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Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil

Soheil S. Mahmoud[1], Matthew Williams, Rodney Croteau*

Institute of Biological Chemistry, Washington State University, PO Box 646340, Pullman, WA 99164-6340, USA

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Abstract

cDNA clones encoding limonene synthase and limonene-3-hydroxylase, both driven by the CaMV 35S promoter, were independently transformed into peppermint (*Mentha×piperita*) to alter the production and disposition of (−)-limonene, the first committed intermediate of essential oil biosynthesis in this species. Although both genes were constitutively expressed in leaves of transformed plants, the corresponding enzyme activities were not significantly increased in the glandular trichome sites of essential oil biosynthesis; thus, there was no effect on oil yield or composition in the regenerated plants. Cosuppression of the hydroxylase gene, however, resulted in the accumulation of limonene (up to 80% of the essential oil compared to about 2% of the oil in wild type plants), without influence on oil yield. These results indicate that limonene does not impose negative feedback on the synthase, or apparently influence other enzymes of monoterpene biosynthesis in peppermint, and suggests that pathway engineering can be employed to significantly alter essential oil composition without adverse metabolic consequences.

Keywords: Peppermint; *Mentha×piperita*; Lamiaceae; Limonene synthase; Limonene-3-hydroxylase; Essential oil; Monoterpene biosynthesis; Metabolic engineering

1. Introduction

Monoterpenes are the major essential oil components of the mint (*Lamiaceae*) family, including peppermint (*Mentha×piperita*) which has been developed as a model system for the study of monoterpene metabolism (Gershenzon et al., 2000; McConkey et al., 2000). Monoterpene biosynthesis in mints is localized specifically to the glandular trichomes (Gershenzon et al., 1989, 2000; McCaskill et al., 1992), and the pathway in peppermint leading to the principal oil component (−)-menthol is complex in involving multiple steps and a wide range of reaction types (Fig. 1) (Croteau and Gershenzon, 1994). Following the conversion of the primary metabolites isopentenyl diphosphate and dimethylallyl diphosphate to geranyl diphosphate, the cyclization of this universal monoterpene precursor by limonene synthase yields the first committed intermediate (−)-4*S*-limonene (Alonso et al., 1992, Colby et al., 1993). The cytochrome P450-mediated hydroxylation of limonene by limonene-3-hydroxylase next produces (−)-*trans*-isopiperitenol (Lupien et al., 1999) which, following a sequence of five (mostly redox) steps, affords (−)-menthol, the most abundant (and characteristic) constituent of the commercial essential oil of peppermint (Croteau and Gershenzon, 1994).

With the pathway for the biosynthesis of peppermint monoterpenes well defined (for review see Wise and Croteau, 1999), more recent attention has turned to the developmental and environmental regulation of metabolism of these natural products. Monoterpene production in mint is restricted to developing oil glands of young leaves (Turner et al., 2000), and the correlation between in vitro activity for the eight enzymatic steps of menthol biosynthesis and the rate of biosynthesis measured in vivo suggests that monoterpene production is controlled by the coordinately regulated activity of the relevant biosynthetic enzymes (Gershenzon et al., 2000;

Abbreviations: LS, limonene synthase; LH, limonene-3-hydroxylase; *smls*, spearmint limonene synthase; *pmls*, peppermint limonene synthase; WT, wild type.

* Corresponding author. Tel.: +1-509-335-1790; fax: +1-509-335-7643.
E-mail address: croteau@mail.wsu.edu (R. Croteau).
[1] Present address: Chemistry Department, Okanagan University College, Kelowna, BC Canada V1V 1V7

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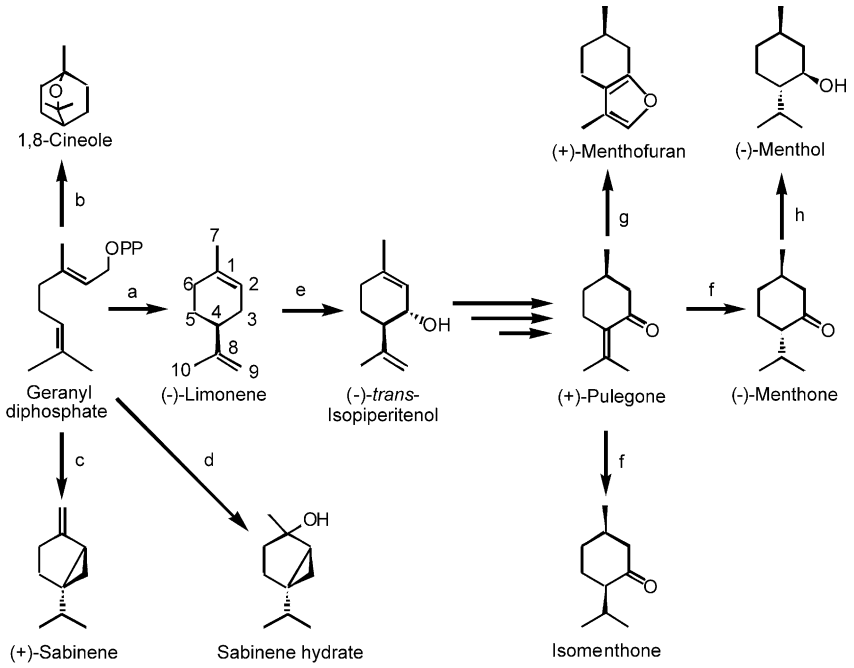

Fig. 1. Pathways for the biosynthesis of peppermint essential oil monoterpenes. The indicated enzymes are: (a) limonene synthase, (b) 1,8-cineole synthase, (c) sabinene synthase, (d) sabinene hydrate synthase, (e) limonene-3-hydroxylase, (f) pulegone reductase, (g) menthofuran synthase, and (h) menthone reductase. Triple arrows indicate multiple steps.

McConkey et al., 2000). These results, supported by measurement of time-dependent transcript abundances and enzyme protein abundances for several pathway steps (McConkey et al., 2000), suggest that oil yield, as well as composition, reflect the simple kinetic consequences of the levels of biosynthetic enzymes present, as determined by transcriptional and translational production of these pathway catalysts and their subsequent turnover.

The synthesis of (−)-4*S*-limonene, in providing the first committed intermediate of the pathway (i.e., the geranyl diphosphate precursor of limonene can be employed in the biosynthesis of other isoprenoids, including other monoterpenes), represents a possible rate limiting step of monoterpene production in peppermint (Gershenzon and Croteau, 1990; Croteau and Gershenzon, 1994). Additionally, the levels of limonene in commercial peppermint oil are very low (<1%), implying that this olefin precursor is rapidly utilized in downstream biosynthetic steps (i.e., that consumption nearly exceeds production). Because of the regulatory implications for this committed step, it was desirable to evaluate in greater detail, and by transgenic means, the metabolic influences of altered production and disposition of this central intermediate.

Two previous attempts have been made (Krasnyanski et al., 1999; Diemer et al., 2001) to constitutively overexpress the limonene synthase gene (Colby et al., 1993) in peppermint. Neither study reported substantive effects on oil yield or consistent influence on oil composition; however, neither study evaluated the expression pattern of the gene in oil glands nor measured any direct parameter of glandular metabolism. In this paper, we describe studies on the constitutive expression of both limonene synthase and limonene-3-hydroxylase, each driven by the CaMV 35S promoter, with specific focus on alterations in monoterpene metabolism in the glandular trichomes of the derived transgenic peppermint plants.

2. Results and discussion

Because limonene synthase (LS) catalyzes the committed, and possibly rate-limiting, step of menthol biosynthesis in peppermint (Gershenzon and Croteau, 1990; Croteau and Gershenzon, 1994), an assessment of the influence of the transgenic overexpression of the *ls* gene is of interest and of potential commercial significance. Two previous attempts to overexpress *ls* in peppermint under the influence of the strong, constitutive CaMV 35S promoter evidenced little or no effect on essential oil composition or yield (Krasnyanski et al., 1999; Diemer et al., 2001); however, neither study directly assessed the influence of the transgene on oil gland metabolism. Our previous success in the sense overexpression of deoxyxylulose phosphate reductoisomerase to promote precursor supply and increase oil yield, and in the antisense expression of menthofuran synthase to alter oil composition (both controlled by the

CaMV 35S promoter) (Mahmoud and Croteau, 2001) encouraged a more detailed reevaluation of *ls* expression from this promoter, as well as an evaluation of the influence of the limonene-3-hydroxylase (*lh*) gene since the latter is responsible for directing limonene into the menthol biosynthesis pathway (Fig. 1) (Lupien et al., 1999).

2.1. Limonene synthase expression

A cDNA clone encoding limonene synthase was first isolated from spearmint (Colby et al., 1993) and subsequently from peppermint (Lange et al., 2000). The two *ls* genes are nearly identical at the deduced amino acid level and, because of its earlier availability and use in the previous studies (Krasnyanski et al., 1999; Diemer et al., 2001), the spearmint gene (*smls*) was employed here. The coding sequence (Colby et al., 1993) was placed under the regulation of the CaMV 35S promoter (construct designated pGALS) as described, and transformed into peppermint using an *Agrobacterium*-based transformation procedure (Niu et al., 1998). Transformation with this construct yielded 36 transgenic plants, most of which maintained wild type appearance and growth habit.

To examine the expression pattern of the transgene, total RNA was extracted from both immature and fully expanded leaves, and analyzed by northern blotting using the coding region of the spearmint limonene synthase as probe. Because the spearmint gene (*smls*) is nearly identical to the peppermint synthase gene (*pmls*), the probe detects transcripts produced from both the transgene and the endogenous peppermint limonene synthase gene. However, because the native limonene synthase is not expressed in fully expanded leaves (after essential oil synthesis is complete) (McConkey et al., 2000), any limonene synthase transcript detected in mature leaves of transgenic plants must originate from the spearmint transgene. Based on this RNA blot data (Fig. 2), nine transformants (including pGALS23, pGALS24, pGALS27 and pGALS33, for which adjusted densitometric signal intensities were 2.21 ± 0.22, 1.67 ± 0.18, 0.59 ± 0.03 and 0.74 ± 0.02, respectively) that ectopically expressed *smls* were identified. The limonene synthase transcript was not detected in the fully expanded leaves of WT and pGALS10.

To confirm that the *smls* mRNA was correctly translated, soluble protein extracts from the pooled leaves of transformed plants were analyzed by immunoblotting using polyclonal antibodies prepared against the spearmint limonene synthase (Alonso et al., 1993). Densitometric analysis of the blots revealed that the apparent overexpressers accumulated 3 to 5 times more limonene synthase protein (signal intensities of 1550 ± 34 and 2030 ± 37 for LS 23 and LS 24, respectively) than did wild type peppermint (signal intensity of 408 ± 28) (Fig. 2).

To evaluate the effects of *smls* overexpression on synthase catalytic capability, limonene synthase activity was examined in soluble protein extracts obtained from whole leaf tissue of the two transformants (pGALS23 and pGALS24) which were the strongest expressers as evidenced by the RNA blot and immunoblot data. The results demonstrated that these protein extracts contained substantially higher limonene synthase activity (313 ± 11.5 and 135 ± 10.4 nmol·leaf^{-1}·h^{-1}, respectively) than did those of wild type plants (75 ± 2.4 nmol·leaf^{-1}·h^{-1}).

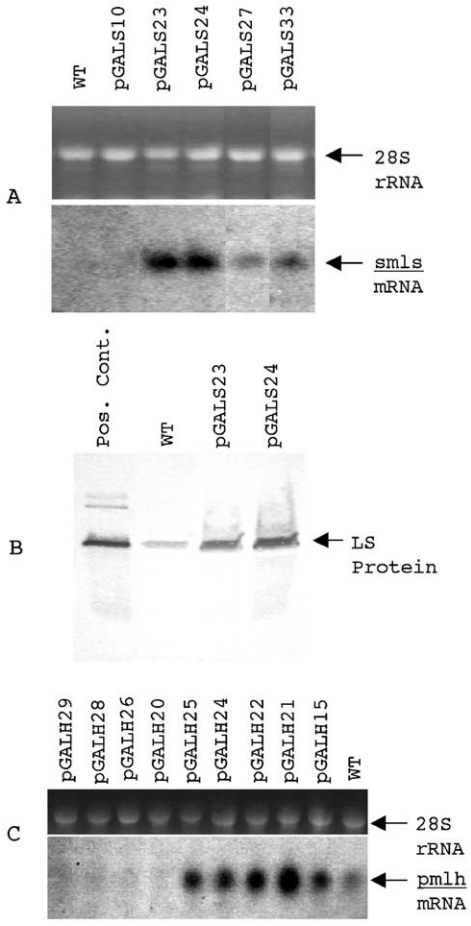

Fig. 2. A and C: Transcript levels for limonene synthase (mature leaves) and limonene-3-hydroxylase (immature leaves) in whole leaf extracts of peppermint plants transformed with spearmint limonene synthase (*smls*), or peppermint limonene-3-hydroxylase (*pmlh*) (pGALS and pGALH, respectively). Transformants pGALS23, pGALS24, pGALS27 and pGALS33 accumulated higher levels of the limonene synthase transcript than did wild type (WT) controls. Transcript levels for *pmlh* were increased in pGALH15, pGALH21, pGALH22, pGALH24, and pGALH25 transformants relative to wild type (WT) controls, but were undetectable in transgenic plants pGALH20, pGALH26, pGALH28, and pGALH29. B: Limonene synthase protein levels in pooled leaf samples of two peppermint plants (pGALS23 and pGALS24) that overexpress limonene synthase. Overexpression of *smls* in all confirmed transgenic plants resulted in increased limonene synthase (LS) protein production compared to wild type (WT) controls. Pos. Cont. is the positive control with recombinant spearmint limonene synthase.

Table 1
Essential oil composition and yield data for representative transgenic peppermint plants transformed with limonene synthase (pGALS23 through pGALS33), and for those that cosuppress (pGALH20 and pGALH28) or overexpress (pGALH22 and pGALH25) limonene-3-hydroxylase

Oil constituent	% Total oil									
	WT	pGALS10	pGALS23	pGALS24	pGALS27	pGALS33	pGALH20	pGALH22	pGALH25	pGALH28
Limonene	2.2	0.9	1.4	0.5	1.3	1.4	79.0	1.7	1.5	77.0
Cineole	6.6	0.5	6.9	3.9	6.3	6.7	6.0	6.0	5.6	7.4
Menthone	47.9	66.4	51.2	57.3	48.4	47.7	5.4	50.7	40.0	5.6
Sabinene hydrate	3.8	2.2	3.3	2.9	3.2	3.0	3.4	3.7	3.1	4.4
Menthofuran	10.7	Trace	6.1	14.8	8.5	7.8	0.6	10.3	15.5	0.6
Isomenthone	4.4	5.3	4.7	4.5	4.6	4.6	0.6	4.2	3.5	1.23
Pulegone	4.1	7.5	2.0	3.4	3.1	1.8	Trace	4.9	7.4	Trace
Menthol	18.2	16.1	22.4	11.1	22.8	24.9	5.2	18.5	23.4	3.86
EO yield (mg/gfWt)	4.7±0.8	4.5±0.9	4.9±0.8	4.7±0.8	5.1±1.0	5.0±0.8	4.9±0.3	4.7±0.2	4.7±0.4	5.6±0.2

Essential oil (EO) yields are calculated in milligrams per gram fresh tissue weight (mg/gfWt). Reported values represent the means of at least three measurements and include the corresponding standard deviations. The sabinene levels were low for all plants and are not included. Note that pGALS10 did not express the synthase transgene but is included to illustrate an apparent insertional effect on oil composition (see text).

Thus, all indications were that spearmint limonene synthase had been successfully, and ectopically, expressed in peppermint. Nevertheless, evaluation of the essential oil composition and yield of the transgenic overexpressers showed no appreciable change compared to wild type plants (Table 1). Transformant pGALS10, which did not express *smls*, is included in the Table to illustrate that essential oil composition can be altered by apparent insertional effects; the phenomenon is not uncommon and has been observed previously (Mahmoud and Croteau, 2001).

The lack of appreciable effect on oil composition and yield, from apparent overexpression of limonene synthase, suggested that either transgene action did not alter metabolism (relative to endogenous flux) in the oil gland sites of synthesis, or that the transgene was not effectively expressed in this locale. To address this question, the expression of limonene synthase was evaluated by northern blotting of RNA obtained specifically from isolated oil glands (Gershenzon et al., 1992) of plants that seemingly overexpressed *smls*. The results (Fig. 3) demonstrated that, compared to wild type peppermint (adjusted signal intensity of 1.81±0.12), limonene synthase transcript abundance *was not* significantly increased ($P>0.30$) in the oil glands of the apparent overexpressers (adjusted signal intensity of 1.94±0.09). Additionally, LS enzyme activity levels were not notably increased in soluble protein extracts of the oil glands of the apparent high level expressers pGALS23 and pGALS24 compared to wild type plants (data not shown). Therefore, in spite of the apparent constitutive overexpression of *ls* (as determined in whole leaf preparations), when isolated oil gland cells were examined there was no appreciable alteration in LS activity relative to the normal, developmentally regulated process of wild type plants (McConkey et al., 2000).

2.2. Limonene-3-hydroxylase expression

Two essentially identical cDNA clones encoding cytochrome P450 (−)-limonene-3-hydroxylase (designated PM2 and PM17) have been isolated from peppermint (Lupien et al., 1999). Heterologous expression of both isoforms in microbes indicated that PM2 afforded higher levels of functional recombinant protein (Haudenschild et al., 2000), and so clone PM2 was utilized. The coding sequence of this gene was placed under the control of the CaMV 35S promoter (construct

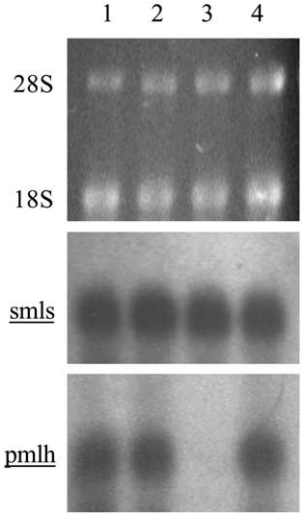

Fig. 3. Transcript levels for limonene synthase (*smls*) and limonene-3-hydroxylase (*pmlh*) in the isolated oil glands of wild type peppermint (lane 1), of plants that overexpress *smls* in leaves (lane 2), of plants that cosuppress *pmlh* (lane 3) and of plants that overexpress *pmlh* in leaves (lane 4). The upper panel illustrates the ethidium bromide stained 18S and 28S ribosomal RNA bands in the corresponding samples which were used to verify equal loading.

designated pGALH) and transformed into peppermint by the *Agrobacterium*-based protocol (Niu et al., 1998) as before.

Analysis of total RNA isolated from immature leaves of plants transformed with pGALH by northern blotting, using the coding region of the gene as probe, showed that transcript levels for limonene-3-hydroxylase were significantly ($P < 0.02$) increased in eight transgenic plants (including pGALH15, pGALH21 pGALH22 pGALH24 and pGALH25, for which the adjusted densitometric signal intensities were 0.85 ± 0.07, 1.95 ± 0.11, 1.39 ± 0.05, 1.12 ± 0.02, and 1.31 ± 0.04, respectively) and were unchanged in seven transformants compared to wild type controls (adjusted signal intensity of 0.66 ± 0.08). The hydroxylase transcripts were barely detectable in the remaining fourteen regenerated plants (adjusted signal intensities of 0.11 ± 0.03 to 0.15 ± 0.04 for all tested plants), suggesting that *lh* was cosuppressed in a high proportion of the transformants (Fig. 2).

Analysis of RNA prepared from the isolated oil glands of these same plants (Fig. 3) showed that the apparent ectopic overexpression of the hydroxylase in leaves did not result in a an increase in transcript abundance in the oil glands of these plants (adjusted signal intensity of 1.6 ± 0.06) relative to the oil glands of untransformed controls (adjusted signal intensity of 2.14 ± 0.12). However, in the apparently cosuppressed plants, the *lh* transcript was not readily detectable in oil gland extracts (Fig. 3) indicating that the gene was effectively silenced in this locale.

Consistent with the results of RNA blot analysis, the essential oil composition and yield was not markedly affected in transgenic plants that constitutively expressed limonene hydroxylase in leaves (but not specifically in oil gland secretory cells) (see Table 1 pGALH22 and pGALH25, for example). However, oil composition was radically altered in transgenic plants in which limonene hydroxylase was silenced (see Table 1 pGALH20 and pGALH28 for example). These cosuppressed plants, in which the limonene hydroxylase message was nearly absent from the oil glands, produced an essential oil that was highly enriched in limonene (to nearly 80% of the oil in pGALH20 and pGALH28, compared to control levels of ~2% in wild type plants) (Table 1), and depleted in the downstream metabolites derived from this intermediate (*cf* Fig. 1). Silencing of the hydroxylase had negligible effect on the production of 1,8-cineole and sabinene hydrate (Table 1), two oxygenated monoterpenes that are derived directly from geranyl diphosphate by other monoterpene synthases (Wise and Croteau, 1999) and that do not involve the intermediacy of limonene (Fig. 1). Interestingly, essential oil yield was unaffected in plants for which *lh* was cosuppressed, indicating that pathway flux control resides upstream of this hydroxylation step, that monoterpene production and accumulation is independent of oil composition, and that limonene synthesis to substantial levels is without feedback effect on limonene synthase and without apparent influence on other cyclization steps (e.g., 1,8-cineole synthase).

3. Conclusions

Transgenic peppermint plants that constitutively, but only moderately, overexpressed *ls* and *lh* were obtained; however, expression under the regulation of the 35S viral promoter was insufficient in all cases to significantly increase production of the targeted enzymes in the glandular trichome sites of essential oil biosynthesis, and, thus, no influence was observed on oil composition or yield of the transformed plants. Although it is possible that the 35S promoter is incapable of detectably promoting expression beyond the relatively high expression levels already present in the oil glands, this outcome may also relate to selection pressure against plants that very strongly express these genes ectopically. Production of monoterpenes in non-specialized cells, which lack the machinery for trafficking and storage of these metabolites, could be toxic. It is reasonable to suppose that ectopic, strong expression of *ls* could lead, by the conversion of endogenous geranyl diphosphate, to levels of limonene that are lethal. Although geranyl diphosphate synthase is very likely restricted to the leucoplasts of mint gland secretory cells (Burke et al., 1999), sufficient geranyl diphosphate, as an intermediate in geranylgeranyl diphosphate synthesis, may be available in the plastids of other cells to allow formation of limonene by the plastid-directed synthase.

In the case of the hydroxylase, the abnormally high rate of cosuppression of this *lh* transgene (>70%) suggests that strong constitutive expression in this case is also not well-tolerated by transformed plants and leads to gene silencing. In this case, the limonene substrate for the hydroxylase is not available (production being restricted to the oil glands); however, the adventitious oxygenation of some other cellular metabolite could have toxic consequences. It also should be noted that the present results do not rule out the possibility that the expression of *lh* and *ls* may be posttranscriptionally regulated in the oil glands of peppermint, opposing any attempt to overexpress these genes.

The present results indicate that effective gene silencing can be achieved using conventional 35S promoter-based approaches to substantially alter an extended metabolic pathway so as to accumulate an intermediate without an appreciable influence on overall flux. However, it is equally clear that the use of strong gland-specific promoters would provide a more direct and precise means of modifying the target monoterpene biosynthetic pathways in commercial mint species.

Environmental influence on the oil composition of peppermint is well-documented (Burbott and Loomis, 1967; Clark and Menary, 1980; Voirin et al., 1990) and classical hybridization studies with fertile *Mentha* species have shown that essential oil composition can be greatly modified in such crosses (for reviews, see Croteau and Gershenzon, 1994; Hefendehl and Murray, 1976), including limonene chemotypes similar to that described here (Hefendehl and Murray, 1973). In a developmental context, it has also been shown that, at the onset of the brief but intense period of essential oil accumulation in the oil glands of immature peppermint leaves, the transient level of limonene can exceed 20% (Gershenzon et al., 2000). The present experiments, however, are the first to demonstrate that altered expression of a single gene (cosuppression of *lh*) leads to the essentially complete replacement of the normal C3-oxygenated monoterpenes (pulegone, menthofuran, menthone and menthol) by the olefin limonene, a monoterpene metabolite of very different polarity and volatility, that is nevertheless adequately managed by the extant glandular trafficking and secretion machinery without effect on overall oil yield. This observation bodes well for biotechnological approaches to exploit *Mentha* oil glands in the transgenic production of a wider range of hydrophobic compounds of industrial, agrochemical and pharmaceutical interest that could not, because of toxicity considerations, be accumulated in non-secretory plant tissues (Mahmoud and Croteau, 2002; McCaskill and Croteau, 1999).

4. Experimental

4.1. Plant material

Peppermint (*M.*×*piperita* L. cv Black Mitcham) plants were propagated from rhizomes and grown in pots (35 cm diam.) containing peat moss:pumice:sand (55:35:10, v/v) under a 26C day (16 h)/16 °C night (8 h) cycle in a greenhouse. Plants were irrigated and fertilized daily with a complete fertilizer (N:P:K, 20:20:20) plus iron chelate and micronutrients.

4.2. Vector assembly and plant transformation

The parent vector pGADXRS (Mahmoud and Croteau, 2001), originally derived from pGA482 (An, 1987), contained the cDNA encoding deoxyxylulose-5-reductoisomerase (*dxr*) inserted between the tandem CaMV 35S promoter with duplicated enhancer and the *Agrobacterium tumefaciens nos* transcriptional terminator. The *dxr* insert was removed by *Eco*RI/*Kpn*I digestion and replaced by ligation with the full-length cDNA encoding the spearmint (−)-limonene synthase (*smls*, which includes the plastidial targeting sequence) (Colby et al., 1993) or the peppermint cytochrome P450 (−)-limonene-3-hydroxylase (*pmlh*) (Lupien et al., 1999), both of which were modified by sticky-end PCR (Zeng, 1998) to encode *Eco*RI and *Kpn*I restriction sites beyond the 5- and 3-termini, respectively. The resulting constructs, designated pGALS and pGALH, were electroporated into *Agrobacterium tumefaciens* strain EHA105 using the MicroPulser (Bio-Rad) according to the manufacturer's protocol. A single transformed colony was isolated in each case, and was grown to log phase and used to transform peppermint leaf explants by an established protocol (Niu et al., 1998).

4.3. RNA and immunoblot analyses

Procedures for the isolation of glandular trichomes from peppermint leaves (Gershenzon et al., 1992; McCaskill et al., 1992), and for the extraction of RNA and protein for immunoblotting from the secretory cells of these structures, have been described previously (McConkey et al., 2000; Mahmoud and Croteau, 2001). Total RNA (5–10 μg) was resolved on a 1.2% agarose gel, transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) and probed with ^{32}P-labeled *smls* or *pmlh* DNA. The probes were prepared using the Ready-To-Go Kit (Amersham Pharmacia Biotech). Pre-hybridization and hybridization were carried out in Rapid-Hyb buffer (Amersham Pharmacia Biotech), and labeled bands were visualized by exposing the membrane to Kodak X-OMAT X-ray film.

Immunoblot analyses were carried out by published protocols (Sambrook et al., 1989) using polyclonal antibodies generated against spearmint limonene synthase in rabbits (Alonso et al., 1993) and ImmunoPure goat anti-rabbit IgG conjugated to alkaline phosphatase (Pierce, Rockford, IL, USA) as secondary antibody. Protein was resolved on a 10% denaturing polyacrylamide gel and transferred to Immobilon-P^{TM} membranes (Millipore) using the Mini-Protean II Cell electrophoresis system (Bio-Rad).

To obtain quantitative estimates of signal intensities, digitized images of all gels and blots were obtained using the Gel Doc 2000^{TM}/Chemi Doc^{TM} gel documentation system (Bio-Rad), and signal intensities were quantified by densitometry using the Scion Image 1.57 software for the Macintosh (National Institute of Health, USA). To correct for loading variations in RNA blots, signals for limonene synthase and limonene-3-hydroxylase were standardized to those of the ethidium bromide stained ribosomal RNA, in the corresponding samples. Multiple (at least 3 replicates) measurements for individual treatment groups were compared to those of controls by performing Student's *t*-test at the 95% confidence level using Microsoft Excel software for the Macintosh.

4.4. Isolation and assay of limonene synthase

Procedures for the isolation of limonene synthase in soluble protein extracts from whole leaf tissue (Croteau and Cane, 1985) and from oil gland secretory cells (Alonso et al., 1992) have been described. The preparations were partially purified to remove phosphatases by adding the soluble extract to powdered hydroxyapatite [1 g pre-washed (potassium phosphate, pH 6.5) matrix per g of extracted tissue], followed by centrifugation to remove the matrix, and assayed by standard procedure for the conversion of [1-^{3}H]geranyl diphosphate to the labeled olefin that is purified by silica gel chromatography (Rajaonarivony et al., 1992). Quantification was by liquid scintillation counting and, typically, less than 20% of the substrate was consumed in the assay.

4.5. Essential oil analysis

Plants were grown to confluence at the flower bud stage as previously described (Mahmoud and Croteau, 2001), and the vegetative parts were harvested, frozen at −20 °C, and manually crushed and mixed to ensure sample uniformity. Essential oil was steam distilled-solvent extracted from multiple 5 g tissue samples, and the distillate was analyzed by gas chromatography (with mass spectrometric confirmation of monoterpene composition) as previously described (Gershenzon et al., 2000; Mahmoud and Croteau, 2001). Camphor (1 mg) was added as the internal standard to all samples prior to the distillation step. Oil yield and compositional data for all plants were obtained by at least six independent measurements over a nine-month period. Note that in commercial practice the harvest would be delayed until the menthol content of the oil exceeds 40%. Time and space constraints necessitated earlier harvest in the present experiments.

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