Supporting Information

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SI Materials and Methods

Plant Materials. A hairy root line for feeding studies was developed by infection of *S. miltiorrhiza* cut leaf and petiole tissue with *Agrobacteria rhizogenes* ATCC 15834 cultured in yeast mannitol broth (YMB) media containing 20 mM acetosyringone. Briefly, seeds were sterilized in 25% (wt/vol) bleach for 10 min, rinsed with sterile water three times, and then plated on MS medium and allowed to germinate and grow for 2 wk. Thereafter, leaf and petiole tissue from several plants were cut into 0.5-cm pieces and placed flat on a new MS medium plate. Following plating, 100 μL of *A. rhizogenes* culture with an optical density at 600nm (OD_{600}) of 1.0 was placed on the surface of the tissue. After 3 d, this tissue was moved to new MS plates containing 200 μg/mL cefotaxime. One week later hairy roots were cut and placed in liquid MS media containing 50 μg/mL cefotaxime. These were subcultured in two separate flasks with cefotaxime three times before exclusion of the antibiotic. One line that exhibited optimal growth and inducible tanshinone production was then selected for use in the feeding studies.

All other analyses were carried out using *S. miltiorrhiza* collected from Laiwu, Shandong province, China. These were used to establish hairy root cultures, which were grown in 6,7-V medium. For analysis, 18 d after subculture, these were elicited with 300 μM $AgNO_3$, which has been shown to induce tanshinone production (1). Samples were taken at 6 h, 12 h, 24 h, 36 h, 72 h, and 120 h postinduction, and these were used for RNA extraction and analysis of tanshinone and ferruginol levels.

Miltiradiene Labeling. Miltiradiene was produced using the C41 OverExpress (Lucigen) strain of *E. coli*, which was transformed with our previously described CPP synthase expression vector pGG*n*C (2), which enables production of the relevant normal stereoisomer of CPP, along with the previously described SmKSL expression vector pDEST14/SmKSLd54 (3), and a previously described pIRS vector containing the genes encoding 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductase, and sopentenyl diphosphate isomerase that increases flux into the endogenous MEP isoprenoid precursor pathway (4). Recombinant cells were selected on NZY (5 g/L yeast extract, 10 g/L casein hydrolysate, 5 g/L NaCL, and 1 g/L $MgSO_4$) plates containing 25 μg/mL carbenicillin, 20 μg/mL chloramphenicol, and 15 μg/mL spectinomycin, and these antibiotics were included in all subsequent growth media. To increase cell growth, media was further supplemented with 1 mM thiamine and 2 μg riboflavin. To optimize diterpene production in minimal media fermentations, the usual recipe (60.2 mM dibasic and 33.0 mM monobasic potassium phosphate, and 7.5 mM ammonium sulfate) was supplemented with 51.3 mM NaCl and 1 mM magnesium sulfate, with adjustment of pH to 7. Carbon was provided by the addition of glucose to 20 mM just before inoculation, with labeling carried out using $^{13}C_6$-glucose (Sigma). After initial studies, we determined that additional supplementation was necessary to increase growth rate, which was accomplished by the addition of a previously described trace mineral solution (5). Cells were grown in 50 mL of the optimized minimal media at 37 °C (200 rpm) to an OD_{600} of 0.7, then moved to a 16 °C shaker for an hour, followed by induction via the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM. After 3 d, cultures were extracted twice with an equal volume of HPLC grade n-hexane overnight at 4 °C. The n-hexane extract was concentrated under nitrogen gas to 2 mL and then loaded on a 2-g silica column and eluted with ethyl acetate/n-hexane (5/95, vol/vol). The sample was dried under nitrogen and then the residue dissolved in 1 mL of n-hexane, and 1 μL injected on a Varian 3900 GC with Saturn 2100 ion trap mass spectrometer (MS) in electron ionization (70 eV) mode. Separation was achieved with a H_2 flow rate of 2 mL/min with a temperature program of 3 min at 50 °C, followed by a gradient from 50 to 300 °C at 14 °C/min with a 3-min hold at 300 °C.

Feeding Studies. Labeled miltiradiene (250 μg) in 50 μL of methanol/DMSO(1/1, vol/vol) was fed to freshly subcultured hairy root cultures (~5 g fresh weight hairy roots in 50 mL MS media in 125-mL flasks) to which AMO1618 (28 μg/mL) and fosmidomycin (20 μM) also had been added. These were then induced with a previously described combination of yeast extract and Ag^+ (6). The cells were incubated at 28 °C for 5 d while shaking at 55 rpm. This time point for harvesting cells was chosen based on the observation that inhibitor efficacy rapidly dropped after day 5, as unfed control cultures were observed to begin secreting the visibly red tanshinones after this time. Hence, hairy roots were removed from the liquid media after 5 d and ground twice in a mortar and pestle under liquid nitrogen. The ground tissue was extracted with 4 mL of methanol for 5 min, centrifuged in a clinical centrifuge, and the extract removed. This methanol extract was filtered through a low-extractable 0.2 μm nylon syringe filter and extracted with an equal volume of n-hexane, which was then dried under nitrogen gas and resuspended in 50 μL of n-hexane, with 3 μL samples subjected to GC–MS analysis. This was carried out using a Varian 3900 GC with Saturn 2100 ion trap mass spectrometer (MS) in electron ionization mode (ferruginol) or chemical ionization (methanol) mode (cryptotanshinone). Separation was achieved with a H_2 flow rate of 2 mL/min with a temperature program of 3 min at 50 °C, followed by a gradient from 50 to 300 °C at 14 °C/min with a 3-min hold at 300 °C. Ferruginol and cryptotanshinone were identified by comparison of retention time and mass spectra to authenticated standards. Labeled extracts were further compared with those from unlabeled induced and noninduced control cultures.

1. Zhang C, Yan Q, Cheuk WK, Wu J (2004) Enhancement of tanshinone production in Salvia miltiorrhiza hairy root culture by Ag^+ elicitation and nutrient feeding. *Planta Med* 70(2):147–151.
2. Cyr A, Wilderman PR, Determan M, Peters RJ (2007) A modular approach for facile biosynthesis of labdane-related diterpenes. *J Am Chem Soc* 129(21):6684–6685.
3. Hillwig ML, et al. (2011) Domain loss has independently occurred multiple times in plant terpene synthase evolution. *Plant J* 68(6):1051–1060.
4. Morrone D, et al. (2010) Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: Comparison of MEV and MEP isoprenoid precursor pathway engineering. *Appl Microbiol Biotechnol* 85(6):1893–1906.
5. Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* 61(2):205–218.
6. Ge X, Wu J (2005) Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^+ and yeast elicitor. *Plant Sci* 168:487–491.

Fig. S1. Alignment of cytochrome P450 reductases (CPRs). Amino acid sequences of *Salvia miltiorrhiza* SmCPR1 (GenBank accession no. CBX24555) and SmCPR2 (GenBank accession no. JX848592) were aligned with those of CPRs from *Catharanthus roseus* (CroCPR2), *Arabidopsis thaliana* (AtATR1 and AtATR2), *Vitis vinifera* (VvCPR2), *Artemisia annua* (AaCPR2) by CLUSTALW (www.genome.jp/tools/clustalw/). The amino acid sequences of CroCPR2, AtATR1, AtATR2, VvCPR2, and AaCPR2 can be found at www.p450.kvl.dk/p450rel.shtml.

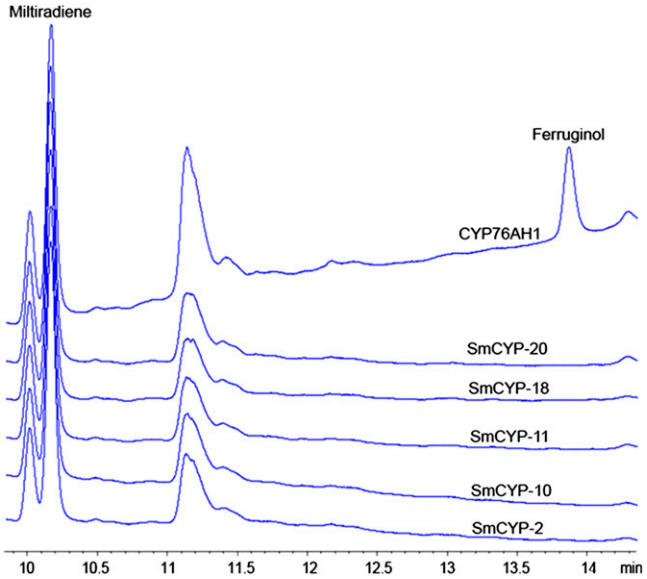

Fig. S2. GC profiles of reaction products of microsome samples of recombinant yeast strains using miltiradiene as substrate. GC analyses were carried out as described in the text.

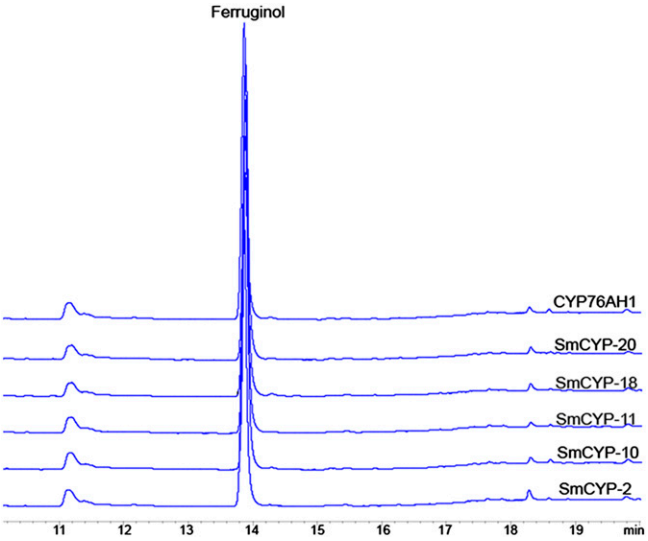

Fig. S3. GC profiles of reaction products of microsome samples of recombinant yeast strains using ferruginol as substrate. GC analyses were carried out as described in the text.

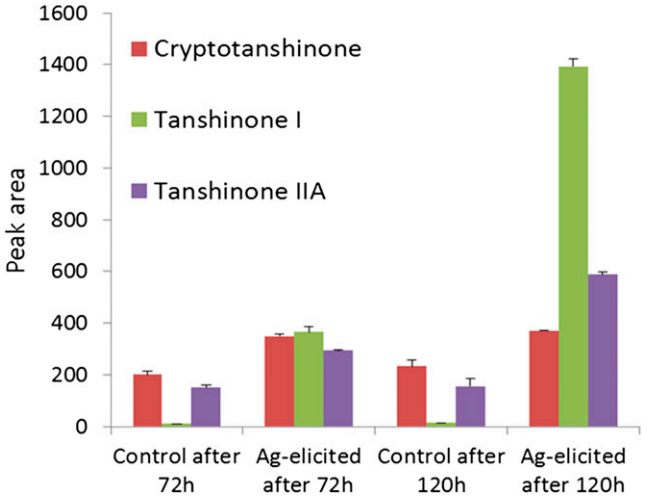

Fig. S4. Accumulation of tanshinones in danshen hairy roots responding to Ag^+ after 72 h and 120 h compared with control. Analysis of tanshinone levels was carried out as previously described with minor modifications (1). Briefly, dried and powered hairy roots elicited with Ag^+ were extracted with a 3:1 (vol/vol) mixture of chloroform/methanol, concentrated fivefold, and subjected to HPLC analysis. HPLC analyses were performed using an Agilent 1200 HPLC system (Agilent Technologies) with an Agilent 5 μm TC-C18 reverse-phase column (4.6 × 250 mm), run isocratically with a 7:3 (vol/vol) mixture of methanol/water (with 0.01% of phosphoric acid) mobile phase, at a flow rate of 1 mL/min, with UV detection at 280 nm. Authentic standards for cryptotanshinone, tanshinone I, and tanshinone IIA were obtained from Jiangxi Herbal Heavenly TechnologyCo.

1. Li MH, Chen JM, Peng Y, Wu Q, Xiao PG (2008) Investigation of Danshen and related medicinal plants in China. *J Ethnopharmacol* 120(3):419–426.

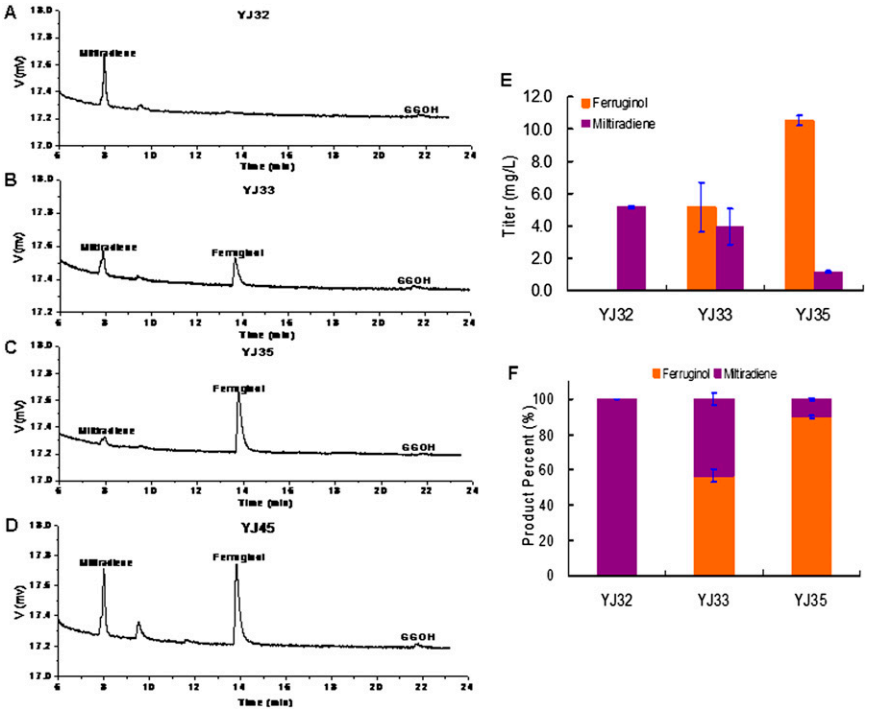

Fig. S5. Inverse correlation between miltiradiene and ferruginol produced by engineered *Saccharomyces cerevisiae* strains. (*A*–*C*) GC profiles of n-hexanae extracts of *S. cerevisiae* YJ32 (*A*), YJ33 (*B*), and YJ35 (*C*); (*D*) GC profiles of n-hexanae extracts produced by engineered *S. cerevisiae* YJ45; (*E*) titer of miltiradiene and ferruginol obtained with different strains; and (*F*) relative contents of miltiradiene and ferruginol in n-hexane extracts from different strains. The terpenoids were extracted as previously described (1) and analyzed by GC using a 7890F system (Techcomp Scientific Instrument Co.) with flame ionization detector, and equipped with an SE-54 column (250 μm × 0.25 μm × 30 m). Column pressure was 0.10 MPa, and carrier gas (N_2) flow rate was 4.5 mL/min. The temperatures for oven, injector, and detector were at 250 °C, 270 °C, and 290 °C, respectively. Miltiradiene was quantified by using ferruginol as a standard.

1. Zhou YJ, et al. (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. *J Am Chem Soc* 134(6):3234–3241.

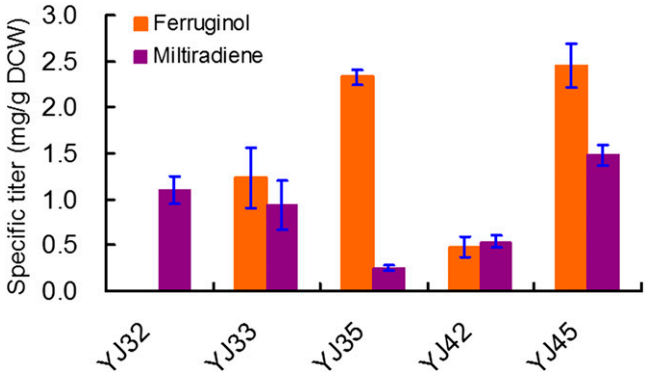

Fig. S6. The specific titers of miltiradiene and ferruginol in engineered *S. cerevisiae* strains. Terpenoids were extracted with n-hexane after cultured for 48 h in YPD medium. The cell dry weight was calculated from a calibration curve relating OD_{600} to dry weight with a factor of one OD_{600} = 0.266 mg dry cell/mL (1).

1. Lee F-J, Hassan H (1987) Biosynthesis of superoxide dismutase and catalase in chemostat culture of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 26(6):531–536.

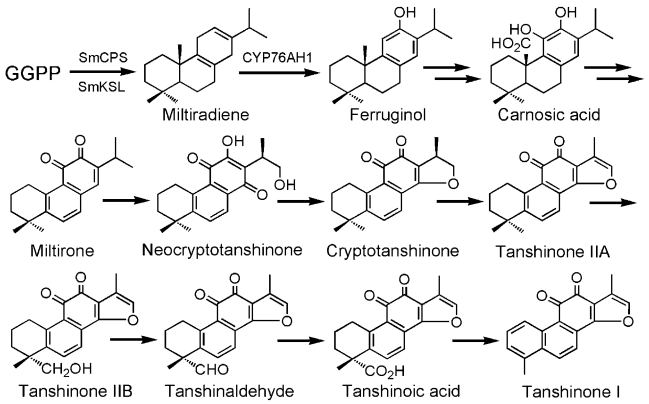

Fig. S7. The production of ferruginol and the hypothesized pathway for tanshinones biosynthesis.

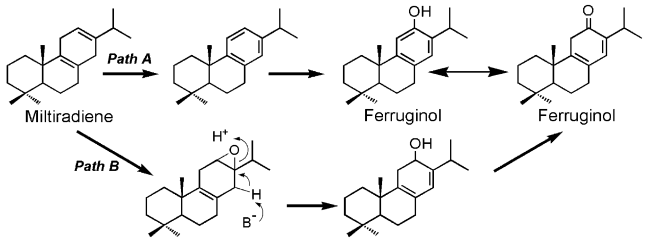

Fig. S8. The proposed mechanism for the conversion of miltiradiene to ferruginol by miltiradiene oxidase CYP76AH1. Path A involves aromatization followed by monooxygenation. Path B involves epoxidation followed by ring-opening and alcohol oxidation.

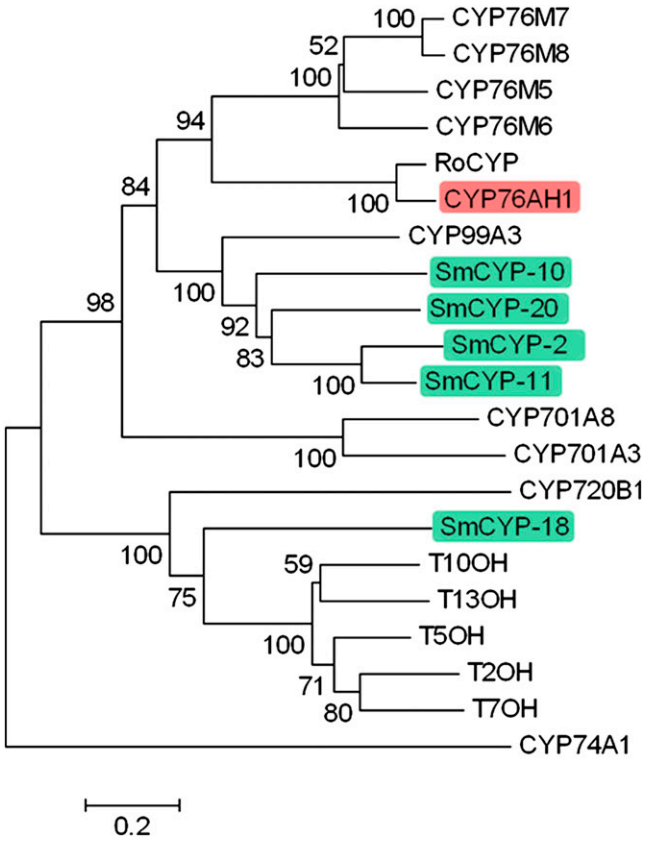

Fig. S9. Molecular phylogenetic analysis of diterpene-modifying cytochrome P450s (CYPs), candidate CYPs from *Salvia miltiorrhiza* and *Rosmarinus officinalis*. *Arabidopsis thaliana* allene oxide synthase (CYP74A1) was used as outgroup. Deduced amino acids of CYPs were aligned by using online CLUSTALW (www.genome.jp/tools/clustalw/). The phylogenetic tree was constructed by the neighbor-joining algorithm using MEGA version 4 with bootstrap of 1000 replicates (1). The CYPs from *Salvia miltiorrhiza* were shown in boxes. The accession nos. of genes used in this analysis are CYP76M5 (AK059010), CYP76M6 (AK101003), CYP76M7 (AK105913), CYP76M8 (AK069701), CYP720B1 (Q50EK6), CYP701A8 (AY579214), CYP701A3 (NP_197962), T2OH (AAS89065), T5OH (AAQ56240), T7OH (AAQ75553), T10OH (AAK00946), T13OH (AAL23619), CYP99A3 (AK071864), and CYP74A1 (CAA63266). The sequence of RoCYP can be downloaded from http://medicinalplantgenomics.msu.edu.

1. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24(8):1596–1599.

Table S1. Primers used in this study

Name	Sequence
TPIp (2, 3, 5)-F	GAATTGGGGATCTACGTATGGTC
FBA1t (2, 3, 5)-R	CTTTAATTTGCGGCCGGTACCCAGTAAGCTACTATGAAAGAC
FBA1t (2, 3, 5)-F	GCAAATAGGTTAATTCAAATTAATTGATATAG
Cyc1t (2, 3, 5)-R	CTTTCATAGTAGCTTACTGGGTACCGGCCGCAAATTAAAGC
TDH3p (2, 3, 5)-F	GTAGAAACATTTTGAAGCTATTCGAGTTTATCATTATCAATAC
TEF1p (2, 3, 5)-F	GATAATGATAAACTCGAATAGCTTCAAAATGTTTCTACTC
TEF1p (2, 3, 5)-R	GGTTGAAGGCGAGCGACATTTTGTAATTAAAACTTAGATTAG
SmCPS (2, 3, 5)-R	GTAACTTAAGGAGTTAAATTCACGCGACTGGCTCGAAAAGC
TDH2t (2, 5,6)-F	CTTTTCGAGCCAGTCGCGTGAATTTAACTCCTTAAGTTACTTTAATG
TDH2t (2, 3, 5)-R	CACCCAATTGTAGATATGCGCGAAAAGCCAATTAGTGTG
ADH1t (2, 3,6)-R	CACTAATTGGCTTTTCGCGCATATCTACAATTGGGTGAAATG
ADH1t (2, 3, 5)-F	CCCAATAGTTAAGTCTTAAGCGAATTTCTTATGATTTATG
CYP76AH1 (2, 3, 5)-R	CATAAATCATAAGAAATTCGCTTAAGACTTAACTATTGGGATAATC
CYP76AH1 (2, 3, 5)-F	CTAATCTAAGTTTTAATTAAAAAATGGATTCTTTTCCTCTCCTC
TEF1p (2, 3, 5)-R	GAGGAGAGGAAAAGAATCCATTTTGTAATTAAAACTTAGATTAG
TEF1p (2, 3, 5)-F3	GATGTTATAATATCTGTGCGTATAGCTTCAAAATGTTTCTA
PGK1p (2, 3, 5)-F	GTAGAAACATTTTGAAGCTATACGCACAGATATTATAACATC
PGK1p (2)-R	CGTAAGCTTGTGGGCCCTATTATATTTGTTGTAAAAAGTAG
pYX212 (2)-F	CTACTTTTTACAACAAATATAATAGGGCCCACAAGCTTACGCGTC
pGK1p (5)-R	CTTCGACGAGGGTTCCATTTTGTTATATTTGTTGTAAAAAGTAG
CPR1 (5)-F	CTTTTTACAACAAATATAACAAAATGGAACCCTCGTCGAAGAAG
CPR1 (5)-R	CGTAAGCTTGTGGGCCCTATTACCATACATCGCGCAAG
pYX212 (5)-F	CTTGCGCGATGTATGGTAATAGGGCCCACAAGCTTACGCGTC
pGK1p (3)-R	CACGGAAGTAGATTCCATTTTGTTATATTTGTTGTAAAAAGTAG
CPR2 (3)-F	CTTTTTACAACAAATATAACAAAATGGAATCTACTTCCGTGAAG
CPR2 (3)-R	CGTAAGCTTGTGGGCCCTATTACCATACATCGCGCAAGTAC
pYX212 (3)-F	GTACTTGCGCGATGTATGGTAATAGGGCCCACAAGCTTACGCGTC
pYX212t (2, 5, 3)-R	TGCCGTAAACCACTAAATCGGAACC
CYP76AH1-EcoRI	GCGAATTCATGGATTCTTTTCCTCT
CYP76AH1-SpeI	AAACTAGTGCCTAAGACTTAACTATTGG
SmCYP-2-EcoRI	GCGAATTCATGGAGTTTAACATCTC
SmCYP-2-SpeI	AAACTAGTGCTCAAGCAGCTGCGGACAAAG
SmCYP-10-EcoRI	GCGAATTCATGGAGAGGGATCTCCCCTT
SmCYP-10-ClaI	AAATCGATTTAGTTGACGGGATTGCAGAG
SmCYP-11-EcoRI	GCGAATTCATGGAGTTCAACATCCCATC
SmCYP-11-SpeI	AAACTAGTTCAAGCAGCTGCACGCAAAGG
SmCYP-18-EcoRI	GCGAATTCATGGAAGTCTTGTACATGTC
SmCYP-18-SpeI	AAACTAGTTCAAGATCGGTGAGGGAAGAG
SmCYP-20-EcoRI	GCGAATTCATGGAGATCCAAATTCCATC
SmCYP-20-SpeI	AAACTAGTGCTTAATTAATAGGCAAAGGTC

Primer names used for engineering yeast construction were expressed in a format of "part name-F" and "part name-R," and the numbers in parentheses represent the strain numbers shown in Fig. 5. For example, "TPIp (2, 3, 5)-F" stands for the forward primer to amplify triosephosphate isomerase (TPI) gene promoter, which is used for the pathway construction of strain YJ32, YJ33, and YJ35. The primer names used for subcloning of the six candidate CYPs into pESC-His were expressed in a format of "gene name-restriction enzyme cutting site."

Table S2. *S. cerevisiae* strains used in this study

Strains	Genotype or characteristic	Source
BY4741	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	ATCC
BY4741(ML)	BY4741complemented with *LEU2* and *MET15*	This study
WAT11U	*MATa; ade2–1; his3–11,-15; leu2–3,-112; ura3–1; canR; cyr*$^{+}$*; AtCPR1*	(1)
YJ32	BY4741 (ML)/ pYX212-(*BTS1-ERG20*)+(*SmKSL-SmCPS*)+CYP76AH1	This study
YJ33	BY4741 (ML)/ pYX212-(*BTS1-ERG20*)+(*SmKSL-SmCPS*)+*CYP76AH1*+*SmCPR2*	This study
YJ35	BY4741 (ML)/ pYX212-(*BTS1-ERG20*)+(*SmKSL-SmCPS*)+*CYP76AH1*+*SmCPR1*	This study
YJ42	WAT11U/ pYX212-(*BTS1-ERG20*)+(*SmKSL-SmCPS*)+*CYP76AH1*	This study
YJ45	WAT11U/ pYX212-(*BTS1-ERG20*)+(*SmKSL-SmCPS*)+*CYP76AH1*+*SmCPR1*	This study

1. Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. *J Biol Chem* 272(31):19176–19186.