Supplementary Information

An artificial pathway for isoprenoid biosynthesis decoupled from native hemiterpene metabolism

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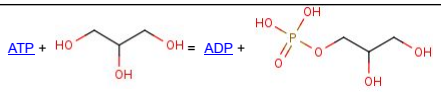
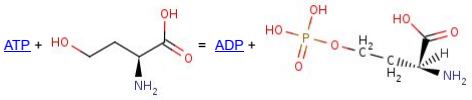
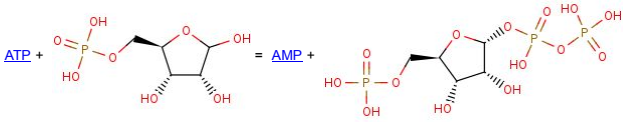
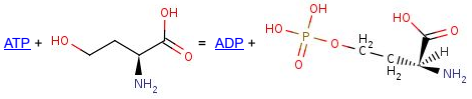
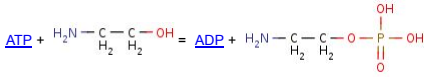
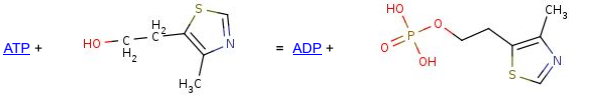
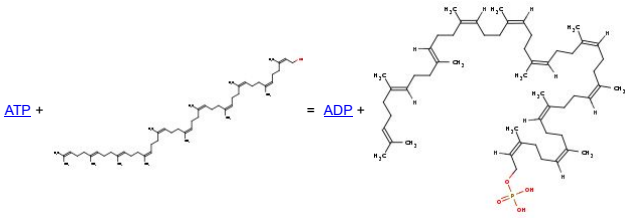
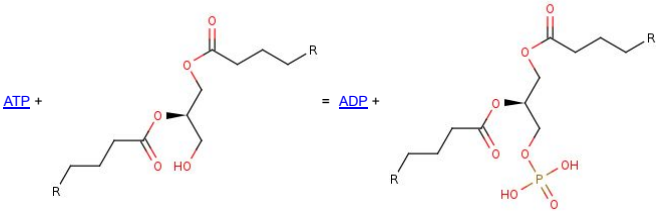
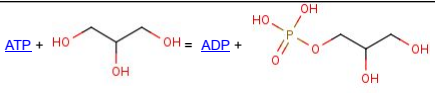
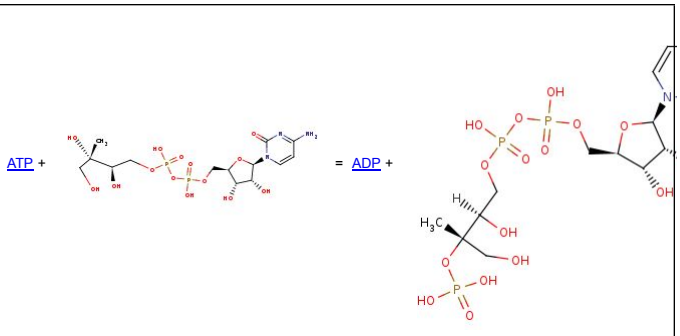
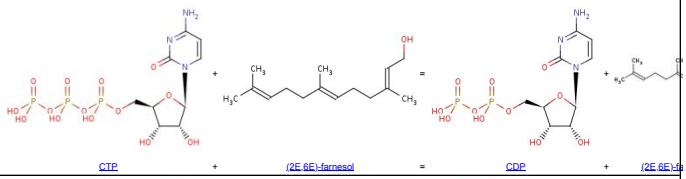
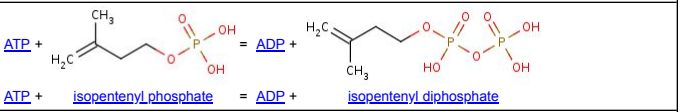
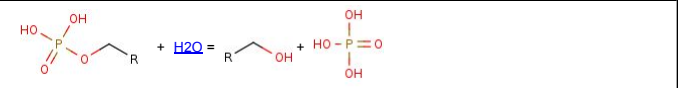
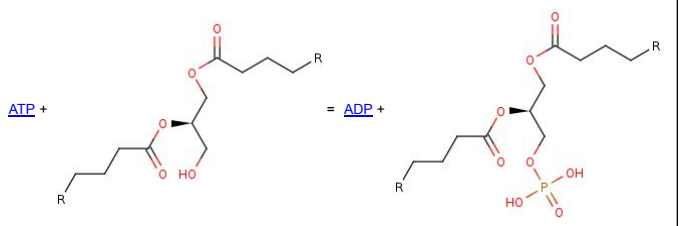
Supplementary Table S1. Codon-optimized gene sequences of proteins used in this study.

Protein	Sequence (5'→3')
IPK from *Thermoplasma acidophilum* codon-optimized for *E. coli*	ATGGATCCGTTCACCATGATGATCCTGAAGATTGGCGGCAGCGTGATTAC CGATAAGAGCGCATATCGCACCGCACGCACCTACGCCATTCGTAGCATCG TGAAAGTGCTGAGCGGCATTGAAGATCTGGTGTGCGTGGTGCATGGCGGT GGTAGCTTTGGCCACATCAAGGCGATGGAGTTTGGTCTGCCGGGTCCGAA AAATCCGCGTAGCAGCATCGGCTACAGCATCGTGCATCGCGACATGGAAA ACCTGGACCTGATGGTGATCGACGCAATGATCGAGATGGGTATGCGCCCG ATTAGCGTGCCGATTAGCGCCCTGCGTTATGATGGTCGCTTTGACTACACC CCGCTGATCCGCTATATTGATGCAGGCTTCGTGCCGGTGAGCTATGGCGA CGTGTATATCAAGGACGAACATAGCTATGGCATCTACAGCGGCGACGATA TTATGGCCGATATGGCCGAACTGCTGAAGCCGGATGTGGCCGTGTTCCTG ACCGATGTGGATGGCATCTATAGCAAGGACCCGAAACGCAATCCGGATGC CGTGCTGCTGCGCGACATCGATACAAACATCACCTTCGATCGCGTGCAGA ACGATGTGACCGGCGGCATTGGCAAGAAATTCGAAAGCATGGTTAAAATG AAAAGTAGCGTTAAAAATGGTGTGTACCTGATTAATGGCAATCACCCGGAG CGCATTGGTGACATCGGCAAGGAGAGCTTCATCGGTACCGTGATTCGC
DGK from *Streptococcus mutans* codon-optimized for *E. coli*	ATGCCGATGGATCTGCGCGACAACAAACAGAGCCAGAAGAAATGGAAAAA CCGCACCCTGACCAGCAGCCTGGAATTTGCCCTGACCGGCATTTTTACCG CCTTCAAAGAAGAACGCAACATGAAAAAACACGCCGTGAGCGCACTGCTG GCCGTGATTGCCGGTCTGGTGTTCAAAGTGAGCGTGATCGAGTGGCTGTT TCTGCTGCTGAGCATCTTCCTGGTGATCACCTTCGAGATCGTGAACAGTGC CATCGAGAATGTGGTGGATCTGGCCAGCGACTATCACTTCAGCATGCTGG CCAAAAACGCCAAAGATATGGCCGCCGGTGCCGTGCTGGTTATTAGCGGT TTTGCCGCCCTGACCGGCCTGATTATTTTTCTGCTGAAAATTTGGTTTCTG CTGTTTCAT
PhoN from *Shigella flexneri* codon-optimized for *E. coli*	ATGCCGATGGATCTGCGCGACAACAAACAGAGCCAGAAGAAATGGAAAAA CCGCACCCTGACCAGCAGCCTGGAATTTGCCCTGACCGGCATTTTTACCG CCTTCAAAGAAGAACGCAACATGAAAAAACACGCCGTGAGCGCACTGCTG GCCGTGATTGCCGGTCTGGTGTTCAAAGTGAGCGTGATCGAGTGGCTGTT TCTGCTGCTGAGCATCTTCCTGGTGATCACCTTCGAGATCGTGAACAGTGC CATCGAGAATGTGGTGGATCTGGCCAGCGACTATCACTTCAGCATGCTGG CCAAAAACGCCAAAGATATGGCCGCCGGTGCCGTGCTGGTTATTAGCGGT TTTGCCGCCCTGACCGGCCTGATTATTTTTCTGCTGAAAATTTGGTTTCTG CTGTTTCAT
FgpaPT2 from *Aspergillus fumigatus* codon-optimized for *E. coli*	ATGAAAGCCGCAAACGCAAGCAGCGCAGAAGCATATCGCGTGCTGAGCC GCGCCTTCCGCTTTGACAACGAGGATCAGAAACTGTGGTGGCACAGCACC GCACCGATGTTTGCAAAGATGCTGGAAACCGCCAACTATACTACCCCGTG CCAGTACCAGTATCTGATCACCTATAAGGAGTGTGTGATCCCGAGCTTAGG CTGCTATCCTACCAATAGCGCACCTCGCTGGCTGAGTATCCTGACCCGTTA CGGTACCCCGTTTGAGCTGAGCCTGAACTGCAGCAACAGCATCGTGCGCT ACACCTTTGAGCCGATTAACCAGCATACCGGCACCGATAAAGACCCGTTC AACACCCATGCCATTTGGGAAAGTCTGCAGCATCTGCTGCCGTTAGAGAA GAGCATCGATCTGGAATGGTTCCGCCACTTCAAACACGACCTGACCCTGA ATAGCGAAGAAAGCGCCTTTCTGGCCCATAACGATCGCCTGGTGGGCGGT ACCATCCGCACCCAGAACAAACTGGCACTGGACCTGAAAGACGGTCGCTT CGCCCTGAAAACCTATATCTACCCGGCCCTGAAAGCCGTGGTGACCGGCA AGACCATTCACGAGCTGGTGTTCGGTAGCGTTCGTCGCTTAGCCGTTCGC GAACCGCGCATTCTGCCGCCGCTGAACATGCTGGAAGAGTATATCCGTAG

	CCGCGGCAGCAAAAGCACCGCAAGTCCGCGTCTGGTTAGCTGTGACCTG ACCAGCCCGGCAAAAAGCCGCATCAAAATCTACCTGCTGGAGCAGATGGT GAGCCTGGAAGCCATGGAAGATCTGTGGACATTAGGCGGCCGTCGCCGT GATGCCAGCACCCTGGAAGGTCTGAGCCTGGTTCGTGAACTGTGGGATCT GATTCAGCTGAGCCCGGGCCTGAAAAGCTACCCTGCCCCGTATCTGCCGC TGGGTGTGATTCCTGACGAGCGTTTACCGCTGATGGCCAATTTTACCCTGC ACCAGAACGATCCGGTGCCGGAACCGCAGGTGTACTTTACAACCTTCGGC ATGAATGACATGGCCGTGGCCGATGCACTGACCACCTTTTTTGAGCGTCG CGGCTGGAGTGAAATGGCACGCACCTATGAAACCACCCTGAAGAGCTACT ACCCGCATGCCGACCATGATAAACTGAACTATTTACATGCCTACATCAGCT TTAGCTATCGCGATCGCACCCCGTATCTGAGTGTGTACCTGCAGAGCTTC GAAACAGGTGACTGGGCCGTGGCAAACCTGAGCGAAAGCAAGGTGAAAT GCCAGGATGCCGCCTGTCAGCCGACAGCACTGCCGCCTGATCTGAGTAAA ACCGGCGTGTACTACAGCGGCCTGCAT

Supplementary Table S2. Summary of wild-type activities of kinases tested for phosphorylation of ISO and DMAA. Data was retrieved using the BRENDA database.[1]

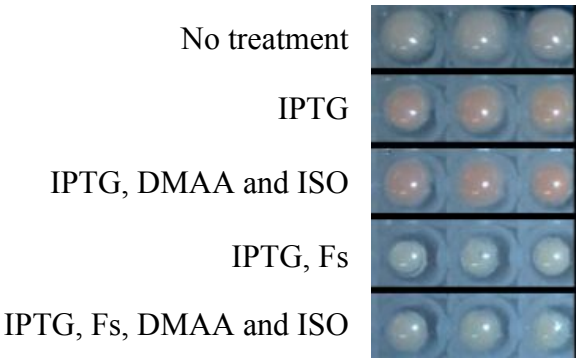
Enzyme	BRENDA Entry	Reaction catalyzed
S. cerevisiae Glycerol kinase	EC2.7.1.30	 ATP + glycerol = ADP + sn-glycerol 3-phosphate
S. cerevisiae Homoserine kinase	EC2.7.1.39	 ATP + L-homoserine = ADP + O-phospho-L-homoserine
E. coli Phosphoribosylpyrophosphate synthetase	EC2.7.6.1	 ATP + D-ribose 5-phosphate = AMP + 5-phospho-alpha-D-ribose 1-diphosphate
E. coli Homoserine kinase	EC2.7.1.39	 ATP + L-homoserine = ADP + O-phospho-L-homoserine
E. coli Ethanolamine kinase	EC2.7.1.82	 ATP + ethanolamine = ADP + O-phosphoethanolamine
E. coli Hydroxyethylthiazole kinase	EC2.7.1.50	 ATP + 4-methyl-5-(2-hydroxyethyl)thiazole = ADP + 4-methyl-5-(2-phosphonooxyethyl)thiazole
E. coli Undecaprenol kinase	EC2.7.1.66	 ATP + undecaprenol = ADP + undecaprenyl phosphate
E. coli Diacylglycerol kinase	EC2.7.1.107	 ATP + 1,2-diacyl-sn-glycerol = ADP + 1,2-diacyl-sn-glycerol 3-phosphate
E. coli Glycerol kinase	EC2.7.1.30	 ATP + glycerol = ADP + sn-glycerol 3-phosphate

E. coli 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE)	EC2.7.1.148	
Arabidopsis thaliana Farnesol kinase	EC2.7.1.216	
Thermoplasma acidophilum Isopentenyl phosphate kinase (IPK)	EC2.7.4.26	
Shigella flexneri Non-specific acid phosphatase (PhoN)	EC3.1.3.2	
Streptococcus mutans Diacylglycerol kinase (DGK)	EC2.7.1.107	

Supplementary Table S3. Primers used in this study.

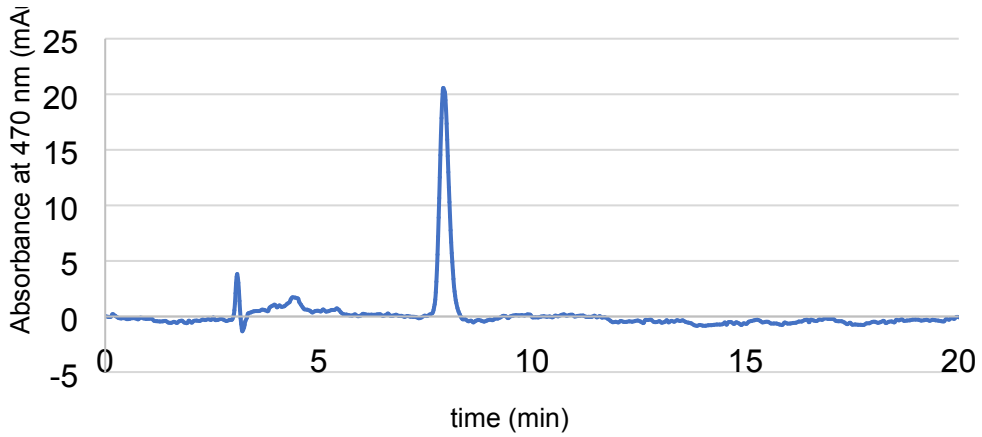
Gene name and organism	NCBI Accession#	Forward (1st) and reverse (2nd) primer (5'→3')
Glycerol kinase, *S. cerevisiae*	NP_011831	1. CATGGGCCGGCCACTTTCCCTCTCTCTTCCGACTTG 2. CATGCTCGAGTTATTGGAAGTTTTCTAGAACCTGTTCG
Homoserine kinase, *S. cerevisiae*	NP_011890.1	1. CATGCATATGGTTCGTGCCTTCAAAATTAAAGTTC 2. CATGCTCGAGTTATCATTGCTGTTCGACGCTAG
Phosphoribosylpyrophosphate synthase, *E. coli*	NP_415725.1	1. CATGCATATGCCTGATATGAAGCTTTTTGCTGG 2. CATGCTCGAGTTAGTGTTCGAACATGGCAGAGAT
Homoserine kinase, *E. coli*	ACX41206	1. CATGCATATGGTTAAAGTTTATGCCCCGGCT 2. CATGCTCGAGTTAGTTTTCCAGTACTCGTGCGC
Ethanolamine kinase, *E. coli*	CP001665.1	1. CATGCATATGGCGCACGACGAACAATG 2. CATGCTCGAGTTATCATTTTGCATATAGCCCCTCC
Hydroxyethylthiazole kinase, *E. coli*	ACT28613.1	1. CATGCATATGCAAGTCGACCTGCTGGGT 2. CATGCTCGAGTTAAGGAGGTGCAGGCATGA
Undecaprenol kinase, *E. coli*	ACA76318	1. CAGTAGATCTCAGCGATATGCACTCGCTG 2. CAGTCTCGAGTTAAAAGAACACGACATACACCG
Diacylglycerol kinase, *E. coli*	ACA79587.1	1.CATGCATATGGCCAATAATACCACTGGATTCAC 2. CATGCTCGAGTTATCCAAAATGCGACCATAAC
Glycerol kinase, *E. coli*	ACT31081.1	1.CATGCATATGACTGAAAAAAAATATATCGTTGCGC 2. CATGCTCGAGTTATTCGTCGTGTTCTTCCCAC
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *E. coli*	AF179284	1. CCATGG CG ATGCGGACACAGTGGCCCTC 2. CTCGAGTTAAGCATGGCTCTGTGCAATGG
Farnesol kinase, *Arabidopsis thaliana*	NM_125242	1. CGATGGATCCGATGGCAACTACTAGTACTACTACAAAGCTC 2. CGATGCGGCCGCTTAGAAGAGTAAGAATCCGGCC
Isopentenyl diphosphate isomerase idi from *E. coli* (cloned into pCDFDuet-GGPP)	BAE76954.1	1. GTACAGATCTTATGCAAACGGAACACGTCAT 2. GTACCTCGAGTTAATTGTGCTGCGCGAAA
IspA Y80D from *E. coli* (cloned int pCDFDuet-GGPP)	NP_414955	1. GTACCCATGGCGATGGACTTTCCGCAGCAAC 2. GTACGCGGCCGCTTATTTATTACGCTGGATGATGTAGTCC
Phytoene desaturase CrtI from *Pantoea ananatis* (cloned into pACYCDuet-Lyc)	D90087	1. GTACCCATGGGCATGAAACCAACTACGGTAATTGGTG 2. GTACAAGCTTTTAAATCAGATCCTCCAGCATCAA
Phytoene synthase crtB from *Pantoea ananatis* (cloned into pACYCDuet-Lyc)	D90087	1. GTACAGATCTCATGGCAGTTGGCTCGAAAAG 2. GTACCTCGAGTTAGAGCGGGCGCTGC
Isopentenyl phosphate kinase (IPK), *Thermoplasma acidophilum* (cloned in pETDuet)	CAC11251.1	1.GTACCATATGATAGATCCGTTCACCATGATGATCC 2. GTACCTCGAGTTAGCGAATCACGGTACCGAT
Isopentenyl phosphate kinase (IPK), *Thermoplasma acidophilum* (cloned in pET28a)	CAC11251.1	1. GTACCATATGGATCCGTTCACCATGATGATCC 2. GTACCTCGAGTTAGCGAATCACGGTACCGAT
Non-specific acid phosphatase (PhoN), *Shigella flexneri*	BAA11655.1	1. CTAGGGATCCGATGAAACGTCAGCTGTTTACC 2. CTAGGCGGCCGCTTATTTTTTCTGATTATTGGCGAAT
Diacylglycerol kinase (DGK), *Streptococcus mutans*	AAN59259.1	1. ATAGGATCCGATGCCGATGGATCTGC 2. CGATGCGGCCGCTTAATGAAACAGCAGAAACCAAATT
Dimethylallyl tryptophan synthase (FgaPT2), *Aspergillus fumigatus* (cloned in pET28a)	AX08549.1	1. ATACATATGAAAGCCGCAAACG 2. TATAAGCTTATGCAGGCCGC
Dimethylallyl tryptophan synthase (FgaPT2), *Aspergillus fumigatus* (cloned in pCDFDuet)	AX08549.1	1. TATAGGATCCGATGAAAGCCGCAAACG 2. ATATAAGCTTATGCAGGCCGC

Supplementary Figure S1: Effect of fosmidomycin (Fs) on lycopene production in the absence of kinase overexpression. *E. coli* BL21Tuner(DE3) harboring pCDFDuet-GGPP, pACYCDuet-Lyc, and 'empty' pETDuet in wells of a microplate were treated with combinations of IPTG (to induce expression of heterologous genes), DMAA and ISO (potential substrates for hemiterpene production), and Fs (to knock-down DXP-dependent hemiterpene production). No DXP-independent lycopene production was observed when the cells were treated with IPTG, Fs, and provided DMAA/ISO, indicating that the activity of endogenous kinases were not sufficient to support lycopene production.

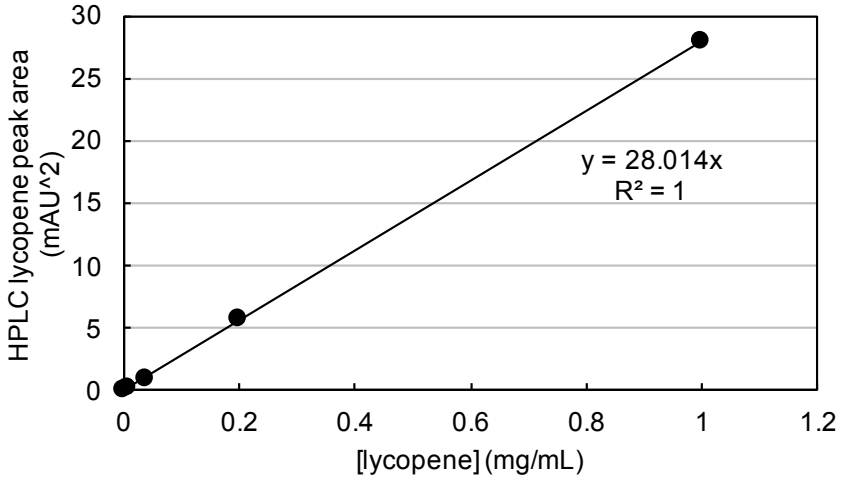


Supplementary Figure S2. Extraction and quantification of lycopene. (**A**) Lycopene was extracted from *E. coli* Novablue(DE3) pAC-LYCipi and analyzed by HPLC. Lycopene eluted at 8.5 min. (**B**) Lycopene standard curve.

A



B



Cloning Candidate Kinase Genes

Genes were amplified from *E. coli* BL21 and *S. cerevisiae* EBY100 genomic DNA by taking 100 µL of cell pellet, adding 200 µL of water, followed by boiling in a 1.5 mL tube for 15 min. The cell debris was pelleted and 1 µL of the supernatant was used for PCR from genomic DNA using the primers listed in **Table S3**. Farnesol kinase from *A. thaliana* was PCR amplified from a cDNA library gifted from the lab of Dr. José M. Alonso (NC State, Department of Genetics) using the primers listed in **Table S3**. The PCR reaction contained 5x Phire II buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 µL Phire II DNA polymerase, and 1 µL of genomic DNA, in a total volume of 50 µL. The cycling parameters used were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 66 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following amplification, the amplified products were gel purified, digested with *Bam*HI and *Not*I, and ligated into similarly treated 'empty' pETDuet and pETDuet-IPK (in MCS2). Ligation mixtures were transformed into chemically competent *E. coli* NovaBlue (DE3) cells (Novagen) and plated on LB agar supplemented with 50 µg/mL kanamycin for incubation overnight at 37 °C. Colonies were then screened for the appropriate size insert by colony PCR using primers annealing to the T7 promoter and T7 terminator. Those colonies with correct sized inserts were then picked and grown in 3 mL LB supplemented with 50 µg/mL kanamycin for incubation overnight at 37 °C. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing.

Screening of Kinases for Phosphorylation of ISO and DMAA by LC-MS Analysis

Each plasmid containing a cloned kinase gene was transformed into chemically competent *E. coli* BL21(DE3) Tuner cells and plated on LB agar supplemented with 50 µg/mL kanamycin for incubation overnight at 37 °C. Colonies were picked the following day and used to inoculate 3 mL LB supplemented with 50 µg/mL kanamycin for incubation overnight at 37°C. A 1 mL portion of this culture was then used to inoculate 100 mL of LB supplemented with 50 µg/mL kanamycin and grown at 37 °C at 250 rpm until the culture reached OD_{600} of ~0.2 before the temperature was reduced to 18 °C and IPTG was added to a final concentration of 1 mM. The culture was incubated for 18 hours at 250 rpm. The culture was pelleted at 4,000 rpm for 10 min, the supernatant decanted, and the cell pellet resuspended in 5 mL of lysis buffer (100 mM Tris, 300 mM NaCl, 10% glycerol, pH 8.0) and lysed by sonication. The debris was then pelleted at 4,500 rpm for 20 min, decanted, and the soluble protein was spun down additionally at 15,000 rpm for 1 h. The resulting soluble fraction was then purified using loose Ni^{2+} resin from GE Healthcare. 200 µL of resin was added to the soluble fraction of protein and incubated on ice for 1 hour with intermittent agitation to suspend the resin. The resin was then spun down for 10 min at 4,500 rpm

at 4 °C and the lysate was removed. The resin was then resuspended in 1 mL of wash buffer (50 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 8.0) and transferred to a 1.5 mL tube. The mixture was allowed to incubate on ice for 10 min before the resin was spun down again as before. This washing procedure was repeated 4 more times before the protein was eluted with 200 µL of elution buffer (50 mM Tris, 500 mM NaCl, 200 mM imidazole, pH 8.0). The protein was then directly assayed and purity was verified by SDS-PAGE with comparison to the soluble fraction of *E. coli* BL21 (DE3) Tuner cells not harboring any plasmid. The assay consisted of 10 µL of purified protein in a total volume of 100 µL containing 5 mM ATP, 1 mM of ISO and DMAA (stock of 100 mM in DMSO), 50 mM Tris at pH 7.5, and 2.5 mM $MgCl_2$. The reaction was incubated overnight at 37 °C before being quenched with an equal volume of methanol. The mixture was then analyzed by low-resolution LC-MS along with a synthetic standard of isopentenyl phosphate and dimethylallyl phosphate. LC-MS experiments were conducted using a Shimadzu LC-MS 2020 single quadrupole instrument with a Phenomenex Kinetex UPLC C18 column (2.1 X 50 mm, 2.6 µm particle, 100 Å pores) column. An aliquot (5 µL) was injected onto and separated using a series of linear gradients developed from 0.1% formic acid in H_2O (A) to 0.1% formic acid in acetonitrile (B) at 0.2 mL/min using the following protocol: 0-2.2 min, 95-1% A; 2.21-2.6 min, 1% A; 2.61-2.62 min, 1-95% A; 2.63-3.5 min, 95% A.

Synthesis of IPP and DMAPP

The synthesis of IPP and DMAPP followed a published procedure.[2] To 4 mmol of the neat alcohol (IPO or DMAA) in a 50 mL polypropylene tube was added trichloroacetonitrile (10 mL) and the mixture was allowed to incubate at room temperature for 5 min. Bis-triethylammonium phosphate (TEAP) solution was prepared by slowly adding solution A (25 mL phosphoric acid, 94 mL acetonitrile) to solution B (110 mL triethylamine, 100 mL acetonitrile) to generate a solution that was 38% solution A and 62% solution B. To the mixture of alcohol and trichloroacetonitrile was added 10 mL of TEAP solution. The mixture was then incubated in a 37 °C water bath for 5 min before another addition of TEAP. A total of three additions of TEAP solution were added and incubated. The mixture was then separated by column chromatography using 6:2.5:0.5 iPrOH: conc. NH_4OH:H_2O with silica as the stationary phase. Prior to loading the column, the reaction mixture was diluted to 20% vol/vol with chromatography buffer and the resulting precipitate was pelleted by centrifugation prior to loading of the flash column. Fractions were analyzed by using a Shimadzu single quadrupole LCMS-2020 and those containing the diphosphorylated compound ($[M-H]^-$), free of tri- or monophosphorylated were pooled. The pooled fractions were then concentrated in vacuo to remove isopropanol and acetonitrile. The concentrated mixture was then

filtered using 0.2 µm cellulose filter and frozen at -80 ˚C. After being frozen overnight, the sample was lyophilized yielding a salt. The triammonium salt was then characterized and stored frozen in 250 µL aliquots at 25 mM each. Dimethylallyl diphosphate (DMAPP): ^{1}H NMR (400 MHz, D_2O) δ 5.43 (t, J = 7.0 Hz, 1H), 4.43 (dd, $J_{H,P}$ = 7.0 Hz, $J_{H,H}$ = 7.0 Hz, 2H), 1.76 (s, 3H), 1.71 (s, 3H); ^{13}C NMR (101 MHz, D_2O) δ 140.1, 119.7 (d, $J_{C,P}$ = 8.2 Hz), 62.7 (d, $J_{C,P}$ = 5.4 Hz), 25.0, 17.3; ^{31}P NMR (162 MHz, D_2O) δ -6.04 (d, J = 21.7 Hz), -9.38 (d, J = 21.6 Hz); HRMS *m/z* calculated for $C_5H_{12}O_7P_2$ $[M\text{-}H^+]^-$ 244.9985, found: 244.9986. Isopentenyl diphosphate (IPP): ^{1}H NMR (400 MHz, D_2O) δ 4.02 – 3.92 (m, 2H), 2.30 (t, J = 6.7 Hz, 2H), 1.68 (s, 3H); ^{13}C NMR (101 MHz, D_2O) δ 143.8, 111.6, 64.1 (d, $J_{C,P}$ = 6.0 Hz), 37.9 (d, $J_{C,P}$ = 7.6 Hz), 21.7; ^{31}P NMR (162 MHz, D_2O) δ -6.22 (d, J = 21.6 Hz), -9.54 (d, J = 21.5 Hz); HRMS *m/z* calculated for $C_5H_{12}O_7P_2$ $[M\text{-}H^+]^-$ 244.9985, found: 244.9985.

Preliminary Characterization of IPK via Lycopene Colorimetric Assay

Colonies of *E. coli* BL21Tuner(DE3) harboring pCDFDuet-GGPP (see below) and pACYCDuet-Lyc (see below) were used to inoculate separate wells of a deep-well plate containing 1 mL of LB media supplemented with ampicillin (150 µg/mL), chloramphenicol (35 µg/mL), and streptomycin (200 µg/mL). The deep-well plate was incubated overnight in a rotary shaker at 37 °C with orbital shaking at 350 rpm. Then, 50 µL of the culture was used to inoculate 400 µL of LB media supplemented with ampicillin (120 µg/mL), chloramphenicol (28 µg/mL), and streptomycin (160 µg/mL). After 3 h of incubation at 37°C at with shaking at 350 rpm, the OD_{600} of the culture was approximately 0.1, and IPTG, DMAA/ISO, and Fs were each added to give final concentrations of 1 mM, 5 mM, and 0.5 µM, respectively, to bring the cultures to a final volume of 500 µL. For controls that lacked one or more of these components, LB media was added instead. Plates were then incubated in the dark in an incubator/shaker at 30 °C with shaking at 350 rpm for 48 h. Then, the deep-well plate was centrifuged at 3,000 rpm for 7 min to pellet the cells. After removal of the growth media from each well, the pellets were resuspended in 1 mL of phosphate buffer saline buffer with vigorous vortexing and the resuspended pellets were visualized and photographed.

Construction of pCDFDuet-GGPP and pACYCDuet-Lyc

pCDFDuet-GGPP contained a mutated version of the *E. coli* IspA gene (Y80D) and *idi* from *E. coli* that were subcloned sequentially into pCDFDuet. Briefly, *ispA* and *idi* were each PCR amplified from *E. coli* DH5α using the primers listed in **Supplemental Table S3**. Each PCR reaction mixture contained 5x Phire II buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 µL Phire II DNA polymerase, and 1 µL of template DNA in a total volume of 50 µL. The cycling parameters

used were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 63 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following amplification, the products were purified by gel electrophoresis and digested with *Nco*I and *Not*I for *ispA* and *Bg*lII for *idi* and ligated into the appropriately treated pCDFDuet vector. Each ligation mixture was transformed into chemically competent *E. coli* DH5α and plated on an LB agar plate containing 200 µg/mL streptomycin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing. The mutation Y80D was introduced by site-directed mutagenesis as described previously.[3]

pACYCDuet-Lyc contains the *CrtEBI* operon genes from *Pantoea ananatis* that were sub-cloned sequentially using pACmod-crtE-crtB-crtI as a template (a gift from the Prof. Schmidt-Dannert, University of Minnesota).[4] Briefly, *crtB* and *crtI* were each PCR amplified from the template pACmod-crtE-crtB-crtI using the primers listed in **Supplemental Table S3**. Each PCR reaction mixture contained 5x Phire II buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 µL Phire II DNA polymerase, and 1 µL of template DNA, in a total volume of 50 µL. The cycling parameters used were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 63 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following amplification, the products were purified by gel electrophoresis and digested with *Bg*lII and *Xho*I for *crtB* and *Nco*I and *Hin*dIII for *crtI* and ligated into the appropriately treated pCDFDuet vector. Each ligation mixture was transformed into chemically competent *E. coli* DH5α and plated on an LB agar plate containing 35 µg/mL chloramphenicol. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing.

Cloning of IPK, PhoN, and DGK

The sequence of *ipk* (**Table S2**) codon-optimized for heterologous expression in *E. coli* was synthesized by IDT and PCR amplified using the primers listed in **Supplemental Table S3**. The PCR reactioj mixture contained 5x Phire II buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 µL Phire II DNA polymerase, and 1 µL of template DNA, in a total volume of 50 µL. The cycling parameters used were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 63 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following amplification, the product was purified by gel electrophoresis, digested with *Nde*I and *Xho*I and ligated into similarly treated pETDuet-1 (into multi-cloning site two). The ligation mixture was transformed into chemically competent *E. coli* DH5α and plated on LB agar containing 100 µg/mL of ampicillin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing using the primer ‘DuetUP2’

(5'-TTGTACACGGCCGCATAATC-3'). For cloning into pET28a, the *ipk* gene was PCR amplified using the primers listed in **Supplemental Table S3** and the same PCR conditions as described above. Following amplification, the product was purified by gel electrophoresis and was digested with *Nde*I and *Xho*I and ligated into similarly treated pET28a. The ligation mixture was then transformed into chemically competent *E. coli* DH5α and plated on LB agar containing 50 µg/mL kanamycin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing.

The sequence of *phoN* (**Table S2**) codon-optimized for expression in *E. coli* was synthesized by IDT and PCR amplified using the primers listed in **Table S2**. The PCR reaction contained 5x Phire II buffer, 0.2 mM dNTPs, 0.5 µM each primer, 1 µL Phire II DNA polymerase, and 20 ng of template DNA, in a total volume of 50 µL. The cycling parameters used were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 66 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following amplification, the amplified product was gel purified, digested with *Bam*HI and *Not*I, and ligated into similarly treated 'empty' pETDuet and pETDuet-IPK (into MCS1). The ligation mixture was transformed into chemically competent *E. coli* DH5α and plated on LB agar containing 100 µg/mL of ampicillin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing using the primer 'pET Upstream' (ATGCGTCCGGCGTAGA).

The sequence of DGK from *Streptococcus mutans* codon optimized for expression in *E. coli* (**Table S2**) was synthesized and subcloned into pET28a according to the same protocol for cloning candidate kinase genes above, using the primers listed in **Table S3**.

Expression and Protein Purification of PhoN

pETDuet-PhoN was transformed into chemically competent *E. coli* BL21(DE3) for protein expression. An overnight 3 mL culture in LB broth of PhoN-pETDuet containing 100 µg/mL of ampicillin was grown at 37 °C and 270 rpm. A 1 L culture in LB broth was inoculated with 1 mL of the overnight culture and grown at 37 °C and 250 rpm to an OD_{600} of 0.6. The culture was cooled to 18 °C, and protein expression was induced by the addition of IPTG to a final concentration of 1 mM. The culture was incubated at 18 °C and 200 rpm for an additional 20 h. The culture was spun down into a pellet which was stored at -20 °C until purification. The cell pellets were thawed and resuspended in 20 mL of lysis buffer (250 mM sodium chloride, 50 mM sodium phosphate, 10 mM imidazole, pH 7.9). The cells were lysed by sonication and spun down. The cell lysate was

then separated from the insoluble cell debris, and the His_6-tagged proteins were purified from the lysate using Ni^{2+} beads on agarose using a low-imidazole buffer (50 mM Tris, 300 mM NaCl, 20 mM imidazole, pH 8.0) as the wash buffer and a high-imidazole buffer (50 mM Tris, 300 mM NaCl, 200 mM imidazole, pH 8.0) to isolate the protein. Then, a 10 kDa spin filter was used to concentrate and buffer exchange the protein into storage buffer (50 mM Tris-HCl, 500 mM NaCl, 20% glycerol, pH 7.4). The concentration of protein was determined using a Bradford assay, and small aliquots of protein were stored at -80 °C until needed.

Expression and Protein Purification of IPK

pET28a-IPK was transformed into chemically competent competent *E. coli* BL21(DE3) for protein expression. An overnight 3 mL culture in LB broth supplemented with kanamycin (50 µg/mL) was grown at 37 °C with shaking at 270 rpm. A 1 L culture in LB broth was inoculated with 1 mL of the overnight culture and grown at 37 °C with shaking at 250 rpm to an OD_{600} of 0.6. The culture was cooled to 30 °C and protein expression was induced by the addition of IPTG to a final concentration of 1 mM. The culture was incubated at 30 °C and 200 rpm for an additional 20 h. The culture was spun down into a pellet which was stored at -20 °C until purification. Cell pellets were thawed and resuspended in 25 mL lysis buffer (250 mM sodium chloride, 50 mM sodium phosphate, 10 mM imidazole, pH 7.9). The cells were lysed by sonication and centrifuged. The cell lysate was then separated from the insoluble cell debris, and the His_6-tagged proteins were purified from the lysate using the Bio-Rad Profinia system and Bio-Scale Mini Nuvia IMAC Ni-Charged 5-mL columns. Following loading of the sample onto the column, the system washed first with 6 column volumes of 2x Native IMAC Wash 1 solution (1 M NaCl, 100 mM Tris, 10 mM imidazole, pH 8.0) and then with 6 column volumes of 2x Native IMAC Wash 2 solution (1 M NaCl, 100 mM Tris, 40 mM imidazole, pH 8.0). The sample was eluted in 3 column volumes of 2x Native IMAC Elution buffer (1 M NaCl, 100 mM Tris, 500 mM imidazole, pH 8.0). Then the eluent was concentrated and buffer exchanged into protein storage buffer (50 mM Tris-HCl, 500 mM NaCl, 20% glycerol, pH 7.4) using 10 kDa molecular-weight cutoff filters. The concentration of protein was determined using a Bradford assay and small aliquots of protein were stored at -80 °C until needed.

Extraction and Quantification of Lycopene

Each aliquot (600 µL) of culture was subjected to acetone extraction and quantification of lycopene by HPLC. The remaining 500 µL was centrifuged at 10,000 rpm, and the supernatant removed. The cell pellets were then dried using a speed vacuum without heat until the pellets

were dry. Then, 200 µL of acetone was added to the pellets, and the tubes were sonicated at 37 °C for 20 min before incubation at 55 °C for 30 min. The tubes were then sonicated again as before. The pellets were spun down and 100 µL was removed for HPLC analysis. HPLC was performed by injecting 10 µL of the clarified extract onto a Phenomenex Kinetex EVO C18 column (250 x 4.6 mm, 5 µm, 100 Å pores) with an isocratic elution buffer consisting of 8: 1.5: 0.5 isopropanol: acetonitrile: methanol over 20 min. Lycopene was assayed at 470 nm. Areas were extracted and compared to the standard curve for quantification.

Lycopene Standard Curve

A standard curve for lycopene was derived by adding various known amounts of commercial lycopene standard to *E. coli* Novablue(DE3) cell pellets, extracting, and quantified as outlined above.

Cloning of FgaPT2

The sequence of *fgaPT2* codon-optimized for expression in *E. coli* (**Table S2**) was synthesized by IDT and PCR amplified using the primers listed in **Table S3**. The 50 µL reaction for amplification of *fgaPT2* contained 5x Phire buffer, 0.2 mM dNTPs, 0.25 µM each primer, 1 µL Phire II DNA polymerase, and 1 µL template DNA. The cycling parameters used for each were as follows: 1) 98 °C, 30 s; 2) 98 °C, 5 s; 3) 64 °C, 15 s; 4) 72 °C, 20 s; 5) repeat steps 2-4 34 times; 6) 72 °C, 1 min; 7) 4 °C, hold. Following PCR amplification, the amplified product was purified by gel electrophoresis, digested with *Hin*dIII and *Nde*I and ligated into similarly treated pET28a to generate pET28a-FgaPT2. The ligation mixture was then transformed into chemically competent *E. coli* DH5α and plated on LB agar containing 50 µg/mL of kanamycin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing using the T7 promoter primer. For cloning into pCDFDuet (used in conjunction with the ADH module in pETDuet-PhoN-IPK), *fgaPT2* was PCR amplified using the same conditions as above but with different primers also listed in **Table S3**. Following amplication, the product was digested with *BamH*I and *Hin*dIII and ligated into similarly treated pCDFDuet. The ligation mixture was then transformed into chemically competent *E. coli* DH5α and plated on LB agar plates containing 100 µg/mL of streptomycin. Plasmid was prepared from a single colony and the gene sequence confirmed by DNA sequencing using the DuetUP2 and T7 teminator primers.

Expression and Protein Purification of FgaPT2

pET28a-FgaPT2 was transformed into chemically competent Rossetta PLysS cells for expression. An overnight 3 mL culture of these cells containing 50 µg/mL of kanamycin and 25 µg/mL of chloramphenicol in LB media was grown at 37 °C and 270 rpm. A 1 L culture in terrific broth containing 30 µg/mL of kanamycin and 35 µg/mL of chloramphenicol was inoculated with 2 mL of overnight culture and grown at 37 °C and 250 rpm to an OD_{600} of 0.6. Once OD was reached, the culture was cooled to 24 °C and induced by the addition of IPTG to 0.5 mM final concentration. The culture was incubated for 24 h at 24 °C. The culture was pelleted and stored in two aliquots at -20 °C until purification. Cell pellets were thawed and resuspended in 25 mL lysis buffer (250 mM sodium chloride, 50 mM sodium phosphate, 10 mM imidazole, pH 7.9). The cells were lysed by sonication and spun down. The cell lysate was then separated from the insoluble cell debris, and the His_6-tagged proteins were purified from the lysate using the Bio-Rad Profinia system and Bio-Scale Mini Nuvia IMAC Ni-Charged 5-mL columns. Following loading of the sample onto the column, the system washed first with 6 column volumes of 2x Native IMAC Wash 1 solution (1 M NaCl, 100 mM Tris, 10 mM imidazole, pH 8.0) and then with 6 column volumes of 2x Native IMAC Wash 2 solution (1 M NaCl, 100 mM Tris, 40 mM imidazole, pH 8.0). The sample was eluted in 3 column volumes of 2x Native IMAC Elution buffer (1 M NaCl, 100 mM Tris, 500 mM imidazole, pH 8.0). Then the eluent was concentrated and buffer exchanged into protein storage buffer (50 mM Tris-HCl, 500 mM NaCl, 20% glycerol, pH 7.4) using 50 kDa molecular-weight cutoff filters. The concentration of protein was determined using a Bradford assay, and small aliquots of protein were stored at -80 °C until needed.

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