TABLE OF CONTENTS

FINAL REPORT

Research over the last four years has progressed fairly closely along the lines initially proposed, with progress-driven expansion of Objectives 1, 2 and 3. Recent advances have developed from three research thrusts: 1. Random sequencing of an enriched peppermint oil gland cDNA library has given access to a large number of potential pathway and regulatory genes for test of function; 2. The availability of new DNA probes and antibodies has permitted investigation of developmental regulation and organization of terpenoid metabolism; and 3. The development of a transformation system for peppermint by colleagues at Purdue University has allowed direct transgenic testing of gene function and added a biotechnological component to the project. The current status of each of the original research objectives is outlined below.

Objective 1. The Mevalonate-Independent Pathway. Although we were the first group to clone from a plant source the first two genes and the last gene of this new pathway for isoprenoid biosynthesis [now called the methylerythritol phosphate (MEP) pathway], and amongst the first to utilize these genes in transgenic plant applications, colleagues working with *E. coli*, with its superior genetics and mutation capabilities, completed the biochemical characterization and gene cloning of this seven step pathway.

The evolutionary history of the enzymes involved in the classical mevalonate (MVA) and MEP pathways to terpenoids and the phylogenetic distribution of their genes across genomes have been evaluated. The results indicate that the MVA pathway is germane to eubacteria, and that eukaryotes have inherited their genes for this pathway from prokaryotes. The occurrence of genes specific to the MEP pathway is restricted to plastid-bearing eukaryotes, indicating that these genes were acquired from the cyanobacterial ancestor of plastids. The results suggest that lateral gene transfer between eubacteria subsequent to the origin of plastids has played a major role in the evolution of this pathway.

Studies with inhibitors of IPP metabolism to promote accumulation of MEP pathway intermediates indicated that tight feedback control of the pathway prevents the accumulation of late-stage metabolites. The methods developed here for metabolite profiling of peppermint oil gland secretory cells did prove useful for herbicide target analysis, and these studies further indicated that three steps may be of significance in the control of carbon flux toward monoterpene biosynthesis. Two of these steps comprise the first two reactions of the MEP

pathway catalyzed by deoxyxylulose phosphate synthase (DXPS) and deoxyxylulose phosphate reductoisomerase (DXR) that are involved in precursor supply, and the third is the branch-point prenyltransferase geranyl diphosphate synthase (GPPS) for specifically directing these isoprenoid precursors to the monoterpenes. The corresponding genes have been isolated with DOE support, and each was used in transgenic applications in peppermint to evaluate flux control and increase essential oil yield (see Objective 4. below).

Objective 2. Enzymes and Genes of Terpenoid Metabolism. The peppermint oil gland EST project was completed at about 1600 acquisitions. This random sequencing effort has yielded candidate clones for nearly all of the remaining enzymes of the (–)-menthol biosynthetic pathway, and most of the redox enzymes have now been confirmed by functional expression in *E. coli* by a method that involves administration of intermediates to cultures of transformed bacteria harboring the test gene followed by steam distillation to isolate product(s) and GC-MS analysis. Of the eight enzymatic steps from primary metabolism to (–)-menthol, the combination of reverse genetic cloning and mining of the EST library by functional expression has yielded all but two of the target genes (isopulegone isomerase and menthone reductase). The EST project has also yielded two new monoterpene synthases and two new sesquiterpene synthases.

Developmental studies (see Objective 3. below) indicated that menthone reductase, catalyzing the last step of menthol biosynthesis, does not appear until quite late in oil gland development, implying a delayed round of transcriptional and translational activity in these secretory structures. Because the EST project was based on mid-stage oil glands in secretory phase (when most of the biosynthetic machinery is highly active), menthone reductase transcripts were not represented in this gland library. A new oil gland library from mature (post secretory) structures was prepared, and a homology based screen has now yielded the menthone reductase gene. The purified recombinant enzyme has been characterized, and used to prepare antibodies for immunolocalization and regulatory studies. This work is now being prepared for publication.

The last remaining gene of the menthol biosynthetic pathway, that for isopulegone isomerase, does not appear to resemble (based on the absence of such sequences in the EST library) ketosteroid isomerase which the enzyme does resemble in mechanism of action. Isolation of this gene requires a reverse genetic approach. The isomerase is being purified from peppermint oil glands. This work is proceeding smoothly, and we expect to obtain sufficient pure protein for microsequencing and correlation to an EST sequence to acquire the target clone. We anticipate the completion of this cloning effort within the next few months. This isopulegone isomerase is of additional interest because it also appears to isomerize isomenthone to menthone (the immediate precursor of menthol) and so has implications for peppermint oil quality.

Objective 3. Organization and Regulation of Metabolism. Studies on the regulation of monoterpene accumulation in peppermint have confirmed that the rate of biosynthesis is the principal determinant of yield and that catabolic losses and losses by volatilization are of minor significance. Using oil glands isolated from peppermint leaves of different ages, *in vitro* assay of the eight sequential enzymes responsible for the biosynthesis of the principal monoterpene (–)-menthol indicated that all but one biosynthetic enzyme had a very similar developmental profile. The exception, (–)-menthone redcutase, the last step of the pathway, exhibited a much delayed peak of activity. RNA-blot analysis indicated that the genes encoding enzymes of most

of the pathway steps are transcriptionally activated in a coordinated fashion, with a time course superimpossible with activity measurements and immunoblot data. These results demonstrating coincidental temporal changes in enzyme activities, enzyme protein levels, and steady-state transcript abundances indicate that most of the monoterpene biosynthetic enzymes in peppermint are developmentally regulated at the level of gene expression.

The pattern of oil gland initiation and ontogeny on expanding peppermint leaves has been defined by population survey at several developmental stages. Remarkably, only 20 to 30 h of secretory activity is required for filling of the gland storage compartment with essential oil. Cryofixation methods were next employed to examine the ultrastructure of developing oil glands, and these results were correlated to the previous chemical, biochemical and molecular studies on monoterpene production and allowed inferences about the mechanism of essential oil secretion.

Immunocytochemical studies were used to define the organization of terpene metabolism in peppermint oil glands. Only the glandular secretory cells (not the basal or stalk cell) are active in monoterpene biosynthesis. The essential precursor of the monoterpenes, geranyl diphosphate, arises in plastids of the secretory cells, and the first committed step of the menthol biosynthetic pathway, limonene synthase, is also localized to this organelle. The next step of the pathway, catalyzed by cytochrome P450 limonene hydroxylase (to produce isopiperitenol), is localized to the endoplasmic reticulum (determined by classical subcellular fractionation studies as well as immunocytochemistry), and the next step, catalyzed by isopiperitenol dehydrogenase, is quite remarkably entirely localized to the matrix of gland cell mitochondria. The remaining (mostly redox) steps are localized to the cytoplasm with the exception of cytochrome P450 menthofuran synthase (that directs a side-route, dead-end pathway) which is localized to endoplasmic reticulum. The subcellular organization of monoterpene metabolism is thus quite complex in involving four participating sites, a finding that has raised the issue of the mechanism of hydrophobic metabolite trafficking between organelles. Immunolocalization studies are now being prepared for publication.

Objective 4. Flux Control of Precursor Supply. Using isolated oil glands as an experimental system, we were able to demonstrate that the cytosolic MVA pathway to terpenoids went inactive very early in gland development due to the diminution of HMG-CoA reductase (the regulatory step of the pathway). Thus, both cytosolic sesquiterpene biosynthesis and plastid-originating monoterpene biosynthesis depend entirely on terpenoid precursors supplied by the plastidial MEP pathway. Our efforts to determine flux of intermediates through the pathway and its subsidiary branches in isolated peppermint oil glands, by the methods of metabolic control analysis, were limited by our inability to specifically inhibit the sesquiterpene (C_{15}) branch of the pathway. Thus, input from the MEP pathway can be perturbed with fosmidomycin, and the monoterpene (C_{10}) branch can be severely inhibited with the potent, irreversible mechanism-based inhibitor of monoterpene cyclases cyclopropyl geranyl diphosphate but the available competitive inhibitors of the sesquiterpene (C_{15}) cyclases are insufficiently potent or, in the case of farnesyl *bis*-phosphonate, inhibit both monoterpene and sesquiterpene biosynthesis. Nevertheless, inhibition of plastidial monoterpene biosynthesis resulted in only marginal (~10%) increase in the rate of cytosolic sesquiterpene formation, and overexpression of IPP isomerase to establish a more favorable IPP:DMAPP ratio had similar, minor effect. These results suggest

that the rate of cytosolic sesquiterpene biosynthesis is limited primarily by the rate of export from the plastids of the C_5 isoprenoid precursors to the cytosol.

Using a transformation system developed by colleagues at Purdue University, homologous copies of the genes encoding DXPS, DXR and IPPI were independently installed in peppermint for overexpression under the control of the CaMV 35S promoter. The transformed DXR plants consistently showed a 40-60% increase in essential oil yield compared to controls, without change in monoterpene composition. The transformed DXPS plants and IPPI plants exhibited 10-15% oil yield increase without compositional change. This lesser influence on yield was not too surprising in view of the prior result that demonstrated that wildtype plants are compromised at the DXR step upstream of both DXPS and IPPI. The antisense approach with menthofuran synthase was also used with peppermint to reduce, by about 50%, the essential oil content of this undesirable monoterpene. The expression of limonene synthase, limonene hydroxylase, and menthone reductase genes has also been manipulated in transgenic peppermint. Field trials with these transgenic plants are ongoing for consideration of commercial release, and gene stacking of the other genes into the DXR plants is in progress to evaluate additive yield effects.

It is clear that the downstream branch of the isoprenoid pathway is controlled by geranyl diphosphate synthase (GPPS). We cloned these genes with the help of microsequence information from the purified protein and oil gland EST mining, and have studied the subunit interactions and regulatory features of this unusual heterodimeric recombinant enzyme. It is the small subunit that determines chain-length specificity of prenyl transfer. Thus, for example, the binding of the small subunit is capable of converting the normally homodimeric geranylgeranyl diphosphate (C_{20}) synthase into a functional geranyl diphosphate (C_{10}) synthase heterodimer.

A cloning effort using the mint GPPS small subunit and large subunit as probes with the grand fir cDNA library did not yield a small subunit homolog but did provide a large subunit homolog that was catalytically functional as a GPPS synthase (as a homodimer with kinetics comparable to the mint heterodimer). This quite surprising finding suggests that there exist two fundamentally different types of geranyl diphosphate synthases with potentially different regulatory roles. Homodimeric types and similar heterodimeric types of GPPS have since been found by others in angiosperms, and homodimeric types in other gymnosperms. Because the homodimeric form is easier to manipulate in a biotechnological sense, the grand fir GPPS was overexpressed in wildtype (DXR limited) peppermint, resulting in a 10-15% oil yield increase without change in composition. To achieve a more favorable yield increase, GPPS has been stacked into DXR enhanced plants.

Following our demonstration that the small subunit of the heterotetrameric GPPS from *Mentha* controls chain-length distribution, we have attempted to make this kinetically competent enzyme more amenable to transgenic manipulation by functionally fusing the two subunits. Construction of such a single open reading frame involves the two possible variants in the order of translation as well as variation in the size and sequence of the linker between large and small subunits. This work has led to the design of a kinetically competent fusion protein. We have also examined the question of whether GPPS interacts with immediately downstream terpenoid cyclization enzymes (both are plastidial) by using an affinity-based approach. Two examples were tested, involving the *Mentha* GPPS and *Mentha* limonene synthase, and the grand fir GPPS and grand

fir pinene synthase. Although such association would seem to have kinetic advantage, no evidence for the binding of GPPS with cyclization enzymes was obtained.

Objective 5. Metabolite Trafficking and Secretion. Both oil gland ultrastructural and immunocytochemical studies have indicated that the subcellular organization of monoterpene metabolism is quite complex in involving multiple participating sites. Thus, the biosynthetic pathway arises in plastids and involves movement of intermediates to the endoplasmic reticulum, then mitochondria, then cytosol, before secretion of end product to the extracellular storage cavity via the lumen of the endoplasmic reticulum. The oil gland EST project revealed abundant lipid transfer proteins, and several types of binding proteins, transporters and pumps presumed to be involved in trafficking between organellar sites of synthesis and secretion of these very hydrophobic metabolites for storage. To set the foundation for this work, we proposed to correlate expression profiles for genes of the various protein players with oil gland development, to immunolocalize the relevant players, and then to determine their specificity, stoichiometry and binding affinity for monoterpenes. This approach requires suitable expression systems for the various protein types in *E. coli*, yeast and insect cells as necessary, and these systems were in development at the end of the grant period. The overall contribution of trafficking/secretion to flux control and the ultimate yield of terpenoid natural products is uncertain; however, it is very likely that these processes will become a major focus of future research in the plant natural products area.

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RESEARCH PERSONNEL

	Effective Dates	Effort on Project	DOE Support
Rodney Croteau, Principal Investigator	3/00 – 3/04	18%	0%
Glenn Turner, Postdoctoral Associate	3/00 – 3/04	100%	100%
Markus Lange, Postdoctoral Associate	3/00 – 3/01	100%	15%
Charles Burke, Postdoctoral Associate	3/02 – 3/03	100%	100%
Soheil Mahmoud, Postdoctoral Associate	3/01 – 3/03	50%	15%
David Williams, Postdoctoral Associate	6/03 – 10/04	30%	30%
Marie McConkey, Graduate Student	3/00 – 3/02	50%	50%
Kerry Ringer, Graduate Student	3/00 – 3/04	25%	10%
Eric Stauber, Undergraduate Assistant	3/01 – 3/02	25%	25%
Chris Sanchez, Undergraduate Assistant	3/02 – 3/03	25%	25%
Matthew Williams, Undergraduate Assistant	3/02 – 3/04	25%	10%