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Molecular evaluation of a spearmint mutant altered in the expression of limonene hydroxylases that direct essential oil monoterpene biosynthesis

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Abstract

Gamma irradiation of Scotch spearmint created a mutant line, 643-10-74, which has an altered essential oil reminiscent of peppermint because the monoterpene metabolites in the oil glands of the mutant are predominantly oxygenated at the C3 position of the *p*-menthane ring instead of the C6 position normally found in spearmint. The limonene hydroxylase genes responsible for directing the regiochemistry of oxygenation were cloned from Scotch spearmint and mutant 643 and expressed in *Escherichia coli*. The limonene bydroxylase from the wild-type parent hydroxylated the C6 position while the enzyme from the mutant oxygenated the C3 position. Comparison of the amino acid sequences with other limonene hydroxylases showed that the mutant enzyme was more closely related to the peppermint limonene-3-hydroxylases than to the spearmint limonene-6-hydroxylases. Because of the sequence differences between the Scotch spearmint and mutant 643 limonene hydroxylases, it is most likely that the mutation did not occur within the structural gene for limonene hydroxylase but rather at a regulatory site within the genome that controls the expression of one or the other regiospecific variants.

Keywords: *Mentha* × *gentilis*; *Lamiaceae*; Mint; Monoterpene biosynthesis; Cytochrome P450; Limonene hydroxylase; *Trans*-carveol; *Trans*-isopiperitenol

1. Introduction

The monoterpene constituents of the essential oils of the genus *Mentha* (family *Lamiaceae*) are distinguished by the position of oxygenation on the *p*-menthane ring (Lawrence, 1981). Of the agronomically important mints (Lawrence, 1985), peppermint (*M.* × *piperita* L.) produces almost exclusively monoterpenes bearing an oxygen function at C3 such as menthol **1** (responsible for the cooling sensation of peppermint), whereas the spearmint types such as native spearmint (*M. spicata* L.) and Scotch spearmint (*M.* × *gentilis* var *cardiaca* (Gerard *ex* Baker) Briq.; syn *M.* × *gracilis* Sole) produce almost exclusively monoterpenes bearing an oxygen function at C6, typified by carvone **2** (responsible for the typical spearmint note) (Fig. 1). The genetic basis of the C3- and C6-oxygenation patterns in fertile *Mentha* species was the target of extensive studies by Murray and associates from the mid-l950s through the mid-1970s (reviewed in Croteau and Gershenzon, 1994), and was shown to be determined by two closely linked diallelic loci designated *Lm* and *C*. The dominant *Lm* allele was postulated to prevent C3-oxygenation whereas the recessive *lm* allele was thought to allow this conversion. In contrast, the dominant *C* allele was proposed to stimulate C6-oxygenation while its recessive counterpart *c* does not promote this conversion (Hefendehl and Murray, 1976). The two dominant alleles must be tightly linked to account for the observation that the C3- and

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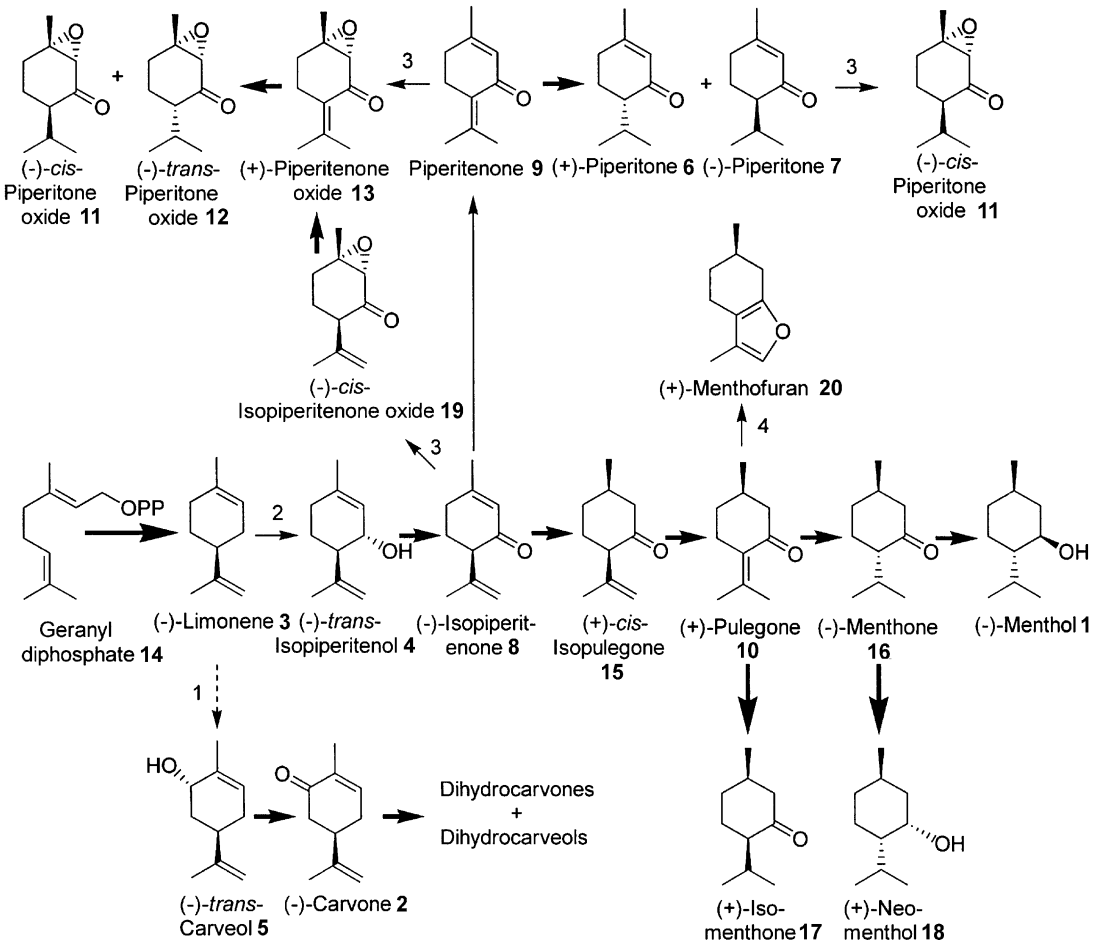

Fig. 1. Enzymatic activities observed only in wild type Scotch spearmint (dashed light arrows), only in mutant 643 (light arrows), and both wild type and mutant (heavy arrows) are indicated (Croteau et al., 1991). The enzymes numbered in the pathway are: (1) limonene-6-hydroxylase, (2) limonene-3-hydroxylase, and (3) terpenone-$\Delta^{1,2}$-epoxidase. The reaction catalyzed by menthofuran synthase (4) in peppermint is also indicated.

C6-oxygenation patterns are mutually exclusive and that C6-oxidation is dominant to C3-oxidation. It has been suggested that *Lm* represents a regulatory gene that controls the expression of C3-hydroxylation and that *C* is a structural or regulatory gene that controls C6-hydroxylation (Croteau and Gershenzon, 1994). However, the roles of *Lm* and *C* in *Mentha* cannot be clarified until the structures of the hydroxylase genes, and of the *Lm* and *C* genes and gene products, are defined, and the molecular level interactions between these entities deciphered.

Biosynthetic investigations have demonstrated that regiospecificity is established very early in the monoterpene biosynthetic pathways in which (−)-4*S*-limonene **3**, the common precursor of both oxygenated series (Kjonaas and Croteau, 1983), undergoes cytochrome P450-mediated hydroxylation at C3 to yield (−)-*trans*-isopiperitenol **4** (in peppermint-type species) or at C6 to afford (−)-*trans*-carveol **5** (in spearmint-type species) (Karp et al., 1990) (Fig. 1). The remaining enzymatic machinery responsible for the subsequent redox transformations of isopiperitenol **4** to menthol **1** is present in both peppermint and spearmint species; however, carveol **2** is a poor substrate for these reactions, with the consequence that only the dehydrogenation product, carvone, accumulates in spearmint types (Croteau et al., 1991).

cDNAs encoding the cytochrome P450 (−)-limonene-6-hydroxylase from native spearmint and the cytochrome P450 (−)-limonene-3-hydroxylase from peppermint have been isolated (Lupien et al., 1999), and both of these regiospecific oxygenases have been functionally expressed in *Escherichia coli* and *Saccharomyces cerevisiae* (Haudenschild et al., 2000). Structure–function studies with these closely enzymes have established the mechanism and steric constraints of these oxygenation reactions (Wüst et al., 2001; Wüst and Croteau, 2002) and demonstrated that the alteration of a single amino acid (F3631) is sufficient to convert the regiochemistry of the spearmint (−)-limonene hydroxylase from a C6-type to a kinetically-competent peppermint-type C3-hydroxylase (Schalk and Croteau, 2000).

An interesting observation, that bears on the genetic control of essential oil monoterpene biosynthesis in mints, derives from biochemical evaluation of a γ-irra-

diation-induced mutant of the sterile hybrid Scotch spearmint (*M.* × *gentilis*) that produces a peppermint-type oil (C3-oxygenation pattern), unlike the spearmint-type oil (C6-oxygenation pattern) found in the wild type (Croteau et al., 1991). Cell-free assay of all of the enzymes responsible for the production of both C3-oxygenated and C6-oxygenated families of monoterpenes derived from (−)-limonene **3** indicated that both the mutant (designated mutant 643) and wild type Scotch spearmint possess a virtually identical complement of catalysts, with the exception of the microsomal, cytochrome P450-dependent (−)-limonene hydroxylase; the C6-hydroxylase producing (−)-*trans*-carveol **5** in the wild type had been entirely replaced by a C3-hydroxylase producing (−)-*trans*-isopiperitenol **4** in the mutant (Fig. 1). Notably, both wild type and mutant possess all of the remaining enzymatic machinery (at roughly comparable levels) for the production of carvone **2** (from carveol **5**) and of menthol **1** (from isopiperitenol **4**), thereby accounting for the specific production of C3-oxygenated monoterpene derivatives in the mutant.

These results suggest that irradiation may have resulted in either a mutation of the C6-hydroxylase structural gene, of the type recently constructed by site-directed mutagenesis to modify regiochemistry (Schalk and Croteau, 2000), or, alternatively, a mutation of a regulatory gene (perhaps *Lm*) to a form which suppresses C6-hydroxylation and activates a nascent C3-hydroxylase (in a monoterpene biosynthetic background that remains otherwise identical). In this paper, we report the cDNA cloning of the C6-hydroxylase from wild type Scotch spearmint and of the C3-hydroxylase from mutant 643. The two cytochrome P450 hydroxylases are sufficiently different to suggest that mutant 643 bears a mutation in a regulatory gene that silences the wild-type limonene-6-hydroxylase and promotes the sole expression of a previously quiescent limonene-3-hydroxylase.

2. Results

2.1. cDNA isolation and expression

Glandular trichomes are the exclusive site of monoterpene biosynthesis in *Mentha* species, and the secretory cells of these structures are thus highly enriched in the relevant biosynthetic enzymes and their corresponding messages (Gershenzon et al., 1992). To avoid the complications of screening a whole leaf cDNA library, oil gland-specific cDNA libraries for both Scotch spearmint and mutant 643 were constructed using protocols previously developed for other mint species (Lupien et al., 1999).

Based upon the assumption that both the C6-hydroxylase of Scotch spearmint and the presumptive mutant C3-hydroxylase of mutant 643 would closely resemble the cytochrome P450 (−)-limonene-6-hydroxylase of native spearmint (Lupien et al., 1999), both libraries were screened by standard hybridization methods (Sambrook et al., 1989) using ^{32}P-labelled cDNA encoding the spearmint C6-hydroxylase as the probe. This strategy yielded 23 positive clones from the wild type Scotch spearmint oil gland library (0.023% abundance), from which two distinct full-length cDNAs were acquired. Both encoded cytochrome P450 enzymes with high levels of sequence identity to the known mint limonene hydroxylases, yet exhibited significant sequence differences between each other (Table 1). One of these cytochrome P450 clones (designated scotch-5B-2; Genbank accession number AY281025) revealed 100% sequence identity with the spearmint limonene-6-hydroxylase (SM12; CYP71D18) at the amino acid level (Table 1), and also at the DNA level including the 3′- and 5′- untranslated regions. The SM12 P450 has been previously shown to hydroxylate (−)-*S*-limonene **3** exclusively at the C6-*trans*-position (Haudenschild et al., 2000). The finding of a limonene-6-hydroxylase in *M.* × *gentilis,* that is identical to that of native spearmint, is not surprising because *M. spicata* is a parent of Scotch spearmint, a sterile hybrid of very recent origin (Tucker and Fairbrothers, 1990; Tucker et al., 1991).

The second cytochrome P450 cDNA clone (designated scotch-10B-2; Genbank accession number AY281026), contained 1482 bp, encoded a protein of 494 residues, and shared 86.3% amino acid sequence identity with the scotch-5B-2 clone and the limonene-6-hydroxylase of native spearmint, and about 67% amino acid sequence identity with the two limonene-3-hydroxylases from peppermint (PM2, CYP71D15; and PM17, CYP71D13) (Table 1). For functional characterization, the 10B-2 clone was expressed in *E. coli* JM109 cells using the pCWori+ expression plasmid (Haudenschild et al., 2000). Microsomes prepared from

Table 1
Sequence identity (%) at the deduced amino acid sequence level of clones 643-9B-2, scotch-10B-2, scotch-5B-2, the spearmint limonene-6-hydroxylase (SM12) and the two peppermint limonene-3-hydroxylases (PM2 and PM17)[a]

	PM17	PM2	SM12	Scotch-5B-2	Scotch-10B-2
643-9B-2	92.2 (89.0)	88.9 (89.0)	70.6 (76.5)	70.6 (76.5)	67.7 (75.0)
Scotch-10B-2	67.0 (74.6)	66.8 (73.6)	86.3 (91.6)	86.3 (91.6)	
Scotch-5B-2	69.2 (76.1)	68.8 (75.4)	100.0 (100.0)		
SM12	69.2 (76.1)	68.8 (75.1)			
PM2	92.0 (92.1)				

[a] Sequence identity (%) at the DNA level in the open reading frame is shown in parentheses.

several independent cultures at different culture periods were shown to contain in excess of 350 pmol P450 per mg protein as measured by CO difference spectrometry (Omura and Sato, 1964). However, when reconstituted with NADPH-dependent cytochrome P450 reductase and evaluated in a standard limonene hydroxylase assay (Haudenschild et al., 2000), no oxygenated metabolite was observed. Given the close sequence similarity of clone 10B-2 to other mint limonene hydroxylases, it may be inferred that this clone encodes a monoterpene oxygenase. Because cytochrome P450-dependent epoxidation of monoterpenoids had been observed with Scotch spearmint microsomes (Croteau et al., 1991), (±)-piperitone **6**,**7**, (−)-isopiperitone **8**, piperitenone **9**, and (+)-pulegone **10** (Fig. 1) were tested as likely substrates for epoxidation by the recombinant scotch-10B-2 microsomal enzyme; however, none of these monoterpenoids was detectably metabolized, and so the substrate for this cytochrome P450 oxygenase remains undefined.

Screening of the mutant 643 oil gland cDNA library with the same C6-hydroxylase probe as above revealed a smaller number of positives (0.015% abundance), from which a single, unique cytochrome P450 clone was obtained. This full-length sequence (designated 643-9B-2) of 1493 bp encoded a protein of 497 residues (Genbank accession number AY281027) and was shown to resemble those of the (−)-limonene-6-hydroxylases of native spearmint and the wild type Scotch spearmint (77% at the nucleotide level) but to be more closely related to the (−)-limonene-3-hydroxylases of peppermint (Genbank accession numbers AF124817 and AF124816) (~92% identity at the nucleotide level) (Table 1). Functional expression of this clone in *E. coli,* followed by isolation, reconstitution and assay as above, revealed the microsomal recombinant cytochrome P450 to convert (−)-limonene **3** to (−)-*trans*-isopiperitenol **4** as the only product (Fig. 2). Assays of the reconstituted 643-9B-2 clone with piperitone (**6**, **7**), piperitenone **9**, and isopiperitenone **8**, as before, did not reveal any epoxidized products (data not shown).

3. Discussion

The present work reports the cDNA isolation and functional expression of a limonene-6-hydroxylase (scotch-5B-2) and limonene-3-hydroxylase (643-9B-2) from oil gland libraries of Scotch spearmint and Scotch spearmint mutant 643, respectively. Functional expression of these two regiospecific hydroxylases correlates with previous studies which showed that these two plant lines produce C6-oxygenated and C3-oxygenated *p*-menthane monoterpenes, respectively, from the common precursor (−)-limonene (Croteau et al., 1991). The deduced amino acid sequence of the mutant 643 gene was more similar to those of the limonene-3-hydroxylases of peppermint than to those of the limonene-6-hydroxylases from native spearmint and Scotch spearmint (Table 1). Regardless of the origin of the mutant 643 limonene-3-hydroxylase gene (there are far too many substitutions for this gene to have arisen by irra-

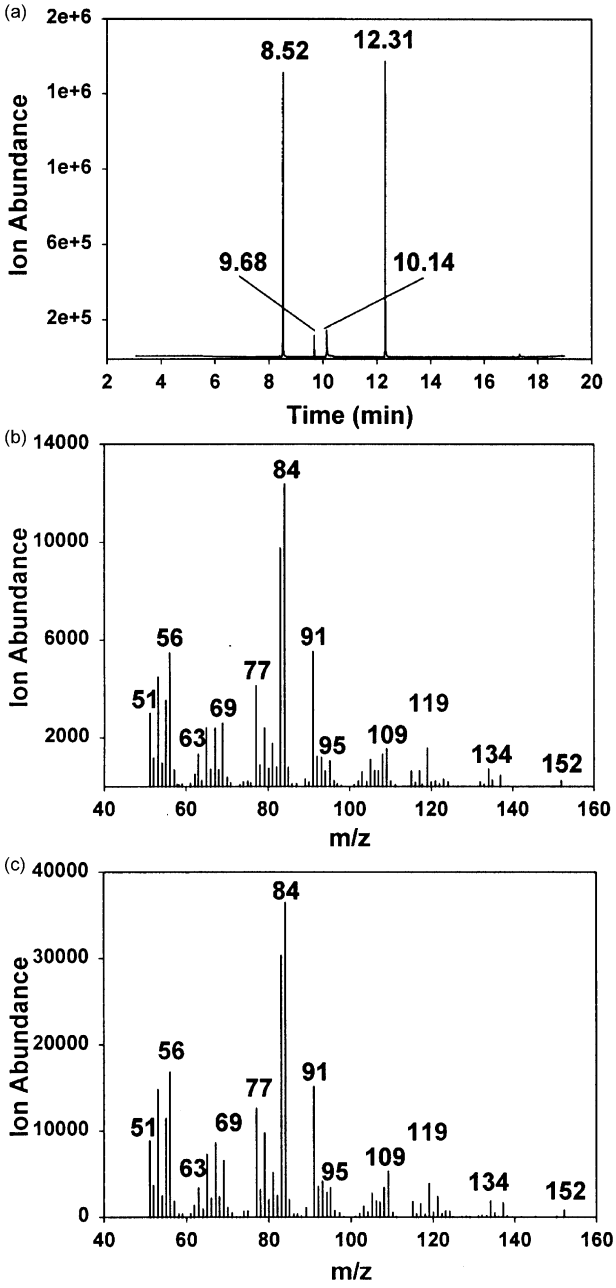

Fig. 2. GC–MS analysis of the reaction products generated by clone 643-9B2-2 in the standard limonene hydroxylase assay. (a) Gas chromatogram (FID) of the diethyl ether-soluble products indicating residual limonene **3** (R_t = 8.52 min), the internal standard camphor (R_t = 9.68 min), (−)-*trans*-isopiperitenol **4** (R_t = 10.14 min) and butylated hydroxytoluene (R_t, = 12.31 min) from the diethyl ether extraction procedure. (b) The mass spectrum and retention time of the product with R_t = 10.14 min were identical to those of authentic *trans*-isopiperitenol **4** (c).

diation-induced mutation of the limonene-6-hydroxylase gene), the crucial amino acid residue at position 363 is isoleucine (not phenylalanine), which is sufficient to convert the regiochemistry of the limonene-6-hydroxylase to a limonene-3-hydroxylase (Schalk and Croteau, 2000).

A second cytochrome P450 clone was also isolated from Scotch spearmint (scotch-10B-2) which closely resembles limonene-6-hydroxylase, but for which a relevant monoterpene substrate could not be defined. Comparison of the 10B-2 sequence to other limonene hydroxylases showed the presence of Ala300 in the I-helix, a position at which Thr is conserved in almost all cytochrome P450 enzymes (Fig. 3). This Thr residue is involved in activation of molecular oxygen during the hydroxylation reaction mediated by cytochrome P450 enzymes. The presence of an Ala residue at this position in the I-helix of the scotch-10B-2 clone could indicate that this cytochrome P450 catalyzes a different type of oxygenation reaction. Because members from the CYP74 family also contain Ala at this position and use fatty acid hydroperoxides as substrates (for a review, see Howe and Schilmiller, 2002), it is conceivable that scotch-10B-2 utilizes limonene hydroperoxide as a substrate to produce limonene epoxide, as this reaction would not require binding and activation of molecular oxygen. Vaz et al. (1998) mutated the I-helix conserved threonine residue of cytochrome P450 2E1 to an alanine and showed that the altered enzyme had enhanced

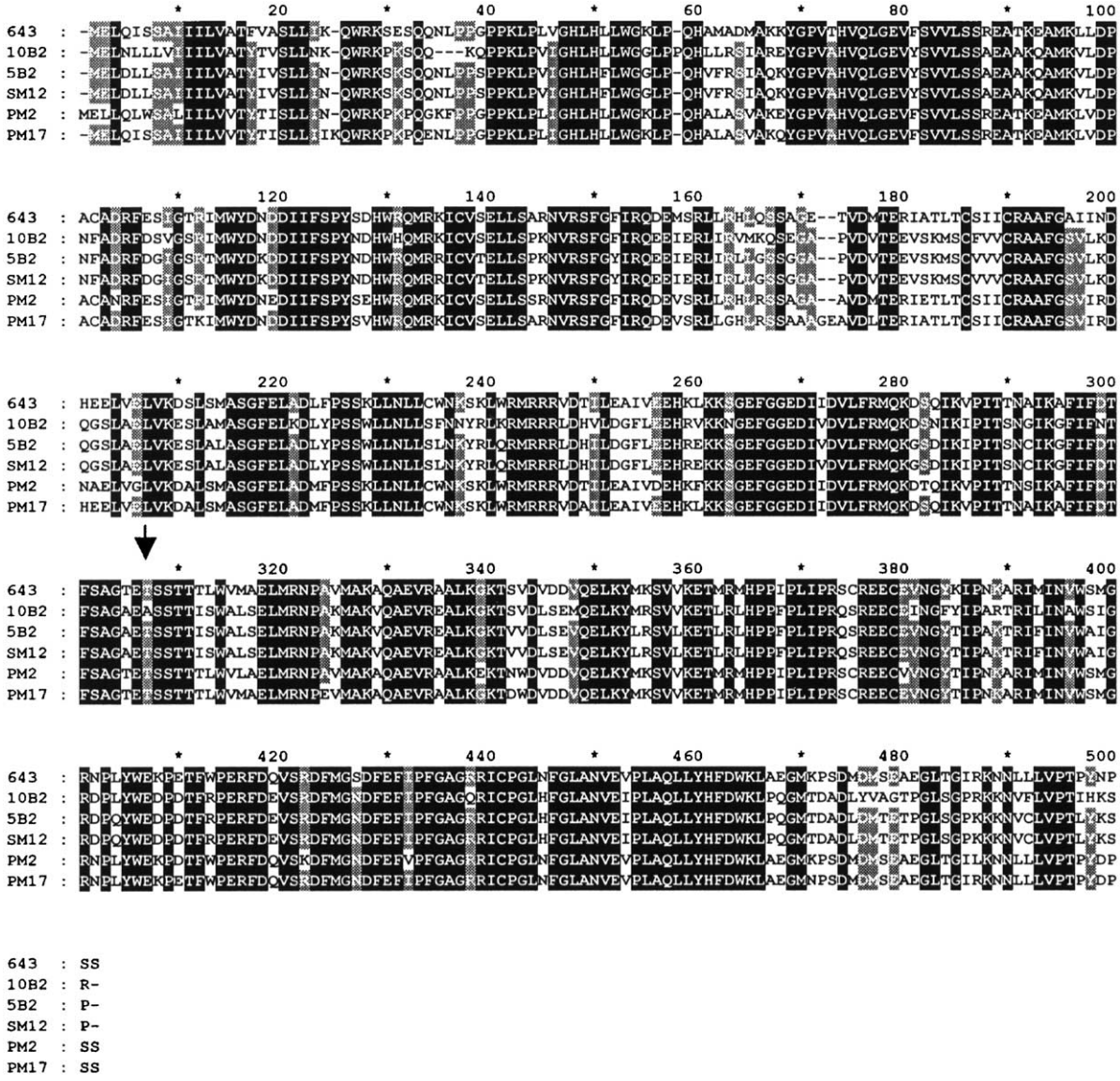

Fig. 3. Sequence alignment of clones 643-9B-2, scotch-10B-2, and scotch-5B-2 with the previously reported limonene hydroxylases SM12 (Genbank accession number AF124815), PM2 (Genbank accession number AF124817), and PM17 (Genbank accession number AF124816) using the ClustalW program (Thompson et al., 1994). Identical residues for all six sequences are shown in black; identical residues for five of the six sequences are shown in grey. The alanine for threonine substitution in clone scotch-10B-2 clone is indicated by the arrow at position 307.

epoxidase activity; they also proposed a reaction mechanism involving hydroperoxo-iron as an oxidant. However, in the same paper, the corresponding mutation in cytochrome P450 2B4 resulted in a decrease in both hydroxylase and epoxidase activities. Thus, although the Thr→Ala mutation may enhance epoxidase activity, this mutation is not in itself sufficient to confer epoxidase activity in the absence of additional tertiary structure features or suitably activated substrates. More work is necessary to determine the substrate and reaction mechanism employed by the scotch-10B-2 encoded protein.

Although monoterpene biochemistry in mints is well understood at the pathway and enzyme levels (Fig. 1), relatively little is known about the regulation of metabolic flux in these systems. Previous chemical analysis of mutant 643 showed that the monoterpene profile had been greatly altered from 70% (−)-carvone **2** (C6-OH) in the oil of wild type Scotch spearmint to over 85% C3-oxygenated monoterpenes in the oil of the mutant, with more than 60% of the product as various forms of 3-keto-1,2-oxides, such as *cis*-piperitone oxide **11** (1.9%), *trans*-piperitone oxide **12** (8.6%), and piperitenone oxide **13** (53%) (Croteau et al., 1991). In addition, the mutant exhibited a 6-fold reduction in the level of limonene (compared to 15% of the oil measured in the parent Scotch spearmint plants), and the oil yields from the leaves of mutant plants were often 50% less than those of wild-type leaves. Enzymatic characterization of the Scotch spearmint and 643 mutant lines showed that the enzymes that convert *trans*-isopiperitenol **4** to menthol **1** were present in both lines; however, the Scotch spearmint parent lacked the limonene-3-hydroxylase activity that would supply the critical substrate (*trans*-isopiperitenol **4**) necessary for these enzymes. In mutant 643, the newly expressed limonene-3-hydroxylase and the terpenone epoxidase activities were located in the microsomal fraction and were inhibited to similar degrees by CO and clotrimazole, suggesting that the mutation may have generated a single bifunctional cytochrome P450 enzyme which can perform both C3-hydroxylation and 1,2-epoxidation. There is precedent for cytochrome P450 enzymes that function as epoxidases (Kroetz and Zeldin, 2002); however, in the present case, the 643-9B-2 limonene-3-hydroxylase did not possess detectable epoxidase activity with the substrates tested.

The appearance of an anomalous epoxidase activity has also been observed in transgenic peppermint plants in which cytochrome P450 menthofuran synthase was cosuppressed (Mahmoud and Croteau, 2003). In this case, the transgenic plants produced 5–10% of the essential oil in the form of *trans*-piperitone oxide **12** compared to undetectable levels of this metabolite in wild-type plants. The results obtained from mutant 643 Scotch spearmint and transgenic peppermint plants indicate that the regulation of monoterpene metabolism in mint peltate oil glands is incompletely understood, and that seemingly simple alterations in the production of a single intermediate can result in drastic and unpredictable changes in metabolite profiles.

In summary, the large number of differences in primary structure between the mutant 643 C3-hydroxylase gene and the Scotch spearmint C6-hydroxylase gene suggests that the mutation did not occur in the limonene hydroxylase structural gene. Rather, the data suggest that the mutation occurred in a regulatory gene, which results in the novel expression of a previously silent limonene-3-hydroxylase and the repression of the native limonene-6-hydroxylase, with major consequences for essential oil composition.

4. Experimental

4.1. Plant materials and cDNA library construction

Scotch spearmint and mutant number 643-10-74 were obtained from C.E. Homer (Oregon State University, Corvallis, OR) and were grown under greenhouse conditions described previously (Croteau et al., 1991). Scotch spearmint (*M.* × *gentilis* var *cardiaca* (Gerard *ex* Baker) Briq.) is a sterile cross between *M. arvensis* and *M. spicata; M.* × *gentilis* is synonomous with *M.* × *gracilis* Sole and *M.* × *cardiaca* (Grey) Baker (Harley and Brighton, 1977).

Oil gland-specific cDNA libraries from young leaves of both Scotch spearmint and mutant 643 plants were constructed using protocols previously published for other mint species (Lupien et al., 1999).

4.2. cDNA isolation and sequencing analysis

Both cDNA libraries were screened by standard hybridization methods (Sambrook et al., 1989). For each library, 10^5 plaque-forming units grown in *E. coli* XL 1-Blue MRF′ were transferred to duplicate filters and hybridized for 24 h at 65 °C with a ^{32}P-labelled 490-bp nucleotide fragment (LH-2), obtained by PCR amplification of the 5′-end of the spearmint limonene-6-hydroxylase cDNA (Lupien et al., 1999), as the probe in a solution of 5 × SSPE (1 × SSPE = 150 mM NaC1, 10 mM sodium phosphate, and 1 mM EDTA), 5 × Denhardt's, 1% SDS and 100 μg/ml sheared salmon sperm DNA. Blots were washed twice with 2 × SSPE at room temp., twice with 2 × SSPE containing 2% SDS for 45 min at 65 °C, and, finally, twice with 0.1 × SSPE for 15 min at room temperature.

Of the 15 plaques affording positive signals from the Scotch spearmint mutant 643 enriched gland library, all were purified through two additional cycles of hybridization. Seven pure clones were isolated and excised as

Bluescript SK$^-$ phagemids. Insert size was determined by PCR, and the termini were sequenced. One unique full-length cytocbrome P450 cDNA clone was obtained from the seven identical acquisitions, and the cDNA was fully sequenced and designated 643-9B-2. A BLAST search (Altschul et al., 1990) revealed the sequence to resemble those of other cytochrome P450 limonene hydroxylases.

Of the 23 plaques affording positive signals from the Scotch spearmint cDNA library, all were purified through two additional cycles of hybridization. Eleven pure clones were isolated and excised as Bluescript SK$^-$ phagemids. Two different full-length cytochrome P450 cDNA clones (over four acquisitions each) were isolated and designated scotch-5B-2 and scotch-10B-2, respectively. A BLAST search (Altschul et al., 1990) revealed the sequences to resemble those of other cytochrome P450 limonene hydroxylases.

4.3. Functional expression in E. coli

For expression from the pCWori+ vector (Barnes, 1996), the 643-9B-2 clone was modified to introduce appropriate restriction sites and to substitute the bovine 17α-hydroxylase N-terminus to optimize expression in the bacterial host (Barnes, 1996; Sandhu, 1994; Halkier et al., 1995; Haudenshild et al., 2000). These modification were introduced by PCR using the original 643-9B2 cDNA in pBSSK(−) as template and the forward primer 5′-GGAATTCCATATGGCTCTGTTATTAG-CAGTTTTTCTGACATTCGTCGCATCCCTCC-3′ to introduce an *NdeI* site (including the start codon) and to replace the N-terminal 14 residues of the membrane anchor with the MALLLAVF sequence, and the reverse primer 5′-CATTGGGAAGCTTTCATGAGGAAG GATTGTAGGGTGTGGG-3′ to introduce a *Hind*III site downstream of the stop codon. The gel-purified PCR product was digested by *Nde*I and *Hind*III and ligated into similarly digested pCWOri+ plasmid between the UV5*lac-tac* promoter and the TrpA terminator, and the sequence-verified plasmid was used to transform *E. coli* JM109 cells by standard protocols (Sambrook et al., 1989).

The scotch-10B-2 clone was similarly modified for expression in pCWori+ by introducing *Nde*I and *Hind*III restriction sites and by substituting the MALLLAVF sequence for the five N-terminal residues using the original clone as template and PCR amplification with the forward primer 5′-GGAATTCCA TATGGCTCTGTTATTAGCAGTTTTTTTGTTGGT AATTATAATCCTCGTGGC-3′ and reverse pruner 5′-CATTGGGAAGCTTAACGACTTTTATGGATGGG -3′. The gel-purified PCR product was digested and ligated, as before, and the sequence-verified plasmid was used to transform *E. coli* JM109 cells by standard protocols (Sambrook et al., 1989).

Single colonies of transformed *E. coli* JM109 cells were used to inoculate liquid cultures in 5 ml Luria-Bertani broth–ampicillin medium. The culture was incubated overnight at 37 °C and then used to inoculate 300 ml Terrific Broth (TB) medium containing 100 μg ampicillin/l, 1 mM thiamine, 75 mg δ-aminolevulinic acid/l, and 1 mM isopropyl β-D-thiogalactopyranoside. These cultures were then incubated for 36 h at 28 °C (200 rpm).

4.4. Enzyme isolation and assay

The host cells incubated as above were chilled on ice for 20 min, and a 2 ml aliquot of the suspended material was retained for measurement of cytochrome P450 content by CO-difference spectrometry (Omura and Sato, 1964), while the remaining cells were harvested by centrifugation at 7000 *g* for 10 min for microsome preparation by a protocol adapted from Halkier et al. (1995). The pelleted cells were resuspended in 0.1 M Tris–HC1 buffer (pH 7.5) containing 20% (v/v) glycerol and 0.5 mM EDTA. Following the addition of lysozyme (0.2 mg/ml) and 1:1 dilution with cold water, the cells were gently stirred for 10 min at 4 °C. The resulting spheroplasts were pelleted at 7000 *g* for 10 min and resuspended in 15 ml of the same buffer. The spheroplast suspension was frozen at −80 °C for 1 h and then thawed at room temp. A few crystals of PMSF were added during thawing, the volume was adjusted to 50 ml with the above extraction buffer, and the cells were then lysed by sonication (3 × 30 s at power setting of 6 with a 1/2 inch probe on a Virtis VirSonic 475 (Gardiner, New York, USA). The lysate was centrifuged at l0,000 *g* for 10 min, and the resulting supernatant was further centrifuged at 150,000 *g* for 2 h. The pelleted membranes were resuspended in 6 ml of same buffer as above supplemented with 1 mM DTT, and the suspension was homogenized using a chilled glass Tenbroeck homogenizer and stored as aliquots at −80 °C until used.

For reconstitution by the method of Haudenschild et al. (2000), 5–40 μl of the suspended membranes were combined with 200 mU of purified spearmint cytochrome P450-reductase (Ponnamperuma and Croteau, 1995) in a final volume of 125 μL assay buffer (50 mM Tris–HC1, pH 7.4, containing 1 mM EDTA and 0.1 mM DTT) supplemented with 250 mM KC1 and 50 mM $MgCl_2$. The mixture was agitated by gentle rotation at room temp. for 20 min, then diluted to 1 ml in assay buffer containing 200 μM (−)-limonene **3**, 2 mM glucose 6-phosphate, 0.8 U glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, 5 μM FAD and 5 μM FMN. The reaction was allowed to proceed for 2 h at 30 °C with gentle shaking, and was then stopped by chilling on ice and extracting with three 1-ml portions of diethyl ether after the addition of 25 nmol (+)-camphor as an internal standard. The solvent was evaporated under a stream of N_2, and the concentrate was analyzed.

Capillary GC was performed on a Hewlett-Packard 5890A Series II gas chromatograph with a 3392A Integrator using a 0.25 mm i.d. by 30 m fused silica capillary column coated with AT-1000 (Alltech, Deerfield, IL) and H_2 as carrier gas (13.5 psi). Samples were analyzed by flame ionization detection using cool on-column injection with the oven programmed from 35 to 45 °C at 50 °C/min (5 min hold), and then at 10 °C/min to 230 °C (10 min hold). Compound identification was based on retention time coincidence with the authentic standard, with quantification by electronic integration based on the internal standard.

To confirm product identification, samples were analyzed by combined capillary GC–MS using a Hewlett-Packard 6890 GC-quadrupole mass selective detector system, equipped with a 0.25 mm i.d. × 30 m fused silica column coated with a 0.25 μm film of HP 5MS (Hewlett-Packard). The oven was programmed from 40 °C (5 min hold) to 320 °C at 20 °C/min at 10 psi He, and EI spectra were recorded at 70 eV with an electron multiplier voltage of 2200 V. Spectra were compared to those of authentic standards.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Barnes, H.J., 1996. Maximizing expression of eucaryotic cytochrome P450s in *Escherichia coli*. Methods Enzymol. 272, 3–14.

Croteau, R., Karp, F., Wagschal, K.C., Satterwhite, D.M., Hyatt, D.C., Skotland, C.B., 1991. Biochemical characterization of a spearmint mutant that resembles peppermint in monoterpene content. Plant Physiol. 96, 744–752.

Croteau, R., Gershenzon, J., 1994. Genetic control of monoterpene biosynthesis in mints (*Mentha*: *Lamiaceae*). In: Ellis, B.E. (Ed.), Genetic Engineering of Plant Secondary Metabolism. Plenum Press, New York, pp. 193–229.

Gershenzon, J., McCaskill, D., Rajaonarivony, J.I.M., 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.

Halkier, B.A., Nielsen, H.L., Koch, B., Møller, B., 1995. Purification and characterization of recombinant cytochrome P450TYR expressed at high levels in *Escherichia coli*. Arch. Biochem. Biophys 322, 369–377.

Harley, R.M., Brighton, C.A., 1977. Chromosome numbers in the genus *Mentha* L. Bot. J. Linn. Soc. 74, 71–96.

Haudenschild, C., Schalk, M., Karp, F., Croteau, R., 2000. Functional expression of regiospecific cytochrome P450 limonene hydroxylases from mint (*Mentha* spp.) in *Escherichia coli* and *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. 379, 127–136.

Hefendehl, F.W., Murray, M.J., 1976. Genetic aspects of the biosynthesis of natural odors. Lloydia 39, 39–52.

Howe, G.A., Schilmiller, A.L., 2002. Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. 5, 230–236.

Karp, F., Mihaliak, C.A., Harris, J.L., 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. Biopbys. 220, 79–89.

Kroetz, D.L., Zeldin, D.C., 2002. Cytochrome P450 pathways of arachidonic acid metabolism. Curr. Opin. Lipid. 13, 273–283.

Lawrence, B.M., 1981. Monoterpene interrelationships in the *Mentha* genus: A biosynthetic discussion. In: Mookherjee, B.D., Mussinan, C.J. (Eds.), Essential Oils. Allured Publishing Corporation, Wheaton, IL, pp. 1–81.

Lawrence, B.M., 1985. A review of the world production of essential oils. Perfum. Flavor. 13, 2–16.

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 (−)-4S-limonene-3-hydroxylase and (−)-4S-limonene-6-hydroxylase. Arch. Biochem. Biophys. 368, 181–192.

Mahmoud, S. S., Croteau, It., 2003. Menthofuran regulates essential oil biosynthesis in peppermint by controlling a downstream monoterpene reductase. Proc. Natl. Acad. Sci. U.S.A. (in press).

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370–2378.

Ponnamperuma, K., Croteau, R., 1995. Purification and characterization of an NADPH-cytochrome P450 (cytochrome c) reductase from spearmint (*Mentha spicata*) glandular trichomes. Arch. Biochem. Biophys. 329, 9–16.

Sambrook, J., Fritsch, E.F., Maniatus, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Sandhu, P., Guo, Z., Baba, T., Martin, M.V., Tukey, R.H., Guengerich, F.P., 1994. Expression of modified human cytochrome P450 1A2 in *Escherichia coli*: stabilization, purification, spectral characterization, and catalytic activities of the enzyme. Arch. Biochem. Biophys. 309, 168–177.

Schalk, M., Croteau, R., 2000. A single amino acid substitution (F363I) converts the regiochemistry of the spearmint (−)-limonene hydroxylase from a C6- to a C3-hydroxylase. Proc. Natl. Acad. Sci. U.S.A. 97, 11948–11953.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

Tucker, A.O., Fairbrothers, D.E., 1990. The origin of *Mentha* × *gracilis* (*Lamiaceae*). I. Chromosome numbers, fertility, and three morphological characters. Econ. Bot. 44, 183–213.

Tucker, A.O., Hendriks, H., Bos, R., Fairbrothers, D.E., 1991. The origin of *Mentha* × *gracilis* (*Lamiaceae*). II. Essential oils. Econ. Bot. 45, 200–215.

Vaz, A.D.N., McGinnity, D.F., Coon, M.J., 1998. Epoxidation of olefins by cytochrome P450: Evidence from site-specific mutagenesis for hydroperoxo-iron as an electrophilic oxidant. Proc. Natl. Acad. Sci. U.S.A. 95, 3555–3560.

Wüst, M., Little, D.B., Schalk, M., Croteau, R., 2001. Hydroxylation

of limonene enantiomers and analogs by recombinant (−)-limonene 3- and 6-hydroxylases from mint (*Mentha*) species: evidence for catalysis within sterically constrained active sites. Arch. Biochem. Biophys. 387, 125–136.

Wüst, M., Croteau, R., 2002. Hydroxylation of specifically deuterated limonene enantiomers by cytochrome P450 limonene-6-hydroxylase reveals the mechanism of multiple product formation. Biochemistry 41, 1820–1827.