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# A systems biology approach identifies the biochemical mechanisms regulating monoterpene essential oil composition in peppermint

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The integration of mathematical modeling and experimental testing is emerging as a powerful approach for improving our understanding of the regulation of metabolic pathways. In this study, we report on the development of a kinetic mathematical model that accurately simulates the developmental patterns of monoterpene essential oil accumulation in peppermint (*Mentha × piperita*). This model was then used to evaluate the biochemical processes underlying experimentally determined changes in the monoterpene pathway under low ambient-light intensities, which led to an accumulation of the branchpoint intermediate (+)-pulegone and the side product (+)-menthofuran. Our simulations indicated that the environmentally regulated changes in monoterpene profiles could only be explained when, in addition to effects on biosynthetic enzyme activities, as yet unidentified inhibitory effects of (+)-menthofuran on the branchpoint enzyme pulegone reductase (PR) were assumed. Subsequent *in vitro* analyses with recombinant protein confirmed that (+)-menthofuran acts as a weak competitive inhibitor of PR ( $K_i = 300 \mu\text{M}$ ). To evaluate whether the intracellular concentration of (+)-menthofuran was high enough for PR inhibition *in vivo*, we isolated essential oil-synthesizing secretory cells from peppermint leaves and subjected them to steam distillations. When peppermint plants were grown under low-light conditions, (+)-menthofuran was selectively retained in secretory cells and accumulated to very high levels (up to 20 mM), whereas under regular growth conditions, (+)-menthofuran levels remained very low ( $<400 \mu\text{M}$ ). These results illustrate the utility of iterative cycles of mathematical modeling and experimental testing to elucidate the mechanisms controlling flux through metabolic pathways.

kinetic modeling | monoterpene biosynthesis | isoprenoid | menthofuran | pulegone reductase

The commercially valuable essential oil of peppermint (*Mentha × piperita*) consists primarily of *p*-menthane-type monoterpenes (1), which are synthesized and accumulated in leaf protuberances termed peltate glandular trichomes (2, 3). Within these trichomes, the biosynthesis of monoterpenes is restricted to nonphotosynthetic secretory cells that are arranged in an eight-celled disk (4). The secretory cells exude essential oil into an emerging cavity formed by the separation of a preformed layer of cuticular material (5). Modification of a general protocol for the isolation of peppermint secretory cells (6) enabled the extraction of high-quality mRNA, the generation of cDNA libraries, the sequencing of randomly selected clones, and the functional testing of cDNAs by expression of recombinant proteins in microbial hosts (7). In combination with developmental studies at the microscopic, molecular, and biochemical levels, these functional genomics approaches have yielded a wealth of information about the biochemical properties of the individual biosynthetic enzymes and the regulation of the monoterpene pathway as a whole (reviewed in ref. 8).

In peppermint secretory cells, the precursors of monoterpenes are derived exclusively from the leucoplast-localized meval-

onate-independent pathway (9, 10). The first committed step of the monoterpene pathway, the conversion of geranyl diphosphate to (–)-limonene, is catalyzed by (–)-limonene synthase (11, 12), which is also localized to leucoplasts (13) (Fig. 1). After translocation to the endoplasmic reticulum, (–)-limonene undergoes a cytochrome P450-dependent hydroxylation, catalyzed by (–)-limonene 3-hydroxylase (14), to form (–)-transisopiperitenol. After import into mitochondria, (–)-transisopiperitenol is oxidized to (–)-isopiperitenone by a specific NAD<sup>+</sup>-dependent short-chain dehydrogenase (15, 16). A double-bond reduction, catalyzed by (–)-isopiperitenone reductase (17), followed by an isomerization of (+)-*cis*-isopulegone (18) generates the branchpoint intermediate (+)-pulegone in the cytosol. Cytosolic (+)-pulegone reductase (PR) synthesizes both (–)-menthone and (+)-isomenthone from (+)-pulegone (17). Further conversions in the main monoterpene pathway are catalyzed by the bifunctional cytosolic activities of (–)-menthone:(–)-*(3R)*-menthol reductase and (–)-menthone:(–)-*(3S)*-neomenthol reductase (19). The side product (+)-menthofuran can be formed from (+)-pulegone by the action of (+)-menthofuran synthase (MFS), an endoplasmic reticulum-localized cytochrome P450-dependent monooxygenase (20). The highest rates of monoterpene biosynthesis were measured during the period of maximal leaf expansion (12–20 days after leaf initiation) (21). Based on <sup>14</sup>CO<sub>2</sub> incorporation experiments with subsequent radio-GC analysis, the rate of monoterpene biosynthesis appeared to be the most important factor controlling leaf monoterpene levels. Subsequent studies established that the transcript levels of genes involved in the peppermint monoterpene pathway and the corresponding enzyme activities correlated well with the rate of monoterpene biosynthesis (22), indicating that flux might be coordinately regulated at the level of gene expression.

High-quality oils are characterized by a complex compositional balance of monoterpenes with high (–)-menthol, moderate (–)-menthone, and low (+)-pulegone and (+)-menthofuran quantities (23, 24). Under adverse environmental conditions, such as low light intensity, water deficit, and/or high night temperatures, elevated quantities of (+)-pulegone and (+)-menthofuran accumulate (25, 26), thus rendering an oil of unsatisfactory quality. Mahmoud and Croteau reported that transgenic plants with decreased MFS transcript levels accumulated vastly reduced (+)-pulegone and (+)-menthofuran amounts under regular and stress conditions (27). Further studies indicated that PR transcript levels decreased in the

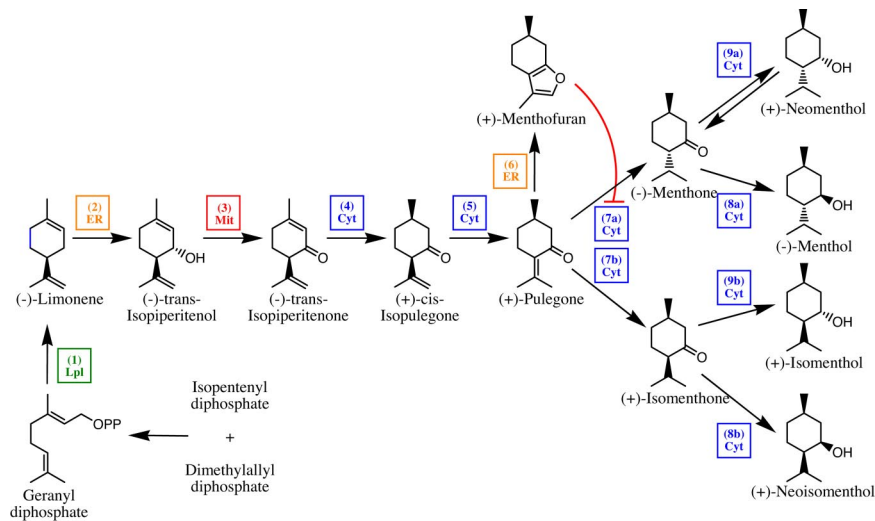
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**Fig. 1.** Outline of *p*-menthane monoterpene metabolism in peppermint glandular trichomes. The following enzymes are involved in this pathway: indicated by "(1)," (-)-limonene synthase; indicated by "(2)," (-)-limonene 3-hydroxylase; indicated by "(3)," (-)-*trans*-isopiperitenol dehydrogenase; indicated by "(4)," (-)-*trans*-isopiperitenone reductase; indicated by "(5)," (+)-*cis*-isopulegone isomerase; indicated by "(6)," (+)-menthofuran synthase; indicated by "(7a)," (+)-pulegone reductase [(-)-menthone-forming activity]; indicated by "(7b)," (+)-pulegone reductase [(+)-isomenthone-forming activity]; indicated by "(8a)," (-)-menthone: (-)-menthol reductase [(-)-menthol-forming activity]; indicated by "(8b)," (-)-menthone: (-)-menthol reductase [(+)-neoisomenthol-forming activity]; indicated by "(9a)," (-)-menthone: (+)-neomenthol reductase [(+)-neomenthol-forming activity]; indicated by "(9b)," (-)-menthone: (+)-neomenthol reductase [(+)-isomenthol-forming activity]. The subcellular compartmentation of *p*-menthane metabolic enzymes is color-coded: Cyt (blue), cytosol; ER (orange), endoplasmic reticulum; Lpl (green), leucoplasts; Mit (red), mitochondria. The inhibition of PR by (+)-menthofuran, as demonstrated in the present manuscript, is indicated by a red arc with an orthogonal red line.

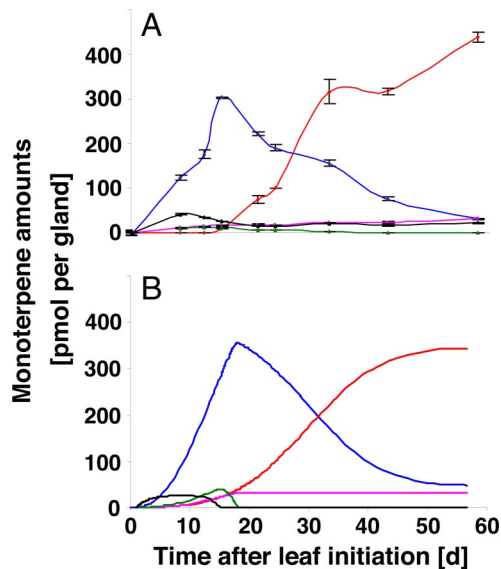
presence of (+)-menthofuran, thus resulting in a decreased PR activity and increased (+)-pulegone amounts (28). Because of these regulatory complexities and the occurrence of branch-points, the fine-tuning of monoterpene biosynthesis cannot be understood intuitively. Here, we report on the development of a kinetic mathematical model, based on the available experimental data, that accurately describes the behavior of the peppermint monoterpene biosynthetic pathway under various experimental conditions. Model predictions were used to generate nontrivial, testable hypotheses regarding poorly understood regulatory mechanisms, and modeling-guided follow-up experiments were used to demonstrate an as yet unidentified role for (+)-menthofuran as a competitive inhibitor of PR. These results indicate that gene expression and posttranslational modulation of enzyme activity both are important factors in regulating peppermint monoterpene biosynthesis.

## Results and Discussion

**Development of a Mathematical Model Simulating Monoterpene Biosynthesis in Peppermint Oil-Gland Secretory Cells.** The filling of peppermint glandular trichomes with monoterpenoid essential oil is a complex process. A mathematical model simulating this process needs to account for various levels of regulation. Several estimates were made to allow linking microscopic variables (e.g., monoterpene composition in individual glandular trichomes) with macroscopic measurements (e.g., leaf monoterpene composition). The number of glandular trichomes per leaf depends on environmental and developmental parameters. Under greenhouse conditions, the number of biosynthetically active glandular trichomes increases from  $\approx 2,500$  (at day 5 after leaf initiation) to  $\approx 13,000$  (at day 18 after initiation) (ref. 29 and R.R.-E., G.W.T., and B.M.L., unpublished data), whereas under reduced light intensity ( $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), the maximum number of glandular trichomes is  $\approx 7,500$  (30) [details in [supporting information \(SI\) Appendix](#)]. The volume of the cluster of eight secretory cells of each individual glandular trichome, which does not depend on environmental conditions, can be determined

based on microscopic size measurements (average diameter of the secretory cell disk is  $60 \mu\text{m}$ , height is  $16 \mu\text{m}$ ). We approximated the shape of the secretory cell cluster as a frustum of a cone, the volume of which can be calculated as  $\frac{1}{3} \pi h (R^2 + Rr + r^2)$ , thus resulting in a calculated average secretory cell disk volume of  $2.35 \times 10^{-5} \mu\text{l}$  at maturity. Morphometric measurements taken by using microscopic images at different developmental stages were combined with stereological approaches (31) to calculate the volume densities of subcellular compartments in peppermint oil-gland secretory cells, thus allowing us to estimate concentrations of enzymes based on prior knowledge regarding their organellar distribution (details in [SI Appendix](#)). We used two independent methods to calculate the amounts of monoterpenes produced per individual glandular trichome. First, monoterpene amounts obtained from gas chromatography–flame ionization detection (GC-FID) analyses of steam-distilled leaves were divided by the number of oil-bearing glandular trichomes. Second, the volume of the essential oil-filled subcuticular cavity of mature glandular trichomes was calculated based on the approximation of its shape as a hemisphere ( $\frac{2}{3} \pi r^3$ ) with a diameter of  $65 \mu\text{m}$  (determined with morphometric measurements taken by using microscopic images). The volume ( $7.53 \times 10^{-5} \mu\text{l}$ ) was then multiplied by the known essential oil density ( $0.9 \text{ mg}/\mu\text{l}$ ) to obtain the monoterpene amount per gland. An average monoterpene molecular weight of  $150 \text{ g}/\text{mol}$  was assumed for converting these values into molar amounts per gland (for details, see [SI Appendix](#)). Both approaches yielded very similar results with monoterpene amounts between 470 and 570 pmol per gland at maturity, indicating that the essential oil in peppermint glandular trichomes consists almost exclusively of monoterpenes.

Kinetic constants for monoterpene biosynthetic enzymes were obtained from the literature (11, 15, 17–19, 32). Michaelis–Menten rate equations were used to describe the kinetic behavior of individual biosynthetic enzymes. These expressions were used in a set of ordinary differential equations to account for the time dependence of the metabolite concentration (details in



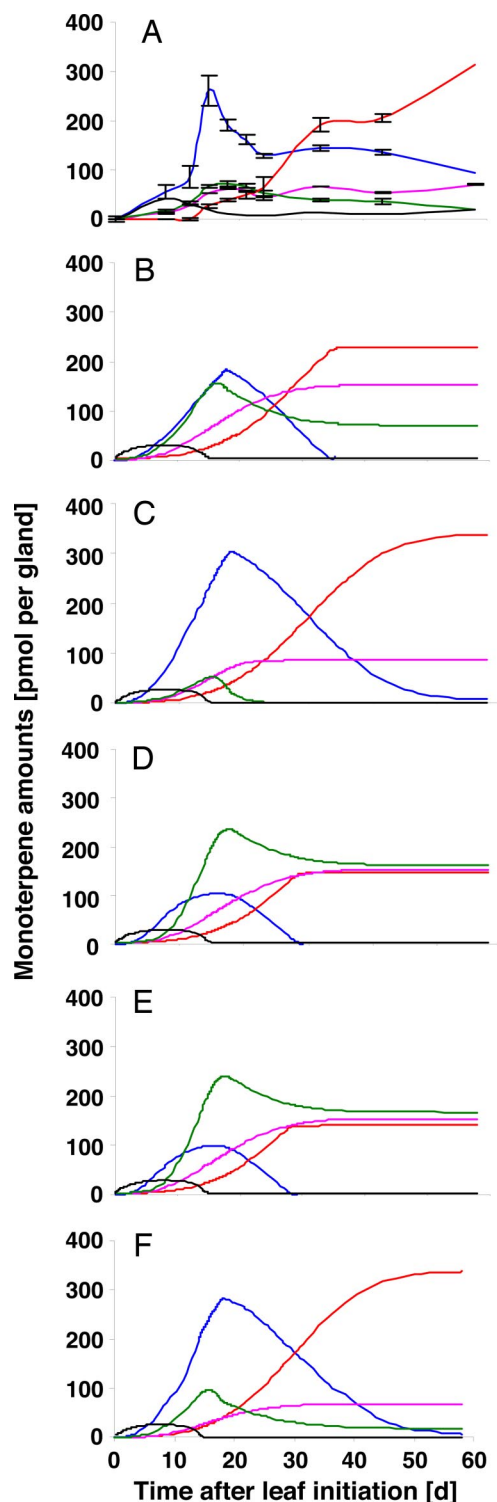
**Fig. 2.** Experimentally determined monoterpene profiles of peppermint plants grown under greenhouse conditions (A) and computer simulation based on a kinetic mathematical model of mint monoterpene biosynthesis (B). The following colors are used for indicating monoterpene profiles: black, (–)-limonene; pink, (+)-pulegone; green, (+)-menthofuran; blue, (–)-menthone; red, (–)-menthol.

*SI Appendix*). In addition, we accounted for changes in enzyme concentration during the course of leaf development by approximating the shape of previously reported enzyme activity patterns (22) with Gaussian functions (details in *SI Appendix*). We did not account for diurnal changes in enzyme activities and the effects of day/night temperature changes on enzyme kinetics; thus, the kinetic and enzyme activity values in our model represent “daily averages.” A dynamic simulation of monoterpene profiles was performed by simultaneously solving the system of modified ordinary differential equations (expressing both enzyme properties and expression patterns) by using the `ode15s` function of the MATLAB software package. Because enzyme concentrations and the variables used for the Gaussian function representing developmental enzyme activity patterns could only be estimated, iterative optimizations of the initial model parameters were performed by using the experimentally measured monoterpene profiles as constraints, until the best fit of modeling results and experimental data were achieved. Our modeling simulated an early accumulation of high levels of (–)-menthone (maximum at 15 days), which is converted to (–)-menthol during the essential oil maturation phase (15–55 days), a transient low-level accumulation of (–)-limonene and (+)-pulegone, and a sigmoidal time course of (+)-menthofuran accumulation (low levels). These simulations were in excellent agreement with monoterpene profiles obtained with greenhouse-grown plants (Fig. 2). The fact that monoterpene profiles under regular growth conditions could be simulated successfully by considering only the kinetic properties and developmental expression patterns of biosynthetic enzymes confirmed previous conclusions that monoterpene biosynthesis is determined by the rate of biosynthesis (21), most likely regulated at the level of gene expression (22).

**Mathematical Modeling Suggests that Posttranslational Regulation Determines Monoterpene Profiles Under Environmental Stress Conditions.** Peppermint oil of the highest commercial quality can only be produced with plants grown in certain geographical regions with hot days and cool nights. It has also been known for many

years that essential oil yield and composition vary widely among growing regions and are affected by numerous environmental and agronomic factors (reviewed in ref. 33). To evaluate the suitability of mathematical modeling for understanding environmental effects on essential oil biosynthesis, we subjected peppermint plants to a series of environmental stresses, measured monoterpene profiles, and tested several hypotheses regarding biochemical and regulatory mechanisms underlying the accumulation of undesirable essential oil components, in particular (+)-pulegone and (+)-menthofuran, based on the degree of convergence between these experimental data and computational simulations. Under all environmental conditions tested (reduction of water/fertilizer to 50%, lowering of light intensity, or increase of night temperatures), a reduction of total oil yield was measured, which correlated with smaller leaves and a lower number of glandular trichomes per leaf (R.R.-E., G.W.T., and B.M.L., unpublished results). In certain experiments (low light and high night temperatures), we also detected an accumulation of (+)-pulegone and (+)-menthofuran (data not shown), the simulation of which required testing various hypotheses computationally. Here, we are going to use the low-light experiment as an example to illustrate the process of computational hypothesis testing.

Control plants were grown in a greenhouse with additional lighting from sodium-vapor lights ( $850 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation), a 16-h photoperiod and a temperature cycle of  $27^\circ\text{C}/21^\circ\text{C}$  (day/night), whereas the experimental treatment involved plants kept in a growth chamber at reduced light intensity ( $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation) but under otherwise identical conditions as controls. Total essential oil yield was  $\approx 50\%$  lower in plants grown under low light conditions ( $480\text{--}630 \mu\text{g}$  per leaf at maturity) compared with controls ( $1,160\text{--}1,270 \mu\text{g}$  per leaf at maturity) (Fig. 3A). Cultivation under low-light conditions led to a transient increase of (+)-pulegone, with a maximum of 70 pmol per glandular trichome at 18 days and a hyperbolic accumulation of (+)-menthofuran of  $\approx 60$  pmol per glandular trichome. Mahmoud and Croteau have reported that (+)-pulegone and (+)-menthofuran increased and decreased, respectively, in concert under stress conditions (27, 28). Stem-feeding experiments with (+)-menthofuran led to a dose-dependent decrease in the expression of the gene encoding PR, the enzyme responsible for the conversion of (+)-pulegone into (–)-menthone, by an as yet unidentified mechanism (28). Furthermore, in transgenic lines with increased expression levels of the gene encoding MFS and higher (+)-menthofuran amounts in the essential oil, (+)-pulegone amounts were higher than in controls, which led to the hypothesis that the metabolic fate of (+)-pulegone is controlled by a (+)-menthofuran-mediated transcriptional down-regulation of PR levels (28). To test whether these assumptions could guide simulations of the monoterpene profiles observed in the present experiments with low-light-grown plants, we initially increased the levels of MFS and decreased PR levels (2-fold up and 2-fold down, respectively) in our mathematical model (*SI Appendix*). Simulations using these model adjustments indicated that, compared with controls, the peak levels of (+)-pulegone and (+)-menthofuran should be dramatically increased (160 and 150 pmol per gland, respectively), whereas (–)-menthone and (–)-menthol levels would be drastically reduced (190 and 220 pmol per gland, respectively) (Fig. 3B). We then simulated numerous other reasonable combinations with increased MFS and decreased PR levels (1.5- to 2.5-fold up and down, respectively), but a satisfactory simulation of the measured monoterpene profiles could not be obtained. Because these simulations were only in partial agreement with experimentally determined values, alternative hypotheses regarding the biochemical mechanisms of environmental variation in peppermint essential oil composition had to be considered.



**Fig. 3.** Effect of stress on monoterpene metabolism. Monoterpene profiles of peppermint plants maintained in growth chambers under low-light conditions (A) and computer simulation considering a reduction of PR and increase in MFS transcript levels (as suggested in ref. 28) (B). Simulations assuming an inhibition of PR by (+)-menthofuran by a competitive (C), uncompetitive (D) or noncompetitive (E) mechanism. Simulation of monoterpene profiles under low-light conditions after model optimization (F). Color-coding of monoterpene profiles is as in Fig. 2.

As a plausible and as yet unexplored possibility for peppermint monoterpene pathway regulation, we simulated the essential oil composition if inhibitory effects of (+)-menthofuran on PR

were assumed. Using our model, we tested for competitive, uncompetitive, and noncompetitive inhibition by using assumed  $K_i$  values of  $10 \mu\text{M}$  (Matlab code in *SI Appendix*). Simulations assuming a competitive inhibition mechanism were in excellent agreement with monoterpene values measured experimentally (Fig. 3C), whereas simulations for uncompetitive or noncompetitive inhibition of PR by (+)-menthofuran predicted very high accumulation levels for (+)-pulegone and (+)-menthofuran (Fig. 3D and E). Based on these computational predictions, we then tested experimentally whether (+)-menthofuran exerted inhibitory effects on PR activity.

**(+)-Menthofuran Is a Competitive Inhibitor of PR.** Recombinant PR was expressed in *Escherichia coli*, partially purified, and assayed as described previously (17). The kinetic constants we determined for (+)-pulegone as a substrate [ $K_m = 40 \mu\text{M}$ ,  $V_{max} = 185 \text{ pmol/s}$ , and  $\text{IC}_{50}$  (substrate inhibition) =  $150 \mu\text{M}$ ] were very similar to those reported previously, although our  $K_m$  value was a bit higher (17). After completing these preliminary studies to establish the appropriate assay conditions, PR enzyme activity was measured with (+)-pulegone as a substrate ( $0\text{--}100 \mu\text{M}$ ), NADPH as a cofactor ( $500 \mu\text{M}$ ), and varying concentrations of the putative inhibitor (+)-menthofuran ( $0\text{--}400 \mu\text{M}$ ). We observed a dose-dependent decrease in PR activity in the presence of (+)-menthofuran (Fig. 4A). To evaluate the mechanism of inhibition, we used the Lineweaver–Burk method (34), in which  $1/V$  ( $V = \text{velocity}$ ) is plotted against  $1/[S]$  ( $[S] = \text{substrate concentration}$ ) (Fig. 4B). The lines obtained for different inhibitor concentrations had a common intercept with the  $1/V$  axis, but the slopes of the lines increased with rising inhibitor concentrations. The  $V_{max}$  value (determined based on intercept with the  $1/V$  axis) remained the same in the presence of different inhibitor amounts, whereas the  $K_m$  value (determined based on the intercept with the  $1/[S]$  axis) increased with rising inhibitor concentrations, thus indicating a competitive inhibition mechanism. The inhibition constant ( $K_i$ ), which defines the competition of substrate and inhibitor for the same active site of PR, was determined by using two independent approaches: (i) with the Dixon method (plotting  $1/V$  against  $[I]$  ( $[I] = \text{inhibitor concentration}$ ),  $K_i$  was determined by linear regression analysis (35); and (ii) with the  $K_{m(\text{app})}$  method (plotting  $K_{m(\text{app})}/V_{max}$  against  $[I]$ ),  $K_i$  was obtained by a nonlinear regression analysis (36). In both cases, a  $K_i$  for (+)-menthofuran as a competitive inhibitor of  $\approx 300 \mu\text{M}$  was calculated. Using the same methods, we estimated a  $K_i$  of  $112 \mu\text{M}$  for substrate inhibition by (+)-pulegone. Because (+)-menthofuran appeared to be a relatively weak competitive inhibitor [ $K_i$  value for (+)-menthofuran  $\approx 7.5$ -fold higher than the  $K_m$  value for (+)-pulegone as substrate], we tested whether (+)-menthofuran concentrations in peppermint secretory cells were sufficiently high to give rise to relevant inhibitory effects.

**(+)-Menthofuran Is Preferentially Retained in Peppermint Oil-Gland Secretory Cells.** Leaves from greenhouse-grown peppermint plants were harvested at 20 and 50 days, secretory cells were isolated and steam distilled, and monoterpene profiles were analyzed by GC-FID (modified from ref. 17). Secretory cells from plants grown in the greenhouse contained primarily (–)-menthone at 20 days and substantial amounts of (–)-menthol at 50 days, whereas only small amounts of (+)-menthofuran and negligible amounts of (+)-pulegone were detected (details in *SI Appendix*). In contrast, secretory cells obtained from plants grown under stress conditions (low light intensity) accumulated (+)-menthofuran as the principal metabolite (up to  $20 \text{ mM}$ , accounting to  $\approx 90\%$  of total monoterpenes). High amounts of (+)-menthofuran had been detected in secretory cells previously when grown under comparable growth conditions (3), but it had not been recognized that this was due to stress conditions (low



constant ( $K_i$ ) for (+)-menthofuran was determined by using the Dixon method (35) and nonlinear regression analysis (36).

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