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Cool-Burn: In Silico Design of Combined TRPV1 and TRPM8 Activation in Capsicum annuum

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Research Article

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SCOVILLE SPLICE RESEARCH SERIES

Cool-Burn: In Silico Design of Combined TRPV1 and TRPM8 Activation in Capsicum annuum

A computed design that adds the six-gene Mentha menthol pathway to Capsicum annuum fruit, with cassettes, a knock-in template at a chromosome 2 site and a plastid GPP budget.

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Patents: US 64/172,827; US 64/188,197; US 64/192,062; US 64/199,105 Figshare: figshare/35015821

OSF: osf.io/mrw7a Hugging Face: capsaicinoid-data GitHub: paper-09 GenBank: QB274225–QB274229

Website: cool-burn-menthol

Package record. 106 claims traced to a file or a cited source (77 computed, 9 definition, 11 design assumption, 3 design target, 6 literature value). 17 tables, 8 figures, 47 data files. Data fingerprint `d2559046b450`. Rerun every result with `bash run_all.sh`.



Phoenix fruit. Scoville Splice LLC image library.

ABSTRACT

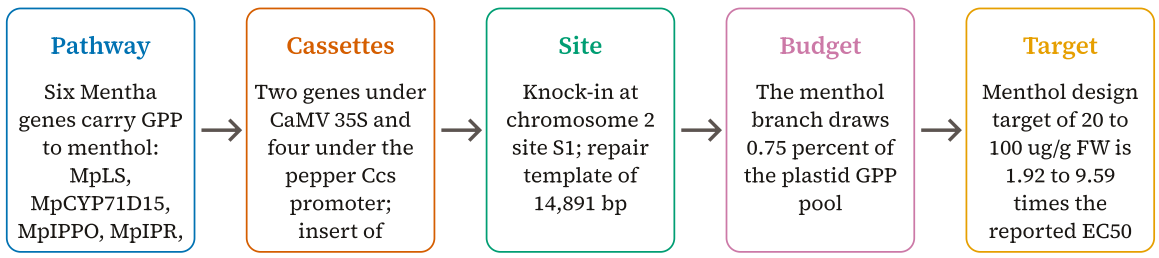
Capsaicin activates the heat receptor TRPV1, and menthol activates the cold receptor TRPM8. This paper describes a design that adds the six-gene menthol pathway of *Mentha* × *piperita* to *Capsicum annuum* fruit: MpLS, MpCYP71D15, MpIPPO, MpIPR, MpPIPR and MpMR. Two cassettes use the CaMV 35S promoter and four use a piece of 920 bp of the pepper Ccs promoter. The six cassettes total 13,891 bp, and the repair template for knock-in at site S1 on chromosome 2 is 14,891 bp. A plastid geranyl diphosphate (GPP) budget with the pool held fixed gives a menthol-branch draw of 0.75% of the pool. The stated limonene route, built from design assumptions with no source, gives 1.72 to 9.18 ug/g FW of menthol (11.0 to 58.7 uM). The design target is 20 to 100 ug/g FW, which is 1.92 to 9.59 times the reported EC50 of 66.7 uM at the cloned TRPM8 channel. At the stated GPP flux of 0.90 umol/gDW/h, the central target of 85 ug/g FW is reached in 6.32 h (5.04 to 7.37 h over the dry-weight range). The heat side is the Phoenix target of 2,956,310 SHU. All results are computed. No experimental data are reported in this paper.

Keywords: Capsicum annuum · menthol · TRPM8 · TRPV1 · limonene synthase · Ccs promoter · plastid GPP budget · Iyengar Flavor Index

KEY POINTS

- Six *Mentha* genes form an insert of 13,891 bp; the repair template at site S1 on chromosome 2 is 14,891 bp.
- Each of the six coding sequences translates to its UniProt sequence at every position.
- The stated limonene route gives 1.72 to 9.18 ug/g FW of menthol; the design target is 20 to 100 ug/g FW.
- The design target is 1.92 to 9.59 times the reported TRPM8 EC50; the stated flux reaches the central target in 6.32 h.
- The menthol branch draws 0.75% of the plastid GPP pool. All results are computed from stated assumptions.

Marks under numbers: computed · design target · design assumption · literature value · definition. The evidence table in Appendix A lists the source file of every one.



Graphical abstract. The design in five steps: six genes, six cassettes, one knock-in site, a GPP budget and the menthol design target beside the reported EC50. Drawn from the steps file. Data: `data/figures/GA.csv`; script: `code/03_supplementary.py`.

1. Introduction

Capsaicin activates TRPV1, the heat- and pain-sensing channel of sensory neurons (Caterina et al., 1997). Menthol activates TRPM8, the cold receptor channel identified in 2002, which opens below about 28 °C (McKemy et al., 2002; Peier et al., 2002). Topical

menthol on human skin is reported to give cold pain and cold hyperalgesia (Wasner et al., 2004).

Peppermint makes (−)-menthol from GPP in a pathway that Croteau et al. (2005) list as eight enzymatic steps. The cDNAs of several of its enzymes were cloned from mint species (Lupien et al., 1999; Ringer et al., 2003, 2005; Davis et al., 2005), and the route has been run with enzymes in *Escherichia coli* (Toogood et al., 2015). Transgenic monoterpene production in fruit is reported for geraniol in tomato (Davidovich-Rikanati et al., 2007), and a lemon limonene synthase and a *Mentha* limonene-3-hydroxylase act in tobacco (Lücker et al., 2004a, 2004b).

This paper designs the six-gene pathway for *Capsicum annuum* fruit. It reports six cassettes built from real sequence records, a repair template for the chromosome 2 site S1 that is shared with the brazzein design (10.5281/zenodo.24511316) and the kokumi design (10.5281/zenodo.24511332), a plastid GPP budget, and menthol design targets set beside the reported EC50 at TRPM8. The heat side is the Phoenix cultivar design (10.5281/zenodo.24511384). Concentrations are compared with the proposed IFI-M reference of the Iyengar Flavor Index (10.5281/zenodo.24511320). The index scores one compound against a reference assay, so the ratios here are concentration ratios and not index scores.

2. Methods

2.1 Computational basis

Genome coordinates are those of the RefSeq assembly GCF_002878395.1 (*Capsicum annuum* UCD10Xv1.1; Hulse-Kemp et al., 2018). The analysis steps run on Python 3.9 or newer and use only the standard library; `bash run_all.sh` reruns them. The solver route of the GPP budget in the research record uses COBRApy 0.32.1 (Ebrahim et al., 2013) with the GLPK solver, and the protein masses in the research record come from Biopython 1.88. Every sequence used by the steps is stored in `inputs/sequences/`, and the public records they come from are listed in `inputs/input_sources.csv`.

2.2 Genes and proteins

The design carries six genes of *Mentha* × *piperita* (Table 1), in pathway order: MpLS (limonene synthase), MpCYP71D15 (cytochrome P450 71D15, limonene-3-hydroxylase), MpIPPO (isopiperitenol dehydrogenase), MpIPR (isopiperitenone reductase), MpPIPR (pulegone reductase) and MpMR (menthone:menthol reductase). Each gene is a UniProt entry, and the reaction column of Table 1 is the annotation of that record. The coding

sequence of each gene is the native sequence of a GenBank mRNA record whose translation equals the UniProt sequence; the MpMR coding sequence is bases 60 to 995 of its record. The sequences are not recoded, and each keeps its native start and stop codon. Protein mass is the sum of the average residue masses plus water (Table 2).

2.3 Promoters and terminator

MpLS and MpCYP71D15 use the CaMV 35S promoter, a piece of 835 bp of the pBI121 vector record, bases 4,974 to 5,808 of that record. MpIPPO, MpIPR, MpPIPR and MpMR use the pepper capsanthin/capsorubin synthase (Ccs) promoter, bases 1,085 to 2,004 of GenBank record Y14165.1, 920 bp in all. The piece ends at the base before the Ccs start codon and so keeps the native leader. The Ccs gene is LOC107875664 on chromosome 6. The genome copy of the piece is bases 219,129,838 to 219,130,749 of NC_061116.1 (912 bp) and aligns to the record piece at 96.4% identity in a semi-global alignment; the last 66 bases before the start codon are identical in the record and the genome (Table S3). Ccs is a pepper fruit gene with a reported promoter activity in pepper fruit: Tian et al. (2017) fused Ccs promoter pieces to the GUS reporter and injected them into mature green fruit, and every piece that held at least one 176-bp repeat gave staining, and Li et al. (2013) reported a 920-bp promoter product with three 176-bp repeats in red *C. annuum* lines that matches Y14165. The terminator is the nopaline synthase (NOS) terminator of 256 bp from the same pBI121 record, bases 4,022 to 4,277. The orientation of the 35S piece was checked by searching a 29-base core of its TATA region on both strands (Table S2).

2.4 Cassettes, insert and repair template

A cassette joins a promoter piece, a coding sequence and the NOS terminator in one orientation on the plus strand without linkers. The insert is the six cassettes back to back in pathway order, and the repair template is the left arm, the insert and the right arm (Table 3, Figure 1). The sequences were checked for reading frame, stop codons, start codons inside the promoter pieces, G plus C content, the number of exact copies of each piece and the recognition sites of BsaI, BsmBI and BpiI on both strands (Table S2). A second sum of the lengths was evaluated with `bc` and a second build of the cassettes is in the research record.

2.5 Site S1 and guides

Site S1 is the window NC_061112.1:131,429,594 to 131,432,593 on chromosome 2 of UCD10Xv1.1, the knock-in site of the brazzein design (10.5281/zenodo.24511316) and the kokumi design (10.5281/zenodo.24511332). The insertion point is 131,431,093. The two homology arms are 500 bp each: the left arm is 131,430,500 to 131,430,999 and the right

arm is 131,431,101 to 131,431,600. Two guides cut on either side of the insertion point, so the 101 bases between the cuts are replaced by the insert. The guides were scanned against the whole genome with our own C scanner (strict NGG PAM, both strands, up to four mismatches) and scored with our own implementation of the Hsu et al. (2013) MIT score. The nearest genes are LOC107859470 (38,000 bp from the insertion point) and LOC107858201 (8,292 bp); both are protein coding. The stored scan output is in `inputs/site_S1_guides.csv` (Table S4).

2.6 Plastid GPP budget

The budget is one node: the plastid GPP pool, its three endogenous sinks (diterpenes and chlorophyll, endogenous monoterpenes, other sinks) and the menthol branch. The pool is held fixed. The menthol branch draws a flux D, and the three endogenous sinks share the rest in proportion to their wild-type fluxes, so each is scaled by $(S - D)$ / (the sum of the wild-type sink fluxes), where S is the pool. Table 4 lists every input; the pool of 120 umol/gDW/h, the wild-type sinks of 95.2, 22.5 and 2.3 umol/gDW/h, and the draws of 0.2 umol/gDW/h for the two-gene design and 0.9 umol/gDW/h for the six-gene design are design assumptions with no source. The budget was computed in closed form (`code/02_pool_model.py`), with the solver route of COBRApy in the research record, and with an independent recomputation (Table S9). It is arithmetic under stated assumptions and is not a genome-scale flux model.

2.7 Stated route, design targets and concentrations

The stated limonene route is limonene in tissue times the conversion to product times the molar-mass ratio of product to limonene. The limonene amount of 5 to 10 ug/g FW and the conversions of 30 to 80% to menthol and 60 to 80% to isopiperitenol are design assumptions with no source. The menthol design target is 20 to 100 ug/g FW with a central value of 85 ug/g FW, and it has no literature basis. Concentration in uM is ug/g FW × 1,000 divided by the molar mass, taking 1 g of tissue as 1 mL; the molar masses are 156.27, 152.23 and 136.24 g/mol. The reference concentrations are the menthol EC50 of 66.7 uM at the cloned TRPM8 channel and 80 uM in trigeminal neurons (McKemy et al., 2002), and the IFI-M convention of 0.1 uM for menthol at TRPM8. The IFI-C reference for capsaicin at TRPV1 is 0.5 uM.

2.8 Titre from the stated flux

The GPP-to-limonene flux readings are 0.18, 0.20 and 0.90 umol/gDW/h (design assumptions with no source). The accumulation rate with no loss, in ug/g FW per hour, is flux × dry-weight fraction × molar mass, and the hours to a level are the level divided by that rate. The dry-weight fraction is 0.082, 0.0957 and 0.120 (Table 4); the values are

the low, mean and high of three USDA FoodData Central pepper records. Each input moved across its range with the others at mid-range for the sensitivity analysis (Table S8).

3. Results

3.1 Proteins and coding sequences

The six proteins are 265 to 599 residues long and 27.2 to 69.7 kDa in mass (Table 2). The coding sequence of each gene translates to its UniProt sequence at every position: 599 of 599, 498 of 498, 265 of 265, 314 of 314, 342 of 342 and 311 of 311 positions. Each coding sequence starts with ATG, ends with a stop codon and has 0 internal stop codons (Table S2). The record of MpIPR annotates (+)-*cis*-isopulegone as its product, and the record of MpPIPR starts from (+)-pulegone; the isomerization between them is one of the eight steps of the reported pathway and is not a gene of this design.

Table 1. The six menthol-pathway genes. The six genes in pathway order with the enzyme, the UniProt entry, the reaction as annotated in the record and the promoter of the design. MpLS and MpCYP71D15 use CaMV 35S; the other four use the pepper Ccs promoter. Data: `data/tables/T01.csv` *; script:* `code/01_cassettes.py` *.*

Step	Gene	Enzyme (UniProt record name)	UniProt	Reaction in the record	Promoter
1	MpLS	Limonene synthase	B0F4G4	GPP -> (-)-limonene	CaMV 35S
2	MpCYP71D15	Cytochrome P450 71D15 ((-)-limonene-3-hydroxylase)	Q9XHE6	(-)-limonene -> (-)-trans-isopiperitenol	CaMV 35S
3	MpIPPO	(-)-Isopiperitenol/(-)-carveol dehydrogenase	Q5C9I9	(-)-isopiperitenol -> (-)-isopiperitenone	Ccs
4	MpIPR	(-)-Isopiperitenone reductase	Q6WAU1	(-)-isopiperitenone -> (+)-cis-isopulegone	Ccs

Step	Gene	Enzyme (UniProt record name)	UniProt	Reaction in the record	Promoter
5	MpPIPR	(+)-Pulegone reductase	Q6WAU0	(+)-pulegone -> menthone	Ccs
6	MpMR	Menthone:(-)-menthol reductase	Q5CAF4	menthone -> (-)-menthol	Ccs

Table 2. Proteins and coding sequences. Length and mass of each protein (sum of average residue masses plus water) and the GenBank record and positions of its native coding sequence. The last column counts the positions at which the translation of the coding sequence equals the UniProt sequence. Data: `data/tables/T02.csv`; *script:* `code/01_cassettes.py`.

Gene	UniProt	Length (aa)	Mass (kDa)	Coding sequence (GenBank record, positions)	Coding sequence (bp)	Positions identical to UniProt
MpLS	B0F4G4	599	69.7	EU108697.1, 1 to 1800	1,800	599 of 599
MpCYP71D15	Q9XHE6	498	56.5	AF124817.1, 1 to 1497	1,497	498 of 498
MpIPPO	Q5C9I9	265	27.2	AY641428.1, 1 to 798	798	265 of 265
MpIPR	Q6WAU1	314	34.4	AY300162.1, 1 to 945	945	314 of 314
MpPIPR	Q6WAU0	342	37.9	AY300163.1, 1 to 1029	1,029	342 of 342
MpMR	Q5CAF4	311	34.1	AY288138.1, 60 to 995	936	311 of 311

3.2 Cassettes and repair template

The six cassettes run from 1,974 to 2,891 bp, and the insert has 13,891 bp with a G plus C content of 43.2% (Table 3, Figure 1). The repair template has 14,891 bp. The Ccs piece holds 0 start codons on the plus strand and 7 matches of the core of its repeat unit. The insert holds 4 exact copies of the Ccs piece, 2 of the 35S piece and 6 of the NOS terminator. The 35S core is found on the plus strand of its piece and is not found on the opposite strand. The insert holds 3, 4 and 8 recognition sites of BsaI, BsmBI and BpiI (Table S2). Table S1 lists every part with its source record and its coordinates in the repair template.

Table 3. Cassettes, insert and repair template. Length of each part of each cassette, and the totals. Insert positions run from the first base of the first promoter piece. The cassettes lie head to tail on the plus strand with no linkers. Data: `data/tables/T03.csv`*; script:* `code/01_cassettes.py`*.*

Cassette	Promoter	Promoter (bp)	Coding sequence (bp)	Terminator (bp)	Cassette (bp)	Insert positions
MpLS	35S	835	1,800	256	2,891	1 to 2,891
MpCYP71D15	35S	835	1,497	256	2,588	2,892 to 5,479
MpIPPO	Ccs	920	798	256	1,974	5,480 to 7,453
MpIPR	Ccs	920	945	256	2,121	7,454 to 9,574
MpPIPR	Ccs	920	1,029	256	2,205	9,575 to 11,779
MpMR	Ccs	920	936	256	2,112	11,780 to 13,891
Insert (six cassettes)					13,891	1 to 13,891
Repair template (arms 500 + 500)					14,891	1 to 14,891

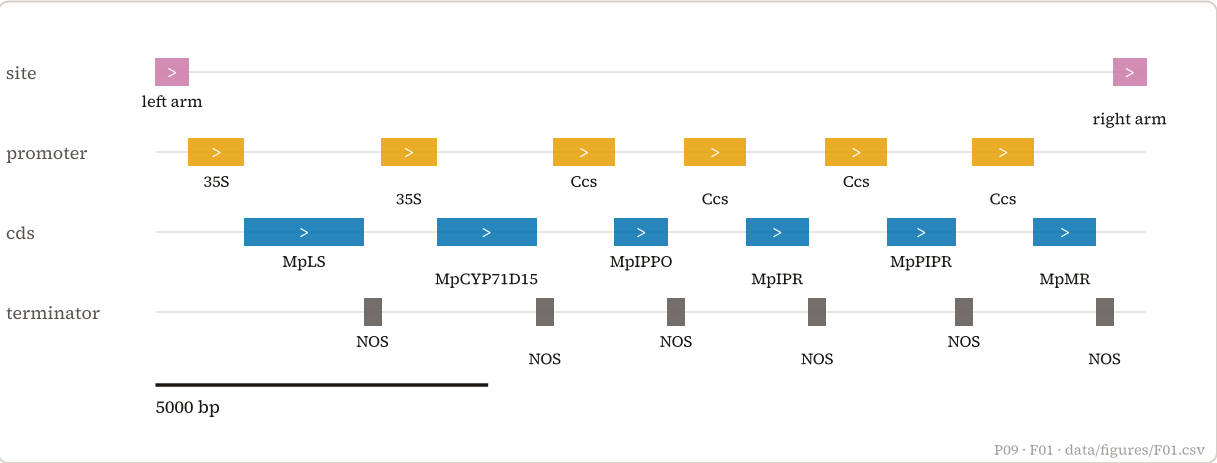


Figure 1. Repair template at site S1. The left homology arm, the six cassettes in pathway order (promoter, coding sequence and NOS terminator of each) and the right homology arm, along the 14,891-bp repair template. The scale bar is in base pairs. Positions are template coordinates computed from the part lengths. Data: `data/figures/F01.csv`; script: `code/01_cassettes.py`.

3.3 Site S1

The window NC_061112.1:131,429,594 to 131,432,593 holds the two guides and the two arms (Figure 2, Table S4). The guides have MIT scores of 64.0 and 70.3, and each has 1 perfect genomic site. The two cuts lie 101 bp apart. The 2,001-bp region around the insertion region hold no soft-masked base and no ambiguous base, and the G plus C content is 46.9%.

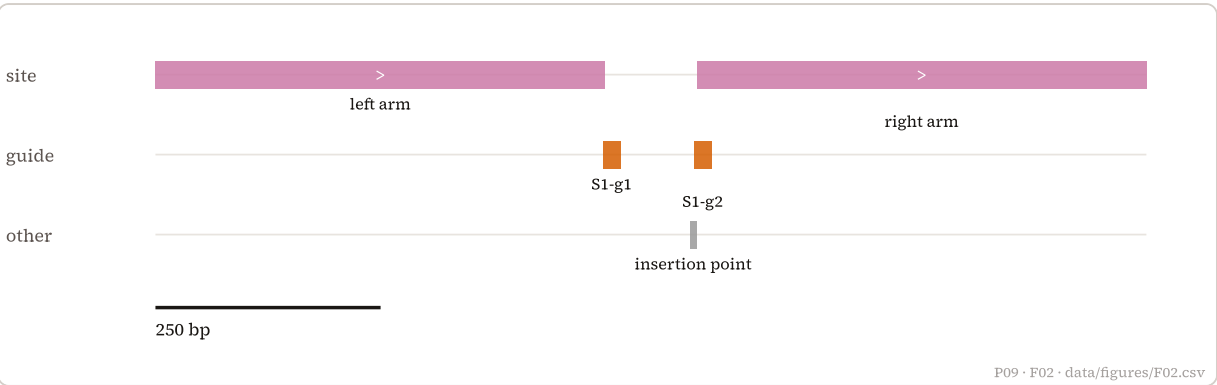


Figure 2. Site S1 on chromosome 2. The 3,000-bp site window with the two homology arms, the two guides and the insertion point. Positions are counted from the first base of the window; the scale bar is in base pairs. Positions are computed from the genome coordinates of site S1. Data: `data/figures/F02.csv`; script: `code/01_cassettes.py`.

3.4 Plastid GPP budget

Table 6 gives the budget. The six-gene draw of 0.9 umol/gDW/h is 0.75% of the pool, and the two-gene draw of 0.2 umol/gDW/h is 0.17%. The sinks sum to the pool of 120 umol/gDW/h in every scenario. Each endogenous sink falls by 0.75% in the six-gene design and by 0.17% in the two-gene design, which gives 94.49, 22.33 and 2.28 umol/gDW/h for the three sinks. If the whole six-gene draw came from one sink alone, the largest reductions would be 0.95%, 4.00% and 39.13% for diterpenes and chlorophyll, endogenous monoterpenes and other sinks (Figure 8). The GPP carbon drawn at the 0.90 umol/gDW/h flux is 0.31% to 0.60% of the sucrose carbon import (Table S7).

Table 6. Plastid GPP budget. Sink fluxes (umol/gDW/h) of the wild type and of the two designs when the 120 umol/gDW/h pool is held fixed and the menthol-branch draw is taken from the three endogenous sinks in proportion to their wild-type fluxes. Data: `data/tables/T06.csv`; script: `code/02_pool_model.py`.

Scenario	Diterpenes and chlorophyll	Endogenous monoterpenes	Other sinks	Menthol branch	Total	Menthol draw (% of pool)	Change of each endogenous sink (%)
Wild type	95.20	22.50	2.30	0.00	120.00	0.00	0.00
Two-gene design (MpLS, MpCYP71D15)	95.04	22.46	2.30	0.20	120.00	0.17	-0.17
Six-gene design	94.49	22.33	2.28	0.90	120.00	0.75	-0.75

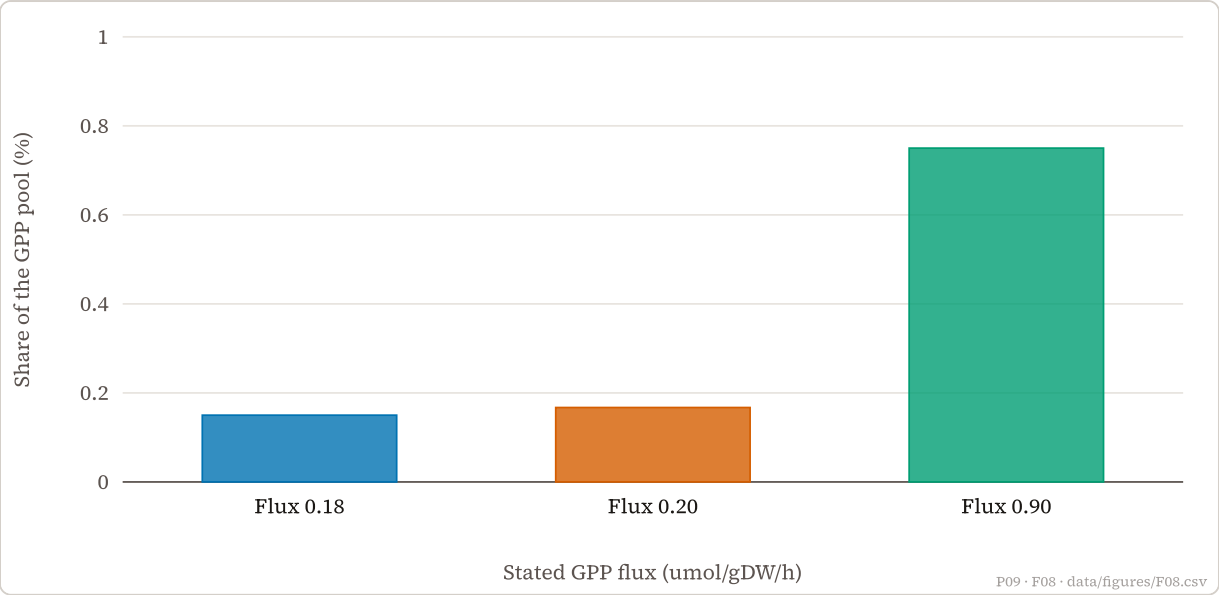


Figure 8. Menthol-branch draw as a share of the GPP pool. The menthol-branch draw at each of the three stated fluxes divided by the plastid GPP pool of 120 umol/gDW/h. Draw / pool x 100. Data: `data/figures/F08.csv`; script: `code/02_pool_model.py`.

3.5 Menthol from the stated route and at the design targets

Table 5 and Figure 3 give the levels. The stated limonene route gives 1.72 to 9.18 ug/g FW of menthol, which is 11.0 to 58.7 uM and 0.17 to 0.88 times the reported cloned-channel EC50. For the two-gene design the same route gives 3.35 to 8.94 ug/g FW of isopiperitenol (22.0 to 58.7 uM). The design target of 20 to 100 ug/g FW is 128.0 to 639.9 uM, which is 1.92 to 9.59 times the cloned-channel EC50 and 1.60 to 8.00 times the EC50 in trigeminal neurons (Figure 4). The central target of 85 ug/g FW is 543.9 uM, 8.15 times the cloned-channel EC50 and 5,439 times the IFI-M convention of 0.1 uM; the target range is 1,280 to 6,399 times the convention. Against the stated route the targets are 2.2 to 10.9 times the highest route value and 11.6 to 58.1 times the lowest. The ratios compare concentrations and are not receptor responses or index scores.

Table 5. Menthol levels and ratios. Menthol from the stated limonene route and at the design targets, as ug/g FW and as uM (1 g FW taken as 1 mL), with the ratio to the reported 66.7 uM cloned-channel EC50 and to the 0.1 uM IFI-M convention. The last two columns divide each target by the highest and the lowest route value. The ratios compare concentrations and are not IFI scores. Data: `data/tables/T05.csv`*; script:* `code/02_pool_model.py`*.*

Level	Menthol (ug/g FW)	Menthol (uM)	Ratio to 66.7 uM	Ratio to 0.1 uM	Times the highest route value	Times the lowest route value
Stated limonene route, lowest	1.72	11.0	0.17	110		
Stated limonene route, highest	9.18	58.7	0.88	587		
Design target, lowest	20	128.0	1.92	1280	2.2	11.6
Design target, central	85	543.9	8.15	5439	9.3	49.4
Design target, highest	100	639.9	9.59	6399	10.9	58.1

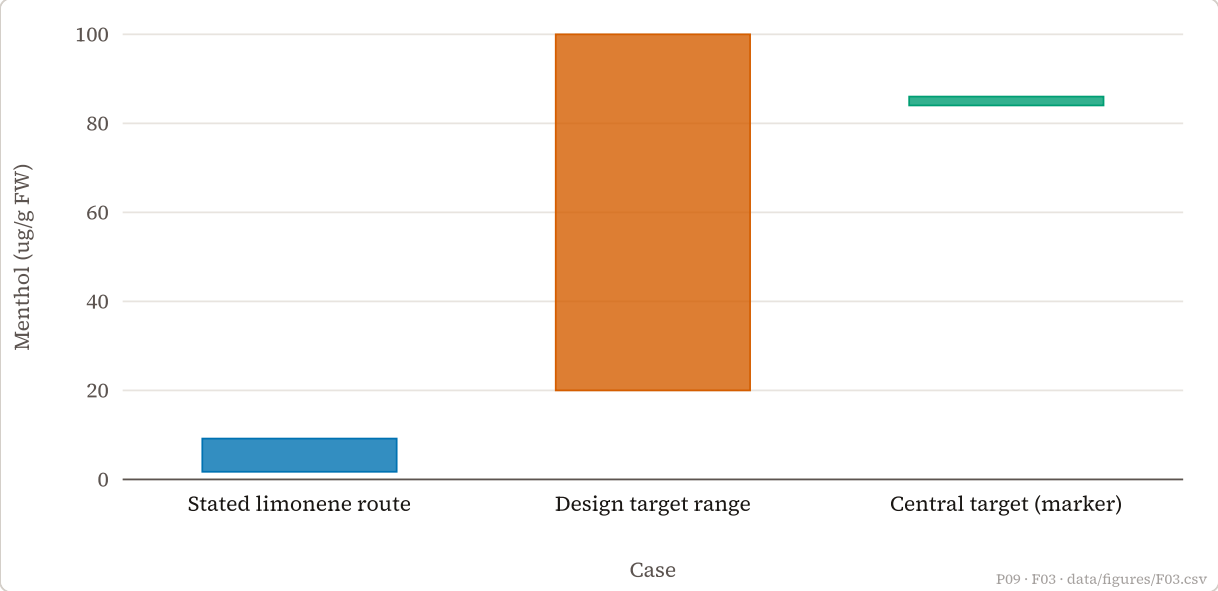


Figure 3. Menthol from the stated route and at the design targets. Bars span the lowest to the highest value: the stated limonene route (design assumptions with no source), the design target range of 20 to 100 ug/g FW, and a narrow marker at the central target of 85 ug/g FW. Closed-form arithmetic of limonene x conversion x molar-mass ratio. Data: `data/figures/F03.csv`; script: `code/02_pool_model.py`.

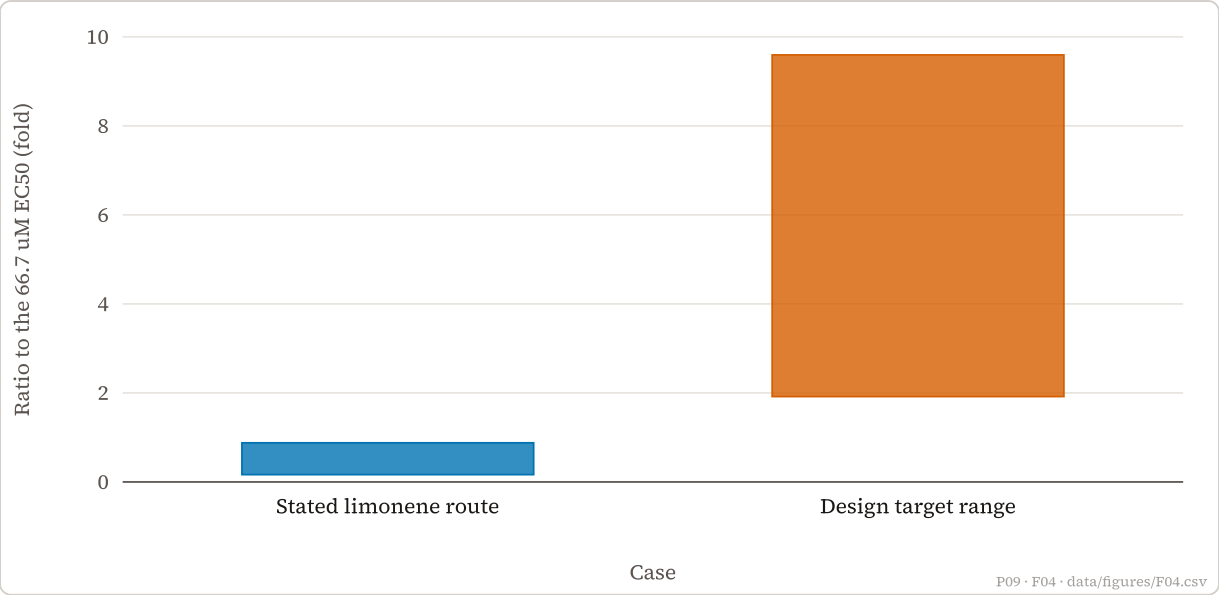


Figure 4. Menthol concentration against the reported cloned-channel EC50. Bars span the lowest to the highest concentration of the stated route and of the design target range, each divided by the reported EC50 of 66.7 uM at the cloned TRPM8 channel. Concentration = ug/g FW x 1000 / 156.27, taking 1 g FW as 1 mL. Data: `data/figures/F04.csv`; script: `code/02_pool_model.py`.

3.6 Time for the stated flux to supply the targets

Table 7 and Figure 5 give the hours. At the 0.90 umol/gDW/h flux the central target is reached in 6.32 h (5.04 to 7.37 h over the dry-weight range), and at the 0.18 umol/gDW/h flux in 31.58 h (25.18 to 36.85 h over the dry-weight range). Over seven days the 0.90 umol/gDW/h flux can supply 2,261 ug/g FW with no loss, so the flux supplies the target in hours while the stated limonene route gives 1.72 to 9.18 ug/g FW. The central target is 9.3 times the highest value of the stated route. Table S5 and Table S6 give every combination.

Table 7. Hours to reach the menthol levels. Hours of flux needed to reach 20, 85 and 100 ug/g FW at the three stated GPP fluxes with no loss. The first number uses the mean dry-weight fraction; the range in brackets runs from the highest to the lowest dry-weight fraction. Data: `data/tables/T07.csv`; script: `code/02_pool_model.py`.

GPP flux (umol/gDW/h)	Hours to 20 ug/g FW	Hours to 85 ug/g FW	Hours to 100 ug/g FW
0.18	7.43 (5.93 to 8.67)	31.58 (25.18 to 36.85)	37.15 (29.63 to 43.35)
0.20	6.69 (5.33 to 7.80)	28.42 (22.66 to 33.17)	33.43 (26.66 to 39.02)
0.90	1.49 (1.19 to 1.73)	6.32 (5.04 to 7.37)	7.43 (5.93 to 8.67)

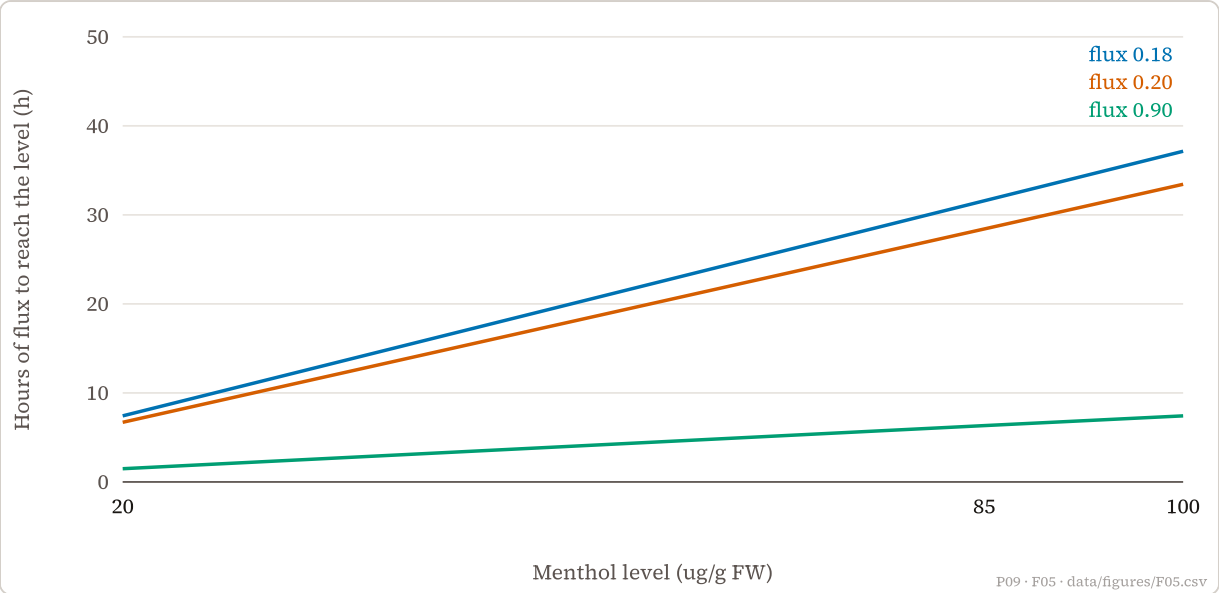


Figure 5. Time for the stated flux to supply a menthol level. Hours needed to reach 20, 85 and 100 ug/g FW at the three stated GPP fluxes (umol/gDW/h), at the mean dry-weight fraction and with no loss. Hours = level / (flux x dry-weight fraction x molar mass). Data: `data/figures/F05.csv`; script: `code/02_pool_model.py`.

3.7 Sensitivity

For the stated route, the conversion moves the result from 2.58 to 6.88 ug/g FW and the limonene amount from 3.15 to 6.31 ug/g FW, around a centre of 4.73 ug/g FW (Figure 6). For the hours to the central target at the 0.20 umol/gDW/h flux, the menthol level moves the result from 6.69 to 33.43 h, the flux from 31.58 to 6.32 h and the dry-weight fraction from 33.17 to 22.66 h, around a centre of 28.42 h (Figure 7, Table S8).

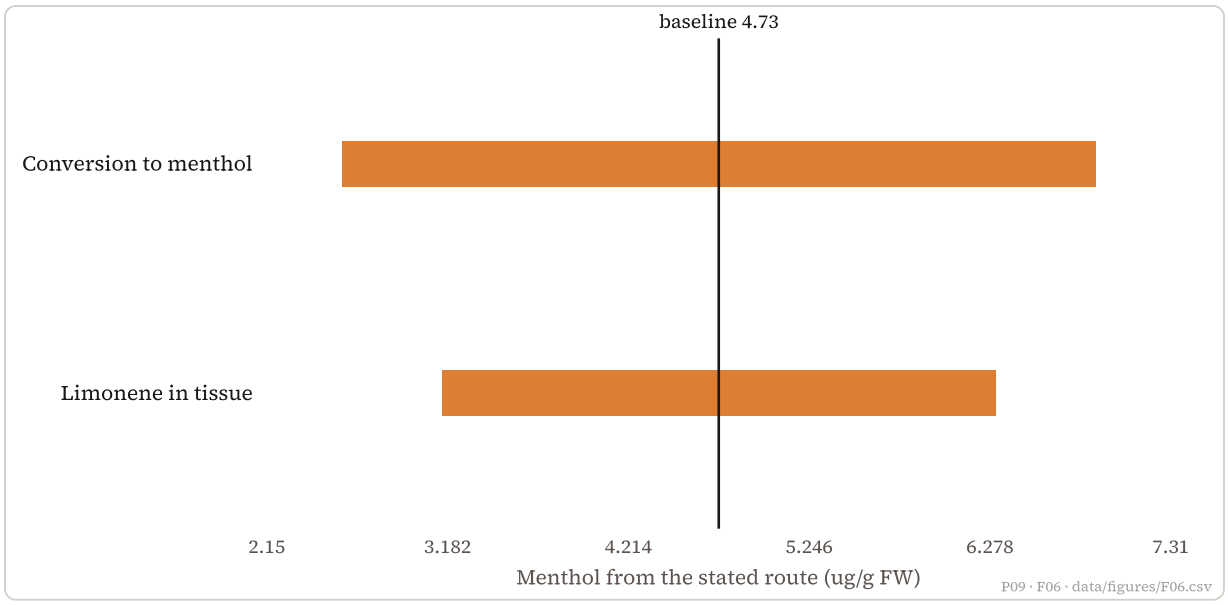


Figure 6. Sensitivity of the stated route to each input. Each bar spans the result when one input moves across its range (limonene 5 to 10 ug/g FW; conversion 30 to 80 percent) and the other stays at mid-range. One-at-a-time analysis of limonene x conversion x molar-mass ratio. Data: `data/figures/F06.csv`; script: `code/02_pool_model.py`.

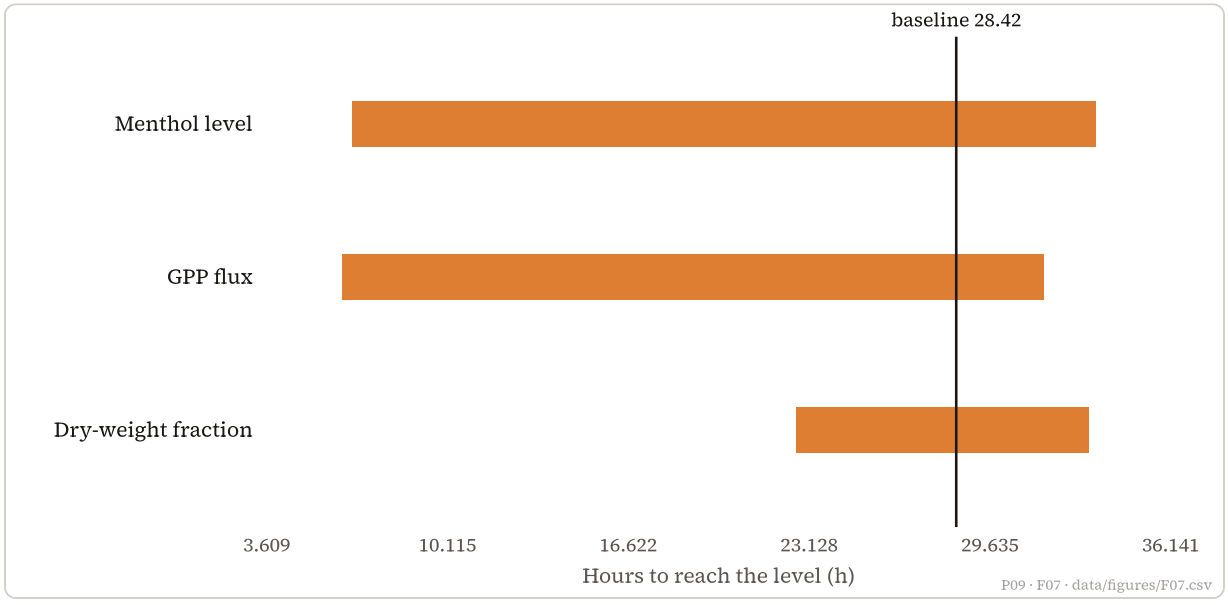


Figure 7. Sensitivity of the time to level to each input. Each bar spans the hours needed when one input moves across its range (flux 0.18 to 0.90 umol/gDW/h; dry-weight fraction 0.082 to 0.120; level 20 to 100 ug/g FW); the baseline is the 0.20 flux, the mean dry-weight fraction and the central 85 ug/g FW target. One-at-a-time analysis of the hours-to-level formula. Data: `data/figures/F07.csv`; script: `code/02_pool_model.py`.

3.8 Agreement of the model routes

The closed-form results and the solver route agree for 265 of 265 quantities; the largest relative difference outside the molar-mass rows is 2.11e-13. The independent

recomputation covers 62 quantities with a largest relative difference of 1.78e-16 (Table S9).

4. Limitations

The GPP budget is a single-node balance, and its pool, sinks, draws and fluxes are design assumptions with no source. The limonene amount and the conversion fractions are design assumptions with no source, the menthol target has no literature basis, and the tobacco reports cited concern emission and the first oxidation step. The six genes cover six of the eight enzymatic steps of the reported pathway. The insert holds four copies of one Ccs piece, whose activity in pepper fruit is reported from transient assays. The ratios compare concentrations with reported EC50 values and are not receptor responses or perceived intensity.

5. Data and code availability

Everything behind this paper is in the data package: the claims table, the data for every table and figure, the scripts that regenerate them, the inputs, the research record and a backup. `bash run_all.sh` reruns every step and compares each regenerated file with the stored file; `bash verify.sh` checks the checksums. The cassette and repair-template sequences are FASTA files in `data/results/`. The paper and data are licensed under CC BY 4.0 and the code under the MIT licence. The public input records are listed in `inputs/input_sources.csv`.

Public records of this paper: Zenodo 10.5281/zenodo.24511340; Figshare: figshare/35015821; OSF: osf.io/mrw7a; Hugging Face: capsaicinoid-data; GitHub: paper-09; GenBank: QB274225–QB274229; Website: cool-burn-menthol.

Intellectual Property. The engineered constructs, cultivar designs, and methodologies described in this paper are the subject of pending United States patent applications (USPTO Provisional Application Nos. 64/172,827; 64/188,197; 64/192,062; and 64/199,105).

Experimental status. All results reported in this paper are computational. Experimental work on these designs is under way at the authors' laboratory facilities in Montecito, California, and Geneva, Switzerland. Those data are not part of this paper, are shared only with commercial partners under agreement, and are planned for publication next year.

Active experiments for this design include a two-gene proof of concept with limonene synthase and limonene-6-hydroxylase, TRPM8 cell assays with menthol, isopiperitenol and pathway intermediates, sensory panel screening, training

and taster-status genotyping, cool-burn and layered pungency with menthol and cinnamaldehyde alongside capsaicin, and E8 promoter activity in pepper fruit (reporter assay, transient then stable).

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Supplementary data

The supplementary tables hold the full data behind the paper. Table S1 lists every part of the repair template with its source record and coordinates. Table S2 holds the sequence checks of the insert and of each gene, Table S3 the Ccs promoter piece and its genome copy, and Table S4 site S1 with its arms, guides and neighbouring genes. Table S5 gives the hours to level at every combination of flux, dry-weight fraction and level, Table S6 the titre the stated flux can supply over time, and Table S7 the GPP carbon against the sucrose carbon import. Table S8 gives the sensitivity ranges, Table S9 the agreement of the model routes and Table S10 the reported values and findings the design refers to. Every table is stored as a CSV file in `data/tables/`, and the scripts that write them are in `code/`. The file map below lists every data, input, code and research-record file.

Table S1. All parts of the repair template. Every part of every cassette with its source record, source positions, template coordinates and length, and the two homology arms. Data: `data/tables/S01.csv`; script: `code/01_cassettes.py`.

Cassette	Part	Source record	Source positions	Template start	Template end	Length (bp)
MpLS	35S promoter piece	AF485783	4,974 to 5,808	501	1,335	835
MpLS	MpLS coding sequence (B0F4G4)	EU108697.1	1 to 1800	1,336	3,135	1,800
MpLS	NOS terminator	AF485783	4,022 to 4,277	3,136	3,391	256
MpCYP71D15	35S promoter piece	AF485783	4,974 to 5,808	3,392	4,226	835
MpCYP71D15	MpCYP71D15 coding sequence (Q9XHE6)	AF124817.1	1 to 1497	4,227	5,723	1,497
MpCYP71D15	NOS terminator	AF485783	4,022 to 4,277	5,724	5,979	256
MpIPPO	Ccs promoter piece	Y14165.1	1,085 to 2,004	5,980	6,899	920

Cassette	Part	Source record	Source positions	Template start	Template end	Length (bp)
MpIPPO	MpIPPO coding sequence (Q5C9I9)	AY641428.1	1 to 798	6,900	7,697	798
MpIPPO	NOS terminator	AF485783	4,022 to 4,277	7,698	7,953	256
MpIPR	Ccs promoter piece	Y14165.1	1,085 to 2,004	7,954	8,873	920
MpIPR	MpIPR coding sequence (Q6WAU1)	AY300162.1	1 to 945	8,874	9,818	945
MpIPR	NOS terminator	AF485783	4,022 to 4,277	9,819	10,074	256
MpPIPR	Ccs promoter piece	Y14165.1	1,085 to 2,004	10,075	10,994	920
MpPIPR	MpPIPR coding sequence (Q6WAU0)	AY300163.1	1 to 1029	10,995	12,023	1,029
MpPIPR	NOS terminator	AF485783	4,022 to 4,277	12,024	12,279	256
MpMR	Ccs promoter piece	Y14165.1	1,085 to 2,004	12,280	13,199	920
MpMR	MpMR coding sequence (Q5CAF4)	AY288138.1	60 to 995	13,200	14,135	936
MpMR	NOS terminator	AF485783	4,022 to 4,277	14,136	14,391	256
Left arm	Site S1 left homology arm	NC_061112.1	131,430,500 to 131,430,999	1	500	500
Right arm	Site S1 right homology arm	NC_061112.1	131,431,101 to 131,431,600	14,392	14,891	500

Table S2. Sequence checks. Insert and repair-template length, promoter and terminator pieces, orientation of the 35S piece, start codons, repeat core hits, exact copies of each piece, restriction sites and, for each gene, reading frame, stop codons, identity of the translation with UniProt and G plus C content. Data: `data/tables/S02.csv`*; script:* `code/01_cassettes.py`*.*

Scope	Check	Value	Basis
insert	insert length bp	13891	sum of the six cassettes
insert	repair template length bp	14891	left arm plus insert plus right arm
insert	number of cassettes	6	one per gene
insert	GC percent insert	43.2	G plus C
promoters	35S piece length bp	835	AF485783 positions 4,974 to 5,808

Scope	Check	Value	Basis
promoters	Ccs piece length bp	920	Y14165.1 positions 1,085 to 2,004
promoters	NOS terminator length bp	256	AF485783 positions 4,022 to 4,277
promoters	35S TATA region on plus strand	yes	29-base core CTATATAAGGAAGTTCATTTCATTTGGAG found on the plus strand of the 35S piece
promoters	35S TATA region on minus strand	no	the same core searched on the opposite strand
promoters	ATG in Ccs piece plus strand	0	start codons inside the Ccs piece, plus strand
promoters	ATG in 35S piece plus strand	12	start codons inside the 35S piece, plus strand
promoters	Ccs repeat core hits	7	matches of a 28-base core of the 176-bp repeat with at most 3 mismatches
promoters	Ccs repeat core positions	101 183 270 358 446 534 622	start positions in the Ccs piece
copies	exact copies of Ccs piece in insert	4	exact plus-strand copies of the 920-bp piece
copies	exact copies of 35S piece in insert	2	exact plus-strand copies of the 835-bp piece
copies	exact copies of NOS in insert	6	exact plus-strand copies of the 256-bp terminator
sites	BsaI sites in insert	3	recognition sites GGTCTC on both strands
sites	BsmBI sites in insert	4	recognition sites CGTCTC on both strands

Scope	Check	Value	Basis
sites	BpiI sites in insert	8	recognition sites GAAGAC on both strands
MpLS	coding sequence length bp	1800	3 x 599 + 3
MpLS	start codon	ATG	first codon
MpLS	stop codon	TGA	last codon
MpLS	internal stop codons	0	stop codons before the last codon
MpLS	translation identical to UniProt positions	599 of 599	translation of the coding sequence against the UniProt sequence
MpLS	GC percent coding sequence	45.3	G plus C
MpLS	GC percent cassette	43.9	G plus C of the cassette
MpCYP71D15	coding sequence length bp	1497	3 x 498 + 3
MpCYP71D15	start codon	ATG	first codon
MpCYP71D15	stop codon	TGA	last codon
MpCYP71D15	internal stop codons	0	stop codons before the last codon
MpCYP71D15	translation identical to UniProt positions	498 of 498	translation of the coding sequence against the UniProt sequence
MpCYP71D15	GC percent coding sequence	58.2	G plus C

Scope	Check	Value	Basis
MpCYP71D15	GC percent cassette	51.2	G plus C of the cassette
MpIPPO	coding sequence length bp	798	3 x 265 + 3
MpIPPO	start codon	ATG	first codon
MpIPPO	stop codon	TGA	last codon
MpIPPO	internal stop codons	0	stop codons before the last codon
MpIPPO	translation identical to UniProt positions	265 of 265	translation of the coding sequence against the UniProt sequence
MpIPPO	GC percent coding sequence	67.5	G plus C
MpIPPO	GC percent cassette	47.0	G plus C of the cassette
MpIPR	coding sequence length bp	945	3 x 314 + 3
MpIPR	start codon	ATG	first codon
MpIPR	stop codon	TAA	last codon
MpIPR	internal stop codons	0	stop codons before the last codon
MpIPR	translation identical to UniProt positions	314 of 314	translation of the coding sequence against the UniProt sequence
MpIPR	GC percent coding sequence	46.8	G plus C

Scope	Check	Value	Basis
MpIPR	GC percent cassette	39.2	G plus C of the cassette
MpPIPR	coding sequence length bp	1029	3 x 342 + 3
MpPIPR	start codon	ATG	first codon
MpPIPR	stop codon	TAA	last codon
MpPIPR	internal stop codons	0	stop codons before the last codon
MpPIPR	translation identical to UniProt positions	342 of 342	translation of the coding sequence against the UniProt sequence
MpPIPR	GC percent coding sequence	44.6	G plus C
MpPIPR	GC percent cassette	38.5	G plus C of the cassette
MpMR	coding sequence length bp	936	3 x 311 + 3
MpMR	start codon	ATG	first codon
MpMR	stop codon	TAA	last codon
MpMR	internal stop codons	0	stop codons before the last codon
MpMR	translation identical to UniProt positions	311 of 311	translation of the coding sequence against the UniProt sequence

Scope	Check	Value	Basis
MpMR	GC percent coding sequence	44.3	G plus C
MpMR	GC percent cassette	38.1	G plus C of the cassette

Table S3. The Ccs promoter piece and its genome copy. Position of the piece in the GenBank record, its genome copy in UCD10Xv1.1 and the alignment of the two. Data: `data/tables/S03.csv`*; script:* `code/01_cassettes.py`*.*

Item	Value	Source
Ccs gene	LOC107875664	RefSeq annotation of UCD10Xv1.1
Ccs gene position	NC_061116.1:219128341-219129837 (minus strand)	RefSeq annotation
Y14165.1 promoter piece	positions 1085 to 2004 (920 bp)	inputs/sequences
Genome copy of the piece	NC_061116.1:219129838-219130749 (912 bp)	research_record/outputs/ccs_piece_output.txt
Identity of the genome copy to the piece	96.4%	semi-global alignment, research_record/outputs/ccs_piece_output.txt
Leader bases identical in record and genome	66	bases before the start codon, research_record/outputs/ccs_piece_output.txt

Table S4. Site S1, arms and guides. Coordinates of the knock-in site, the two homology arms, the two guides with their genome-wide site counts and MIT scores, and the neighbouring genes. Data: `data/tables/S04.csv`; *script:* `code/01_cassettes.py`.

Item	Value	Detail
Site window	NC_061112.1:131429594-131432593	3,000 bp
Insertion point	131431093	
Left arm	NC_061112.1:131430500-131430999	500 bp
Right arm	NC_061112.1:131431101-131431600	500 bp
Bases replaced between the two cuts	101	bp
Guide S1-g1	GTTGGAGCGAGTACTATGCA (- strand, PAM AGG)	cut after 131430999; perfect sites 1; sites with 2, 3 and 4 mismatches 9, 25 and 186; MIT score 64.0
Guide S1-g2	ACCTAATGGGTTGCAAGGAA (- strand, PAM TGG)	cut after 131431100; perfect sites 1; sites with 2, 3 and 4 mismatches 4, 40 and 182; MIT score 70.3
Neighbouring gene, left	LOC107859470	
Neighbouring gene, right	LOC107858201	

Table S5. Hours to level at every combination. Hours of flux needed to reach each level at every combination of flux and dry-weight fraction, for menthol and for isopiperitenol, with no loss. Data: `data/tables/S05.csv`*; script:* `code/02_pool_model.py`*.*

Product	Flux (umol/gDW/h)	Dry-weight fraction	Level (ug/g FW)	Hours to level
menthol	0.18	low	20	8.67
menthol	0.18	low	85	36.85
menthol	0.18	low	100	43.35
menthol	0.18	mid	20	7.43
menthol	0.18	mid	85	31.58
menthol	0.18	mid	100	37.15
menthol	0.18	high	20	5.93
menthol	0.18	high	85	25.18
menthol	0.18	high	100	29.63
menthol	0.20	low	20	7.80
menthol	0.20	low	85	33.17
menthol	0.20	low	100	39.02
menthol	0.20	mid	20	6.69
menthol	0.20	mid	85	28.42
menthol	0.20	mid	100	33.43
menthol	0.20	high	20	5.33
menthol	0.20	high	85	22.66
menthol	0.20	high	100	26.66
menthol	0.90	low	20	1.73
menthol	0.90	low	85	7.37
menthol	0.90	low	100	8.67

Product	Flux (umol/gDW/h)	Dry-weight fraction	Level (ug/g FW)	Hours to level
menthol	0.90	mid	20	1.49
menthol	0.90	mid	85	6.32
menthol	0.90	mid	100	7.43
menthol	0.90	high	20	1.19
menthol	0.90	high	85	5.04
menthol	0.90	high	100	5.93
isopiperitenol	0.18	low	4	1.78
isopiperitenol	0.18	low	8	3.56
isopiperitenol	0.18	mid	4	1.53
isopiperitenol	0.18	mid	8	3.05
isopiperitenol	0.18	high	4	1.22
isopiperitenol	0.18	high	8	2.43
isopiperitenol	0.20	low	4	1.60
isopiperitenol	0.20	low	8	3.20
isopiperitenol	0.20	mid	4	1.37
isopiperitenol	0.20	mid	8	2.75
isopiperitenol	0.20	high	4	1.09
isopiperitenol	0.20	high	8	2.19
isopiperitenol	0.90	low	4	0.36
isopiperitenol	0.90	low	8	0.71
isopiperitenol	0.90	mid	4	0.31
isopiperitenol	0.90	mid	8	0.61

Product	Flux (umol/gDW/h)	Dry-weight fraction	Level (ug/g FW)	Hours to level
isopiperitenol	0.90	high	4	0.24
isopiperitenol	0.90	high	8	0.49

Table S6. Titre supplied by the flux. Maximum menthol titre the stated flux can supply over 1 to 50 days at every combination of flux and dry-weight fraction, with no loss. Data: `data/tables/S06.csv`*; script:* `code/02_pool_model.py`*.*

Product	Flux (umol/gDW/h)	Dry-weight fraction	Days	Titre supplied by the flux, no loss (ug/g FW)
menthol	0.18	low	1	55
menthol	0.18	low	3	166
menthol	0.18	low	7	387
menthol	0.18	low	14	775
menthol	0.18	low	30	1661
menthol	0.18	low	50	2768
menthol	0.18	mid	1	65
menthol	0.18	mid	3	194
menthol	0.18	mid	7	452
menthol	0.18	mid	14	904
menthol	0.18	mid	30	1938
menthol	0.18	mid	50	3230
menthol	0.18	high	1	81
menthol	0.18	high	3	243
menthol	0.18	high	7	567
menthol	0.18	high	14	1134
menthol	0.18	high	30	2430

Product	Flux (umol/gDW/h)	Dry-weight fraction	Days	Titre supplied by the flux, no loss (ug/g FW)
menthol	0.18	high	50	4051
menthol	0.20	low	1	62
menthol	0.20	low	3	185
menthol	0.20	low	7	431
menthol	0.20	low	14	861
menthol	0.20	low	30	1845
menthol	0.20	low	50	3075
menthol	0.20	mid	1	72
menthol	0.20	mid	3	215
menthol	0.20	mid	7	502
menthol	0.20	mid	14	1005
menthol	0.20	mid	30	2154
menthol	0.20	mid	50	3589
menthol	0.20	high	1	90
menthol	0.20	high	3	270
menthol	0.20	high	7	630
menthol	0.20	high	14	1260
menthol	0.20	high	30	2700
menthol	0.20	high	50	4501
menthol	0.90	low	1	277
menthol	0.90	low	3	830
menthol	0.90	low	7	1937

Product	Flux (umol/gDW/h)	Dry-weight fraction	Days	Titre supplied by the flux, no loss (ug/g FW)
menthol	0.90	low	14	3875
menthol	0.90	low	30	8304
menthol	0.90	low	50	13839
menthol	0.90	mid	1	323
menthol	0.90	mid	3	969
menthol	0.90	mid	7	2261
menthol	0.90	mid	14	4522
menthol	0.90	mid	30	9691
menthol	0.90	mid	50	16151
menthol	0.90	high	1	405
menthol	0.90	high	3	1215
menthol	0.90	high	7	2835
menthol	0.90	high	14	5671
menthol	0.90	high	30	12152
menthol	0.90	high	50	20253

Table S7. GPP carbon against sucrose carbon. GPP carbon drawn by the menthol branch as a percent of the sucrose carbon imported, for each flux, dry-weight fraction and sucrose import. Data: `data/tables/S07.csv`*; script:* `code/02_pool_model.py`*.*

GPP flux (umol/gDW/h)	Dry-weight fraction	Sucrose import	GPP carbon draw (% of sucrose carbon import)
0.18	low	low	0.08
0.18	low	high	0.06
0.18	mid	low	0.10

GPP flux (umol/gDW/h)	Dry-weight fraction	Sucrose import	GPP carbon draw (% of sucrose carbon import)
0.18	mid	high	0.07
0.18	high	low	0.12
0.18	high	high	0.09
0.20	low	low	0.09
0.20	low	high	0.07
0.20	mid	low	0.11
0.20	mid	high	0.08
0.20	high	low	0.13
0.20	high	high	0.10
0.90	low	low	0.41
0.90	low	high	0.31
0.90	mid	low	0.48
0.90	mid	high	0.36
0.90	high	low	0.60
0.90	high	high	0.45

Table S8. Sensitivity. One input moves across its range while the others stay at mid-range: menthol from the stated route, and hours to reach the central target at the 0.20 flux. Data: `data/tables/S08.csv` *; script:* `code/02_pool_model.py` *.*

Model	Input	Range	Result at low value	Result at high value	Result at mid-range
Menthol from the stated route (ug/g FW)	Conversion to menthol	30 to 80 percent	2.58	6.88	4.73
Menthol from the stated route (ug/g FW)	Limonene in tissue	5 to 10 ug/g FW	3.15	6.31	4.73
Hours to reach a menthol level at the 0.20 flux (h)	Menthol level	20 to 100 ug/g FW	6.69	33.43	28.42
Hours to reach a menthol level at the 0.20 flux (h)	GPP flux	0.18 to 0.90 umol/gDW/h	31.58	6.32	28.42
Hours to reach a menthol level at the 0.20 flux (h)	Dry-weight fraction	0.082 to 0.120	33.17	22.66	28.42

Table S9. Agreement of the model routes. Counts and largest relative differences of the closed-form results against the solver route, an independent recomputation and the closed-form script of the research record. Data: `data/tables/S09.csv` *; script:* `code/03_supplementary.py` *.*

Measure	Value
quantities compared, closed form against solver route	265
quantities that agree within 1 percent	265
largest relative difference without the molar-mass rows	2.11e-13
largest relative difference, molar mass from atomic masses against structure	1.82e-16
quantities compared, independent recomputation	62
largest relative difference, independent recomputation	1.78e-16

Measure	Value
quantities compared, closed-form script of the research record	265
quantities that agree within 1 percent, closed-form script of the research record	265

Table S10. Reported values and findings used. Literature values and findings that the design refers to, with their sources. Data: `data/tables/S10.csv`*; script:* `code/03_supplementary.py`*.*

Reported item	Reported value or finding	Source
Menthol EC50 at the cloned TRPM8 channel	66.7 uM (s.e.m. 3.3)	McKemy et al. (2002), Nature 416:52-58
Menthol EC50 in trigeminal neurons	80 uM (s.e.m. 2.4)	McKemy et al. (2002), Nature 416:52-58
Ccs promoter pieces carrying at least one 176-bp repeat, fused to GUS and injected into mature green pepper fruit	staining reported for every such piece	Tian et al. (2017), Physiol Mol Biol Plants 23:685-691
Ccs promoter product in red C. annuum lines	920 bp with three 176-bp repeats, matching Y14165	Li et al. (2013), PLoS ONE 8:e61996
Limonene in tobacco expressing a lemon limonene synthase	emission measured as headspace rate	Lücker et al. (2004), Plant Physiol 134:510-519
Limonene to isopiperitenol in tobacco with a Mentha limonene-3-hydroxylase	first oxidation step reported; emission measured	Lücker et al. (2004), Plant J 39:135-145
Geraniol in tomato fruit under a fruit promoter	transgenic monoterpene accumulation in fruit reported	Davidovich-Rikanati et al. (2007), Nat Biotechnol 25:899-901
Pathway of (-)-menthol from GPP	eight enzymatic steps listed	Croteau et al. (2005), Naturwissenschaften 92:562-577

Supplementary data: file map

Path	Contents
`data/tables/T01.csv`	Table 1: The six menthol-pathway genes
`data/tables/T02.csv`	Table 2: Proteins and coding sequences
`data/tables/T03.csv`	Table 3: Cassettes, insert and repair template
`data/tables/T04.csv`	Table 4: Inputs of the budget and titre model
`data/tables/T05.csv`	Table 5: Menthol levels and ratios
`data/tables/T06.csv`	Table 6: Plastid GPP budget
`data/tables/T07.csv`	Table 7: Hours to reach the menthol levels
`data/tables/S01.csv`	Table S1: All parts of the repair template
`data/tables/S02.csv`	Table S2: Sequence checks
`data/tables/S03.csv`	Table S3: The Ccs promoter piece and its genome copy
`data/tables/S04.csv`	Table S4: Site S1, arms and guides
`data/tables/S05.csv`	Table S5: Hours to level at every combination
`data/tables/S06.csv`	Table S6: Titre supplied by the flux
`data/tables/S07.csv`	Table S7: GPP carbon against sucrose carbon
`data/tables/S08.csv`	Table S8: Sensitivity
`data/tables/S09.csv`	Table S9: Agreement of the model routes
`data/tables/S10.csv`	Table S10: Reported values and findings used
`data/figures/GA.csv`	Data behind the graphical abstract
`data/figures/F01.csv`	Data behind Figure 1
`data/figures/F02.csv`	Data behind Figure 2
`data/figures/F03.csv`	Data behind Figure 3
`data/figures/F04.csv`	Data behind Figure 4
`data/figures/F05.csv`	Data behind Figure 5
`data/figures/F06.csv`	Data behind Figure 6
`data/figures/F07.csv`	Data behind Figure 7
`data/figures/F08.csv`	Data behind Figure 8
`data/results/cassettes_P09.fasta`	The six cassettes (promoter piece, native coding sequence, NOS terminator) with record and positions in each header
`data/results/insert_P09.fasta`	The insert: six cassettes back to back in pathway order (13891 bp)
`data/results/repair_template_P09.fasta`	The repair template at site S1: left arm, insert, right arm (14891 bp)
`data/results/cassette_parts.csv`	Every part of every cassette with its source record, positions and coordinates in the cassette, the insert and the repair template
`data/results/cassette_totals.csv`	Length of each cassette and its start and end in the insert and in the repair template

Path	Contents
`data/results/protein_table.csv`	Length and mass of each protein, coding-sequence record and identity of its translation with the UniProt sequence
`data/results/sequence_checks.csv`	Reading frame, start and stop codons, identity with UniProt, G plus C, promoter and terminator checks, repeated pieces and restriction sites
`data/results/promoter_record.csv`	The Ccs promoter piece, its genome copy and its alignment
`data/results/site_S1_table.csv`	Site S1: window, arms, guides and neighbouring genes
`data/results/model_results.csv`	Every quantity of the GPP budget and titre model (closed form): id, quantity, value, units
`data/results/model_headline.csv`	Headline quantities of the model
`data/results/gpp_budget.csv`	GPP sink fluxes of the wild type and the two designs, the menthol draw and the change of each endogenous sink
`data/results/sensitivity_route.csv`	Menthol from the stated route when one input moves over its range
`data/results/sensitivity_hours.csv`	Hours to reach the central target when one input moves over its range
`data/results/route_agreement.csv`	Closed form against the solver route for every quantity, with relative differences
`data/results/route_agreement_summary.csv`	Counts and largest differences of the agreement checks
`data/figures/*`	Data behind the figures (one CSV per figure or panel)
`data/results/single_sink_worst_case.csv`	Largest reduction of one endogenous sink if the whole menthol-branch draw came from that sink alone, at each stated draw
`data/results/menthol_levels.csv`	Menthol from the stated route and at the design targets, as ug/g FW, uM and ratios (cloned-channel EC50, trigeminal EC50, IFI-M convention)
`data/wet_lab_active.csv`	Experiments active in the authors' laboratories for this paper: register identifier (WL-01 to WL-84), experiment name and stage code (S0 paperwork, S1 calibration and cheap decisive tests, S2 transient and in vitro mechanism tests, S3 stable lines, S4 tests on engineered material, S5 optional and long-lead)
`inputs/model_inputs.csv`	Every input of the GPP budget and titre model with unit and kind (design assumption, design target, literature value or definition)
`inputs/ifi_reference_values.csv`	Reference EC50 of the capsaicinoid axis (IFI-C, capsaicin at TRPV1) and the proposed cooling axis (IFI-M, menthol at TRPM8)
`inputs/pathway.csv`	The six genes in pathway order with enzyme, UniProt entry, reaction in the record, promoter and coding-sequence record
`inputs/menthol_proteins_records.csv`	UniProt name and review status of the six proteins
`inputs/input_sources.csv`	Public records used: genome, GenBank records, pBI121 parts and UniProt entries
`inputs/ccs_gene.csv`	Position of the pepper Ccs gene in UCD10Xv1.1
`inputs/site_S1_features.csv`	Site S1 window, insertion point, arm coordinates and neighbouring genes
`inputs/site_S1_guides.csv`	The two guides at site S1 with positions, cuts, genome-wide site counts and MIT scores (stored scan output)

Path	Contents
`inputs/sequences/Ccs_promoter_920_Y14165.1_1085-2004.fasta`	Ccs promoter piece, 920 bp
`inputs/sequences/CaMV35S_promoter_835_AF485783_4974-5808.fasta`	CaMV 35S promoter piece, 835 bp
`inputs/sequences/NOS_terminator_256.fasta`	NOS terminator, 256 bp
`inputs/sequences/S1_arm_left_500.fasta`	Left homology arm at site S1, 500 bp
`inputs/sequences/S1_arm_right_500.fasta`	Right homology arm at site S1, 500 bp
`inputs/sequences/cds_*.fasta`	Native coding sequences of the six genes (GenBank record and positions in the header)
`inputs/sequences/menthol_pathway_proteins.faa`	Amino-acid sequences of the six proteins (UniProt accession in the header)
`code/01_cassettes.py`	Cassettes, insert, repair template, sequence checks, promoter record and site S1 table
`code/02_pool_model.py`	GPP pool budget, titre and time to level, stated route, concentration ratios, sensitivity tables and figure data
`code/03_supplementary.py`	Agreement of the model routes, reported values and graphical-abstract data
`research_record/scripts/run_model.py`	Solver route of the GPP budget (COBRApy) and titre calculations; writes research_record/outputs/model_results.csv
`research_record/scripts/check_model.py`	Closed-form route and value-by-value agreement check; writes research_record/outputs/agreement.csv
`research_record/scripts/protein_check.py`	Length and mass of the six proteins (Biopython); writes research_record/outputs/protein_lengths_masses.csv
`research_record/scripts/fetch_cds.py`	Fetches GenBank records for each protein and keeps the coding sequence whose translation equals the UniProt sequence
`research_record/outputs/model_results.csv`	Raw output of the solver route
`research_record/outputs/agreement.csv`	Agreement of the two routes, value by value
`research_record/outputs/protein_lengths_masses.csv`	Raw output of the protein length and mass script
`research_record/outputs/fetch_cds_output.txt`	Raw output of the coding-sequence search
`research_record/outputs/cds_summary.json`	Coding sequence found for each UniProt entry
`research_record/outputs/arithmetic_python.txt`	Raw output of the research build of the cassette lengths
`research_record/outputs/arithmetic_bc.txt`	Raw output of the bc sums of the research build

Path	Contents
`research_record/outputs/ccs_piece_output.txt`	Raw output of the Ccs promoter piece cut and its alignment to the genome copy
`research_record/outputs/site_S1_context.txt`	Raw output of the site S1 context: arms, window and neighbouring genes
`research_record/outputs/site_S1_guide_pairs.txt`	Raw output of the guide scan and pair choice at site S1
`research_record/second_script_rerun/p09_recompute.py`	Independent recomputation of the headline model quantities by direct arithmetic
`research_record/second_script_rerun/p09_recompute_output.csv`	Output of the independent recomputation
`research_record/second_script_rerun/cassette_arithmetic_bc.sh`	Second route for the cassette lengths: sums evaluated with bc
`research_record/second_script_rerun/cassette_arithmetic_bc_output.txt`	Output of the bc sums

Cite this paper

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C10	Phoenix: In Silico Design of a Capsicum annuum Cultivar with a Pun1/AT3 Double Variant, Targeting 2,956,310 Scoville Heat Units	10.5281/zenodo.24511384

Appendix A. Evidence table

Claim ID	Claim	Value	Kind	Source file	Location
P09-C001	The Ccs promoter piece is 920 bp	920 bp	computed	`data/results/sequence_checks.csv`	check Ccs_piece_length_bp
P09-C002	The six cassettes together form an insert of 13,891 bp	13,891 bp	computed	`data/results/sequence_checks.csv`	check insert_length_bp
P09-C003	Site S1 is on chromosome 2 (NC_061112.1)	2	computed	`inputs/site_S1_features.csv`	row chromosome

Claim ID	Claim	Value	Kind	Source file	Location
P09-C004	The repair template is 14,891 bp	14,891 bp	computed	`data/results/sequence_checks.csv`	check repair_template_length_bp
P09-C005	The six-gene menthol-branch draw is 0.75 percent of the GPP pool	0.75%	computed	`data/results/model_headline.csv`	quantity six_gene_draw_percent_of_pool
P09-C006	The stated limonene route gives 1.72 to 9.18 ug/g FW of menthol	1.72 to 9.18 ug/g FW	computed	`data/results/model_headline.csv`	quantities menthol_stated_route_low and _high
P09-C007	The stated route gives 11.0 to 58.7 uM of menthol	11.0 to 58.7 uM	computed	`data/results/model_headline.csv`	quantities menthol_stated_route_uM_low and _high
P09-C008	The menthol design target is 20 to 100 ug/g FW	20 to 100 ug/g FW	design target	`inputs/model_inputs.csv`	rows menthol_level_low and menthol_level_high
P09-C009	The design target is 1.92 to 9.59 times the reported cloned-channel EC50	1.92 to 9.59 times	computed	`data/results/menthol_levels.csv`	column ratio_to_cloned_channel_EC50, rows Design target, lowest and highest
P09-C010	The reported menthol EC50 at the cloned TRPM8 channel is 66.7 uM	66.7 uM	literature value	`literature`	McKemy, Neuhausser and Julius 2002, Nature 416:52-58, doi 10.1038/nature719
P09-C011	The highest stated GPP-to-limonene flux reading is 0.90 umol/gDW/h	0.90 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row gpp_flux_reading_high
P09-C012	The central menthol design target is 85 ug/g FW	85 ug/g FW	design target	`inputs/model_inputs.csv`	row menthol_level_mid
P09-C013	At the 0.90 flux the central target is reached in 6.32 h (5.04 to 7.37 h over the dry-weight range)	6.32 h (5.04 to 7.37 h over the dry-weight range)	computed	`data/tables/T07.csv`	row 0.90, column Hours to 85 ug/g FW
P09-C014	The Phoenix (SS-0096) heat design target is 2,956,310 SHU	2,956,310 SHU	design target	`inputs/model_inputs.csv`	row phoenix_shu_design_target

Claim ID	Claim	Value	Kind	Source file	Location
P09-C015	At the 0.90 flux the central target is reached in 6.32 h at the mean dry-weight fraction	6.32 h	computed	`data/tables/T07.csv`	row 0.90, column Hours to 85 ug/g FW
P09-C016	TRPM8 is reported to open below about 28 C	28 °C	literature value	`literature`	McKemy, Neuhausser and Julius 2002, Nature 416:52-58, doi 10.1038/nature719
P09-C017	The menthol pathway from primary metabolism has eight enzymatic steps	eight enzymatic steps	literature value	`literature`	Croteau, Davis, Ringer and Wildung 2005, Naturwissenschaften 92:562-577, doi 10.1007/s00114-005-0055-0
P09-C018	The reference genome is Capsicum annuum UCD10Xv1.1 (RefSeq assembly GCF_002878395.1)	GCF_002878395.1	definition	`inputs/input_sources.csv`	row genome
P09-C019	The steps run on Python 3.9 or newer	3.9	definition	`code/LIBRARIES.txt`	line Quick mode
P09-C020	The solver route uses COBRApy 0.32.1	0.32.1	definition	`code/LIBRARIES.txt`	line Solver route
P09-C021	The protein masses in the research record use Biopython 1.88	1.88	definition	`code/LIBRARIES.txt`	line Protein masses
P09-C022	The design carries six genes: two with the CaMV 35S promoter and four with the Ccs promoter	six	definition	`inputs/pathway.csv`	rows 1 to 6, column promoter
P09-C023	The MpMR coding sequence is bases 60 to 995 of GenBank record AY288138.1	60 to 995	computed	`inputs/pathway.csv`	row MpMR, column record_positions
P09-C024	The CaMV 35S piece is 835 bp	835 bp	computed	`data/results/sequence_checks.csv`	check 35S_piece_length_bp
P09-C025	The 35S piece is bases 4,974 to 5,808 of the pBI121 record AF485783	4,974 to 5,808	computed	`data/results/cassette_parts.csv`	row MpLS, part 35S promoter piece
P09-C026	The Ccs piece is bases 1,085 to 2,004 of GenBank record Y14165.1	1,085 to 2,004	computed	`data/results/promoter_record.csv`	row Y14165.1 promoter piece

Claim ID	Claim	Value	Kind	Source file	Location
P09-C027	The Ccs gene LOC107875664 is on chromosome 6 (NC_061116.1)	6	computed	inputs/ccs_gene.csv	row chromosome
P09-C028	The genome copy of the Ccs piece is NC_061116.1 bases 219,129,838 to 219,130,749	219,129,838 to 219,130,749	computed	data/results/promoter_record.csv	row Genome copy of the piece
P09-C029	The genome copy is 912 bp	912 bp	computed	data/results/promoter_record.csv	row Genome copy of the piece
P09-C030	The genome copy aligns to the record piece at 96.4 percent identity	96.4%	computed	data/results/promoter_record.csv	row Identity of the genome copy to the piece
P09-C031	The last 66 bases before the Ccs start codon are identical in the record and the genome	66	computed	data/results/promoter_record.csv	row Leader bases identical in record and genome
P09-C032	Ccs promoter pieces with at least one 176-bp repeat gave GUS staining in injected mature green pepper fruit	one 176-bp repeat	literature value	literature	Tian, Li, Li, Shah and Gong 2017, Physiol Mol Biol Plants 23:685-691, doi 10.1007/s12298-017-0437-8
P09-C033	A 920-bp Ccs promoter product with three 176-bp repeats in red C. annuum lines matches Y14165	920-bp promoter product with three 176-bp repeats	literature value	literature	Li, Wang, Gui, Chang and Gong 2013, PLoS ONE 8:e61996, doi 10.1371/journal.pone.0061996
P09-C034	The NOS terminator is 256 bp	256 bp	computed	data/results/sequence_checks.csv	check NOS_terminator_length_bp
P09-C035	The NOS terminator is bases 4,022 to 4,277 of the pBI121 record	4,022 to 4,277	computed	data/results/cassette_parts.csv	row MpLS, part NOS terminator
P09-C036	Site S1 is the 3,000-bp window NC_061112.1:131,429,594 to 131,432,593	NC_061112.1:131,429,594 to 131,432,593	computed	data/results/site_S1_table.csv	row Site window

Claim ID	Claim	Value	Kind	Source file	Location
P09-C037	The insertion point is 131,431,093	131,431,093	computed	`data/results/site_S1_table.csv`	row Insertion point
P09-C038	Each homology arm is 500 bp	500 bp	computed	`data/results/site_S1_table.csv`	rows Left arm and Right arm
P09-C039	The left arm is NC_061112.1 bases 131,430,500 to 131,430,999	131,430,500 to 131,430,999	computed	`data/results/site_S1_table.csv`	row Left arm
P09-C040	The right arm is NC_061112.1 bases 131,431,101 to 131,431,600	131,431,101 to 131,431,600	computed	`data/results/site_S1_table.csv`	row Right arm
P09-C041	101 bases lie between the two cuts and are replaced by the insert	101 bases	computed	`data/results/site_S1_table.csv`	row Bases replaced between the two cuts
P09-C042	The nearest gene on the left, LOC107859470, is 38,000 bp from the insertion point	38,000 bp	computed	`inputs/site_S1_features.csv`	row neighbour_left
P09-C043	The nearest gene on the right, LOC107858201, is 8,292 bp from the insertion point	8,292 bp	computed	`inputs/site_S1_features.csv`	row neighbour_right
P09-C044	The plastid GPP pool is 120 umol/gDW/h	120 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row gpp_pool_total_wt
P09-C045	The wild-type sinks are 95.2, 22.5 and 2.3 umol/gDW/h	95.2, 22.5 and 2.3 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	rows wt_sink_diterpene_chlorophyll, wt_sink_endogenous_monoterpene, wt_sink_other
P09-C046	The two-gene menthol-branch draw is 0.2 umol/gDW/h	0.2 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row two_gene_menthol_branch_draw

Claim ID	Claim	Value	Kind	Source file	Location
P09-C047	The six-gene menthol-branch draw is 0.9 umol/gDW/h	0.9 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row six_gene_menthol_branch_draw
P09-C048	Limonene in tissue is taken as 5 to 10 ug/g FW	5 to 10 ug/g FW	design assumption	`inputs/model_inputs.csv`	rows limonene_yield_low and limonene_yield_high
P09-C049	The conversion of limonene to menthol is taken as 30 to 80 percent	30 to 80%	design assumption	`inputs/model_inputs.csv`	rows conversion_limonene_to_menthol_low and _high (0.30 to 0.80)
P09-C050	The conversion of limonene to isopiperitenol is taken as 60 to 80 percent	60 to 80%	design assumption	`inputs/model_inputs.csv`	rows conversion_limonene_to_ipol_low and _high (0.60 to 0.80)
P09-C051	The molar masses are 156.27 (menthol), 152.23 (isopiperitenol) and 136.24 (limonene) g/mol	156.27, 152.23 and 136.24 g/mol	definition	`inputs/model_inputs.csv`	rows mw_menthol, mw_isopiperitenol, mw_limonene
P09-C052	The reported menthol EC50 in trigeminal neurons is 80 uM	80 uM	literature value	`literature`	McKemy, Neuhausser and Julius 2002, Nature 416:52-58, doi 10.1038/nature719
P09-C053	The IFI-M reference EC50 of menthol at TRPM8 is the convention 0.1 uM	0.1 uM	definition	`inputs/ifi_reference_values.csv`	row IFI-M
P09-C054	The IFI-C reference EC50 of capsaicin at TRPV1 is 0.5 uM	0.5 uM	definition	`inputs/ifi_reference_values.csv`	row IFI-C
P09-C055	The stated GPP-to-limonene flux readings are 0.18, 0.20 and 0.90 umol/gDW/h	0.18, 0.20 and 0.90 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	rows gpp_flux_reading_low, _mid, _high
P09-C056	The dry-weight fraction of fresh weight is 0.082, 0.0957 and 0.120 (low, mean, high of three USDA records)	0.082, 0.0957 and 0.120	definition	`inputs/model_inputs.csv`	rows dw_fraction_low, dw_fraction_mid, dw_fraction_high
P09-C057	The six proteins are 265 to 599 residues long	265 to 599 residues	computed	`data/results/protein_table.csv`	column length_aa

Claim ID	Claim	Value	Kind	Source file	Location
P09-C058	The six proteins have masses of 27.2 to 69.7 kDa	27.2 to 69.7 kDa	computed	`data/results/protein_table.csv`	column mass_kDa
P09-C059	Each coding sequence translates to its UniProt sequence at every position (599 of 599, 498 of 498, 265 of 265, 314 of 314, 342 of 342 and 311 of 311)	599 of 599, 498 of 498, 265 of 265, 314 of 314, 342 of 342 and 311 of 311 positions	computed	`data/results/protein_table.csv`	columns length_aa and identical_positions_with_translation
P09-C060	No coding sequence has an internal stop codon (0 in each)	0	computed	`data/results/sequence_checks.csv`	checks internal_stop_codons
P09-C061	The six cassettes are 1,974 to 2,891 bp long	1,974 to 2,891 bp	computed	`data/results/cassette_totals.csv`	column cassette_bp
P09-C062	The G plus C content of the insert is 43.2 percent	43.2%	computed	`data/results/sequence_checks.csv`	check GC_percent_insert
P09-C063	The Ccs piece holds 0 start codons on the plus strand	0	computed	`data/results/sequence_checks.csv`	check ATG_in_Ccs_piece_plus_strand
P09-C064	The Ccs piece holds 7 matches of the 28-base repeat core (at most 3 mismatches)	7	computed	`data/results/sequence_checks.csv`	check Ccs_repeat_core_hits
P09-C065	The insert holds 4 exact copies of the Ccs piece	4	computed	`data/results/sequence_checks.csv`	check exact_copies_of_Ccs_piece_in_insert
P09-C066	The insert holds 2 exact copies of the 35S piece	2	computed	`data/results/sequence_checks.csv`	check exact_copies_of_35S_piece_in_insert

Claim ID	Claim	Value	Kind	Source file	Location
P09-C067	The insert holds 6 exact copies of the NOS terminator	6	computed	`data/results/sequence_checks.csv`	check exact_copies_of_NOS_in_insert
P09-C068	The insert holds 3 BsaI, 4 BsmBI and 8 BpiI recognition sites	3, 4 and 8	computed	`data/results/sequence_checks.csv`	checks BsaI_sites_in_insert, BsmBI_sites_in_insert, BpiI_sites_in_insert
P09-C069	The MIT scores of the two guides are 64.0 and 70.3	64.0 and 70.3	computed	`data/results/site_S1_table.csv`	rows Guide S1-g1 and S1-g2
P09-C070	Each guide has 1 perfect genomic site	1	computed	`data/results/site_S1_table.csv`	rows Guide S1-g1 and S1-g2, perfect sites
P09-C071	The two cuts lie 101 bp apart	101 bp	computed	`data/results/site_S1_table.csv`	row Bases replaced between the two cuts
P09-C072	The region of 2,001 bp around the insertion region holds no soft-masked base and no N	2,001-bp region	computed	`research_record/outputs/site_S1_context.txt`	line window +-1 kb
P09-C073	The G plus C content of that region is 46.9 percent	46.9%	computed	`research_record/outputs/site_S1_context.txt`	line window +-1 kb
P09-C074	The two-gene draw is 0.17 percent of the pool	0.17%	computed	`data/results/model_headline.csv`	quantity two_gene_draw_percent_of_pool
P09-C075	Each endogenous sink falls by 0.75 percent in the six-gene design	0.75%	computed	`data/results/gpp_budget.csv`	row Six-gene design, column change_of_each_endogenous_sink_percent

Claim ID	Claim	Value	Kind	Source file	Location
P09-C076	Each endogenous sink falls by 0.17 percent in the two-gene design	0.17%	computed	`data/results/gpp_budget.csv`	row Two-gene design, column change_of_each_endogenous_sink_percent
P09-C077	The six-gene sinks are 94.49, 22.33 and 2.28 umol/gDW/h	94.49, 22.33 and 2.28 umol/gDW/h	computed	`data/results/gpp_budget.csv`	row Six-gene design
P09-C078	The largest single-sink reductions at the 0.90 draw are 0.95, 4.00 and 39.13 percent	0.95%, 4.00% and 39.13%	computed	`data/results/single_sink_worst_case.csv`	rows draw 0.90
P09-C079	The GPP carbon drawn at the 0.90 flux is 0.31 to 0.60 percent of the sucrose carbon import	0.31% to 0.60%	computed	`data/tables/S07.csv`	rows flux 0.90
P09-C080	The stated-route concentrations are 0.17 to 0.88 times the cloned-channel EC50	0.17 to 0.88 times	computed	`data/results/menthol_levels.csv`	column ratio_to_cloned_channel_EC50, rows Stated limonene route
P09-C081	The stated route gives 3.35 to 8.94 ug/g FW of isopiperitenol	3.35 to 8.94 ug/g FW	computed	`data/results/model_headline.csv`	quantities isopiperitenol_stated_route_low and _high
P09-C082	The isopiperitenol route values are 22.0 to 58.7 uM	22.0 to 58.7 uM	computed	`data/results/model_headline.csv`	quantities isopiperitenol_stated_route_uM_low and _high
P09-C083	The design target of 20 to 100 ug/g FW is 128.0 to 639.9 uM	128.0 to 639.9 uM	computed	`data/results/menthol_levels.csv`	column menthol_uM, rows Design target, lowest and highest
P09-C084	The design target is 1.60 to 8.00 times the EC50 in trigeminal neurons	1.60 to 8.00 times	computed	`data/results/menthol_levels.csv`	column ratio_to_trigeminal_EC50
P09-C085	The central target of 85 ug/g FW is 543.9 uM	543.9 uM	computed	`data/results/menthol_levels.csv`	row Design target, central

Claim ID	Claim	Value	Kind	Source file	Location
P09-C086	The central target is 8.15 times the cloned-channel EC50	8.15 times	computed	`data/results/menthol_levels.csv`	row Design target, central, column ratio_to_cloned_channel_EC50
P09-C087	The central target is 5,439 times the IFI-M convention	5,439 times	computed	`data/results/menthol_levels.csv`	row Design target, central, column ratio_to_IFI_M_reference
P09-C088	The target range is 1,280 to 6,399 times the IFI-M convention	1,280 to 6,399 times	computed	`data/results/menthol_levels.csv`	column ratio_to_IFI_M_reference, rows Design target, lowest and highest
P09-C089	The targets are 2.2 to 10.9 times the highest route value	2.2 to 10.9 times	computed	`data/results/menthol_levels.csv`	column times_highest_route_value
P09-C090	The targets are 11.6 to 58.1 times the lowest route value	11.6 to 58.1 times	computed	`data/results/menthol_levels.csv`	column times_lowest_route_value
P09-C091	The lowest stated flux reading is 0.18 umol/gDW/h	0.18 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row gpp_flux_reading_low
P09-C092	At the 0.18 flux the central target is reached in 31.58 h (25.18 to 36.85 h over the dry-weight range)	31.58 h (25.18 to 36.85 h over the dry-weight range)	computed	`data/tables/T07.csv`	row 0.18, column Hours to 85 ug/g FW
P09-C093	Over 7 days the 0.90 flux can supply 2,261 ug/g FW of menthol with no loss at the mean dry-weight fraction	2,261 ug/g FW	computed	`data/tables/S06.csv`	row menthol, 0.90, mid, 7 days
P09-C094	The central target is 9.3 times the highest stated-route value	9.3 times	computed	`data/results/menthol_levels.csv`	row Design target, central, column times_highest_route_value
P09-C095	The conversion moves the stated-route menthol from 2.58 to 6.88 ug/g FW	2.58 to 6.88 ug/g FW	computed	`data/results/sensitivity_route.csv`	row Conversion to menthol

Claim ID	Claim	Value	Kind	Source file	Location
P09-C096	The limonene amount moves the stated-route menthol from 3.15 to 6.31 ug/g FW	3.15 to 6.31 ug/g FW	computed	`data/results/sensitivity_route.csv`	row Limonene in tissue
P09-C097	The centre of the stated-route sensitivity is 4.73 ug/g FW	4.73 ug/g FW	computed	`data/results/sensitivity_route.csv`	column baseline_result
P09-C098	The middle stated flux reading is 0.20 umol/gDW/h	0.20 umol/gDW/h	design assumption	`inputs/model_inputs.csv`	row gpp_flux_reading_mid
P09-C099	The menthol level moves the hours to the central target at the 0.20 flux from 6.69 to 33.43 h	6.69 to 33.43 h	computed	`data/results/sensitivity_hours.csv`	row Menthol level
P09-C100	The flux moves the hours from 31.58 to 6.32 h	31.58 to 6.32 h	computed	`data/results/sensitivity_hours.csv`	row GPP flux
P09-C101	The dry-weight fraction moves the hours from 33.17 to 22.66 h	33.17 to 22.66 h	computed	`data/results/sensitivity_hours.csv`	row Dry-weight fraction
P09-C102	The centre of the hours sensitivity is 28.42 h	28.42 h	computed	`data/results/sensitivity_hours.csv`	column baseline_result
P09-C103	The closed-form and solver-route results agree within 1 percent for 265 of 265 quantities	265 of 265	computed	`data/results/route_agreement_summary.csv`	rows quantities compared and agree

Claim ID	Claim	Value	Kind	Source file	Location
P09-C104	The largest relative difference outside the molar-mass rows is 2.11e-13	2.11e-13	computed	`data/results/route_agreement_summary.csv`	row largest relative difference without the molar-mass rows
P09-C105	The independent recomputation covers 62 quantities	62	computed	`data/results/route_agreement_summary.csv`	row quantities compared, independent recomputation
P09-C106	The largest relative difference of the independent recomputation is 1.78e-16	1.78e-16	computed	`data/results/route_agreement_summary.csv`	row largest relative difference, independent recomputation

Appendix B. Glossary, abbreviations and units

Term	Meaning	Type
TRPV1	Transient receptor potential vanilloid 1: the ion channel that capsaicin activates	abbreviation
TRPM8	Transient receptor potential melastatin 8: the cold and menthol receptor channel	abbreviation
GPP	Geranyl diphosphate: the ten-carbon precursor of monoterpenes, made in the plastid	abbreviation
MEP pathway	The plastid route that makes the five-carbon building blocks of terpenoids	term
EC50	Concentration that gives half of the maximal response in an assay	abbreviation
IFI	Iyengar Flavor Index: scores one compound against a reference assay at one receptor	abbreviation
IFI-C	The capsaicinoid pungency axis of the IFI (capsaicin at TRPV1)	abbreviation
IFI-M	The proposed cooling axis of the IFI (menthol at TRPM8); its reference EC50 is a convention	abbreviation

Term	Meaning	Type
SHU	Scoville heat unit: the unit of pungency	abbreviation
CaMV 35S	Cauliflower mosaic virus 35S promoter: a constitutive plant promoter	abbreviation
Ccs	Capsanthin/capsorubin synthase: a pepper fruit gene whose promoter drives four of the six cassettes	abbreviation
NOS	Nopaline synthase terminator: the transcription terminator of every cassette	abbreviation
MpLS	Mentha x piperita limonene synthase: makes (-)-limonene from GPP	abbreviation
MpCYP71D15	Mentha x piperita cytochrome P450 71D15 (limonene-3-hydroxylase): makes isopiperitenol from limonene	abbreviation
MpIPPO	Mentha x piperita isopiperitenol dehydrogenase: makes isopiperitenone from isopiperitenol	abbreviation
MpIPR	Mentha x piperita isopiperitenone reductase	abbreviation
MpPIPR	Mentha x piperita pulegone reductase: makes menthone from pulegone	abbreviation
MpMR	Mentha x piperita menthone:menthol reductase: makes menthol from menthone	abbreviation
Cassette	One expression unit: promoter piece, coding sequence and terminator	term
Insert	The six cassettes joined head to tail	term
Repair template	The insert flanked by a left and a right homology arm, used for knock-in at a cut site	term
Homology arm	A stretch of genome sequence at each end of the repair template that matches the cut site	term
Site S1	The chromosome 2 site shared by the knock-in designs of this series	term
MIT score	Hsu et al. (2013) aggregate specificity score of a guide, 0 to 100	term

Term	Meaning	Type
UCD10Xv1.1	The Capsicum annuum reference assembly (RefSeq GCF_002878395.1)	term
FW	Fresh weight	abbreviation
DW	Dry weight	abbreviation
uM	Micromolar: micromoles per litre	unit
mM	Millimolar: millimoles per litre	unit
ug/g FW	Micrograms per gram of fresh weight	unit
umol/gDW/h	Micromoles per gram of dry weight per hour	unit
bp	Base pairs	unit
aa	Amino-acid residues	unit
kDa	Kilodalton: 1,000 daltons	unit
g/mol	Grams per mole: molar mass	unit
h	Hours	unit
GC content	Share of G and C bases in a sequence	term
CDS	Coding sequence: the bases from the start codon to the stop codon	abbreviation
UniProt	The public protein sequence database	term
GenBank	The public nucleotide sequence database	term
RefSeq	The NCBI reference sequence collection	term
GUS	Beta-glucuronidase: a reporter enzyme that stains tissue blue	abbreviation

Appendix C. Computational environment

Item	Value
Python	3.15.2
Platform	Darwin x86_64
Libraries	standard library only
Steps and run time	1 code/01_cassettes.py: 1.6 s; 2 code/02_pool_model.py: 0.1 s; 3 code/03_supplementary.py: 0.1 s
Input files	21 files, SHA-256 in `code/environment.json`
Data fingerprint	`d2559046b4501c4147653c5722bb975af9a6a6ced539329856776c13c07112de`

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