

Genetic engineering of essential oil production in mint

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New approaches directed to unraveling monoterpene metabolism and secretion and recent progress in transformation protocols have set the stage for the systematic genetic engineering of essential oil production. This article focuses on specific strategies to improve the quality and quantity of mint essential oils.

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Abbreviations

DXP	1-deoxy-D-xylulose-5-phosphate
EST	expressed sequence tag
FPP	farnesyl diphosphate
GAP	glyceraldehyde-3-phosphate
GGPP	geranylgeranyl diphosphate
GPP	geranyl diphosphate
IPP	isopentenyl diphosphate

Introduction

Mints have been used and valued as aromatic herbs for thousands of years. Mint is the common name of approximately 25 perennial species of the genus *Mentha* of the Lamiaceae family. Mints are sources of essential oils which are used for flavoring, perfume production and medicinal purposes. The chemical constituents associated with the typical olfactory characteristics of these oils are monoterpenes and, to a lesser extent, sesquiterpenes, which both belong to a structurally diverse group of natural products known as isoprenoids. Given the high commercial value of mint oils (\$160 million farm gate value which is converted to \$5.5 billion in finished products (US alone); 1998 Production Statistics, US Mint Industry Research Council), the processes involved in the metabolism of monoterpenes and their storage in specialized tissues are attractive targets for genetic engineering. Recent advances in understanding these developmentally controlled events, particularly in the case of peppermint (*Mentha x piperita*) as an experimental model system, and technical progress in transformation techniques, have provided the basis for the biotechnological manipulation of essential oil production. Where possible, we emphasize research directly related to essential oil formation and describe the potential of genetic engineering techniques for mint.

Tissue localization

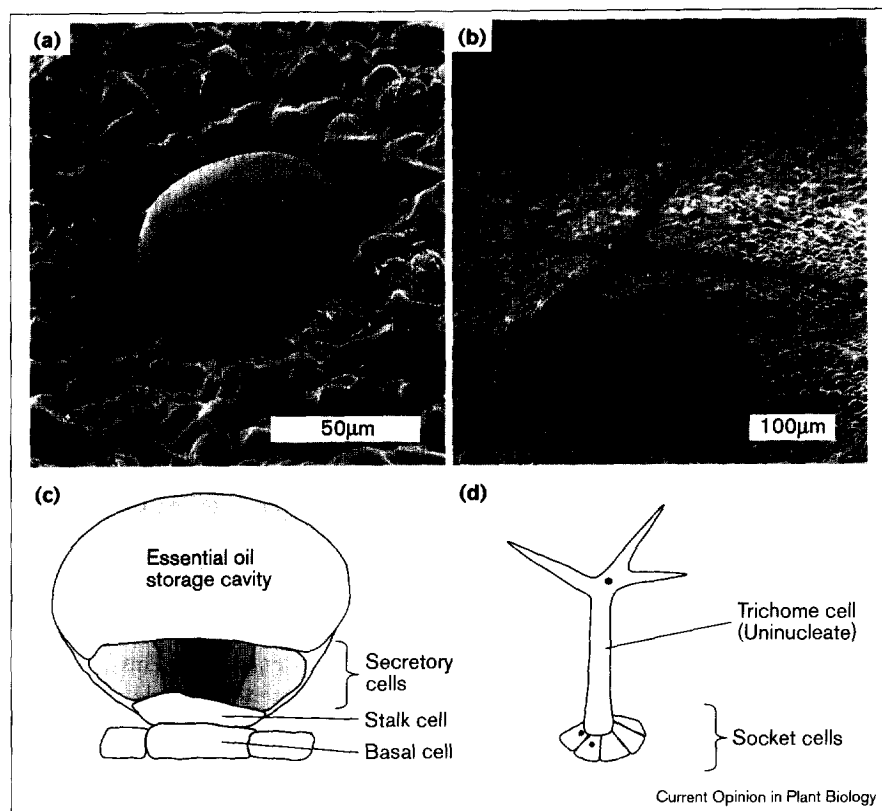
The essential oil of mint is synthesized and stored in modified epidermal leaf hairs called (peltate) glandular trichomes (Figure 1a), which consist of a single basal cell, a stalk cell, and a radial cluster of secretory cells (Figure 1c). In these highly-specialized structures, material produced

by the secretory cells accumulates within a large subcuticular cavity formed by expansion of the surmounting cuticle [1]. It appears that glandular trichomes have evolved to allow the large-scale synthesis and storage of compounds which require segregation from the rest of the plant tissue due to their cytotoxicity [2]. Studies with isolated secretory cells from oil glands of peppermint have established that this cell type, which is non-photosynthetic, expresses genes encoding the complete enzymatic machinery necessary to produce monoterpenes from imported sucrose [3,4].

Isoprenoid biosynthesis

Isoprenoid biosynthesis in mints may be divided into four stages. Stage 1 comprises the synthesis of the C₅ intermediate isopentenyl diphosphate (IPP), the central building block of isoprenoids (Figure 2). In plants, two distinct pathways are utilized to produce IPP. The cytosolic compartment contains the enzymes of the acetate/mevalonate pathway [5]. Plastid-derived isoprenoids of plants, however, including carotenoids and the prenyl side chains of chlorophyll and plastoquinone, as well as monoterpenes and diterpenes, are synthesized via the recently discovered pyruvate/glyceraldehyde-3-phosphate (GAP) pathway, which also operates in several eubacteria [6*]. In secreting peppermint (*Mentha x piperita*) oil glands, the acetate/mevalonate pathway is essentially blocked, most likely at the stage of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a key regulatory enzyme of this route. The IPP used for both monoterpene and sesquiterpene formation is exclusively synthesized in the plastids [7] via the pyruvate/GAP pathway, as established recently for several monoterpenes [8]. The initial step of this pathway is the formation of 1-deoxy-D-xylulose-5-phosphate (DXP) by condensation of pyruvate and GAP with loss of CO₂. In the second step, rearrangement and reduction of DXP yield 2-C-methyl-D-erythritol-4-phosphate [6*]. The subsequent steps towards IPP have not yet been elucidated, and their definition is currently a very active field of research. Stage 2 begins with the isomerization of IPP to dimethylallyl diphosphate, which then serves as the reactive starter unit for sequential elongation reactions with one, two or three additional IPP units to form geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅) or geranylgeranyl diphosphate (GGPP, C₂₀), respectively [9]. In stage 3, these prenyl diphosphates undergo a range of cyclization reactions to produce the parent skeletons of the different classes of monoterpenes from GPP [10], sesquiterpenes from FPP [11], and diterpenes from GGPP [12]. The cyclic parent compounds are then transformed in stage 4 by redox, isomerization and conjugation reactions to yield the various isoprenoid end-products such as those typical of the essential oils (Figure 3) [10]. Since the vast majority of the components of mint essential oils are monoterpenes of the *p*-menthane type, the

Figure 1



Scanning electron micrographs of (a) a peppermint peltate glandular trichome and (b) a mature *Arabidopsis* trichome (courtesy of G Turner). Trichome anatomy of a mature (c) mint glandular trichome and (d) *Arabidopsis* non-glandular trichome is illustrated.

metabolism of these monoterpenoids provides the focus of this review.

As part of an ongoing effort to isolate genes of isoprenoid metabolism, a cDNA library has been constructed that was derived from peppermint oil gland secretory cells, a cell type highly specialized for essential oil formation [13] and, thus, greatly enriched in monoterpene biosynthetic mRNA species [4]. The sequencing of 400–900 bases of 1500 randomly selected clones of this cDNA library allows estimation that up to 8–10% of these expressed sequence tags (ESTs) represent enzymes comprising all four stages of monoterpene metabolism. Among the clones obtained are DXP synthase [14^{••}], and DXP reductoisomerase (BM Lange, R Croteau; unpublished results) (stage 1), IPP isomerase [4], GPP synthase, FPP synthase and GGPP synthase (stage 2; MR Wilding, C Burke, R Croteau; unpublished data) limonene synthase (A Cromwell, J Crock, BM Lange, R Croteau; unpublished data) and limonene-3-hydroxylase (stage 4; [15]). Two biosynthetic genes from spearmint (*Mentha spicata*), limonene synthase [16] and limonene-6-hydroxylase [15], have also been characterized (stage 3,4). The peppermint oil gland EST sequencing project has also revealed additional candidate genes with sequence homologies to dehydrogenases (stage 1, stage 4), kinases (stage 1), dehydratases (stage 1), isomerases (stage 4), as well as acyl transferases and glycosyl transferases (stage 4); these are

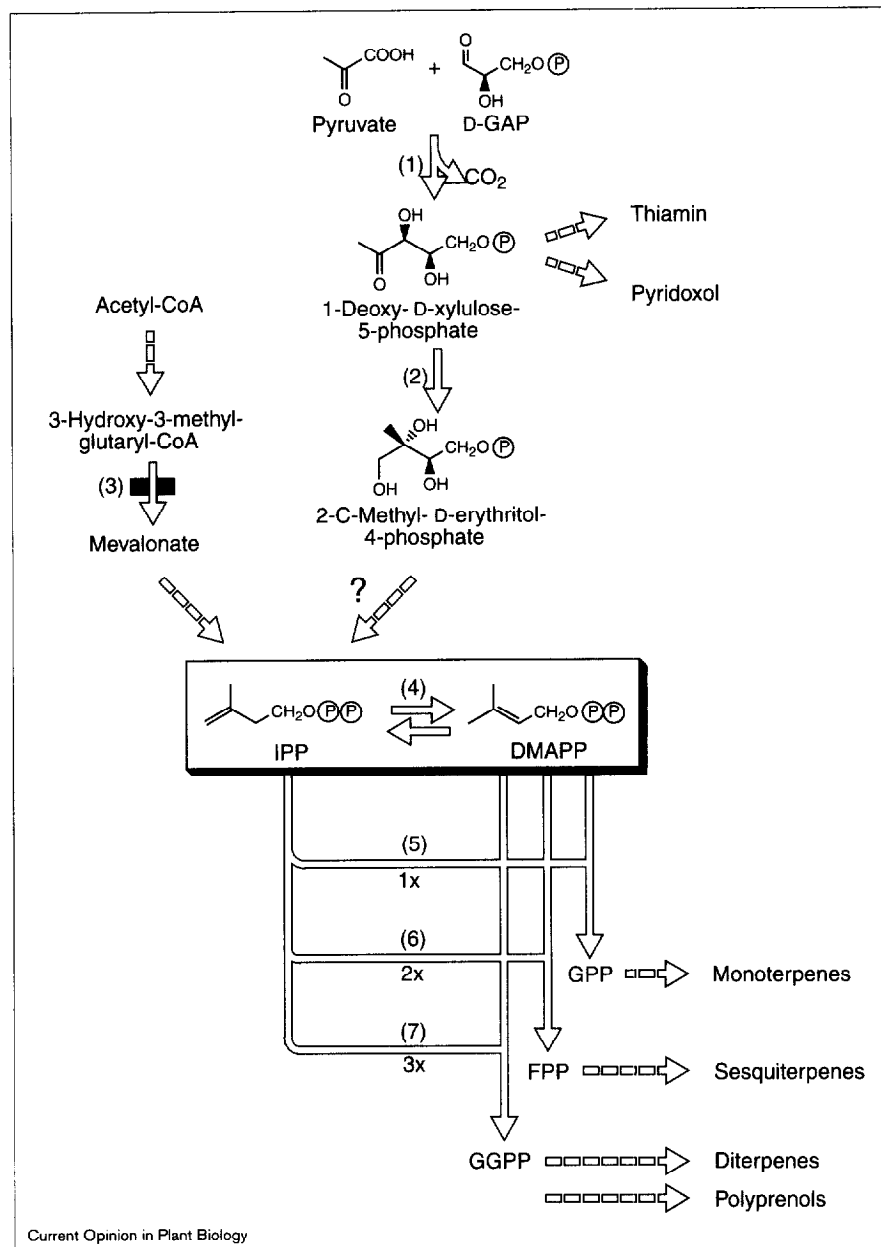
now being functionally expressed to investigate their potential roles in monoterpene biosynthesis.

Tissue-specific expression of transgenes

In recent years, *Agrobacterium tumefaciens*-based transformation protocols have been developed for peppermint, but only transformed calli [17], teratomas [18], or transient reporter gene expression [19] have been described. In 1998, two groups independently reported improved regeneration systems that yielded the first transgenic peppermint plants [20^{••},21]. Before molecular engineering technology can be efficiently applied to essential oil production in mint, however, an additional prerequisite has to be met; thus, to insure that transgenes are not expressed ectopically with attendant, potential complications (cytotoxicity, [2]), appropriate promoter elements must be identified that direct glandular trichome-specific expression. Although several plant epidermis-specific promoters have been reported [22–24,25[•]], including two promoters that direct gene expression to non-glandular trichomes of *Arabidopsis thaliana* [26] and both glandular and non-glandular trichomes of tobacco [27], a promoter that selectively regulates gene expression in glandular trichomes remains to be discovered. Promoter regions of a number of genes that encode trichome-localized enzymes of monoterpene biosynthesis are currently being analyzed in search of regulatory elements that provide the desired tissue specificity.

Figure 2

Overview of isoprenoid biosynthesis in plants. The enzymes indicated are: (1) 1-deoxy-D-xylulose-5-phosphate synthase; (2) 1-deoxy-D-xylulose-5-phosphate reductoisomerase; (3) 3-hydroxy-3-methylglutaryl coenzyme A reductase; (4) IPP isomerase; (5) GPP synthase; (6) FPP synthase; and (7) GGPP synthase. Dashed arrows indicate multiple enzymatic steps; the question mark indicates that the enzymes involved in these steps are presently unknown. The acetate/mevalonate pathway in secreting peppermint oil glands is blocked, most likely at position (3).

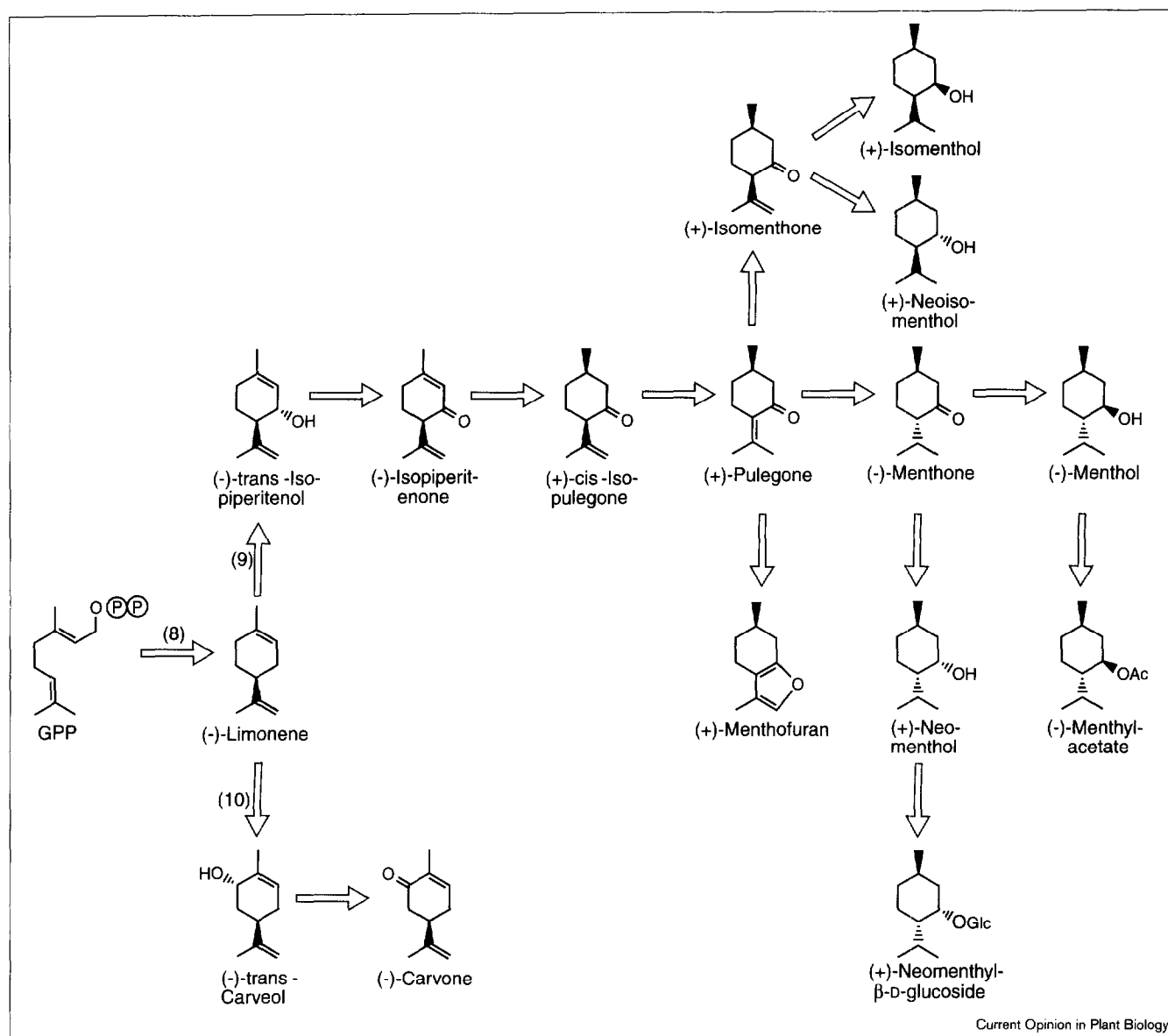


Redirection of metabolic flux

Although major advances have been made in cloning genes involved in the biosynthesis of the *p*-menthane monoterpenes of mint, molecular genetic approaches to increase the overall yield of monoterpene essential oils by increasing flux through the target pathway are still in the future. Knowledge of the various developmental factors that control essential oil formation, of the potential importance of metabolic branch points in control of flux, of possible feedback regulatory properties of intermediates, and of transport mechanisms required for the secretion of essential oils into specialized storage structures is presently lacking. It is likely that the manipulation of several enzyme activities will be required to redistribute metabolic

flux towards monoterpene biosynthesis. Additionally, redirecting significant flux towards monoterpene production may lead to deprivation of essential compounds formed from common intermediates; for example, both thiamin and pyridoxol are also derived from DXP (Figure 2) [28,29], as are diterpenes (e.g. gibberellins as phytohormones), tetraterpenes (e.g. carotenoids as photosynthetic pigments) and several polyterpenes (e.g. sidechain of the electron carrier plastoquinone). As an example of such unintended consequences, Fray *et al.* [30] reported that the constitutive overexpression of a phytoene synthase gene (carotenoid (C_{40}) biosynthesis) in transgenic tomato plants caused a dwarf phenotype, most probably by redirecting metabolite flux away from the

Figure 3



Monoterpene metabolism in peppermint and spearmint glandular trichomes. The enzymes indicated are: (8) (-)-limonene synthase; (9) (-)-limonene-3-hydroxylase (peppermint); and (10) (-)-limonene-6-

hydroxylase (spearmint). The conversion of (-)-menthone to (+)-neomenthyl-β-D-glucoside is a catabolic transformation, the site of occurrence of which is unknown.

gibberellin (growth hormone; C₂₀) and phytol (chlorophyll side chain; C₂₀) biosynthetic pathways.

Manipulation of monoterpene product profiles

Recent progress in the molecular biology of terpene synthases [31•] offers a tool for modifying the aroma profiles of essential oil plants by expression of foreign terpene synthase genes in transgenic plants to yield oils with novel properties. The ornamentals industry has embraced these genetic engineering approaches; however, the flavor and fragrance industry has been cautious since their markets are directed to human food and cosmetics. It seems likely that, at least in the near future, transgenic approaches to generate higher yields of desirable flavor and fragrance

compounds, or to lower the amounts of undesirable essential oil components, will be better received than those efforts directed to creation of novel metabolites.

An area where genetic engineering would certainly be of a commercial interest is the manipulation of *p*-menthane monoterpene metabolism in peppermint (Figure 3). Thus, once the gene that encodes the dehydrogenase which converts (-)-menthone to (-)-menthol becomes available, overexpression should lead to channeling of (-)-menthone toward the commercially desirable (-)-menthol. Alternatively, using antisense technology, the expression of the dehydrogenase responsible for the epimeric reduction of (-)-menthone to (+)-neomenthol could be reduced,

thereby decreasing catabolic losses of (–)-menthone via this route to the corresponding glucoside [32,33]. In a second approach, the less desirable oil components (+)-pulegone and (+)-menthofuran, the formation of which is determined by environmental factors beyond the control of the grower [34,35], could be diminished by repression of the putative cytochrome P450 oxygenase responsible for the conversion of (+)-pulegone to (+)-menthofuran and by overexpression of the gene encoding (+)-pulegone reductase leading to (–)-menthone (Figure 3).

Altering expression of genes related to trichome formation

A fourth goal of genetic engineering technology is to increase the tissue density of mint glandular trichomes in which the essential oil is produced and stored. This approach is based on the assumption that developmental processes involved in the formation of both glandular and non-glandular trichomes (Figure 1a–d) are similar. Four loci that affect the initiation of non-glandular trichomes of *Arabidopsis* have been characterized (*glabra-1* (*gl1*) [36], *transparent testa glabra* (*tig*) [37], *Triptychon* (*Try*) [38] and *Reduced Trichome Number* (*RTN*) [39]), but only one of the corresponding genes has been described. The *GL1* gene encodes a transcriptional regulator of the *myb* gene family [40], the expression of which is directed to leaf primordia and developing trichomes [26]. The *TTG* gene has not yet been cloned, but, interestingly, all *tig* defects are reversed by expression of the maize *R* gene, which belongs to the *myc* family of regulatory factors [41]. *GL1* expression alone is not sufficient to trigger the induction of trichome formation in cells that normally do not form these specialized anatomical structures. The constitutive overexpression of both *GL1* and the maize *R* gene (the functional complement of the *Arabidopsis* *tig* mutation), however, causes trichomes to develop on all epidermal surfaces [42]. The ubiquitous expression of *GL1* in the absence of the negative regulator *TRY* also leads to ectopic trichome formation on additional organs [43]. As a first step toward the biotechnological modification of (glandular) trichome surface density, *GL1* and *R* could be co-expressed under the control of a constitutive promoter in transgenic mint plants. In parallel, the isolation of mint orthologs of genes known to regulate trichome formation in *Arabidopsis* (*GL1*; *TTG*, *TRY*, *RTN*) should allow generation of more efficient constructs for the transgenic induction of glandular trichome formation in mint.

Conclusions

This review has described approaches for the genetic engineering of essential oil production with special emphasis on monoterpene metabolism in mint glandular trichomes. The specific aims for future research in this area will likely involve three sets of objectives. Short term goals include cloning of all the remaining genes of *p*-menthane monoterpene biosynthesis, defining promoter regions of genes specifically expressed in glandular trichomes, testing constructs for directing glandular trichome-specific expression of transgenes, constitutively overexpressing available

Arabidopsis *GL1* and maize *R* genes in transgenic peppermint plants to investigate potential roles in inducing (glandular) trichome formation, and isolating mint orthologs to *Arabidopsis* regulatory genes involved in trichome formation. Mid term goals are represented by optimization of vectors to adjust expression levels of transgenes as needed for the improvement of mint essential oil quality and quantity, and transformation of mint with constructs containing homologous regulatory genes. The ultimate goals are tissue-specific, regulated gene expression for multiple monoterpene biosynthetic enzymes to achieve the desired changes in essential oil composition and yield, and utilization of transgenic mint to investigate the regulatory processes involved in the control of pathway fluxes, metabolic profiles and monoterpenoid secretion.

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