sequence of 342 amino acid residues and a predicted monomeric protein size of about 38 kDa [72]. PR belongs to the medium-chain dehydrogenase/reductase (MDR) superfamily and is highly similar (60–68 % sequence identity) to double-bond reductases (some functionally characterized to act on structurally and biosynthetically unrelated substrates) across the angiosperm lineage. There is only very low sequence identity (below 20 %) between this MDR and the SDRs (ISPD, ISPR, MMR, and MNR) of peppermint *p*-menthane monoterpene biosynthesis. Based on immunohistochemical evidence, PR is localized to the cytosol of glandular secretory cells [85]. Partially purified, native PR (eluting as 45 kDa protein from size-exclusion chromatography) generates a mixture of *l*-menthone (70 %) and *d*-isomenthone (30 %) with (+)-(1*R*)-pulegone as a substrate and NADPH as redox cofactor (no activity with NADH; [72]. Other double-bond–containing *p*-menthane monoterpenes (e.g., (+)-(1*R*,4*R*)-*cis*-isopulegone or (+)-(4*S*)/(−)-(4*R*)-piperitone) were not suitable substrates and the reverse reaction (oxidation of *l*-menthone or *d*-isomenthone with $NADP^+$ as oxidant) was not observed. Interestingly, the partially purified, recombinant PR (assayed under the same conditions as the native enzyme with (+)-(1*R*)-pulegone and NADPH) yields *l*-menthone and *d*-isomenthone in a 55:45 ratio. As possible reasons for this discrepancy, a lack of posttranslational modifications of the recombinantly expressed protein or the involvement of a yet to be characterized epimerase converting *d*-isomenthone to *l*-menthone, have been discussed before [19], but the issue has remained unresolved. PR has a high affinity for the substrate and redox cofactor (K_m values of 2.3 and 6.9 μM for (+)-(1*R*)-pulegone and NADPH, respectively) and its activity is characterized by a relatively high reaction rate (k_{cat} of 1.8 s^{-1}). (+)-(1*R*)-Pulegone accumulates in the oil of peppermint (usually at 0.5–3.0 % in field-grown plants) which, considering the rather high enzymatic efficiency, likely reflects the relatively low amount of PR enzyme (when compared to the preceding enzymes ISPD and ISPR) present in glandular trichomes [59].

The last set of reactions of the *p*-menthane monoterpene biosynthetic pathway in peppermint is catalyzed by NADPH-dependent monoterpenoid keto reductases (Fig. 1; [42]). MMR and MNR are of similar size (1,096 and 1,131 nucleotide ORFs; 311 and 324 residue polypeptides; molecular weights of approximately 34 and 36 kDa, respectively). The sequences of MMR and MNR share 73 % identity and, as mentioned before, are almost equally similar to ISPR (63 and 64 %, respectively). Recombinant MMR converts *l*-menthone to *l*-menthol (95 %) and (+)-(1*R*,3*S*,4*S*)-neomenthol (syn. *d*-neomenthol; 5 %; K_m values of 3.0 and 0.12 μM for the substrate and redox cofactor, respectively; k_{cat} value of 0.6 s^{-1}), whereas (+)-(1*R*,3*S*,4*R*)-neoisomenthol (syn. *d*-neoisomenthol; 87 %) and *d*-isomenthol (13 %) are formed from *d*-isomenthone as a substrate (K_m value of 41 μM; [21]). The reactions catalyzed by MMR are irreversible (no oxidation of alcohols in the presence of $NADP^+$).

MNR catalysis leads to an essentially opposite product profile, when compared to that of MMR, but with dramatically reduced substrate affinity and reaction velocity. When *l*-menthone is used as a substrate in in vitro assays with the recombinant MNR enzyme (K_m value of 647 μM; k_{cat} value of 0.06 s^{-1}), *d*-neomenthol is the major product (94 %) and *l*-menthol a side product (6 %). With *d*-isomenthone as a substrate (K_m value of >1 mM), *d*-isomenthol (86 %) and *d*-neoisomenthol (14 %) are generated [21]. MMR is localized to the cytosol and nuclei of secretory cells in glandular trichomes at the later stages of peppermint leaf development [86]. This is consistent with the gene expression and enzyme activity profiles, which both indicate a late appearance of MMR during essential oil accumulation [21, 45, 59]. A combination of substrate availability (concentration of *l*-menthone is significantly higher than that of *d*-isomenthone in secretory cells) and the catalytic properties of the keto reductases (enzymatic efficiency of MMR is substantially higher than that of MNR) lead to the accumulation of predominantly *l*-menthol in the essential oil of peppermint, whereas the other alcohols are only minor constituents [74, 75].

***Eucalyptus p*-Menthane Monoterpene Pathway**. The principal monoterpene of *Eucalyptus* essential oils is 1,8-cineole (Fig. 4), which accumulates to >50 % (v/v)

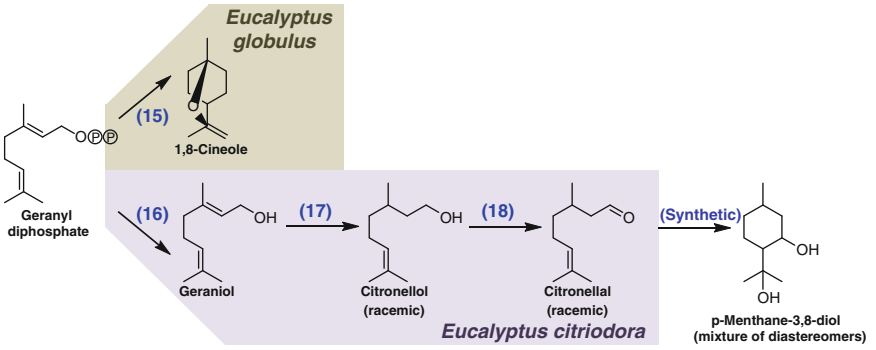


Fig. 4 *p*-Menthane monoterpene biosynthesis in *Eucalyptus* species. The enzymes involved in this pathway are *15* 1,8-cineole synthase; *16* geranyl diphosphate diphosphatase; *17* geraniol reductase; and *18* citronellol dehydrogenase

in commercial cultivars. A cDNA clone for a 1,8-cineole synthase was obtained recently and characterized by functional expression in *E. coli*. [41]. The recombinant protein released a mixture of monoterpenes with 1,8-cineole and linalool as major products, and α-terpineol, limonene, myrcene, sabinene, and ocimene (in decreasing amounts) as minor products. Interestingly, these metabolites also accumulate in the oil, although not in the same proportions as produced by recombinant 1,8-cineole synthase [41]. Lemon eucalyptus accumulates high levels of a mixture of (+)-(*R*)- and (−)-(*S*)-citronellal. Commercially, these metabolites are converted, by synthetic chemistry, into insecticidal *p*-menthane-3,8-diol (PMD; [97]. As a first step, the biosynthetic pathway involves the hydrolysis of the diphosphate group from geranyl diphosphate (Fig. 4). Geraniol synthases catalyzing this reaction have been cloned and characterized from various angiosperms [37, 38, 81, 94] but not within the Myrtaceae. The following double-bond reduction is catalyzed by geraniol reductase, the activity of which has been detected in the Rosids and Vitales [3, 26, 51], but the gene coding for this enzyme is still awaiting discovery. Finally, the (+)-(*R*)/(−)-(*S*)-citronellol mixture is oxidized to the corresponding aldehydes by citronellol dehydrogenase. Such an activity has been detected in several members of the genus *Pseudomonas*, which can utilize citronellal and other monoterpenes as carbon sources [30], but to date has not been described in plants.

4 Metabolic Engineering of the *p*-Menthane Monoterpene Biosynthetic Pathway

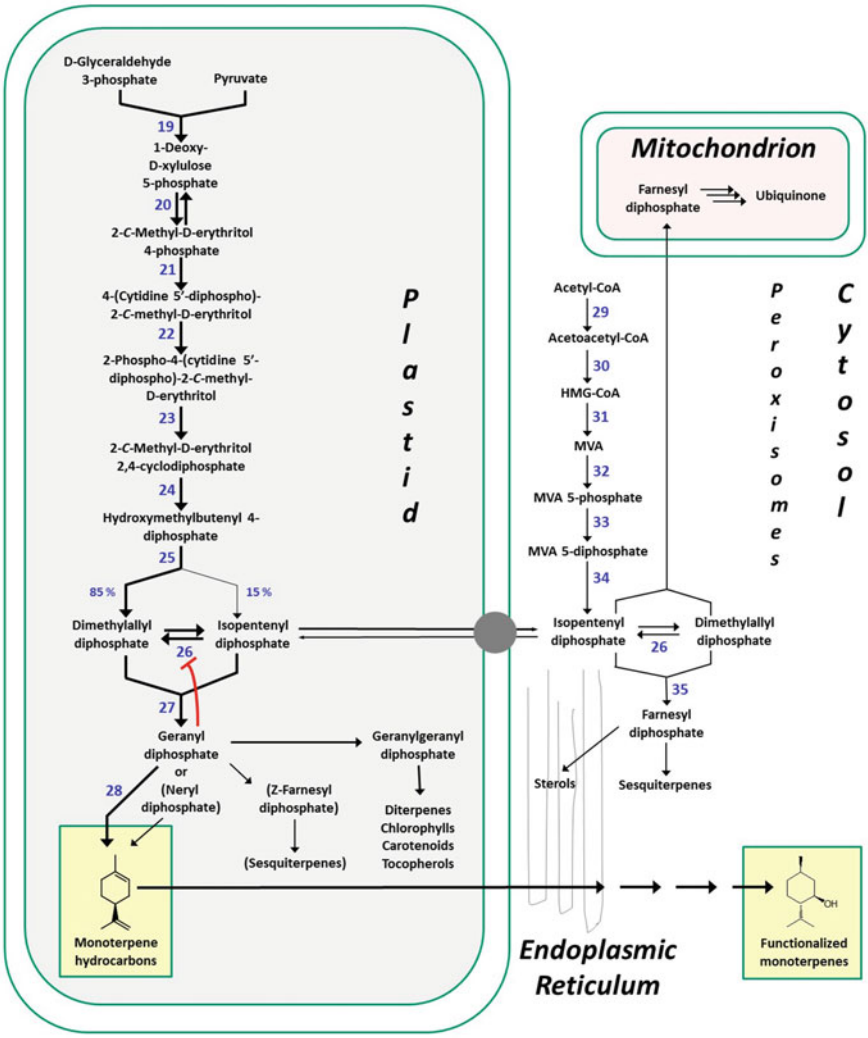
Fundamental Challenges with Monoterpene Storage. Larger amounts of volatile and semivolatile plant terpenoids (C_5 to C_{20} metabolites) are generally accumulated in intraorganellar structures that are surrounded by a membrane (e.g., oil bodies; [68]), larger extracellular storage structures (e.g., resin ducts, laticifers, secretory cavities, or glandular trichomes; [28]), or are emitted into the atmosphere [31]. These sequestration mechanisms likely evolved because of the cytotoxicity of many terpenoids, which can play important roles in plant defense but need to be separated from cellular metabolism. Monoterpenes have been demonstrated to affect the properties of both soluble and membrane-associated enzymes negatively, interfere with the action of intra- and intercellular transporters, and increase the permeability of membranes [80]. In most plant cells, monoterpenes are derived from precursors synthesized in plastids via the methylerythritol 4-phosphate (MEP) pathway. The same pathway operates in the majority of eubacteria (including *E. coli* as a common host for synthetic biology). In contrast, yeast terpenoids are derived from the mevalonate pathway, which is also the primary pathway for the biosynthesis of sterols and sesquiterpenes in plants ([34]; Fig. 5). Metabolic crosstalk, that is, the exchange of terpenoid intermediates between plastidial and cytosolic pathways, has been described in plants, but the extent, direction of carbon flux, and relevance of

their intracellular transport for specific terpenoid end products is highly dependent on the plant species, the tissue of the cell type under investigation, and the environmental context [34].

The functionalization of monoterpene hydrocarbons usually commences with oxygenation reactions, often catalyzed by ER-localized cytochrome P450-dependent oxygenases [47]. Further modifications, including, among others, redox, desaturation, and conjugation reactions, occur mostly through catalysis by cytosolic or ER-associated enzymes (or less commonly mitochrondrial proteins). The final step, which involves either the volatilization or secretion/storage of monoterpenes, is only poorly understood [47]. In summary, there are three critical issues that need to be addressed when attempting to increase the amounts of specific monoterpenes in plants or microbes using biotechnological approaches: (1) volatility/storage, (2) cytotoxicity, and (3) precursor supply/compartmentation. In the following paragraphs, I highlight successes, but also mention limitations, of metabolic engineering efforts to increase the accumulation of monoterpenes.

Transformation of Commercial Cultivars to Accumulate High-Value *p*-Menthane Monoterpenes. Methods for the transformation of plant cultivars that naturally accumulate *p*-menthane monoterpenes were first developed in the 1990s. Most of these protocols (only early pioneering studies of gene introduction are listed here) involved the cocultivation of explants with *Agrobacterium* and the subsequent regeneration of transgenic plants, and were successful with *Mentha* [4, 11, 23, 64], *Carum* [44], *Citrus* [61, 88], and *Eucalyptus* [35, 62, 63]. Transient transformations using biolistic treatments were also reported [11, 76, 79, 96]. Although these methods are viable on an experimental scale, the transformation efficiency with these plant genera is universally low and the regeneration of transgenic plants with desirable traits fairly slow.

Despite these challenges, significant progress has been made with engineering mint for improved essential oil characteristics. Genes involved in precursor supply and at the interface toward monterpene end products (MEP and early *p*-menthane pathways) (Fig. 5) were overexpressed to increase flux and thereby increase oil yield. For several genes this approach was successful (1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), isopentenyl diphosphate isomerase, geranyl diphosphate synthase), whereas increased transcript abundance in transgenic plants was not associated with yield gains for other genes (1-deoxy-D-xylulose 5-phosphate synthase and (−)-limonene 3-hydroxylase; Table 3; [24, 46, 56, 57]; all in peppermint). Results for (−)-limonene synthase overexpression were variable, with some authors reporting yield enhancements ([24]; in peppermint), whereas others did not detect improvements in yield (Table 3; [43], peppermint; [24], cornmint; [46], peppermint). The Croteau laboratory pursued compositional adjustments by decreasing the expression levels of genes with relevance for the formation of undesirable side products. The expression of the MFS gene (gene (6) in Fig. 1) in antisense orientation (MFS7A line) led to a decrease in the concentrations of, as expected, (+)-(*R*)-menthofuran but also, unexpectedly, (+)-(1*R*)-pulegone in transgenic peppermint plants (Table 3; [56]). It seemed counterintuitive that the concentration of the substrate for a downregulated enzyme would decrease and a



plausible explanation did not emerge until my laboratory developed a kinetic mathematical model of monoterpenoid essential oil biosynthesis [74]. Simulations indicated that the observed monoterpene profiles could potentially be explained if (+)-(*R*)-menthofuran acted as a competitive inhibitor of (+)-pulegone reductase, an hypothesis that was subsequently confirmed by experimental testing [74]. A second surprising finding was that the MFS7A line had an increased essential oil yield [56], although this gene was the only one found to be modulated in MFS7A and appeared to be unrelated to overall carbon flux into monoterpenes. Simulations with a second-generation mathematical model, which we had developed also to assess the determinants of yield, suggested that differences in the size distribution of glandular trichomes could explain the observed yield gain [75].

◀**Fig. 5** Outline of terpenoid biosynthesis in plants with an emphasis on the cellular context of *p*-menthane monoterpene formation. The generally higher flux observed for the plastidial methylerythritol phosphate (MEP) pathway, in comparison to the mevalonate (MVA) pathway, which is localized the cytosol, ER, and peroxisomes, is indicated by *thicker reaction arrows* (peroxisome not depicted for clarity). A transporter for the exchange of isoprenoid intermediates across the plastidial membrane is indicated by a *gray circle*. Abbreviations for metabolites: *CoA* coenzyme A; *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA; *MVA* mevalonate. Enzyme numbering: *19* 1-deoxy-D-xylulose 5-phosphate synthase; *20* 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *21* 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; *22* 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase; *23* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *24* (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate synthase; *25* (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; *26* isopentenyl diphosphate isomerase; *27* geranyl diphosphate synthase; *28* monoterpene synthase; *29* acetoacetyl-CoA thiolase; *30* 3-hydroxy-3-methyl-glutaryl-CoA synthase; *31* 3-hydroxy-3-methylglutaryl-CoA reductase; *32* mevalonate kinase; *33* phosphomevalonate kinase; *34* mevalonate diphosphate decarboxylase; *35* (*E*,*E*)-farnesyl diphosphate synthase. Plastidial pathways for the biosynthesis of monoterpenes via neryl diphosphate and certain sesquiterpenes via (*Z*,*Z*)-farnesyl diphosphate, as recently identified in the Solanaceae, are depicted as well. However, the relevance of these pathways for the biosynthesis of commercially harvested *p*-menthane monoterpenes has not yet been established [47]. Note that the specialized cell types involved in the biosynthesis of monoterpenes in glandular trichomes and secretory cavities generally have, with only a few exceptions, nongreen plastids that do not produce chlorophylls and carotenoids from plastidial geranylgeranyl diphosphate at the time of terpenoid accumulation

Follow-up experiments demonstrated that critical aspects of the pattern of glandular trichome development were altered in MFS7A, where these structures were more numerous and matured earlier than in wild-type controls, thus leading to higher essential oil amounts independent of gene expression patterns within secretory cells of glandular trichomes [75]. Essential oil yield gains did not correlate directly with the expression patterns of biosynthetic genes but were achieved through the initiation of more glandular trichomes, possibly by an as yet uncharacterized feedback mechanism resulting from gene overexpression. By transforming peppermint with two genes, one demonstrated before to affect yield (DXR) positively and one with beneficial effects on both yield and composition (MFS), we then generated transgenic plants with significant yield increases (up to 80 % higher than wild-type) and highly desirable oil profiles (high *l*-menthone and *l*-menthol; low (+)-(*R*)-menthofuran and (+)-(1*R*)-pulegone), as demonstrated in multiyear field trials (Table 3; [46]).

We have also employed peppermint as a host for biotechnological efforts to accumulate other *p*-menthane monoterpenes that occur only at very low levels in this plant. We were particularly interested to investigate if metabolic engineering was a viable approach to eliminate oxygenated constituents from the oil, thereby generating mixtures of monoterpene hydrocarbons with physicochemical properties desirable for the development of drop-in biofuels. Our strategy took advantage of a line (termed PM20) in which the limonene 3-hydroxylase gene was cosuppressed, thus resulting in an overaccumulation of *l*-limonene in the oil [57]. The PM20 line was transformed with additional constructs for the overexpression of various

terpene synthases to generate transgenic lines with elevated levels of (*S*)-(+)-linalool, (+)-(1*R*,5*R*)-sabinene, and α/β-pinene [46]. Further advances can be expected from the use of glandular trichome-specific promoters (to increase the strength and specificity of transcript abundance in the cell types that are most relevant for essential oil biosynthesis) and global regulators of the *p*-menthane monoterpene pathway (to activate more than one or two genes at a time). Early work in this area has recently been summarized, and comments on opportunities and current constraints have been provided elsewhere [48].

The vast majority of studies with transgenic *Citrus* plants were performed to enhance the resistance to biotic and abiotic stresses [82], which is outside the scope of this review chapter. Very little effort has been spent on modulating the essential oils accumulated in fruit peel. One of the reasons is that, depending on the *Citrus* cultivar, the oil is already enriched in a desirable, high-value *p*-menthane monoterpene (*d*-limonene at up to 97 % of the oil). It is also important to note that monoterpenoid fruit volatiles play critical roles in the interaction between plants and beneficial as well as disease-causing organisms [78]. Interestingly, when the (+)-limonene synthase gene was downregulated in orange- using antisense technology, the resulting transgenic plants, which accumulated dramatically reduced peel oil, were significantly more resistant (when compared to appropriate controls) to a bacterial and a fungal pathogen, and males of the citrus pest medfly were less attracted to the fruit, thus decreasing feeding damage [77].

Ohara et al. [66] compared the effects of transforming *Eucalyptus camaldulensis* with two different constructs for the expression of a (−)-limonene synthase gene from *Perilla frutescens* (Table 3). One construct was designed to direct the gene product to the cytosol, and the other targeted the enzyme to plastids. As mentioned before, the precursors for monoterpenes, geranyl diphosphate (or neryl diphosphate in some plants), are generated in plastids [48]. The essential oil of *Eucalyptus* accumulates primarily 1,8-cineole, which is generated by a plastidial monoterpene synthase [41]. One would thus expect that a plastidial localization of an introduced monoterpene synthase should result in the generation of higher amounts of the corresponding monoterpene when compared to a cytosolic localization that would require translocation of geranyl diphosphate to the cytosol). Surprisingly, Ohara et al. [66] observed a higher (−)-limonene production with the construct for cytosolic localization of the gene product (Table 3). Even more surprisingly, it was also reported that enhanced (−)-limonene concentrations in transgenic plants strongly correlated with an increased accumulation of the plastidially generated *p*-menthane monoterpenes 1,8-cineole and α-pinene [66]. The causes for these highly unexpected findings were not further investigated.

Accumulation of High-Value *p*-Menthane Monoterpenes in Heterologous Plant Hosts. Although some plants that accumulate valuable essential oils might be amenable to metabolic engineering, the transformation efficiencies, as mentioned above, are generally fairly low and alternative plant hosts have been evaluated for biotechnological efforts to produce specific *p*-menthane monoterpenes. A (−)-limonene synthase gene from *Perilla frutescens* was transformed into tobacco using three different constructs designed to direct the gene product to either plastids, the

Table 3 Metabolic engineering of *p*-menthane monoterpene biosynthesis in commercial cultivars (representative examples)

Engineered species	Introduced gene	Promoter	Targeting of gene product	Primary *p*-menthane monoterpene targets	Outcomes	References
Mentha x *piperita* L.	1-Deoxy-D-xylulose 5-phosphate reductoisomerase	CaMV 35S (ubiquitous)	Plastid	(−)-(1*R*,4*S*)-Menthone (−)-(1*R*,3*R*,4*S*)-Menthol	44 % oil yield increase	Mahmoud et al. [56]
	Isopentenyl diphosphate isomerase	CaMV 35S (ubiquitous)	Plastid	(−)-(1*R*,4*S*)-Menthone (−)-(1*R*,3*R*,4*S*)-Menthol	26 % oil yield increase	Lange et al. [46]
	Geranyl diphosphate synthase	CaMV 35S (ubiquitous)	Plastid	(−)-(1*R*,4*S*)-Menthone (−)-(1*R*,3*R*,4*S*)-Menthol	18 % oil yield increase	Lange et al. [46]
	(+)-(*R*)-Menthofuran synthase (antisense orientation)	CaMV 35S (ubiquitous)	n.a.	(+)-(*R*)-Menthofuran (decrease)	35 % oil yield increase (+)-(*R*)-Menthofuran < 3 % of oil (+)-(1*R*)-Pulegone < 1 % of oil	Mahmoud et al. [56]
	1-deoxy-D-xylulose 5-phosphate reductoisomerase and (+)-(*R*)-Mentho-furan synthase (antisense orientation)	CaMV 35S (ubiquitous) CaMV 35S (ubiquitous)	Plastid n.a.	(−)-(1*R*,4*S*)-Menthone (−)-(1*R*,3*R*,4*S*)-Menthol (+)-(*R*)-Menthofuran (decrease)	61 % oil yield increase (+)-(*R*)-Menthofuran < 2 % of oil (+)-(1*R*)-Pulegone < 1 % of oil	Lange et al. [46]
	(−)-Limonene 3-hydroxylase (cosuppression)	CaMV 35S (ubiquitous)	n.a.	(−)-(4*S*)-Limonene	(−)-(4S)-Limonene 79 % of oil (1–2 % in wild-type controls)	Mahmoud et al. [57]
Citrus sinensis L.	(−)-Limonene synthase (antisense orientation)	CaMV 35S (ubiquitous)	n.a.	(−)-(4*S*)-Limonene (decrease)	Enhanced resistance against *Penicillium digitatum*	Rodríguez et al. [77]

(continued)

Table 3 (continued)

Engineered species	Introduced gene	Promoter	Targeting of gene product	Primary *p*-menthane monoterpene targets	Outcomes	References
Eucalyptus camaldulensis Dehnh.	(−)-Limonene synthase	CaMV 35S (ubiquitous)	Cytosol	(−)-(4*S*)-Limonene	(−)-(4*S*)-Limonene 0.033 % of fresh weight (control: 0.001 %) 1,8-Cineole 0.16 % of fresh weight (control: 0.02 %) α-Pinene 0.016 % of fresh weight (control: 0.002 %)	Ohara et al. [66]
		CaMV 35S (ubiquitous)	Plastid	(−)-(4*S*)-Limonene	(−)-(4*S*)-Limonene 0.02 % of fresh weight (control: 0.001 %) 1,8-Cineole 0.08 % of fresh weight (control: 0.02 %) α-Pinene 0.016 % of fresh weight (control: 0.02 %)	

CaMV cauliflower mosaic virus; *n.a.* not applicable

cytosol, or ER membranes (Table 4; [65]). Transgenic plants carrying the construct for the plastidial localization of the enzyme had the highest (−)-limonene levels with up to 143 ng/g fresh weight (corresponding to roughly 0.0001 % of dry weight biomass). Lücker et al. [53] transformed tobacco with three terpene synthase genes from *Citrus* encoding (+)-limonene synthase, γ-terpinene synthase, and (−)-β-pinene synthase. The corresponding monoterpenes, (+)-limonene, γ-terpinene, and (−)-β-pinene, were emitted by transgenic lines (termed TERLIMPIN) at 70–1,200 ng/g fresh weight within a 24-h period (depending on developmental stage) (Table 4). Over longer periods of time, substantial amounts of monoterpenes were produced in these transgenic plants but, due to their volatilization, would not be harvestable in a commercially meaningful fashion. When TERLIMPIN plants were additionally transformed with a putative limonene 3-hydroxylase gene from curly mint, the 3-hydroxylated metabolite (+)-*trans*-isopiperitenol was emitted from flowers at 400 ng/g fresh weight within a 24-h period (Table 4) [54]. Notably, various other *p*-menthane monoterpenes, particularly *p*-cymene and isomers of menthatriene and isopiperitenone, were also formed, by as yet uncharacterized conversions, in these transgenic plants.

In lemon eucalyptus, geraniol is a biosynthetic precursor of (+)-(*R*)/(−)-(*S*)-citronellal, which itself is converted synthetically to PMD. Two independent attempts to produce geraniol in a heterologous plant host have been published. A geraniol synthase gene from sweet basil was transformed into tomato and expressed under control of the fruit-specific polygalacturonidase promoter [20]. A larger number of monoterpenes, including some that are derived from geraniol (e.g., geraniol, geranic acid, citronellol, neral) and others that are not (e.g., myrcene, limonene, and β-ocimene), were produced in fruit of transgenic plants at about 0.0004 % of fruit fresh weight (Table 4). In addition to releasing metabolic turnover products, the transgenic fruit were deficient in carotenoids, most likely due to a diversion of flux from a common precursor (geranyl diphosphate) to monoterpenes (Fig. 5; [20]).

A geraniol synthase from *Lippia dulcis* was expressed under the control of the ubiquitin promoter in transgenic maize plants [95]. Novel volatiles were not generated at detectable levels, but various glycosylated, acetylated, malonylated, and oxidized derivatives of geraniol accumulated (only geranoyl-6-*O*-malonyl-β-D-glucose was quantified at 0.0017 % of leaf fresh weight; Table 4). Fischer et al. [29] expressed a geraniol synthase from sweet basil in several different plant hosts. In *Arabidopsis thaliana*, geraniol was the only newly formed monoterpene in transgenic plants (0.0002 % of fresh weight). In tobacco, linalool, and nerol were found in addition to geraniol, whereas grapevine produced citronellol and nerol as side products (Table 4; [29]). The authors hypothesized that the terpene synthase activity can be modulated depending on the cellular context within the host organism. However, because side products accumulated only at very low levels, it is also conceivable that other endogenous activities were induced in transgenic plants (which was not investigated). In summary, these results indicate that in heterologous plant hosts expressing terpene synthases the initially formed catalytic products can be subjected to as yet unpredictable metabolic turnover.

Table 4 Metabolic engineering of *p*-menthane monoterpene biosynthesis in heterologous plant hosts

Engineered species	Introduced gene	Promoter	Targeting of gene product	Primary *p*-menthane monoterpene targets	Outcomes	References
Nicotiana tabacum L.	(−)-Limonene synthase	CaMV 35S (ubiquitous)	Cytosol	(−)-(4*S*)-Limonene	(−)-(4*S*)-Limonene 0.000004 % of fresh weight (control: n.d.)	Ohara et al. [65]
		CaMV 35S (ubiquitous)	Plastid	(−)-(4*S*)-Limonene	(−)-(4*S*)-Limonene 0.00001 % of fresh weight (control: n.d.)	
		CaMV 35S (ubiquitous)	ER	(−)-(4*S*)-Limonene	(−)-(4*S*)-Limonene n.d.	
	(+)-Limonene synthase	CaMV 35S (ubiquitous)	Plastid	(+)-Limonene	1,200 ng g^{-1} 24 h^{-1} (max. emission)	Lücker et al. [53]
	γ-Terpinene synthase	CaMV 35S (ubiquitous)	Plastid	γ-Terpinene	630 ng g^{-1} 24 h^{-1} (max. emission)	
	(−)-β-Pinene synthase	CaMV 35S (ubiquitous)	Plastid	(−)-β-Pinene	380 ng g^{-1} 24 h^{-1} (max. emission)	
	(+)-Limonene synthase	CaMV 35S (ubiquitous)	Plastid	(+)-Limonene	Roughly the same as in Lücker et al. [53]	Lücker et al. [54]
	γ-Terpinene synthase	CaMV 35S (ubiquitous)	Plastid	γ-Terpinene	Roughly 66 % lower as in Lücker et al. [53]	
	(−)-β-Pinene synthase	CaMV 35S (ubiquitous)	Plastid	(−)-β-Pinene	Roughly the same as in Lücker et al. [53]	
	limonene 3-hydroxylase	CaMV 35S (ubiquitous)	ER	(+)-*trans*-Isopiperitenol	400 ng g^{-1} 24 h^{-1} (max. emission) (other monoterpenes also formed)	
	Geraniol synthase	CaMV 35S (ubiquitous)		Geraniol	Geraniol at 0.001 % of fresh weight (linalool and nerol formed)	Fischer et al. [29]

(continued)

Table 4 (continued)

Engineered species	Introduced gene	Promoter	Targeting of gene product	Primary *p*-menthane monoterpene targets	Outcomes	References
Solanum lycopersicum L.	Geraniol synthase	Polygalacturonase (fruit-specific)	Plastid	Geraniol	Geraniol and other monoterpenes formed at 0.0004 % of fresh weight; carotenoid deficiency	Davidovich-Rikatani et al. [20]
Zea mays subsp. *mays* L.	Geraniol synthase	Ubiquitin (ubiquitous)	Plastid	Geraniol	Only geraniol derivatives (but not geraniol) detected (metabolites mostly not quantified);	Yang et al. [95]
Arabidopsis thaliana L. Heynh.	Geraniol synthase	CaMV 35S (ubiquitous)		Geraniol	Geraniol at 0.0002 % of fresh weight	Fischer et al. [29]
Vitis vinifera L.	Geraniol synthase	CaMV 35S (ubiquitous)		Geraniol	Geraniol at 0.0005 % of fresh weight	Fischer et al. [29]

CaMV cauliflower mosaic virus; *ER* endoplasmic reticulum

Accumulation of High-Value *p*-Menthane Monoterpenes in Microbial Hosts

Tremendous progress has been made in recent years to develop microbial platform strains for the introduction of plant pathways, including those leading to terpenoids [93]. Considering the challenges with the high-level production of monoterpenes in engineered plants, microbial hosts are an obvious choice for monoterpene biotechnology. The genomes of typical engineering strains of *E. coli* and baker's yeast, which are the most common hosts due to the availability of a whole range of tools for synthetic pathway construction, are devoid of genes for the biosynthesis of monoterpenes. The first introduction of genes for an entire plant pathway into *E. coli* was performed by Carter et al. [12]. The authors generated cassettes for the single and combined expression (as polygenic operons) of genes encoding geranyl diphosphate synthase (N-terminally truncated version from *Abies grandis*), (−)-limonene synthase (N-terminally truncated version from *Mentha spicata*), (−)-limonene 6-hydroxylase (from *Mentha spicata*) fused to NADPH:cytochrome P450 reductase (from *Mentha* x *piperita*), and (−)-*trans*-carveol dehydrogenase (*Mentha spicata*), thus transferring the pathway used for the biosynthesis of *l*-carvone in spearmint. An engineered *E. coli* strain expressing only the geranyl diphosphate synthase and (−)-limonene synthase genes released *l*-limonene at 5 mg/l into the culture medium (Table 5; [12]). Interestingly, when all genes were coexpressed in engineered *E. coli*, the results remained the same (5 mg/l *l*-limonene). The authors hypothesized that two factors may have contributed to these undesirable results: (1) the *l*-limonene substrate might not be formed at sufficiently high levels (40 μM at the end of a 24-h growth period) and (2) *l*-limonene is secreted, which further decreases the amounts of available substrate for the hydroxylase. *l*-Limonene was then added to the culture medium (at 1 mM) and small amounts of *l*-carvone (2 μM) were produced. Although a proof-of-concept stage was achieved, the data indicated that secretion of *l*-limonene remained the most problematic challenge [12].

For the higher-level production of a functionalized monoterpene, Alonso-Gutierrez et al. [2] implemented several strain improvements. The supply of terpenoid precursors was enhanced through the introduction of a synthetic MVA pathway (with genes from various sources), thereby evading the tight regulation of the endogenous MEP pathway in the *E. coli* host (Fig. 6). In addition to a construct carrying codon-optimized variants of the genes encoding MVA pathway enzymes, geranyl diphosphate synthase (N-terminally truncated version from *Abies grandis*) and (−)-limonene synthase (N-terminally truncated version from *Mentha spicata*), the authors also introduced a second cassette carrying genes coding for a cytochrome P450-dependent oxygenase previously demonstrated to hydroxylate *l*-limonene at C7 [87], ferredoxin, and ferredoxin reductase (as electron carriers; all genes from *Mycobacterium* HXN 1500; Fig. 6).

In a rich medium containing 1 % glucose as the carbon source, the strain produced *l*-limonene at 400 mg/l and (−)-(4*S*)-perillyl alcohol at 100 mg/l (Table 5; [2]). These levels were significantly higher than those achieved before but the high concentrations of *l*-limonene indicate that secretion of this intermediate and/or inefficient turnover to the desired end product were still limiting factors. For the *E. coli* based production of simple monoterpene hydrocarbons as potential biofuels,

Table 5 Metabolic engineering of *p*-menthane monoterpene biosynthesis in microbial hosts

Engineered species	Introduced genes (Source)	Modifications	Primary *p*-menthane monoterpene targets	Outcomes	References
Escherichia coli	Geranyl diphosphate synthase (*Abies grandis*)	5′-Truncated (gene product lacks plastidial targeting sequence)	(−)-(4*R*)-*trans*-Carvone	(−)-(4*S*)-Limonene at 5 mg/l (no (−)-(4*R*)-*trans*-carvone)	Carter et al. [12]
	(−)-Limonene synthase (*Mentha spicata*)	5′-Truncated (gene product lacks plastidial targeting sequence)			
	(−)-Limonene 6-hydroxylase (*Mentha spicata*)–NADPH:cytochrome P450 reductase (*Mentha* x *piperita*)	Gene fusion			
	(−)-*trans*-carveol dehydrogenase (*Mentha spicata*)				
	Acetoacetyl-CoA thiolase (*Escherichia coli*)		(−)-(4*S*)-Perillyl alcohol	(−)-(4*S*)-Limonene at 400 mg/l; (−)-(4*S*)-Perillyl alcohol at 100 mg/l	van Beilen et al. [87]
	3-Hydroxy-3-methyl-glutaryl-CoA synthase (*S. aureus*)	Codon-optimized			
	3-Hydroxy-3-methylglutaryl-CoA reductase (*S. aureus*)	5′-Truncated; (gene product soluble rather than ER-localized) Codon-optimized			
	Mevalonate kinase (*Saccharomyces cerevisiae*)	Codon-optimized			
	Phosphomevalonate kinase (*S. cerevisiae*)	Codon-optimized			

(continued)

Table 5 (continued)

Engineered species	Introduced genes (Source)	Modifications	Primary *p*-menthane monoterpene targets	Outcomes	References
	Mevalonate diphosphate decarboxylase (*S. cerevisiae*)	Codon-optimized			
	Isopentenyl diphosphate synthase (*E. coli*)				
	Geranyl diphosphate synthase (*Abies grandis*)	5′-Truncated; Codon-optimized			
	(−)-Limonene synthase (*Mentha spicata*)	5′-truncated; Codon-optimized			
	CYP153 (Mycobacterium HXN 1500)				
	Ferredoxin (Mycobacterium HXN 1500)				
	Ferredoxin reductase (Mycobacterium HXN 1500)				
Saccharomyces cerevisiae	Farnesyl diphosphate synthase (*S. cerevisiae*) Geraniol synthase (*Ocimum basilicum*)	Mutated gene sequence	Geraniol	Geraniol at 5 mg/l	Oswald et al. [67] Fischer et al. [29]

Dunlop et al. [25] screened a library of efflux pumps for the secretion of various hydrophobic molecules. In this case, a secretion of nonfunctionalized terpenoids is actually desired. A construct very similar to that mentioned above (carrying genes coding for the MVA pathway enzymes, geranyl diphosphate synthase and *l*-limonene synthase) was combined with the expression of a suitable efflux pump (from *Alcanivorax borkumensis*). Controls (same constructs but lacking the pump) released *l*-limonene at 35 mg/l, whereas strains that also expressed the gene for the efflux pump produced the metabolite at 55 mg/l, indicating that product export is a viable strategy for the increased accumulation of monoterpene hydrocarbons (Table 5). The Karst laboratory explored yeast as an expression host for plant monoterpene biosynthetic genes. To accomplish this goal, a mutant farnesyl diphosphate synthase (FPPS) was developed that releases, in addition to the natural product farnesyl diphosphate, the shorter-chain geranyl diphosphate and derived hydrolysis products (geraniol and linalool; [13]. The release of geraniol from a yeast strain carrying the mutant FPPS was further increased by introducing a geraniol synthase gene from sweet basil [67]. Further optimization of the FPPS to produce high amounts of geranyl diphosphate, while maintaining sufficient farnesyl diphosphate levels for continued growth, resulted in strains that released geraniol at up to 5 mg/l (with linalool and citronellol as side products; Table 5) [29]. Brennan et al. [8] reported on two-phase extractive fermentation systems that trap monoterpenes in an organic phase, thus mitigating issues with the toxicity of these metabolites. It is now instructive to test if this approach is also viable when employed with engineered monoterpene-producing strains.

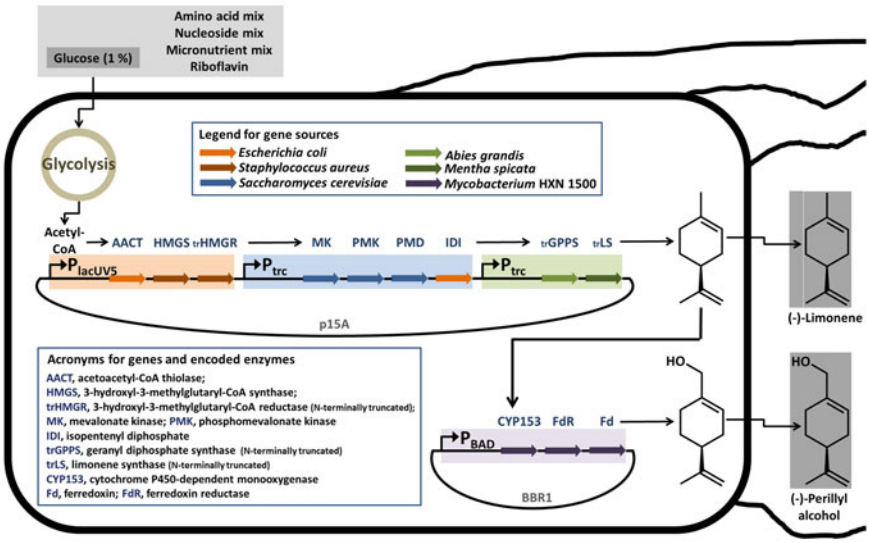


Fig. 6 Metabolic engineering of oxygenated *p*-menthane monoterpene biosynthesis in *E. coli* (modified after Alonso-Gutierrez et al. [2])

5 Conclusions

Numerous approaches have been tested to develop plant and microbial platform cultivars/strains for the high-level accumulation of terpenoids. After decades of research, several trends have emerged that are relevant for the production of *p*-menthane monoterpenes as targets:

- The yields of engineered terpenoids in the native producer (particularly if it is a plant with specialized anatomical structures for terpenoid storage) are generally higher than those in heterologous plant hosts (e.g., *Arabidopsis* or tobacco).
- Metabolic turnover (redox reactions and/or glycosylation) is a common challenge when novel terpenoids are accumulated in heterologous plant hosts. A second problem relates to the volatility of monoterpenes. Unless stored in a specialized anatomical structure (e.g., glandular trichomes or resin ducts) or biochemically conjugated (e.g., as glycosides), terpenoids are likely to be volatilized and are thus not harvestable on a commercial scale.
- Promoters that allow a transgene to be expressed specifically in specialized anatomical structures (e.g., glandular trichomes) have been employed but have not yet delivered a step change in the accumulation of terpenoids by metabolic engineering (for more details see [48]).
- Simple terpenoids can be accumulated to high levels in microbial hosts. However, the yields of highly functionalized plant terpenoids in engineered microbial hosts are typically fairly low. Nevertheless, because of the availability of versatile tools and relative speed of progress, microbial engineering appears to be the most promising near-term alternative for producing *p*-menthane monoterpenes.

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