Bioenergetics of Monoterpenoid Essential Oil Biosynthesis in Nonphotosynthetic Glandular Trichomes 1[OPEN]

Sean R. Johnson, 2 Iris Lange, Narayanan Srividya, and B. Markus Lange 3

Institute of Biological Chemistry and M.J. Murdock Metabolomics Laboratory, Washington State University, Pullman, Washington 99164-6340

ORCID IDs: 0000-0001-8261-9015 (S.R.J.); 0000-0001-7934-7987 (N.S.); 0000-0001-6565-9584 (B.M.L.).

The commercially important essential oils of peppermint (Mentha x piperita) and its relatives in the mint family (Lamiaceae) are accumulated in specialized anatomical structures called glandular trichomes (GTs). A genome-scale stoichiometric model of secretory phase metabolism in peppermint GTs was constructed based on current biochemical and physiological knowledge. Fluxes through the network were predicted based on metabolomic and transcriptomic data. Using simulated reaction deletions, this model predicted that two processes, the regeneration of ATP and ferredoxin (in its reduced form), exert substantial control over flux toward monoterpenes. Follow-up biochemical assays with isolated GTs indicated that oxidative phosphorylation and ethanolic fermentation were active and that cooperation to provide ATP depended on the concentration of the carbon source. We also report that GTs with high flux toward monoterpenes express, at very high levels, genes coding for a unique pair of ferredoxin and ferredoxin-NADP+ reductase isoforms. This study provides, to our knowledge, the first evidence of how bioenergetic processes determine flux through monoterpenoid biosynthesis in GTs.

Plant secondary (or specialized) metabolites are frequently synthesized and accumulated in specialized tissues or cell types. Anatomical structures that harbor specialized metabolic pathways can take many forms and vary widely across the plant lineages. Some examples are resin ducts, secretory cavities, glandular trichomes (GTs), laticifers, and gum ducts (Fahn, 1988). GTs are particularly widespread across the angiosperm lineages and accumulate, among others, the specialized metabolites responsible for the psychoactive effects of cannabis (Cannabis sativa), the bitter flavor of hops (Humulus lupulus), the insect-repellent principles of tomato (Solanum lycopersicum) leaves, the antimalarial activity of sweet wormwood (Artemisia annua), and the essential oils of members of the Lamiaceae (Lange and Turner, 2013).

Peppermint (Mentha x piperita) and its close relatives have a long history as model systems for furthering our understanding of GT structure and function. Pioneering studies in the late 1980s and early 1990s reported on methods for purifying secretory cells of GTs away from other leaf cell types and established that the entire biosynthetic pathway from imported sugars to the monoterpenoid end products is confined to these specialized cell types (Gershenzon et al., 1992; McCaskill et al., 1992; McCaskill and Croteau, 1995). In the mid to late 1990s, these protocols were modified to allow the development of an EST library, the sequencing of which revealed the stunning metabolic specialization of mint GTs (Lange et al., 2000). These data sets also enabled the cloning and characterization of genes encoding the entire complement of enzymes responsible for p-menthane monoterpenoid biosynthesis in peppermint (Lange et al., 1998; Burke et al., 1999; Lange and Croteau, 1999a, 1999b; Ringer et al., 2003, 2005; Davis et al., 2005). Kinetic mathematical models facilitated the discovery of feedback control, GT developmental patterns, and epigenetic processes as critical determinants of metabolic outcomes (Rios-Estepa et al., 2008, 2010; Lange and Rios-Estepa, 2014; Aikami et al., 2015). Protocols for the Agrobacterium tumefaciens-mediated transformation of mint species had been developed in the late 1990s (Berry et al., 1996; Caissard et al., 1996; Diemer et al., 1998; Niu et al., 1998) and were later employed, supported in part by mathematical modeling, to successfully modulate monoterpenoid essential composition and yield in transgenic peppermint plants (Mahmoud and Croteau, 2001; Mahmoud et al., 2004; Lange et al., 2011, 2017).
RESULTS

Reconstruction of Metabolism in Peppermint GTs

To automate various processes relevant to building metabolic models, we developed a software package called YASMEnv. Because of space constraints, the capabilities cannot be outlined in the main body of this article, and extensive details are provided in Supplemental Methods and Data File S1. Further instructions and the entire code are available freely at https://bitbucket.org/seanjohnson/yasmenv.

To assemble the reactions required for central carbon metabolism in peppermint GTs, we surveyed the literature for a particularly well-curated metabolic reconstruction and selected the Arabidopsis Core Model (Arnold and Nikoloski, 2014) to serve as the foundation for our efforts. Additional metabolic reactions were extracted from the AraCyc and MetaCyc databases (Mueller et al., 2003; Caspi et al., 2016). A process termed instantiation (Latendresse et al., 2012) was employed, using an implementation of the relevant algorithm in YASMEnv, to ensure that only mass-balanced reactions were included in the model. Each reaction was associated with a subcellular compartment based on the records in the Arabidopsis Core Model, AraCyc, and MetaCyc, and these associations were confirmed by searches against the TargetP and SUBAcon databases (Emanuelsson et al., 2000; Hooper et al., 2014). Sequences of transcripts represented in recently acquired transcriptome data sets for peppermint GTs (Ahkami et al., 2015) were compared, based on global identity (Myers and Miller, 1988; Tian and Skolnick, 2003), with those associated with reactions in the Arabidopsis Core Model, AraCyc, and MetaCyc. The first iteration of the menpiGT metabolic reconstruction consisted of all reactions of the Arabidopsis Core Model and reactions from AraCyc and MetaCyc with at least one associated peppermint transcript (Supplemental Methods and Data File S1).

The model was refined in five additional stages: (1) completion of pathways to known GT metabolites, (2) removal of thermodynamically infeasible loops, (3) removal of reactions retained from the Arabidopsis Core Model that are not supported by peppermint transcripts, (4) removal of reactions that cannot carry flux because they are not connected to the remainder of the network, and (5) modifications to the model based on the experimental data presented in this article (Supplemental Methods and Data File S1). The final model (termed menpiGT_2015) contains 624 reactions, accounting for 757 expressed transcripts. The menpiGT_2015 model is a reflection of the astounding specialization of secretory cells within peppermint GTs during secretory phase (Fig. 1A). An imported oligosaccharide (raffinose or Suc) is converted, through central carbon metabolism and then dedicated pathways, primarily into monoterpens (94%–95% [v/v] of essential oil), sesquiterpenes (4%), and polymethoxylated flavones (1%–2%; Fig. 1B), while genes involved in other pathways that commonly carry significant flux, such as amino acid conversion into proteins, sugar nucleotide interconversions into cell wall macromolecules, and fatty acid/lipid biosynthesis, are expressed only at fairly low levels (Supplemental Table S1).

Knockout Simulations Suggest Essential Roles for ATP Regeneration and Nonphotosynthetic Fd Reduction

To test the utility of menpiGT_2015 for identifying processes required for maintaining high flux toward monoterpens, we employed a process called simulated reaction deletions (Edwards and Palsson, 2000). Reactions were removed from menpi_2015 one at a time, and the impact on monoterpene formation was evaluated (Supplemental Table S2). Thirty-five reactions were found to be essential for producing (−)-menthol from an imported carbon source (raffinose; Table I). The requirements for catabolism of raffinose (the primary carbon source considered in the model), the exchange of...
small molecules across membranes (balancing C and O budgets), and the reactions involved directly in the biosynthesis of precursors and end products of the monoterpene pathway were expected outcomes of these in silico predictions. Interestingly, the regeneration of nucleotide triphosphates (in particular CTP and ATP) and the recycling of reduced Fd also were returned as essential reactions in the simulated knockout study (Table I). These reactions are linked to the bioenergetics of GTs, which were poorly understood at the outset of our study; therefore, we designed experiments to further test how these processes may affect monoterpene formation.

### Sensitivity of Monoterpene Production to the Inhibition of Oxidative Phosphorylation Depends on the Concentration of Carbon Source

Peppermint GTs are nonphotosynthetic structures (Werker et al., 1985), and one important question pertains to the regeneration of ATP to power their highly active metabolism. In heterotrophic animal cells, oxidative phosphorylation in mitochondria has a critical role in supplying ATP; thus, one would hypothesize that this process also might provide energy equivalents in nonphotosynthetic GTs. Indeed, the genes coding for the enzyme complexes of the oxidative phosphorylation pathway were expressed at fairly high levels in peppermint GTs (Fig. 2A), indicating that this pathway of ATP regeneration is likely operational. Isolated GTs are capable of synthesizing monoterpenes from externally supplied sugar precursors (Gershenzon et al., 1992; McCaskill et al., 1992; McCaskill and Croteau, 1995) and, thus, would seem to be an excellent model system in which to study this process. However, previous feeding experiments employed complex media that contained various enzyme cofactors, including ATP; thus, it was important to test if ATP could be omitted from the incubation buffer. In experiments that evaluated various combinations of cofactors, we determined that the presence of NADP⁺ and ADP is sufficient to support monoterpene production in isolated peppermint GTs (Supplemental Methods and Data File S2).

Two additional experimental considerations need to be mentioned. (1) An alternative pathway for regenerating ATP, independent of oxidative phosphorylation, would be through the activity of adenylate kinase (direct phosphorylation of proffered ADP), and to disable
this potentially cooperative reaction for ATP formation, the incubation buffer was supplemented with AMP, a known inhibitor of the enzyme (Atkinson, 1968). (2) Although raffinose is the primary transport oligosaccharide in many Lamiaceae (and likely imported into plants; therefore, we added the relevant reactions included in version 5 of menpiGT_2015 and indicated that alternative mechanisms for ATP regeneration, particularly at high sugar concentrations, needed to be considered.

One such possibility would be the coupling of ATP recycling to fermentative steps, such as those occurring in microbes and plants under oxygen-limiting conditions. To the best of our knowledge, fermentative reactions have not been included in any metabolic reconstruction for plants; therefore, we added the relevant reactions manually to menpiGT_2015 (version 6). In addition, transcriptome data obtained with isolated peppermint GTs at secretory stage (Ahkami et al., 2015) were incorporated using a modified SPOT algorithm (Kim et al., 2016; 2007), there is no commercial source for the radiolabeled tracer; therefore, we employed [U-14C]Suc in our experiments (Suc is formed during rafﬁnose catabolism).

Isolated GTs were incubated in media containing three different Suc concentrations and subjected to oligomycin (omitted in controls), which inhibits the ATP synthase component of the enzyme complexes performing oxidative phosphorylation (Huijing and Slater, 1961; Penefsky, 1985). At the end of the experimental period, radiolabeled monoterpenes were extracted into hexane/ether (1:1, v/v) and quantified by liquid scintillation counting. Based on data obtained in control experiments (no oligomycin), the maximum incorporation of label from [U-14C]Suc into monoterpenes occurs at 1 mM Suc (5.8 nmol per million gland cells), with a slight decrease at 10 mM (Fig. 2B). Oligomycin had no effect on monoterpene accumulation at 0.02 mM Suc, when the monoterpene yield (and likely the ATP demand) was very low. At 1 mM Suc, the addition of oligomycin resulted in a significant 2-fold decrease in the quantity of monoterpenes produced (from 5.8 to 3.2 nmol per million gland cells). Surprisingly, at 10 mM Suc (the highest concentration tested), oligomycin had no impact on monoterpene production (4.5 nmol per million gland cells). These results could not be explained with the reactions included in version 5 of menpiGT_2015 and indicated that alternative mechanisms for ATP regeneration, particularly at high sugar concentrations, needed to be considered.

Table 1. Reactions returned as being essential for (−)-menthol production from rafﬁnose, as determined by simulated reaction deletions

<table>
<thead>
<tr>
<th>Reaction Description</th>
<th>Pathway</th>
<th>Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Deoxy-D-xylulose 5-phosphate synthase</td>
<td>Methylerythritol phosphate (MEP) pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>1-Deoxy-D-xylulose 5-phosphate reductoisomerase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>4-Phosphopentidyl-2-C-methyl-D-erythritol synthase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>4-Phosphopentidyl-2-C-methyl-D-erythritol kinase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>4-Hydroxy-3-methylbut-2-en-1-yl diphosphate reductase</td>
<td>MEP pathway</td>
<td>Plastid</td>
</tr>
<tr>
<td>Geranyl diphosphate synthase</td>
<td>Prenyl elongation</td>
<td>Plastid</td>
</tr>
<tr>
<td>(−)-Limonene 3-hydroxylase</td>
<td>Monoterpane modifications</td>
<td>Cytosol</td>
</tr>
<tr>
<td>(−)-trans-Isopiperitenol dehydrogenase</td>
<td>Monoterpane modifications</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>(−)-trans-Isopiperitene reductase</td>
<td>Monoterpane modifications</td>
<td>Cytosol</td>
</tr>
<tr>
<td>(+)-cis-Isopulegone isomerase</td>
<td>Monoterpane modifications</td>
<td>Cytosol</td>
</tr>
<tr>
<td>(+)-Pulegone reductase</td>
<td>Monoterpane modifications</td>
<td>Cytosol</td>
</tr>
<tr>
<td>(−)-Menthone:(−)-menthol reductase</td>
<td>Monoterpane modifications</td>
<td>Cytosol</td>
</tr>
<tr>
<td>(−)-Limonene transport</td>
<td>Monoterpane modifications</td>
<td>Plastid to cytosol (ER)</td>
</tr>
<tr>
<td>(−)-trans-Isopiperitenol transport</td>
<td>Monoterpane modifications</td>
<td>Cytosol to mitochondrion</td>
</tr>
<tr>
<td>(−)-trans-Isopiperitene transport</td>
<td>Monoterpane modifications</td>
<td>Mitochondrion to cytosol</td>
</tr>
<tr>
<td>(−)-Menthol export</td>
<td>Monoterpane modifications</td>
<td>Cytosol to oil cavity</td>
</tr>
<tr>
<td>Proton diffusion</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>Plastid to cytosol</td>
</tr>
<tr>
<td>CO2 diffusion</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>Mitochondrion to cytosol</td>
</tr>
<tr>
<td>Water diffusion</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>Cytosol to mitochondrion</td>
</tr>
<tr>
<td>Oxygen diffusion</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>Cytosol to mitochondrion</td>
</tr>
<tr>
<td>Oxygen exchange</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>External to cytosol</td>
</tr>
<tr>
<td>CO2 exchange</td>
<td>O2/CO2/H2O/H diffusion</td>
<td>Cytosol to external</td>
</tr>
<tr>
<td>Raffinose import</td>
<td>Raffinose catabolism</td>
<td>External to cytosol</td>
</tr>
<tr>
<td>UTP:Gal-1-phosphate uridylyltransferase</td>
<td>Raffinose catabolism</td>
<td>Cytosol</td>
</tr>
<tr>
<td>UDP:Gal 4-epimerase</td>
<td>Raffinose catabolism</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Raffinose galactosidase</td>
<td>Raffinose catabolism</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Galactokinase</td>
<td>Raffinose catabolism</td>
<td>Cytosol</td>
</tr>
<tr>
<td>CDP kinase</td>
<td>CTP regeneration</td>
<td>Plastid</td>
</tr>
<tr>
<td>CMP kinase</td>
<td>CTP regeneration</td>
<td>Plastid</td>
</tr>
<tr>
<td>Inorganic phosphatase</td>
<td>CTP regeneration</td>
<td>Plastid</td>
</tr>
<tr>
<td>Respiratory complex I</td>
<td>Oxidative phosphorylation</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>Oxidative phosphorylation</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>Fd-NADP+ reductase</td>
<td>Fd reduction</td>
<td>Plastid</td>
</tr>
</tbody>
</table>
details are outlined in Supplemental Methods and Data File S1). Allowed carbon sources were oligosaccharides, ammonia, oxygen, hydrogen sulfide, sulfate, inorganic phosphate, water, and carbon dioxide. The experimentally derived biomass export reactions, as presented in Figure 1B, were disabled and, therefore, became values to be predicted by the model based on gene expression patterns. Allowed outputs were monoterpenes, sesquiterpenes, polymethoxylated flavones, ethanol, lactate, cellulose, amino acids, water, and carbon dioxide (Supplemental Table S3). Interestingly, our simulations predicted a significant formation of fermentative reaction products (lactate and ethanol; Fig. 3B); thus, we embarked on experiments, described in the following paragraph, to test the hypothesis that fermentation plays a role in energizing peppermint GTs.

**Fermentation Is Active in Peppermint GTs**

In fermentative processes, NADH and pyruvate formed in glycolysis are converted into NAD\(^+\) and an organic molecule (most often ethanol or lactic acid), which is coupled to reactions of the bottom half of glycolysis that regenerate ATP (Fig. 3A). Based on transcriptome data (Ahkami et al., 2015), genes involved in both glycolysis (glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, and pyruvate kinase) and fermentation (pyruvate decarboxylase and alcohol dehydrogenase [ADH]) are expressed at high (in some cases very high) levels in peppermint GTs (Fig. 3A). When isolated GTs were supplied with appropriate reactants, significant ADH activity (assayed with NAD\(^+\), NBT, and ethanol) was detected in about 12% of gland cell clusters (observed as dark staining; Fig. 3, C–E), which corresponds well with the proportion of GTs in secretory phase on young leaves (Turner et al., 2000a, 2000b). Enzyme assays performed with crude protein extracts indicated a fairly high specific ADH activity in GTs isolated from young leaves (0.8 nmol min\(^{-1}\) mg\(^{-1}\) protein) but no measurable activity in GTs obtained from mature leaves (where no monoterpene biosynthesis occurs; Fig. 3F). These data support the hypothesis that fermentation plays an important role in energizing nonphotosynthetic peppermint GTs and justify the inclusion of these reactions in version 6 of the menpiGT_2015 model.

Given that monoterpenol dehydrogenases are among the most highly expressed genes in secretory phase GTs (Lange et al., 2000; Ahkami et al., 2015), one may postulate that the ADH activity we observed is a nonspecific side activity of these enzymes. To test this hypotheses,
we first performed a BLASTx search with the well-characterized ADH1 sequence from potato (Solanum tuberosum; Matton et al., 1990) against the peppermint GT transcriptome data set. This search indicated the presence of two putative orthologs (at roughly 88% identity for the translated peptide sequence) that were expressed at high levels in peppermint GTs (accession nos. MF579445 and MF579446; Supplemental Fig. S1). The cDNAs corresponding to the peppermint ADH candidates were cloned by PCR with gene-specific primers and ligated into an appropriate expression vector. Each vector carrying a peppermint candidate cDNA was transformed into Escherichia coli, the expression of the transgene was induced, and the recombinant protein was purified. The sizes of the proteins encoded by MF579445 and MF579446 were both roughly 41 kD (Fig. 3G). One ADH-like candidate (recombinant protein produced from MF579445) turned out to be inactive in our assays, while the other (produced from MF579446; henceforth referred to as ADH1) had the properties of a typical ADH \[K_m(\text{ethanol}) = 53.9 \text{mM}, k_{\text{cat}} = 603.5 \text{s}^{-1}\] (Fig. 3, H and I). These results confirm the presence of a specific ADH activity, which is independent of monoterpene dehydrogenases, in non-photosynthetic peppermint GTs.

**Mint GTs Contain a Unique Fd/FNR Complex**

The precursors for monoterpene and sesquiterpene biosynthesis in peppermint GTs are provided exclusively by the MEP pathway (McCaskill and Croteau, 1995; Eisenreich et al., 1997; Lange et al., 2001). The genes
involved in the MEP pathway and downstream monoterpene-specific reactions are expressed at very high levels in peppermint GTs (Fig. 4). The electron transfer reactions of reductive steps of the MEP pathway, catalyzed by 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS) and 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR), employ Fd and FNR (Seemann et al., 2006; Rekittke et al., 2013). This cofactor requirement is in accordance with our modeling prediction that Fd recycling is essential for monoterpene biosynthesis (Table I). The nonphotosynthetic isoforms of Fd and FNR have redox potentials that are significantly different from those in photosynthetic cells (Aliverti et al., 2001); thus, electrons can flow in a different direction from NADPH to FNR, then from FNR to Fd, and finally from Fd to the iron-sulfur clusters of HDS and HDR (Fig. 4). Thus far, nonphotosynthetic Fd and FNR isoforms have only been characterized from roots (Hanke et al., 2005; Aliverti et al., 2008; Onda et al., 2000); therefore, it was of interest to evaluate which isoforms are present in nonphotosynthetic GTs.

Public databases were surveyed for data sets covering leaf, root, and GT transcriptomes of angiosperms. Phylogenetic trees were then generated based on alignments of nucleotide sequences of Fd and FNR isoforms (Supplemental Table S4). FNRs expressed in GTs (in some cases more than one isoform) separated into two different clades: those expressed in nonphotosynthetic GTs (cannabis, peppermint, and basil [Ocimum basilicum]) were identical to the root isoform or clustered with root isoforms, whereas those expressed in photosynthetic GTs (tomato and sweet wormwood) clustered with leaf isoforms (Fig. 5A). Generally speaking, similar patterns were observed with GT-expressed isoforms of Fd (separation of leaf from root/GT isoforms). However, there was one significant difference: the major isoforms...
expressed in nonphotosynthetic GTs (particularly in the mint family) formed a subclade that was distinguishable from that of root isoforms (Fig. 5B).

Did GT-specific Fd isoforms evolve in mint? The hybrid hexaploid nature of peppermint makes it difficult to answer this question due to the potential for the presence of many Fd isoforms in genomes retained from three different ancestral species. However, the recently completed draft genome assembly for *M. longifolia*, a diploid species related to one of the ancestors of present-day peppermint (Vining et al., 2017), allowed us to examine all Fd isoforms. Organ- and tissue-specific transcriptome data for *M. longifolia* were recently deposited in public archives (root and GT transcriptomes with National Center for Biotechnology Information [NCBI] short read archive accession nos. SRP083887 and SRR3623199, respectively). The sequence of the minor Fd isoform of *M. longifolia* GTs (average expression of 289 TPM) was identical to that of the root isoform, whereas the major isoform (average expression of 2,586 TPM) was distinct from the root isoform (Fig. 5B). The major and minor isoforms from genus *Mentha* GTs differed in the mature peptide sequence at 12 positions (Fig. 6A). To ensure that these findings were not due to assembly errors from short reads, we PCR amplified and sequenced cDNAs corresponding to both Fd isoforms (KY748231 [GT major] and KY748232 [GT minor/root]) and thereby confirmed the unique sequence of the major GT isoform. The major peppermint GT-specific isoform of Fd was essentially identical to the major isoform of other mint species, and the same sequence conservation across species was observed for the minor GT/root isoform (Fig. 5B). Mapping of the Fd isoforms to the *M. longifolia* genome revealed that they map to different contigs: the major Fd isoform of GTs was mapped to contig 19,491 (refers to genome assembly with National Center for Biotechnology Information BioProject accession no. PRLNA310613), whereas the minor GT isoform mapped to contig 5,531, indicating that these genes likely have separate origins.

We also obtained transcriptome data for GTs isolated from *Perilla frutescens* (short read archive accession no. SRR4106089) and included previously published GT-specific cDNA sequences from basil (Gang et al., 2001) in our phylogenetic analysis, which expands the scope of sequence comparisons to other Lamiaceae with high monoterpenoid essential oil content. The GT-specific Fd isoforms of these species clustered with the major isoform of mint species, indicating that nonphotosynthetic GTs with high flux toward monoterpenes may harbor unique Fd isoforms across the mint family.

To assess the potential implications of a GT-specific Fd isoform in peppermint, we modeled the structures of Fd (GT major isoform), FNR (GT/root isoform), and HDR based on published data for closely related isoforms from plants (Fig. 6). Because of the more speculative nature of this analysis, we are providing the details in “Discussion.”

**DISCUSSION**

MenpiGT_2015 Is the First Genome-Scale Reconstruction of Specialized Metabolism in GTs

Large-scale mathematical models of plant metabolism were initially focused on species for which genome

---

**Figure 5.** A unique Fd/FNR pair occurs in monoterpene-accumulating GTs. A, Maximum likelihood tree of FNR isoforms. B, Maximum likelihood tree of Fd isoforms. Comparisons employ nucleotide sequences corresponding to the mature proteins (excluding plastidial targeting sequences). Scale bars represent the number of substitutions per site. Species abbreviations are as follows: Aann (*Artemisia annua*), Athal (*Arabidopsis thaliana*), Csat (*Cannabis sativa*), Hlup (*Humulus lupulus*), Maqu (*Mentha aquatica*), Marv (*Mentha arvensis*), Mlon (*Mentha longifolia*), Mpip (*Mentha *piperita*), Mspi (*Mentha spicata*), Obas (*Ocimum basilicum*), Pfru (*Perilla frutescens*), Slyc (*Solanum lycopersicum*). Other abbreviations are as follows: L, leaf isoform; maj, major GT isoform; min, minor GT isoform; R, root isoform. Colors indicate isoforms present in leaves (green), roots (gray), GTs (yellow), and both roots and GTs (yellow/gray).
sequences were available (Arabidopsis, rice, maize, and other model organisms), but with the advent of cost-competitive technologies for obtaining transcriptome sequences, metabolic reconstructions coupled with flux balance analysis also have been performed for nonmodel plants (Hay and Schwender, 2011). Most of these models were generated to represent reactions in a major organ, and only a few accurately reflect metabolism specific to a certain tissue (de Oliveira Dal’Molin et al., 2010b; Hay and Schwender, 2011; Pilalis et al., 2011; Mintz-Oron et al., 2012). To our knowledge, the menpiGT_2015 model is the first attempt to gather data from various sources (literature, transcriptomics, metabolomics, and biochemical assays) and capture metabolism in nonphotosynthetic GTs. We decided to start with a well-curated, compartmentalized, metabolic reconstruction recently generated for a model plant (Arabidopsis Core Model; Arnold and Nikoloski, 2014) and then incorporated additional information from Web-based databases focusing on metabolism (AraCyc and MetaCyc) to produce a consensus set of reactions. The YASMEnv toolbox, which we developed specifically to automate various steps in the generation of metabolic models, was invaluable in these efforts, and we hope that others will take advantage of the freely available scripts (https://bitbucket.org/seanrjohnson/yasmenv). Further model development steps included eliminating thermodynamically infeasible loops, ensuring that all reactions are mass balanced, and associating reactions with transcripts (adding reactions unique to mint GTs and eliminating reactions not supported by mint transcripts). These adjustments reduced the total number of reactions in mepiGT_2015 to 624 (from an initial 2,639 reactions; Supplemental Methods and Data File S1) and are reflective of the remarkable specialization of mint GTs.

Since mint GT cells are nonphotosynthetic, an imported carbon source (likely raffinose; McCaskill et al., 1992; Büchi et al., 1998; Olennikov and Tankhaeva, 2007) is converted, via central carbon metabolism, to precursors for terpenoid biosynthesis (which correspond to more than 95% of products accumulated in essential oil). Importantly, precursors for both C10-derived monoterpenes and C15-derived sesquiterpenes are provided exclusively by the plastidial MEP pathway (McCaskill and Croteau, 1995; Eisenreich et al., 1997; Lange et al., 2001), which is reflected in the extremely high expression levels of the relevant genes (whereas expression levels of genes of the cytosolic/peroxisomal mevalonate pathway are low or undetectable; Supplemental Table S1). The pathway toward methoxylated flavones is clearly active at the transcriptional level, with biosynthetic genes being conformation of the FNR-Fd-HDR ternary complex. C, Ribbon diagram of the FNR-Fd interaction. Residues that differ between the major and minor Fd isoforms of GTs are highlighted in red. D, Ribbon diagram of the Fd-HNR interaction. Residues that differ between the major and minor Fd isoforms of GTs are highlighted in red.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Protein-protein interactions in the ternary FNR-Fd-HDR complex of peppermint GTs. A, Alignment of mature peptide sequences of Fd isoforms present in peppermint GTs. Residues that differ between the major and minor isoform are highlighted in yellow. B, Docking conformation of the FNR-Fd-HDR ternary complex. C, Ribbon diagram of the FNR-Fd interaction. Residues that differ between the major and minor Fd isoforms of GTs are highlighted in red. D, Ribbon diagram of the Fd-HNR interaction. Residues that differ between the major and minor Fd isoforms of GTs are highlighted in red.
expressed at appreciable levels (Supplemental Table S1), but the quantities of these specialized metabolites in mint GTs are generally very low (1%–2% of the essential oil, with no significant occurrence in other cell types; Voirin and Bayet, 1992; Sato and Tamura, 2015).

Transcripts related to cellular maintenance reactions (e.g. protein synthesis and degradation, nucleotide biosynthesis, and fatty acid/lipid biosynthesis) are detectable in published mint data sets (Ahkami et al., 2015), but their abundance is very low compared with those involved in terpenoid and polymethoxylated flavone biosynthesis. Some genes involved in nucleotide interconversion reactions are expressed at high levels (e.g. adenosine kinase at 2,122 TPM and adenylate kinase at 788 TPM), but these are linked to the regeneration of ATP, which is needed to support the highly active biosynthetic pathways of GTs (the MEP pathway alone requires six ATP molecules to generate one molecule of geranyl diphosphate [a monoterpene precursor]). The conversions from 1-deoxy-D-xylulose 5-phosphate to one molecule of (−)-menthol require 10 molecules of NADPH, and the high expression levels of genes involved in the oxidative branch of the pentose phosphate pathway (coding for Glc-6-P dehydrogenase, 6-phosphogluconolactonase, and 6-phosphogluconate dehydrogenase; Fig. 4), which support NADPH regeneration (Kruger and von Schaewen, 2003), are in accordance with the expected demands. In summary, menpiGT_2015 captures the specialization of mint GTs at secretory stage and allows us to test initial assumptions about metabolism computationally, which can then be assessed further experimentally.

Iterative Mathematical Modeling and Experimental Testing Provide Insights into the Processes That Power Metabolism in Nonphotosynthetic GTs

Reaction knockout simulations with menpiGT_2015 (version 5) were performed to determine which reactions are essential for (−)-menthol production (Table I; Supplemental Table S2). Surprisingly, of the seven pathways or processes identified by this simulation as being essential, only two, the MEP pathway and the monoterpene biosynthetic pathway, were strongly supported by prior experimental evidence (Lange, 2015). Three other essential processes, Suc/raffinose import and metabolism (carbon source), CTP regeneration (required to provide CTP as reactant in the MEP pathway), and exchange of small molecules across membranes (trivial), did not appear to warrant further exploration at this time. The remaining two essential processes, oxidative phosphorylation and regeneration of reduced Fd, had not been studied at all in GTs and, therefore, were deemed worthy of experimental testing.

Feeding experiments with isolated GTs were employed to assess the contribution of oxidative phosphorylation to the formation of organic soluble products (primarily monoterpenes) from 13C-labeled Suc. One of the surprises in our experimental data was the finding that monoterpene production in the presence of the ATP synthase inhibitor oligomycin was dependent on the concentration of Suc. We observed inhibitory effects at 1 mM Suc but not at 0.02 or 10 mM Suc (Fig. 3). The lack of inhibition at 0.02 mM Suc may be due to a low overall metabolic rate (measured as monoterpene production per unit of time) at such a low Suc concentration. At 1 mM Suc, oligomycin treatment reduced monoterpene production to about half of that observed in the absence of oligomycin, and one might interpret this as incomplete inhibition of ATP synthase. However, this would not explain why there was no effect of the oligomycin treatment on monoterpene accumulation at 10 mM Suc. An alternative explanation was the involvement of processes other than oxidative phosphorylation in regenerating ATP. Adenylate kinase was inhibited by the addition of AMP in our assays (Atkinson, 1968) and, therefore, was unlikely to be a significant contributor to ATP formation, leaving fermentation as the only remaining candidate for providing energy equivalents. After adding fermentative reactions to our model and incorporating gene expression patterns acquired previously (Ahkami et al., 2015), simulations predicted that fermentation products (lactate and ethanol) should indeed accumulate in peppermint GTs. Follow-up biochemical experiments provided evidence that peppermint GTs contain highly active ADH1, the signature enzyme of fermentation (Fig. 3), thereby confirming the model predictions. It is important to note that this enzyme is clearly distinct from monoterpenol dehydrogenases, which were demonstrated previously to not possess an ADH side activity (Kjonaas et al., 1985; Gershenzon et al., 1989).

The next question was if the consideration of fermentation could explain the data obtained in the experiments in which oxidative phosphorylation was inhibited. More specifically, why did we observe a significant reduction of monoterpene production in isolated GTs treated with oligomycin at 1 mM, but not at 10 mM, Suc concentration? In all plant ADH enzymes characterized thus far, the $K_m$ value is always in the millimolar range (Mayne and Lea, 1984; Tong and Lin, 1988; Osterman et al., 1993; Hadizadeh and Keyhani, 2004). We calculated a $K_m$ of 66.3 mM for peppermint ADH1, which is consistent with these earlier studies. Therefore, we hypothesize that acetaldehyde does not reach an intracellular concentration that is sufficiently high to drive ADH activity toward ethanolic fermentation at lower Suc concentrations. Under those conditions, ATP regeneration does not rely on fermentation and is dependent primarily on oxidative phosphorylation (thus, the effect of the ATP synthase inhibitor on monoterpene production at 1 mM Suc). The situation would be different at high Suc concentrations, when intracellular levels of acetaldehyde can be high enough for ADH to operate in the direction of ethanol production, which complements or maybe even substitutes for oxidative phosphorylation (thus, no effect of the ATP synthase inhibitor on monoterpene production at 10 mM Suc). Based on measurements in various plants, the intracellular concentration
of oligosaccharides can be up to 100 mM (Nadwodnik and Lohaus, 2008), indicating that the concentration of acetaldehyde in mint GTs could be sufficiently high to support ethanolic fermentation.

Peppermint GTs synthesize oxygenated monoterpene s at high flux (each individual gland fills within about 24 h after reaching secretory stage; Turner et al., 2000a, 2000b), which places significant demands on the availability of molecular oxygen for biosynthetic cytochrome P450-dependent monoxygenases (Lupien et al., 1999; Bertea et al., 2001). Mint GTs are covered by a thick waxy cuticle (Turner et al., 2000a, 2000b), which may prohibit a direct diffusion of atmospheric oxygen into the cells responsible for monoterpenoid essential oil biosynthesis. Together, these factors may contribute to oxygen-limiting conditions in GTs, which would favor fermentation as a mechanism for ATP regeneration (Fig. 4).

### Occurrence of GT-Specific Fd/FNR Isoforms in Monoterpene-Accumulating Plants

The regeneration of reduced Fd was identified in this study as an essential process by computational knock-out simulations (Table I). This is a logical consequence of the fact that Fd is a cofactor for electron transfer reactions toward two reductive enzymes of the MEP pathway (Seemann et al., 2006; Rekittke et al., 2013) and flavone hydroxylases (Berim et al., 2014), which are involved in the formation of the major products of mint GTs (monoterpenes and methoxylated flavones, respectively). In earlier versions of menpIGT_2015, Fd served as a substrate for FNR, which is the direction of electron flow in photosynthetic cells. With the removal of PSI from the model, due to the lack of support from gene expression data, the direction of FNR had to be reversed in order to maintain the capacity for monoterpenoid production, which is the direction of electron flow suggested to occur in nonphotosynthetic root cells (Aliverti et al., 2001).

A phylogenetic analysis indicated that the most highly expressed FNR isoform of mint GTs clustered with the nonphotosynthetic isoforms of other plants, which was an expected outcome. Surprisingly, however, two different Fd isoforms were found to be expressed in GTs of the genus Mentha. The minor isoform (lower expression levels) of M. piperita and M. longifolia was identical to the isoform most highly expressed in roots of the same species. The major isoform (10-fold higher expression than the minor isoform) was unique to GTs. The major isoform also was conserved across several members of the Lamiaceae. This begs the questions of whether there is a functional consequence to this sequence diversity. One possibility would be that the interaction between the major Fd isoform of GTs and reductive enzymes of the MEP pathway, HDS and HDR, facilitates high flux through these steps and the remainder of the monoterpenoid biosynthetic pathway.

What are the potential implications of a novel Fd isoform specifically expressed in nonphotosynthetic GTs of the genus Mentha (and possibly across the Lamiaceae)? Based on a molecular model of ternary peppermint FNR-Fd-HDR complexes, we asked if there were differences among the sequences of peppermint Fd isoforms (Fig. 6A) that had the potential to affect the interaction(s) with FNR and/or HDR (Fig. 6B). Prior crystal structure data of the electron transfer complex between Fd and FNR provided evidence for a close proximity of the [2Fe-2S] cluster of Fd and the FAD prosthetic group of FNR (Kurisu et al., 2001). The authors suggested that pairs of charged residues form intermolecular salt bridges, which was in accordance with prior biochemical data (for review, see the context of plant Fd-FNR interactions by Aliverti et al., 2008). Two residues implicated previously in the interaction of Fd and FNR were different in peppermint isoforms (Shinohara et al., 2017): Ser-32 and Gly-95 in the GT major isoform corresponded to Thr-32 and Ser-95, respectively, in the minor isoform (numbering based on the mature polypeptide, excluding the plastidial targeting sequence, of the major isoform of peppermint; Fig. 6C).

Crystal structures have been resolved for both HDS and HDR, but only one study modeled the binding with Fd. Rekittke et al. (2013) employed docking calculations to select the energetically most favorable complexes between HDR and the cognate Fd from Plasmodium falciparum. The authors concluded that Fd was most likely to attach to a shallow well formed by the N terminus, helix α1B, and the loops near strands β4A and β4B. Our FNR-Fd-HDR docking simulations indicated that one of the lowest energy ternary complexes had a molecular surface that was very similar to that suggested previously (Rekittke et al., 2013). Interestingly, six residues purportedly involved in the Fd-HDR interaction differed between the major and minor Fd isoforms of peppermint GTs (Glu-54, Lys-55, Thr-57, Lys-69, Glu-73, and Ala-85 in the major isoform corresponded to Val-54, Ser-55, Ser-57, Glu-54, Lys-55, Thr-57, Asn-69, Gln-73, and Ser-85 in the minor isoform, respectively; Fig. 6D).

We hypothesize that the differences between the major and minor Fd isoforms in GTs may affect the stability of the FNR-Fd-HDR complex, which, in turn, may impact flux through the biosynthetic step catalyzed by HDR. The situation would be different in photosynthetic GTs characterized thus far. In type VI GTs of Solanum habrochaites, the accumulation of terpenoids occurs over a longer period of time, while in peppermint GT, filling concludes within a matter of days (Turner et al., 2000a, 2000b). Terpenoids in S. habrochaites GTs are derived from both the mevalonate and MEP pathways (Bergau et al., 2015; Balcke et al., 2017), whereas in peppermint, terpenoids of GTs are produced solely via the MEP pathway and, therefore, the reactions catalyzed by HDS and HDR (McCaskill and Croteau, 1995; Eisenreich et al., 1997; Lange et al., 2001). It is tempting to speculate that the major GT-specific Fd isoform of peppermint (and possibly other Lamiaceae) might have evolved to facilitate the particularly high flux through the reactions catalyzed by HDS and HDR en route to monoterpenoid end products.
MATERIALS AND METHODS

Simulations Using the menpiGT_2015 Model

The step-wise development of a metabolic reconstruction and its data-driven iterations are described in Supplemental Methods and Data File S1. To simulate gene knockouts, upper and lower bounds were set such that CO2 and raffinose were the only permitted carbon sources in the network, oxygen uptake was permitted, but NH4+ import was disabled. The upper and lower flux bounds for menthol export were set to 1, and the object function was set to minimize raffinose import. A reference minimum for raffinose import was calculated with no internal reactions disabled. Upper and lower flux bounds for each reaction were then set to 0, one at a time, and raffinose import was computed. The SPOT algorithm was implemented according to Kim et al. (2016), with the addition of Boolean constraints to prevent reversible reactions from being counted twice in the objective function (referred to as Boolean constrained SPOT). Prior to running Boolean constrained SPOT, boundary fluxes were modified to disable the experimentally derived biomass export reaction and to allow the optional import and export of small molecules. Allowed carbon sources were Suc, Glc, Fru, or raffinose. Additional allowed imports were ammonia, oxygen, hydrogen sulfide, sulfate, inorganic phosphate, water, and carbon dioxide. Allowed exports were monoterpenes, sesquiterpenes, poly- methoxylated flavones, ethanol, lactate, cellulose, amino acids, water, and carbon dioxide. Optimization problems were solved with Gurobi Optimizer (Gurobi Optimization), accessed through YASMEnv, a modeling tool we developed specifically for this study (Supplemental Methods and Data File S1).

Chemicals and Biochemicals

Chemicals were purchased from Sigma-Aldrich, unless noted otherwise. Biochemical reagents were sourced from Thermo Fisher Scientific, unless noted otherwise. [U-14C]Suc (432 mCi mol−1 in 90% aqueous ethanol) was obtained from MP Biomedicals.

GT Isolation

GTs were isolated based on the protocol developed by Gershenson et al. (1992), with modifications. Roughly 20 g of leaves was harvested into a plastic bag on ice. Depending on the purpose of the experiments, young leaves (less than half expanded) and/or mature leaves (fully expanded) were harvested when the first flower buds emerged. Leaves were weighed and transferred into a bead beater chamber containing 100 g of glass beads (0.5 mm diameter) and rinsed with wash buffer (200 mM sorbitol, 10 mM KCl, 2 mM MgCl₂, 25 mM HEPES-[p-N,N,N′-tetraacetic acid], 0.5 mM NaN₃, 0.1 mM NaPO₄, adjusted to pH 7.4). Bead beating was performed as three 1-min pulses, with 1 min of cooling on ice between pulses. The contents of the bead beater chamber were poured onto a nylon mesh (350 µm) and rinsed with wash buffer (containing 10% [v/v] glycerol and 1 mM DTT) and sonicated for three pulses, with 1 min of cooling on ice between pulses. The mixture was centrifuged for 2 min at 15,000 × g and the supernatant was collected and filtered through a 0.22-µm filter and desalted using a Bio-Gel P6 column (Bio-Rad). The concentration of total protein was determined (Bradford, 1976), and ADH activity was measured using the method of Mayer and Arnold (2002) with modifications (an aliquot of the crude protein extract was boiled for 5 min to serve as a negative control). The protein extract (15 µg of protein microtiter plate well for young leaves; 3 µg per well for mature leaves) was diluted with assay buffer to finally contain Tris (50 mM, pH 8), NBT chloride (0.18 mM), NAD⁺ (0.18 mM), phenazine methosulfate (0.018 mM), and gelatin (0.08%, w/v). The reaction was started by the addition of 0.2 uM ethanol and absorption at 580 nm (formation of NADH) measured over a period of 4 h (μ-Quant; BioTek Instruments). The slope of the absorption curve was compared with that of a standard curve generated by adding known quantities of NADH directly to the assay buffer (no cells). The specific activity was obtained by subtracting the absorbance determined in assays performed without adding substrate.

Cloning and Characterization of ADH from Peppermint

The sequence of the well-characterized ADH1 from potato (Solanum tuberosum; Pi-6073; Matton et al., 1990) was used to query our peppermint (Mentha × piperita) transcriptome assembly. Two contigs that contained genes with high identity (~88% at the peptide sequence level) and the corresponding cDNAs were amplified using Phusion DNA polymerase (New England Biolabs) with an annealing temperature of 60°C. The primer sequences were 5′-TGGCTGTC- CAAACAAAAGCAG3′ (ADH1 gene; forward 1), 5′-TATAGCTGCTACAAC- CAAACACG3′ (ADH1 gene; forward 2), 5′-GACTGTTGTAAGGCTGTTAG- CTTAACACG3′ (ADH2 gene; forward 1), 5′-CTTACACATGAACTTTAG3′ (ADH2 gene; reverse 1), 5′-TCCACGAACTTTAG3′ (ADH2 gene; reverse 2), 5′-GATTGCTGCTACAACG3′ (ADH3 gene; forward 1), 5′-TATGCTGCTACAACG3′ (ADH3 gene; forward 2), 5′-GATTGGTACTTATTGCTACAACG3′ (ADH3 gene; forward 1), 5′-TTTAATGCTGCTACAACG3′ (ADH3 gene; forward 2), 5′-CTTACACATGAACTTTAG3′ (ADH4 gene; reverse 1), 5′-CTTACACATGAACTTTAG3′ (ADH4 gene; reverse 2), 5′-TTTAATGCTGCTACAACG3′ (ADH4 gene; forward 1), and 5′-TTTAATGCTGCTACAACG3′ (ADH4 gene; forward 2). The target vector (pSBET; Schenk et al., 1995) was digested with NdeI and BamHI and then mixed with PCR amplicons in a sticky-end ligation reaction. The identity of constructs was confirmed by sequencing. Constructs were transformed into Escherichia coli BL21 cells for recombinant protein production.

A single colony was picked and grown in Luria-Bertani solution (5 mL) containing 50 µg mL−1 kanamycin for 12 h. The mixture was added to a larger Erlenmeyer flask containing 150 mL of Luria-Bertani medium, and cells were grown at 37°C to an optical density of 0.8. Transgene expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside, and the flask was incubated at 16°C for 16 h. The turbid broth was centrifuged in a Sorvall RC5C centrifuge (Thermo Fisher Scientific) at 5,000 g for 10 min. Precipitated cells were suspended in 50 mM 3-morpholino-2-hydroxypropanesulfonic acid buffer (containing 10% [v/v] glycerol and 1 mM DTT) and sonicated for three
cycles of 15 s each on ice. The resulting suspension was centrifuged in a model 4515 D microfuge (Eppendorf) at 13,000 × g for 30 min. The supernatant was collected and loaded onto hydroxyapatite (Sima-Aldrich) in batch mode and incubated for 1 h. After washing with 3-morpholino-2-hydroxypropanesulfonic acid buffer, the sample was eluted with 200 mM phosphate buffer. To assess the purity of the preparation, 15 μL of the eluent was processed by SDS-PAGE and stained using Coomassie Brilliant Blue. Following destaining using a 207:73 (v/v/v) mixture of methanol/acetic acid/water, protein bands were detected by imaging (Alpha Innotech). Activity assays contained 10 μM purified enzyme and varying concentrations of ethanol. The conversion of NAD to NADH was used to monitor the progress of the reaction at 340 nm (Synergy H1 Hybrid Reader; BioTek). The Origin 2017 software package (OriginLab) was employed to fit data and deduce kinetic constants.

FNR and Fd Sequence Comparisons

*Mentha longifolia, Mentha arvensis, and Perilla frutescens* mRNA was purified from isolated GT tissue as described above. For *M. longifolia* and *P. frutescens*, strand-specific pair-end libraries were prepared and sequenced using the HiSeq3000 platform (Illumina). For *M. arvensis*, single-end libraries were sequenced by the HiSeq2000 platform (Illumina). Sequences for other species were obtained from the literature or from publicly accessible databases (Supplemental Table S3). In some cases, the literature data included information on gene expression levels. In other cases, we assembled contigs and determined relative expression levels. To generate contigs, reads were trimmed using Skewer (Jiang et al., 2014) and assembled with either Trinity (Grabherr et al., 2011; in the case of Illumina reads) or MIRA (Chevreux et al., 2004; in the case of 454 reads). Expression levels were estimated as average read coverage in the case of MIRA assemblies or calculated using Bowtie (Langmead et al., 2009) and RSEM (Li et al., 2010) in the case of Trinity assemblies. The identity of constructs was confirmed by sequencing.

Cloning of GT-Specific Isoforms of Peppermint Fd and FNR

GTs were isolated, RNA was purified, and first-strand cDNA was generated as described previously (Ahkami et al., 2015). cDNAs were amplified using Phylogen DNA polymerase (New England Biolabs) with an annealing temperature of 60°C. The primer sequences were 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, major GT isoform, forward), 5′-TCTATGGATCCTCCACATCTGTCGAGAC-3′ (Fd, major GT isoform, reverse), 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, major GT isoform, forward), 5′-TCTATGGATCCTCCACATCTGTCGAGAC-3′ (Fd, minor GT isoform, reverse), 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, minor GT isoform, forward), 5′-TCTATGGATCCTCCACATCTGTCGAGAC-3′ (Fd, minor GT isoform, reverse), 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, minor GT isoform, reverse), 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, minor GT isoform, reverse), 5′-AGTCTGATACAGAAGATGCTCAGAACTC-3′ (Fd, minor GT isoform, reverse), 5′-TCTATGGATCCTCCACATCTGTCGAGAC-3′ (Fd, minor GT isoform, reverse), and 5′-TCTATGGATCCTCCACATCTGTCGAGAC-3′ (Fd, minor GT isoform, reverse). Following digestion with NdeI and BamHI, amplicons were ligated into the pGEM-T Easy vector (Promega), and the identity of constructs was confirmed by sequencing.

Simulating Protein-Protein Interactions in the Peppermint GT FNR-Fd-HDR Complex

The sequences of peppermint Fd (minor GT/root isoform), Fd (major GT isoform), FNR (GT/root isoform), and HDR were submitted to the I-TASSER server for protein structure prediction (Roy et al., 2010; Yang et al., 2013). A structure representation in Protein Data Bank format was returned and employed in structural alignments using the Chimera molecular visualization program (Petterson et al., 2004). Protein Data Bank files of the recently published crystal structures of the root isoforms of maize Fd and FNR (Shinohara et al., 2017) were uploaded into Chimera. A structural alignment was then performed by overlaying the corresponding peppermint Fd and FNR isoforms. Using the Swap function in Chimera, the sequences of the maize Fd and FNR isoforms were changed manually to reflect the residues that are present in the appropriate peppermint Fd and FNR isoforms. The structures of the peppermint Fd and FNR isoforms obtained by this approach (excluding iron-sulfur clusters and ligands) were energy minimized in Chimera, and the iron-sulfur clusters and ligands were subsequently reintroduced by docking. The same method was employed to produce a peppermint HDR structure (following alignment to the published *Plasmidium falciparum* HDR structure; Rekitkke et al., 2013). The structure files for peppermint Fd, FNR, and HDR were submitted to the GRAMM-X server (Tovchigrechko and Vasker, 2006) to assess interactions in a three-protein complex (Fd-FNR as receptor and HDR as ligand). The GRAMM-X algorithm generated multiple docked confirmations. The conformation that most closely maintained the amino acid distances suggested previously to contribute to protein-protein interaction surfaces (Rekitkke et al., 2013; Shinohara et al., 2017) was selected for structure-function studies.

Accession Numbers

Accession numbers are as follows: *M. piperita* Fd cDNA clone (major GT isoform), GenBank KJ748231; *M. piperita* Fd cDNA clone (minor GT/root isoform), GenBank KJ748232; *M. piperita* FNR cDNA clone (GT/root isoform), GenBank KJ748233; *M. piperita* FNR cDNA clone (leaf isoform), GenBank KJ748234; *M. piperita* HDR cDNA sequence, GenBank KY888887; *M. piperita* ADH-like cDNA sequence, GenBank MF579445; *P. frutescens* Fd cDNA sequence, GenBank MF579446; *P. frutescens* GT transcriptome, SRR410089; *M. arvensis* GT transcriptome, SRR1271560 (young leaves) and SRR1271559 (mature leaves); *M. longifolia* (CMEN585 accession) GT transcriptome, SRR3623199.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Alignment of alcohol dehydrogenase sequences.

Supplemental Table S1. Reactions included in various iterations of the menpiGT_2015 model.

Supplemental Table S2. Predicted outcomes of simulated reaction deletions.

Supplemental Table S3. Predicted reaction outcomes of the menpiGT_2015 model using Boolean constrained SPOT and gene expression patterns to estimate flux distribution.

Supplemental Table S4. Sequences and relative expression levels of genes coding for FNR and Fd isoforms across angiosperms that harbor GTs.

Supplemental Methods and Data File S1. Reconstructing mint GT metabolism (all iterations of the menpiGT_2015 model).

Supplemental Methods and Data File S2. Establishing experimental conditions to test the effects of in vivo ATP synthase inhibition on monoterpene formation in isolated peppermint GTs.

ACKNOWLEDGMENTS

We thank Susan Vogtmann for tending plants, Kelly Vining (Oregon State University) for sharing data prior to publication, and Jeremy Jewell, Amber Parrish, and Glenn Turner for helpful discussions and technical assistance.

Received April 21, 2017; accepted August 22, 2017; published August 24, 2017.

LITERATURE CITED


Copyright © 2017 American Society of Plant Biologists. All rights reserved.


Sato A, Yamada H (2015) High antiallergic activity of 5,6,4'-trihydroxy-7,8,3'-trihemisovalene and 5,6-dihydroxy-7,8,3'-tetratemisovalene from eau de cologne mint (Mentha × piperita citrata). Fitoerapia 70: 74–83


Copyright © 2017 American Society of Plant Biologists. All rights reserved.