Supporting Online Material for

Complete biosynthesis of opioids in yeast

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Materials and Methods

Chemicals, media, and strain cultivation

Difco yeast nitrogen base without amino acids and ammonium sulfate (YNB), Bacto peptone, Bacto yeast extract, Luria Broth (LB), LB agar, dextrose, and galactose were obtained from Becton, Dickinson and Company (BD). Kanamycin monosulfate, geneticin sulfate (G418), ampicillin, spectinomycin, amino acids, uracil, adenine hemisulfate, tris(hydroxymethyl)aminomethane hydrochloride, polysorbate 20 (Tween-20), ascorbic acid, and LC-MS grade methanol were obtained from EMD chemicals. LC-MS grade formic acid, acetonitrile, isopropanol, and *n*-hexane were obtained from Thermo Fisher Scientific. 1-(3,4-dihydroxybenzyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol hydrobromide (tetrahydropapaveroline or norlaudanosoline, NL) was obtained from Santa Cruz Biotechnology. Phleomycin was purchased from InvivoGen. Amberlite XAD-4 resin, LC-MS grade diethylamine, and hygromycin B were purchased from Sigma. The authentic chemical standards (*S*)-reticuline perchlorate and salutaridine were obtained from Specs; (*R*)-reticuline and 1,2-dehydroreticulinium iodide from Toronto Research Chemicals; and 3,4-dihydroxy-L-phenylalanine (L-DOPA), dopamine hydrochloride, thebaine, and hydrocodone bitartrate from Sigma.

E. coli strains were selected on LB agar plates with 50 mg/L kanamycin, 50 mg/L ampicillin, or 100 mg/L spectinomycin and grown in LB liquid media with the appropriate antibiotic. Yeast 10x drop out (DO) supplement was prepared as synthetic complete supplement with the desired selection component omitted. *S. cerevisiae* strains were selected on YNB-DO (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose, and 1x DO) agar or on YPAD (1% yeast extract, 2% peptone, 80 mg/L adenine

hemisulfate, and 2% dextrose) agar with 200 mg/L G418, 200 mg/L hygromycin B, or 10 mg/L phleomycin. Yeast were grown in selective YNB-DO media or in YPAD media.

Strains and plasmids

E. coli strain TOP10 (Life Technologies) was used for cloning and amplification of plasmids. Plasmids were recovered using Econospin columns (Epoch Life Sciences) according to manufacturer's instructions. *S. cerevisiae* strain W303α was used as the base strain for engineered strains in Figs. 2-3 and figs. S7-8. *S. cerevisiae* strain CEN.PK2-1Dα was used as the base strain for reticuline-producing platform strains and strains in Figs. 1 and 4 and fig. S3 (table S3). Oligonucleotide primer sequences are provided in table S7. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT) or the Stanford Protein and Nucleic Acid Facility. Heterologous gene sequences were cloned from previously published plasmids or downloaded from Genbank, the 1000 Plants Project, or PhytoMetaSyn, yeast codon-optimized (*35*), and synthesized by Life Technologies or IDT (table S1 and S8). PfuUltraII Fusion HS DNA polymerase (Agilent Technologies) for <3 kb fragments, Platinum Taq PCR SuperMix (Life Technologies) for site-directed mutagenesis, and Expand High Fidelity PCR system for >3 kb fragments (Roche Diagnostics) were used for PCR amplifications according to manufacturer's instructions. PCR products were purified by agarose gel extraction with Zymoclean gel DNA recovery kit (Zymo Research) according to manufacturer's instructions. Restriction enzymes, T4 DNA ligase, and deoxynucleotides were purchased from New England Biolabs.

Strains and plasmids used or constructed in this work are described in table S3. Plasmids pCS3300-3305 and pCS3313-3340 were constructed by amplifying the insert with either a CACC 5' overhang or 5' and 3' BP sequences and using the pENTR/D-TOPO cloning kit or Gateway BP clonase II and pDONR221 (Life Technologies), respectively, to create a Gateway entry vector. The insert was then cloned into a pAG destination vector (pAG416GPD-ccdB, #14148 or pAG416GPD-ccdB-HA, #14244) obtained from Addgene via Susan Lindquist (*43*) using Gateway LR clonase II (Life Technologies). Constructs were verified by sequencing through the inserted region (Elim Biopharmaceuticals). Plasmids were introduced into yeast by the lithium acetate/salmon sperm carrier DNA/polyethylene glycol transformation method (*44*).

Holding vectors (pCS2803, 3028, 3030, 3040, 3041, 3138, 3271-3273, 3289-3298, and 3343-3350) were constructed by amplifying the backbone, including the promoter and terminator, and assembling with the insert via Gibson cloning (*45*). Expression cassettes were amplified using primers with 15 bp overhangs for gap repair in yeast. 100 ng each of the PCR products and linearized pYES1L (Life Technologies) were introduced by electroporation into the desired yeast background strain to make constructs pCS3308-3311 (*13, 46*). Plasmids were isolated from yeast and amplified in *E. coli* as previously described.

Yeast chromosomal modifications were made by the microhomology gene disruption method and DNA assembler (*47, 48*). Expression cassettes were amplified using primers with 15-40 bp overhangs for gap repair in yeast. Each assembly was flanked by an integration homology region of 50-500 base pairs. We selected chromosomal regions from which we expected no growth defect and active expression as

integration loci (*23-25*). 100-300 ng of each of the PCR products were introduced by electroporation into the desired yeast background strain to make strains CSY1055-1061, CSY1064, and CSY1065. All modifications were verified by yeast colony PCR as previously described, with the exclusion of gelatin from buffers (*49*).

Agrobacterium tumefaciens strain GV3101 (Inzé laboratory, Ghent University) and 4-6 week old *Nicotiana benthamiana* plants were provided by the Sattely laboratory (Stanford University), and plant expression vectors pEAQ-HT and pEAQ-HT-GFP (pCS3352) (*50*) were provided by the Lomonossoff laboratory (John Innes Centre). Wild-type *P. somniferum* SalSyn was cloned from GFC55 in pVL1392 (*51*), provided by the Kutchan laboratory (Donald Danforth Plant Center). Please note that opiate-producing yeast strains can only be shared with laboratories that possess appropriate DEA registration and permits.

Yeast cultivation assays for benzylisoquinoline alkaloid production

Single colonies of freshly transformed or freshly streaked yeast strains were inoculated into 3 mL in 16 x 150 mm glass culture tubes or 0.5 mL in a 96-well plate of the appropriate YNB-DO media and grown for 17 h at 30 °C in a shaking incubator at 260 rpm. Overnight cultures were back-diluted 1:20 into assay media composed of YNB-SC or DO media. Where indicated, media lacked ammonium sulfate and/or was supplemented with 10 mM ascorbic acid, 50 mM 2-oxoglutarate, and/or substrate to a total volume of 500 µL in culture tubes or plates. Ascorbic acid is a general antioxidant and was previously reported to stabilize the catecholic molecule norlaudanosoline in *E. coli* cultures (*52*) and BH_4, the tyrosine hydroxylase cofactor in this engineered pathway

(*18, 53*). 4-HPAA is produced by catabolism of L-tyrosine for nitrogen assimilation via the Ehrlich pathway. Therefore, limiting the nitrogen source ammonium sulfate in the media upregulates expression of endogenous yeast genes associated with amino acid catabolism and 4-HPAA production (*54*). Thus, both of these changes to the media are directed toward increasing precursor supply to the pathway. 2-Oxoglutarate is a co-substrate for T6ODM (*55*).

Cultures were grown for 72-120 h and then pelleted by 5 min centrifugation. The supernatant was analyzed by high performance liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (LC-MS/MS MRM).

Analysis of benzylisoquinoline alkaloid production by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Yeast growth media supernatant was analyzed by LC-MS/MS using an Agilent 1260 infinity binary pump HPLC and Agilent 6420 triple quadrupole mass spectrometer with an electrospray ionization source. Mobile phase A was water with 0.1% formic acid (FA), phase B was acetonitrile with 0.1% FA, and the flow rate was 0.4 mL/min. For analysis of L-DOPA and dopamine (method A), chromatography was performed with a Pursuit PFP column (2.1 x 150 mm, 3 μm, Agilent Technologies), 15 μL injection volume, and the following method: 2% B for 0.5 min, 2-60% B for 1 min, 60% B for 2.5 min, and re-equilibrate for 3 min. For all other compounds, 5 μL samples were injected on a a Zorbax Eclipse Plus C18 column (2.1 x 50 mm, 1.8 μm, Agilent Technologies) and separated with one of the following methods: for norlaudanosoline feeding assays (method B), 10-35% B for 4 min, 35-98% B for 0.5 min, held at 98% B for 1 min,

returned to 2% B over 0.6 min, then re-equilibrate with 10% B for 2 min; for *de novo* thebaine production assays (method C), 3% B for 0.1 min, 3-35% B for 4.9 min, 35-98% B for 1 min, hold 98% B for 1 min, re-equilibrate with 3% B for 2 min; and for analysis of opioid drugs (method D), 5-35% B for 5.5 min, 35-90% B for 0.5 min, hold 90% B for 1 min, decrease to 5% B over 0.5 min, the hold for 2 min re-equilibration. The LC eluent was directed to the MS for 1-5 min with ESI source gas temperature 350 °C, gas flow of 11 L/min, nebulizer pressure 40 PSI, capillary voltage of 3500 V, and Delta EMV (+) of 200. Compound identity was confirmed by comparing the retention time and either product ion spectrum or multiple MRM transitions to an authentic standard when available or to published mass fragmentation spectra (*13, 27, 56-58*) using MassHunter Qualitative Analysis v. B.06 (Agilent Technologies). For quantification, the MS was used in MRM mode to monitor the transitions in table S2. These transitions were determined using the MassHunter Optimizer software with standards when available or with samples with high abundance and confirmed with literature. The data was analyzed using MassHunter Quantitative Analysis v. B.07 for QQQ (Agilent Technologies) to identify and integrate peaks with the correct ratio of qualifying and quantifying mass transitions and correct retention times (table S2). Quantifier MRM peak areas were compared to a calibration curve of external standard peak areas to determine concentration. For compound characterization the MS was either used in MRM mode with a constant collision energy to monitor four to eight transitions and generate an MRM spectrum or used in product ion scan mode to detect all fragments resulting from a specified collision energy to generate a product ion spectrum.

For chiral characterization in norlaudanosoline-fed experiments, reticuline was

concentrated from yeast media by pelleting 12.5 mL yeast culture and adding 30 mg XAD-4 resin per mL supernatant, incubating on a rotator overnight at room temperature, and eluting with 100 μL methanol per mL supernatant. For chiral characterization in *de novo* experiments, reticuline was concentrated from yeast media by pelleting 500 mL yeast culture, adjusting the pH to 8 with NH_4OH, and extracting four times with 100 mL dichloromethane. Dichloromethane was removed under vacuum and the residue was resuspended in 5 mL water with 0.1% formic acid. Both concentrates were fractionated by reverse-phase HPLC (Pursuit XRs-C18, 50 mm × 10 mm, 5 μm) with isocratic 15% methanol with 0.1% formic acid over 6.5 min with a flow rate of 5 mL/min and injection volume of 40-60 μL. Peak-based fractions were collected at approximately 4.5 min. Fractions were pooled, freeze-dried, and resuspended in 125 μL isopropanol for fed experiments or 300 μL for *de novo* experiments. Depending on concentration, 1-5 μL were injected onto a chiral column (Phenomenex Lux cellulose-1, 150 mm x 2 mm, 3 μm) and separated with isocratic 72% N-hexane, 28% isopropanol, 0.1% diethylamine with a flow rate of 0.3 mL/min and detection by MS and 250 nm UV. MS detection was performed with an Agilent 6320 Ion Trap mass spectrometer with ESI source gas temperature 350 °C, gas flow of 10 L/min, nebulizer pressure 40 PSI and isolation of *m/z* 330.1 with width 1.0. The retention time of reticuline peaks was compared to that of authentic (*S*)-reticuline and (*R*)-reticuline standards. Individual chromatograms from chiral analysis were smoothed using a 7-point boxcar moving average algorithm (http://terpconnect.umd.edu/~toh/spectrum/smoothing.xls).

Identification of reticuline epimerase and enzyme variants from transcriptomes

A previously published codeinone reductase (COR) virus-induced gene silencing (VIGS) construct (*31*) that resulted in reticuline accumulation was used to query the *P. bracteatum P. setigerum*, *P. somniferum*, *P. rhoeas* 1000 Plants Project (*32*) and *P. bracteatum* PhytoMetaSyn (*33, 34*) transcriptomes and all deposited sequences in Genbank belonging to *Papaveraceae* using blastn. The hits with E<0.1 were in turn used as blastn queries of all Genbank sequences belonging to taxid: Ranunclales. The best reciprocal blast hit for each transcriptome hit was reviewed to determine encoded conserved protein domains. Once one DRS-DRR fusion sequence was observed as a hit, that sequence (Pbr.89405) was translated and the amino acid sequence was used as the query for a second search of both databases with tblastn. A phylogenetic tree of the sequences identified from the initial database search, including those identified as DRS-DRR fusion enzymes, is provided in Fig. 2B. A phylogenetic tree of the additional DRS-DRR variants identified in the second database search is provided in fig. S6. Phylogenetic trees were generated using ClustalX bootstrap NJ tree with 1000 trials, and visualized with FigTree. The sequences of the synthesized codon-optimized variants tested (Pbr.89405, Pbr. 12180, Pbr.4328, and Pso.2062398) are provided in table S8.

The SalSyn variant from *P. bracteatum* (PbSalSyn) was identified by querying the 1000 Plants Project *P. bracteatum* transcriptome with tblastn using the protein sequence for PsSalSyn and identifying the best hit by reciprocal blast. The coding sequence was determined from trancriptome sequence TMWO-2131695, codon-optimized for yeast, synthesized, and cloned to make pCS3335.

Western blot analysis

Western blot analysis of yeast expressing C-terminally human influenza hemagglutinin epitope-tagged (3xHA) salutaridine synthase variants (pCS3313-3321) was performed as previously described (*59*) with Novex NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) in place of Laemmli buffer. Plant samples were prepared from 4-6 week old tobacco (*Nicotiana benthamiana*) leaves 96 h after infusion *with A. tumefaciens* strain GV3101 with a GFP expressing plasmid (pCS3352) or PsSalSyn-3xHA plasmid (pCS3312). Three leaves were homogenized to powder under liquid nitrogen with mortar and pestle and resuspended in 1 mL 30 mM potassium phosphate buffer, pH 8. A volume of this crude lysate equivalent to 80 µg supernatant protein (determined by Bradford assay) was added to 12.5 µL 4x LDS buffer and adjusted with water to 50 µL final volume. Novex sharp pre-stained protein standard and 12 µL samples, equivalent to 0.6 mg yeast wet weight (~20 µg protein) or 20 µg plant supernatant protein, were loaded on a NuPage Novex 4-12% Bis-Tris protein gel and run with MOPS SDS running buffer (Life Technologies) at 150 V for 50-90 min. Semi-dry transfer to a nitrocellulose membrane was performed at 15 V for 15 min with transfer buffer (Life Technologies). The membrane was blocked with 5% BSA and probed overnight at 4 °C with anti-HA horseradish peroxidase (HRP) (ab1188, Abcam) (1:5,000). Chemiluminescence was induced by SuperSignal West Pico substrate (Pierce) and images were acquired by a G:Box Chemi XT4 imaging system (Syngene).

Supplementary Tables

Table S1. Expression cassettes used in this study.

Module	Plasmid template	Cassette	Source organism	GenBank ID (protein)	DNA coding sequence reference or entry in Table S8
1	pCS3028	*ARO4*^{Q166K} (with ARO4 native promoter and terminator)	*S. cerevisiae*	AJQ16674.1	(*60*)
1	pCS3030	*ARO7*^{T226I} (with ARO7 native promoter and terminator)	*S. cerevisiae*	NP_015385	(*61*)
1	pCS2922	*hphNTI* selection marker (Hyg^{R}) flanked by loxP	*K. pneumoniae*	AEG42736	(*62*)
1	pCS784	P_{TEF1}-*ARO10*-T_{CYC1}	*S. cerevisiae*	NP_010668	native
1	pCS3289	P_{TDH3}-*TKL1*-T_{ADH1}	*S. cerevisiae*	NP_015399	native
2	pCS3040	P_{TPI1}-*yRnSpr*-T_{STE2}, P_{TEF1}-*yRnPts*-T_{CYC1}	*R. norvegicus*	AAA42130, AAH59140	(*18*)
2	pCS270	KanMX (G418^{R}) selection marker flanked by loxP	*Tn 903*	AF298793	(*63*)
2	pCS3041	P_{TDH3}-*yRnQdpr*-T_{ADH1}, P_{PGK1}-*yRnPcbd1*-T_{PHO5}	*R. norvegicus*	P11348, NP_001007602	(*18*)
3, 5	pCS3290	P_{TDH3}-*RnTh*^{WR}-T_{ADH1}	*R. norvegicus*	NP_036872	(*64*)
3	pCS3291	P_{TPI1}-*yPpddc*-T_{STE2}	*P. putida*	AAN68161	(*18*)
3	pCS271	*HIS5* selection marker flanked by loxP	*S. pombe*	AF298790	(*63*)
3	pCS3292	P_{TEF1}-*yRnDhfr*-T_{CYC1}	*R. norvegicus*	AF318150	(*18*)
3	pCS3293	P_{PGK1}-*yCjNCS*-T_{PHO5}	*C. japonica*	BAF45338	(*18*)
4, 5	pCS3294	P_{PYK1}-*PsCNMT*-T_{MFa1}	*P. somniferum*		(*11*)
4	pCS3138	P_{PGK1}-*Ps6OMT*-T_{PHO5}	*P. somniferum*	AAP45315	(*11*)
4	pCS3295	P_{TDH3}-*yEcNMCH*-T_{ADH1}	*E. californica*	AF014801	(*18*)
4	pCS274	*LEU2* selection marker flanked by loxP	*K. lactis*	AF298792	(*63*)
4	pCS3296	P_{TEF1}-*yPsCPR*-T_{CYC1}	*P. somniferum*	AAC05021	(*18*)
4	pCS2803	P_{TPI1}-*yPs4'OMT*-T_{STE2}	*P. somniferum*	AAP45314	(*18*)
5	pCS3298	P_{TEF1}-*Ps4'OMT*-T_{CYC1}	*P. somniferum*	AAP45314	(*11*)
5	pCS272	Ble (Phleo^{R}) selection marker flanked by loxP	*Tn 5*	AF298794	(*63*)
6	pCS273	*URA3* selection marker flanked by loxP	*K. lactis*	AF298788	(*63*)
6	pCS3273	P_{TDH3}-*yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-T_{ADH1}	*E. californica* and *P. bracteatum*	B5UAQ8.1 and no ID	(*14*) and S8
6	pCS3272	P_{PGK1}-*yPsSalAT*-T_{PHO5}	*P. somniferum*	Q94FT4.1	(*65*)
6	pCS3271	P_{TPI1}-*yPbSalR*-T_{STE2}	*P. bracteatum*	A4UHT7.1	(*65*)
6	pCS3300	P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}	*P. bracteatum*	no ID	S8
7	pCS2656	P_{TDH3}-*yPsT6ODM*-T_{ADH1}	*P. somniferum*	ADD85329.1	(*13*)
7	pCS2663	P_{PGK1}-*yPpmorB*-T_{PHO5}	*P. putida*	AAC43569.1	(*13*)
n/a	pCS3343	P_{TPI1}-*yPsSalR*^{F104A}-T_{STE2}	*P. somniferum*	3O26_A	(*36, 65*)
n/a	pCS3344	P^{TPI1}-*yPsSalR*^{I275A}-T_{STE2}	*P. somniferum*	3O26_A	(*36, 65*)
n/a	pCS3345	P_{TPI1}-*yPbSalR*^{F104A}-T_{STE2}	*P. bracteatum*	A4UHT7.1	(*36, 65*)
n/a	pCS3346	P_{TPI1}-*yPbSalR*^{I275A}-T_{STE2}	*P. bracteatum*	A4UHT7.1	(*36, 65*)

n/a	pCS3347	P_{PGK1}-*yPbSalAT-1*-T_{PHO5}	*P. bracteatum*	ACI45392	S8
n/a	pCS3348	P_{PGK1}-*yPbSalAT-2*-T_{PHO5}	*P. bracteatum*	ACI45393	S8
n/a	pCS3349	P_{PGK1}-*yPoSalAT*-T_{PHO5}	*P. orientale*	ACI45395	S8
n/a	pCS3350	P_{PGK1}-*yPsAT1*-T_{PHO5}	*P. somniferum*	AFB74620	Yanran Li, unpublished

Table S2. Retention times and MRM transitions used to quantify alkaloids in LC-MS/MS analysis.

			Quantifier MRM Transition			**Qualifier MRM Transition**		
Compound	Method	Rt (min)	Precursor → Product Ion	Fragmentor	CE	Precursor → Product Ion	Fragmentor	CE
L-DOPA	A	2.04	198→152	135	20			
Dopamine	A	2.11	154→91	135	20			
Reticuline	B	2.46	330→192	120	19	330→137	120	31
Thebaine	B	3.11	312→251	102	22	312→221	102	14
3'-Hydroxy-*N*-methylcoclaurine	C	3.05	316→192	135	25			
Reticuline	C	3.92	330→192	120	19	330→177	120	43
1,2-Dehydroreticuline	C	3.61	328→312	172	34	328→284	172	34
Salutaridine	C	3.77	328→237	126	23	328→211	126	23
Thebaine	C	4.47	312→251	102	22	312→221	102	14
Reticuline	D	3.74	330→192	120	19	330→177	120	43
Thebaine	D	4.38	312→251	102	22	312→221	102	14
Hydrocodone	D	3.03	300→199	183	31	300→213	183	31

Table S3. Plasmids and *S. cerevisiae* strains used in this study.

Plasmid	**Description**	**Source**
pCS8	pRS316, CEN/ARS vector, *URA3* selectable marker	(*66*)
pCS2765	YAC vector pYES1L, P_{TDH3}-*T6ODM*-T_{ADH1}+P_{PGK1}-*morB*-$T_{PHO5,}$ *TRP1* selectable marker	(*13*)
pCS3352	pEAQ-HT-GFP	(*50*)
pCS3300	CEN/ARS vector, P_{HXT7}-*yPbDRS-DRR Pbr.89405*-T_{CYC1}, *URA3* selectable marker	this work
pCS3301	CEN/ARS vector, P_{TDH3}-*yPbDRS-DRR Pbr.89405*-T_{CYC1}, *URA3* selectable marker	this work
pCS3302	CEN/ARS vector, P_{HXT7}-*yPsDRS-DRR Pso.2062398*-T_{CYC1}, *URA3* selectable marker	this work
pCS3303	CEN/ARS vector, P_{TDH3}-*yPsDRS-DRR Pso.2062398*-T_{CYC1}, *URA3* selectable marker	this work
pCS3304	CEN/ARS vector, P_{HXT7}-*yPbDRS-DRR Pbr.12180*-T_{CYC1}, *URA3* selectable marker	this work
pCS3305	CEN/ARS vector, P_{TDH3}-*yPbDRS-DRR Pbr.12180*-T_{CYC1}, *URA3* selectable marker	this work

pCS3308	YAC vector pYES1L, P_{TDH3}-*yPsSalSyn*-T_{ADH1}+P_{PGK1}-*yPsSalAT*-T_{PHO5}+P_{TPI1}-*yPbSalR*-T_{STE2}, *TRP1* selectable marker	this work
pCS3309	YAC vector pYES1L, P_{TDH3}-*PsSalSyn*-T_{ADH1}+P_{PGK1}-*yPsSalAT*-T_{PHO5}+P_{TPI1}-*yPbSalR*-T_{STE2}+P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}, *TRP1* selectable marker	this work
pCS3310	YAC vector pYES1L, P_{TDH3}-*yPsSalSyn*-T_{ADH1}+P_{PGK1}-*yPsSalAT*-T_{PHO5}+P_{TPI1}-*yPbSalR*-T_{STE2}+P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}, *TRP1* selectable marker	this work
pCS3311	YAC vector pYES1L, P_{TDH3}- *yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-T_{ADH1}+P_{PGK1}-*yPsSalAT*-T_{PHO5}+P_{TPI1}-*yPbSalR*-T_{STE2}+P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}, *TRP1* selectable marker	this work
pCS3312	pEAQ-*PsSalSyn-3xHA*	this work
pCS3313	CEN/ARS vector, P_{TDH3}-*PsSalSyn-3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3314	CEN/ARS vector, P_{TDH3}-*yPsSalSyn-3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3315	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N105A}-*3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3316	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N331A}-*3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3317	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N105AN331A}-*3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3318	CEN/ARS vector, P_{TDH3}-*yPbSalSyn-3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3319	CEN/ARS vector, P_{TDH3}-*yEcCFS-3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3320	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPsSalSyn*$^{95-505}$-*3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3321	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-*3xHA*-T_{CYC1}, *URA3* selectable marker	this work
pCS3322	CEN/ARS vector, P_{TDH3}-*PsSalSyn*-T_{CYC1}, *URA3* selectable marker	this work
pCS3323	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*-T_{CYC1}, *URA3* selectable marker	this work
pCS3324	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N105A}-T_{CYC1}, *URA3* selectable marker	this work
pCS3325	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N331A}-T_{CYC1}, *URA3* selectable marker	this work
pCS3326	CEN/ARS vector, P_{TDH3}-*yPsSalSyn*^{N105AN331A}-T_{CYC1}, *URA3* selectable marker	this work
pCS3327	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-23}$-*yPsSalSyn*$^{33-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3328	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-26}$-*yPsSalSyn*$^{36-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3329	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-31}$-*yPsSalSyn*$^{42-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3330	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-33}$-*yPsSalSyn*$^{44-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3331	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-40}$-*yPsSalSyn*$^{51-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3332	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-66}$-*yPsSalSyn*$^{78-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3333	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPsSalSyn*$^{95-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3334	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-90}$-*yPsSalSyn*$^{102-505}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3335	CEN/ARS vector, P_{TDH3}-*yPbSalSyn*-T_{CYC1}, *URA3* selectable marker	this work
pCS3336	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-23}$-*yPbSalSyn*$^{30-504}$-T_{CYC1}, *URA3* selectable marker	this work

pCS3337	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-26}$-*yPbSalSyn*$^{33-504}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3338	CEN/ARS vector, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3339	CEN/ARS vector, P_{TDH3}-*BM3*$^{1-6}$-*yPsSalSyn*$^{44-486}$-*BM3*$^{449-1049}$-T_{CYC1}, *URA3* selectable marker	this work
pCS3340	CEN/ARS vector, P_{TDH3}-*BM3*$^{1-6}$-*yPbSalSyn*$^{41-495}$-*BM3*$^{449-1049}$-T_{CYC1}, *URA3* selectable marker	this work

Strain	Genotype	Source
CSY3	W303 (*Matα, ade2–1; ura3–1; his3–11,15; trp1–1; leu2–3,112; can 1–100*)	K. Weis
CSY288	CSY3 *his3Δ*::P_{TEF1}-*Ps6OMT*-T_{CYC1}, *leu2Δ*::P_{TEF1}-*PsCNMT*-T_{CYC1}, *ura3Δ*::P_{TEF1}-*Ps4'OMT*-T_{CYC1}	(*11*)
CSY893	CEN.PK2-1D *(Matα; ura3-52; trp1-289; leu2-3,112; his3Δ1; MAL2-8^{C}; SUC2)*	EUROSCARF acc. No. 30000B
CSY1055	CSY893 *ybr197cΔ::*P_{TPI1}-*yRnSpr*-T_{STE2}, P_{TEF1}-*yRnPts*-T_{CYC1}, *KanMX*, P_{TDH3}-*yRnQdpr*-T_{ADH1}, P_{PGK1}-*yRnPcbd1*-T_{PHO5}	this work
CSY1056	CSY1055 *ydr514cΔ::*P_{PYK1}-*PsCNMT*-T_{MFa1}, P_{PGK1}-*Ps6OMT*-T_{PHO5}, P_{TDH3}-*yEcNMCH*-T_{ADH1}, *LEU2*, P_{TEF1}-*yPsCPR*-T_{CYC1}, P_{TPI1}-*yPs4'OMT*-T_{STE2}	this work
CSY1057	CSY1056 *ymr206wΔ::*P_{TDH3}-*RnTh*^{WR}-T_{ADH1}, P_{TPI1}-*yPpddc*-T_{STE2}, *HIS5*, P_{TEF1}-*yRnDhfr*-T_{CYC1}, P_{PGK1}-*yCjNCS*-T_{PHO5}	this work
CSY1058	CSY1057 *ybl059wΔ::ARO4*^{Q166K}, *ARO7*^{T226I}, *hphNTI*, P_{TDH3}-*TKL1*-T_{ADH1}	this work
CSY1059	CSY1057 *ybl059wΔ::ARO4*^{Q166K}, *ARO7*^{T226I}, *hphNTI*, P_{TEF1}-*ARO10*-T_{CYC1}, P_{TDH3},*TKL1*-T_{ADH1}	this work
CSY1060	CSY1059 *ypl250cΔ::*P_{TDH3}-*RnTh*^{WR}-T_{ADH1}, P_{TEF1}-*Ps4'OMT*-T_{CYC1}, P_{PGK1}-*yCjNCS*-T_{PHO5}, *ble*	this work
CSY1061	CSY1059 *zwf1Δ::* P_{TDH3}-*RnTh*^{WR}-T_{ADH1}, P_{TEF1}-*Ps4'OMT*-T_{CYC1}, P_{PGK1}-*yCjNCS*-T_{PHO5}, *ble*	this work
CSY1064	CSY1060 *trp1Δ::*P_{PGK1}-*yPsSalAT*-T_{PHO5}, P_{TPI1}-*yPbSalR*-T_{STE2}, *URA3*, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-T_{ADH1}, P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}	this work
CSY1065	CSY1061 *trp1Δ::*P_{PGK1}-*yPsSalAT*-T_{PHO5}, P_{TPI1}-*yPbSalR*-T_{STE2}, *URA3*, P_{TDH3}-*yEcCFS*$^{1-83}$-*yPbSalSyn*$^{92-504}$-T_{ADH1}, P_{HXT7}-*yPbDRS-DRR (Pbr.89405)*-T_{CYC1}	this work
CSY1071	CSY288 *trp1Δ::*P_{TEF1}-*yPsCPR*-T_{CYC1}	this work

Table S4. Intermediate analysis for optimization of the reticuline-producing platform strain through pathway and strain engineering. Reticuline in the growth media was analyzed by LC-MS/MS MRM and quantified with an external standard curve. Error is the standard deviation of three biological replicates. This data is depicted in Fig. 1B.

Molecule (μg/L)	CSY1057	1058	1059	1060	1061
L-DOPA	NM	NM	88 ± 16	60 ± 30	70 ± 40

Dopamine	NM	NM	9600 ± 1400	9200 ± 200	12000 ± 2000
3'-Hydroxy-*N*-methylcoclaurine[a]	1.0 ± 0.2	31.2 ± 1.1	28 ± 2	8.5 ± 1.0	14.9 ± 0.4
Reticuline	12.28 ± 0.04	20.7 ± 0.2	20.0 ± 0.3	68 ± 2	82 ± 3

NM indicates not measured.
[a]Relative MRM peak area, where 1 is the peak area for CSY1057.

Table S5. Intermediate analysis for comparison of thebaine produced from SalSyn variants in yeast. Yeast strains were fed 1 mM *rac*-norlaudanosoline, and thebaine in the growth media was quantified by LC-MS/MS MRM with an external standard curve. Error is the standard deviation of at least three biological replicates. This data is depicted in Fig. 3C.

Molecule (μg/L)	CSY1071+pCS3309 (PsSalSyn)	yPsSalSyn	yEcCFS$^{1-83}$-yPbSalSyn$^{92-504}$
Reticuline	660 ± 50	570 ± 40	480 ± 100
1,2-Dehydroreticuline[a]	1.00 ± 0.10	1.02 ± 0.04	0.95 ± 0.11
Salutaridine[a]	1.00 ± 0.10	1.36 ± 0.10	4.4 ± 1.3
Thebaine	16.7 ± 0.8	20.8 ± 1.4	56 ± 18

[a]Relative MRM peak area, where 1 is the peak area for CSY1071+pCS3309.

Table S6. Intermediate analysis for complete biosynthesis of the opiate thebaine and semi-synthetic opioid drug hydrocodone in yeast. Growth media was analyzed for opioids by LC-MS/MS MRM and thebaine was quantified with an external standard curve. Error is the standard deviation of at least three biological replicates. This data is depicted in Fig. 4.

	CSY1064+empty YAC		CSY1064+pCS2765	
Molecule (μg/L)	No 2-oxoglutarate	50 mM 2-oxoglutarate	No 2-oxoglutarate	50 mM 2-oxoglutarate
Reticuline	31.3 ± 1.0	31.2 ± 0.9	34.6 ± 1.4	29.5 ± 0.9
Thebaine	6.4 ± 0.3	2.4 ± 0.9	7.7 ± 0.5	2.4 ± 1.2
Hydrocodone	ND	ND	ND	~0.3

ND indicates not detected.

Table S7. Mutagenesis, fusion, and assembly oligonucleotide primers used in this study.

Primer Name	Primer Sequence (5'→3')
Mutagenesis primers[a]	

PsSalRI275A.R	GCCGTAGTTCATTTCTGTTTTAACCAAACCAGG
PsSalRI275A.F	GAAATGAACTACGGCGCTGGAAATTATACTGCC
PsSalRF104A.R	ACCTGCAACCCCAGCATTGTTTACCAAG
PsSalRF104A.F	GCTGGGGTTGCAGGTGCTTCAGTTGATGCTGATCG
PbSalRF104A.F	GCTGGGGTAGCGGGGGCTAGCGTAGAT
PbSalRF104A.R	CCCCGCTACCCCAGCATTATTCACAAGTATGTCTA
PbSalRI275A.F	AGACAGAAATGAACTACGGTGCTGGTAATTATACAGC
PbSalRI275A.R	ACCGTAGTTCATTTCTGTCTTTACCAAACC
yPsSalSynN39A.Fv3	TTTCGTTTTATCAAAGTGCAGCAACTACTGAA
yPsSalSynN39A.Rv3	TGCACTTTGATAAAACGAAATTTTAGAGA
yPsSalSynN105A.Fv2	AAGGGAGGTCTTGGTCGCCAAAAGTGCTGA
yPsSalSynN105A.Rv2	TCAGCACTTTTGGCGACCAAGACCTCCCTT
yPsSalSynN331A.Rv2	CGTTGTCCACCAGTTCTAGCGTTTATCTCTCT
yPsSalSynN331A.Fv2	TAGAACTGGTGGACAACGTC
Fusion cloning primers[b]	
BM3-D41yPbSalSyn.gibF	CAATGTCTATCAAAGAAATGCCAGAAGGTCCAAAAACCTTGC
D41yPbSalSyn-BM3.gibR	GGAATCTTCTTGGATTTAGCTGGGGTAACCTTAGCTTCCAATG
yPsSS486-Bm3_483.R	AATGGAATCTTCTTGGATTTAGCTAGAATAAAAGATATAGCCTCGCT CAGATCGGGTAG
yPsSS486-Bm3_483.F	AGCGAGGCTATATCTTTTATTCTAGCTAAATCCAAGAAGATTCCATT GGGTGGTATC
EcCFS31-D41yPsSS.Nterm.gibRv2	CAGGCCATTCCATAGTTGAGATAGATGATGATTTCTTTAACAAT
EcCFS31-D41yPsSalSyn.Cterm.gibFv2	CTCAACTATGGAATGGCCTGCTGGACCG
pENTR-EcCFS33.R	CCATTCCATAGTTGAGATAGATGATGATTTCTTTAACAATTTGG
EcCFS33-D43yPsSS.F	CTATCTCAACTATGGAATGGCCTGCTGGACCGAAGACTCTTCCCA
D50yPsSalSyn.fwd	CTAAGAAATTACCCATAATTGGCAATTTGCATCAATTG
EcCFSN50.rev	GCCAATTATGGGTAATTTCTTAGGACCCTTAGGCCATTC
D77yPsSalSyn.fwd	TACATGGTGGTGCCTTTACCATTTGGATTG
EcCFSN77.rev	GTAAAGGCACCACCATGTATTTTGGCTAAATTTGCTAATAC
D94yPsSalSyn.fwd	GTAATCTCTGACATTGATAATGCAAGGGAGGTTTTG
EcCFSN94.rev	GCATTATCAATGTCAGAGATTACAATCATAGGCCTCCAG
D101yPsSalSyn.fwd	CTTGGGAGGTTTTGGTCAACAAAAGTGCTGATTATAG
EcCFSN101.rev	GACCAAAACCTCCCAAGCCTTATCAATGTCAG
EcCFSs-SalSyn.linkF	GTCTTTGCTATCGCCAAATTGTTAAAGTTTTATCAAAGCGCAAATAC TACTGAATG
EcCFSs-SalSyn.linkR	CATTCAGTAGTATTTGCGCTTTGATAAAACTTTAACAATTTGGCGAT AGCAAAGAC
EcCFSl-SalSyn.linkF	CTATCGCCAAATTGTTAAAGAAATCATCAAGCGCAAATACTACTGAA TGGC
EcCFSl-SalSyn.linkR	GCCATTCAGTAGTATTTGCGCTTGATGATTTCTTTAACAATTTGGCG ATAG
D91yPbSalSyn.fwd	GTAATCTCTGATATTGATAACGCCAGAGAAGTTTTGG
EcCFSN94-D91yPbSS.rev	GGCGTTATCAATATCAGAGATTACAATCATAGGCCTCCAG

DNA assembly primers[c]	
YBL059W.Upstream.FW	CACAATTCGAAGCCGTATCTAACGCAG
YBL059W.Upstream.RV	CATCGAAACATACATCATTCACTGATTTACAAGTGCAGTTGAACAAAAGC
YBL059W.Downstream.FW	CTCTACCGGCAGATCTAGTGTTATACCATAGTAGTAGTTTCAATAATATATTCCACTACTTATATGTG
YBL059W.Downstream.RV	CATCATAGCGATAAAGCGGACCATCGG
YBR197C.Upstream.FW	TGAAAAAGGCCACTACTGAGCCAAGC
YBR197C.Upstream.RV	GCTTTTACGAGTTCACATTATTTGTCTTCGTTCGTGCCAGCAAC
YBR197C.Downstream.FW	AAAAGGAAGAGTGAATGACATGTATGGGTTGAAAATATTTAGAGGATGCTAAAAG
YBR197C.Downstream RV	CAACATTGCATAGCATCCAAATACGTGAAAGC
YMR206W.Upstream.FW	CAACATGAAGCGAGAGACTTCATTGCG
YMR206W.Upstream.RV	CTCTACCGGCAGATCCATGGTTATATTCTGTTGTCTTCTTTACTTTAAGGCGAAAG
YMR206W.Downstream.FW	AAAAGGAAGAGTGAATAGTTGATTAGAAGACTATAGCTAAATAATCATTTCATTGGGAAATGAC
YMR206W.Downstream.RV	GTATGCCGATGTTGAATCTAGGGGAGG
YDR514C.Upstream.FW	GTTTGTTTCTTCTTATCTTCAGCTGCTGAG
YDR514C.Upstream.RV	ATCCTAAGAGAATTCACAATAGCTTATAATCTGTGTAGTCAAACTATATACTAGGC
YDR514C.Downstream.FW	GCTTTTACGAGTTCAATCTAGCTAGAAGTTTTGTAGGTATATGTGATTTAAGATATAG
YDR514C.Downstream.RV	CATTATCACGTTGTTTGCCACAAGAATTATTG
YPL250C.Upstream.FW	CTCTCGTCGTCATTAAACAGCGTGAG
YPL250C.Upstream.RV	CTCTACCGGCAGATCCATTGTAAATAATTGTAATTGTATATTATTGTGTGTAAGTTTGGTTGG
YPL250C.Downstream.FW	ATCAGATCCACTAGTGGCCTATGCGTGACGCATTTTACCACATCTCTACTTGTTC
YPL250C .Downstream RV	GATTATTCACAGGAGAAATTGGGGGCAC
ZWF1.Upstream FW	GCGAGCTTTCCGGGTTTAGAAACATC
ZWF1.Upstream RV	CTCTACCGGCAGATCCATCTTGCCTTATGTGGTTTTCTATTCTATTGGATTTAC
ZWF1.Downstream FW	ATCAGATCCACTAGTGGCCTATGCGTAGAAAAATGCAAGCACATTCATTTATCGGCTAAG
ZWF1.Downstream RV	CAGAATACAGGCAAGAAGGGCATTTGG
TRP1.Upstream FW	AGTTAGAGGCGGTGGAGATATTCC
TRP1.Upstream RV	AAAAGGAAGAGTGAATTCACCAATGGACCAGAACTACCT
TRP1.Downstream FW	GAAGGCTTTAATTTGACTGAGTAGTATTTATTTAAGTATT
TRP1.Downstream RV	CTGATGGTGTTTATGCAAAG

[a] Red letters denote mutagenesis sites.
[b] Red letters denote regions of overlap for Gibson assembly of fusions.
[c] Red letters denote regions of overlap with expression cassettes.

Table S8. New codon-optimized DNA sequences used in this study.

Plasmid	Gene	Coding sequence
pCS3335	*yPbSalSyn*	ATGGCCCCAATCAACATCGAAGAAAATGATTTCTGGATGATTGCCTGCACCGTTATTATCGTTTTTGCCTTGATGAAGTTCATGGTTTCCTTCTACCAATCTGCTAACACTACTGAATGGCCAGAAGGTCCAAAAACCTTGCCAATTATTGGTAACTTGCATCAATTGGGTGGTGGTGTTCCATTGCAAGTT

		GCTTTAGCTAATTTGGCTAAAGTTTACGGTGGTGCTTTC ACCATTTGGATTGGTTCTTGGGTTCCAATGATCGTTATC TCCGATATTGATAACGCCAGAGAAGTTTTGGTTAACAA GTCTGCTGATTACTCCGCTAGAGATGTTCCAGATATTTT GAAGATTATTACCGCCAACGGTAAGAACATTGCTGATT GTGATTCTGGTCCATTCTGGCATCATTTGAAGAAGGGT TTACAATCCTGCATCAACCCATCTAACGTTATGTCTTTG TCCAGATTGCAAGAAAAGGACATGCAAAACTTGATTAA GTCCATGCAAGAAAGAGCCTCTCAACAAAACGGTATCT TGAAACCATTGGATCATGCTAAAGAAGCCTCCATCAGA TTATTGTCCAGAGTTATTTTCGGTCAAGACTTCTCCAAC GAAGATTTGGTTATTGGTGTTAAGGATGCCTTGGACGA AATGGTTAGAATTTCTGGTTTGGCTTCTTTGGCTGATGC TTTCAAAATTGCTAAGTACTTGCCATCCCAAAAAAAGAA CATCAGAGATATGTACGCCACCAGAGATAGAGTTTACA ACTTGATTCAACCACACATCGTCAGTAATTTGCCAGCC AATTCATTCTTGCATTTCTTGACCTCTCAAGACTACTCT GACGAAATCATCTACTCCATGGTTTTGGAAATTTTCGGT TTGGGTGTTGATTCTACTGCTGCTACAGCTGTTTGGGC TTTGTCTTTTTTGGTTGGTGAACAAGAAATCCAAGAAAA GTTGTACAGAGAAATCAACAACTTGACCGGTGGTCAAA GACCAGTTAAGGTTGTTGACTTGAAAGAATTGCCATAC TTGCAAGCCGTTATGAAAGAAACCTTGAGAATGAAGCC AATTGCTCCATTGGCTGTTCCACATGTTGCTGCTAAAG ATACCACTTTTAAGGGTAGAAGAATCGTCAAGGGTACT AAGGTTATGGTTAACTTGTACGCCATTCATCACGATCCA AATGTTTTTCCAGCTCCATACAAGTTCATGCCTGAAAGA TTTTTGAAGGGTGTTAACTCCGATGGTAGATACGGTGA TATTAACACCATGGAATCCTCATTGATTCCATTTGGTGC TGGTATGAGAATTTGCGGTGGTGTTGAATTGGCAAAAC AAATGGTTGGTTTTGCTTTGGCCTCTATGGTCAATGAAT TTAAGTGGGATTGTGTCTCCGAAGGTAACTTACCAGAT TTGTCTGAAGCTATCTCCTTCATCTTGTACATGAAGAAT CCATTGGAAGCTAAGGTTACCCCAAGAACTAAGCCATT CGATTCTAGATGA
pCS3273	*yEcCFS*$^{1\text{-}83}$*-yPbSalSyn*$^{92\text{-}504}$	ATGGAAGAGTCTTTATGGGTCGTTACTGCAACTGTTGT AGTCGTCTTTGCTATCGCCAAATTGTTAAAGAAATCATC ATCTATCTCAACTATGGAATGGCCTAAGGGTCCTAAGA AATTACCAATCATCGGTAACTTACACCAGTTAGGTGGA GAAGCATTTCACGTTGTATTAGCAAATTTAGCCAAAATA CATGGTACAGTAATGACTATTTGGGTAGGAGCCTGGAG GCCTATGATTGTAATCTCTGATATTGATAACGCCAGAGA AGTTTTGGTTAACAAGTCTGCTGATTACTCCGCTAGAG ATGTTCCAGATATTTTGAAGATTATTACCGCCAACGGTA AGAACATTGCTGATTGTGATTCTGGTCCATTCTGGCAT CATTTGAAGAAGGGTTTACAATCCTGCATCAACCCATCT AACGTTATGTCTTTGTCCAGATTGCAAGAAAAGGACAT GCAAAACTTGATTAAGTCCATGCAAGAAAGAGCCTCTC AACAAAACGGTATCTTGAAACCATTGGATCATGCTAAA GAAGCCTCCATCAGATTATTGTCCAGAGTTATTTTCGGT CAAGACTTCTCCAACGAAGATTTGGTTATTGGTGTTAAG GATGCCTTGGACGAAATGGTTAGAATTTCTGGTTTGGC TTCTTTGGCTGATGCTTTCAAAATTGCTAAGTACTTGCC ATCCCAAAAAAAGAACATCAGAGATATGTACGCCACCA GAGATAGAGTTTACAACTTGATTCAACCACACATCGTCA GTAATTTGCCAGCCAATTCATTCTTGCATTTCTTGACCT

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		CAGCTGTTTGGGCTTTGTCTTTTTTGGTTGGTGAACAA
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		GATTCCATTTGGTGCTGGTATGAGAATTTGCGGTGGTG
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		CTATGGTCAATGAATTTAAGTGGGATTGTGTCTCCGAA
		GGTAACTTACCAGATTTGTCTGAAGCTATCTCCTTCATC
		TTGTACATGAAGAATCCATTGGAAGCTAAGGTTACCCC
		AAGAACTAAGCCATTCGATTCTAGATGA
pCS3300	*yPbDRS-DRR (Pbr.89405)*	ATGGAATTGCAATACTTCTCCTACTTCCAACCTACCTCT
		TCTGTTGTTGCTTTGTTGTTGGCATTGGTCAGTATCTTG
		TTTTCCGTTGTTGTTTTGAGAAAGACCTTCTCCAACAAC
		TATTCTTCTCCAGCTTCTTCTACTGAAACCGCTGTTTTG
		TGTCATCAAAGACAACAATCTTGCGCCTTGCCAATTTCT
		GGTTTGTTGCATGTTTTCATGAACAAGAACGGTTTGATC
		CATGTTACCTTGGGTAATATGGCTGATAAGTACGGTCC
		AATTTTCTCTTTTCCAACCGGTTCTCATAGAACCTTGGT
		TGTTTCTTCTTGGGAAATGGTCAAAGAATGTTTCACCG
		GTAACAACGATACCGCCTTTTCTAATAGACCAATTCCAT
		TGGCTTTCAAGACCATTTTCTATGCCTGTAGAGGTATC
		GACTCTTACGGTTTATCTTCTGTTCCATACGGTAAATAT
		TGGAGAGAATTGAGAAAGGTTTGCGTCCACAACTTGTT
		GTCCAATCAACAATTATTGAAGTTCAGACACTTGATCAT
		CTCCCAAGTTGATACCTCCTTCAACAAGTTATACGAATT
		GTGCAAGAACTCCGAAGATAATCAAGGTATGGTTAGAA
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		GTAGAATCGTTTGCGGTTTCCAATCTGATCCAAAAACT
		GGTGCTCCATCTAGAGTCGAACAATTCAAAGAAGCTAT
		TAACGAAGCCTCCTACTTCATGTCTACTTCTCCAGTTTC
		TGATAACGTTCCAATGTTGGGTTGGATCGATCAATTGA
		CTGGTTTGACTAGAAACATGACCCATTGTGGTAAGAAG
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		ACAAAAGAGAAGATTCTCCAGAACAAAAGGTGGTGACG
		AAAAGGATGATGAACAAGATGATTTCATCGACATCTGC
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		CAATCCACCAAAAATCCCAATCAAGTCCATCGTTTTGGA
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		TATTTGGACCTTGTCCTTGTTGTTGAACAACCCAAATGT
		TTTGGCTAAGGCCAAACAAGAAGTTGACGCTCATTTTG
		AAACTAAGAAGAGATCTACCAACGAAGCTTCTGTCGTT
		GTTGATTTCGATGATATTGGTAACTTGGTCTACATCCAA
		GCCATTATCAAAGAATCCATGAGATTATACCCAGTCTCC
		CCAGTTGTTGAAAGATTGTCATCTGAAGATTGTGTTGTT
		GGTGGTTTTCATGTTCCAGCTGGTACTAGATTGTGGGC
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GACCAGATTGATCTTGGAATTCGAAATGAAATCTCCATC
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CTTACAAAGTTGTTCCATTGGACATCTTGTTGACCCATA
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GTAAGATTACCATGGATATCCCAGAAGAAGATATCTGC
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TAAGGCTTAA

pCS3302 *yPsDRS-DRR (Pso.2062398)*

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GACAACAACCACTACAGCTGCAGGTATGGTTAGAATCG
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GTTTGACTAGAAACATGAAGCATTGTGGTAAGAAGTTG
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		TCCATCACAAATCCCAATCAAGTCCATCGTTTTGGATAT
		GATTGGTGGAGGTACTGATACCACTAAGTTGACTACAA
		TTTGGACCTTGTCCTTGTTGTTGAACAACCCACATGTTT
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Supplementary Figures

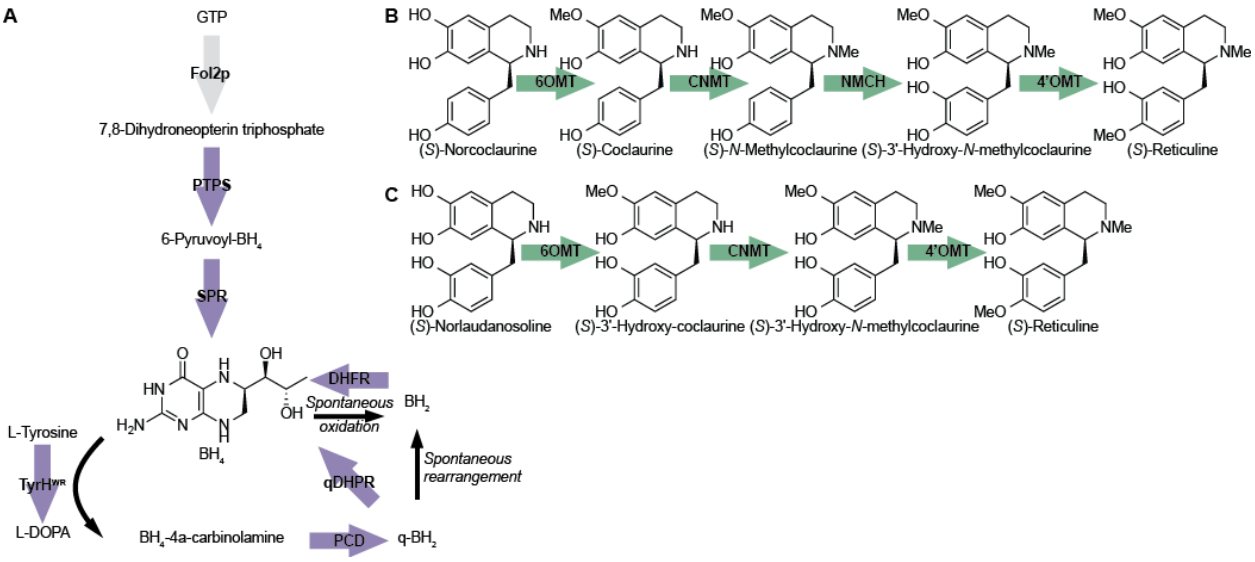

Figure S1. Biosynthetic scheme for 5,6,7,8-tetrahydrobiopterin (BH_4) cofactor biosynthesis and recycling and conversion of (*S*)-norcoclaurine or (*S*)-norlaudanosoline to (*S*)-reticuline. Schemes for **(A)** BH_4 biosynthesis, recycling, and salvage, **(B)** conversion of (*S*)-norcoclaurine to (*S*)-reticuline, and **(C)** conversion of (*S*)-norlaudanosoline to (*S*)-reticuline. Block arrows indicate enzyme-catalyzed steps. Light grey arrows, unmodified yeast enzymes; purple arrows, *Rattus norvegicus* enzymes. GTP, guanosine triphosphate; q-BH_2, quinonoid dihydrobiopterin; BH_2, 7,8-dihydrobiopterin; Fol2p, GTP-cyclohydrolase I; PTPS, 6-pyruvoyl-BH_4 synthase; SPR, sepiapterin reductase; PCD, pterin-4a-carbinolamine dehydratase; qDHPR, q-BH_2 reductase; DHFR, dihydrofolate reductase; 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; NMCH, *N*-methylcoclaurine hydroxylase; 4'OMT, 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase.

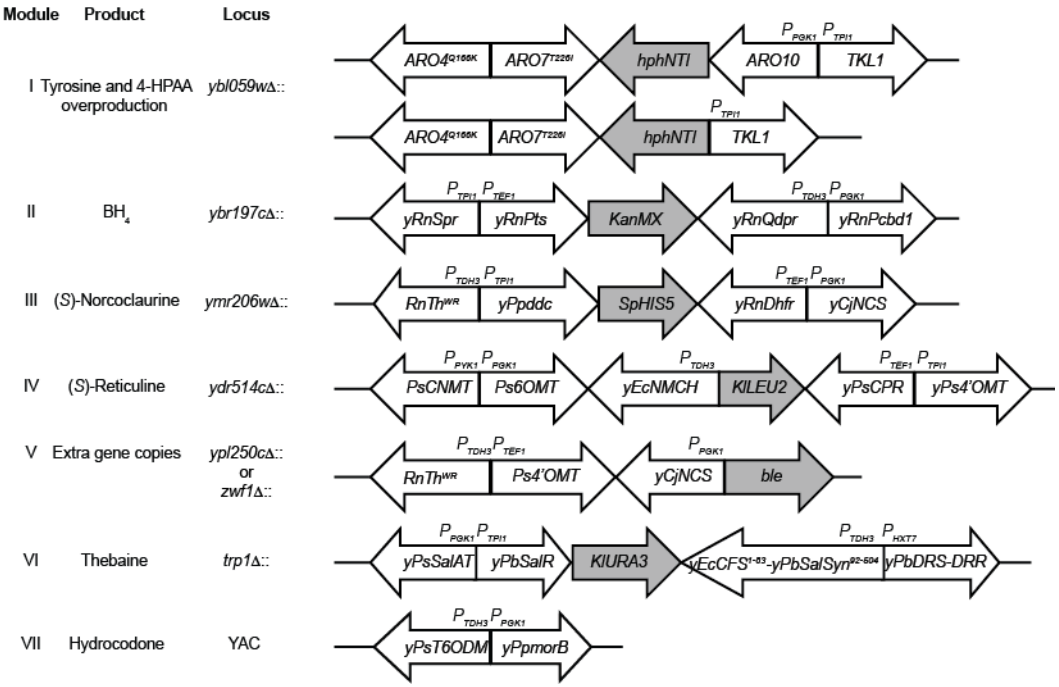

Figure S2. Genetic design of pathway modules for yeast strain construction.

Modules are designed to integrate into one of six chromosomal loci in the yeast genome or for yeast artificial chromosome-based (YAC) expression. White block arrows indicate gene expression cassettes with a promoter, coding sequence, and terminator. Grey block arrows, loxP-flanked selection markers. For non-yeast enzymes, codon-optimization is indicated by a leading "y", and the source genus and species is designated by the two letters immediately preceding the gene symbol. See tables S1, S3, S7, and S8 for expression cassette, strain number, genome assembly primers, and new synthetic coding sequences.

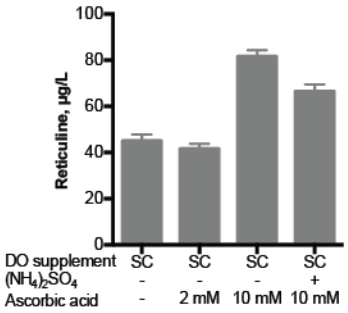

Figure S3. Optimization of strain cultivation.

Optimization of ascorbic acid concentration and nitrogen source for reticuline production. Strain CSY1061 was cultivated in minimal media with the indicated modification for 72 h and reticuline in the growth media was quantified by LC-MS/MS MRM with an external standard curve. Error bars represent standard deviation of three biological replicates.

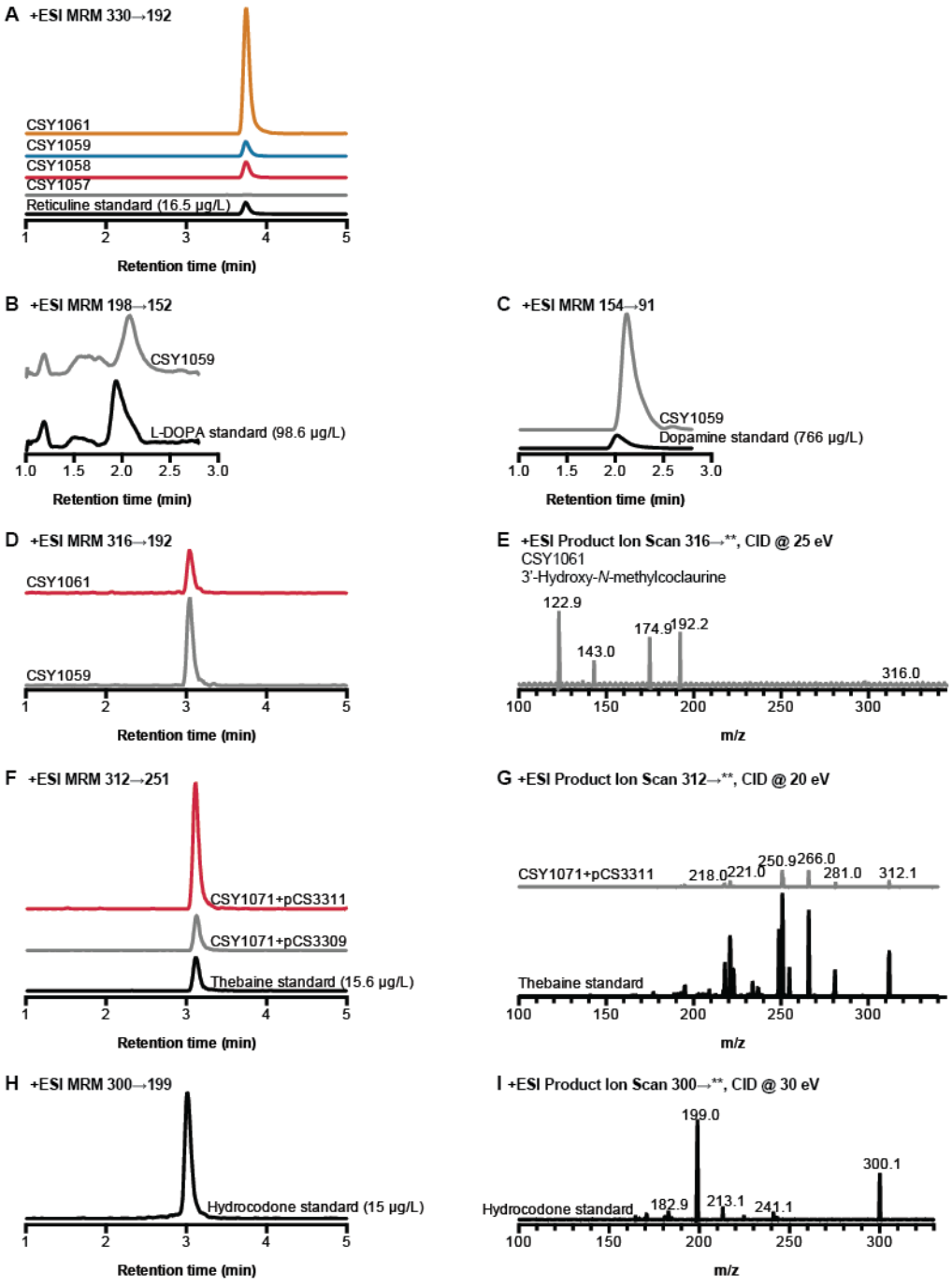

Figure S4. LC-MS/MS characterization of intermediates and products in yeast growth media.

Chromatogram traces of **(A)** reticuline, **(B)** L-DOPA, **(C)** dopamine, and **(D)** 3'-hydroxy-*N*-methylcoclaurine in growth media of indicated strains. **(E)** Product ion spectrum (25 ev) of 3'-

hydroxy-*N*-methylcoclaurine is consistent with a published spectrum (*57*). Chromatogram traces **(F)** and product ion spectra (20 eV, **G**) of thebaine in growth media of CSY1071+pCS3311 and 15.6 μg/L (50 nM) thebaine standard. Chromatogram trace **(H)** and product ion spectra (30 eV, **I**) of 15 μg/L (50 nM) hydrocodone standard. Traces for growth media are representative of at least three biological replicates.

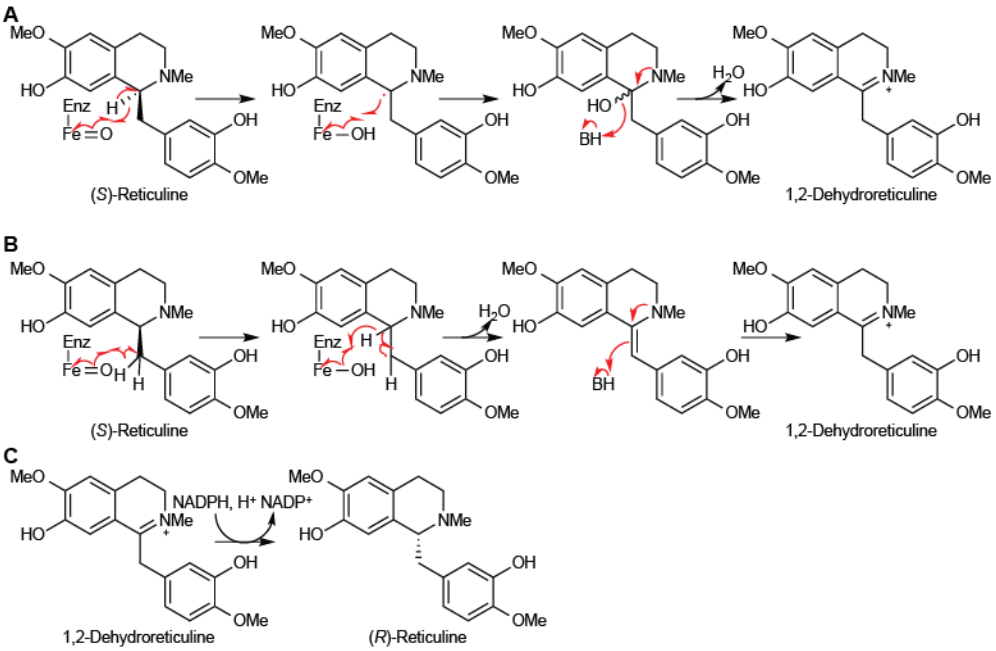

Figure S5. Mechanistic hypothesis for DRS-DRR activity.

The oxidation of (*S*)-reticuline to 1,2-dehydroreticuline may occur via **(A)** a carbinolamine or **(B)** an enamine intermediate. **(C)** 1,2-Dehydroreticuline is then stereospecifically reduced to (*R*)-reticuline.

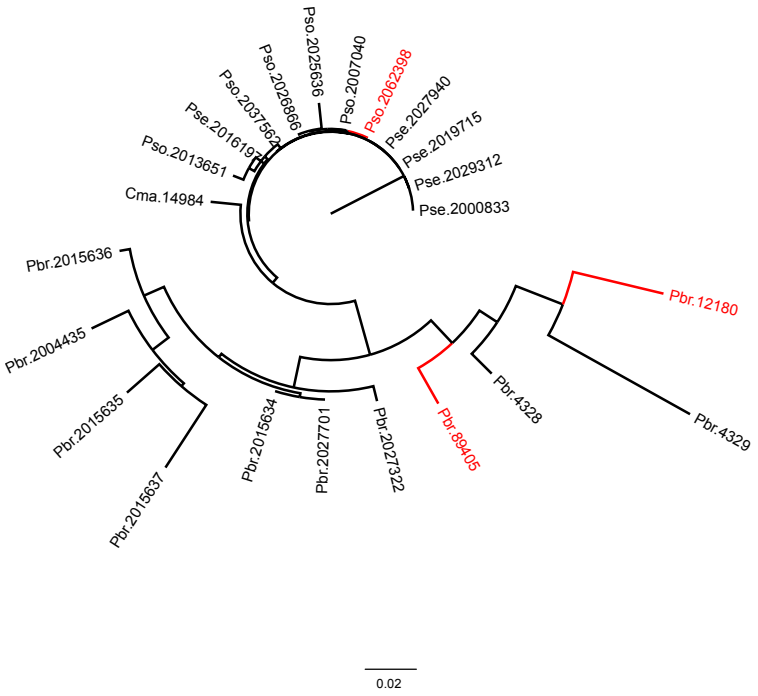

Figure S6. Identification of additional DRS-DRR variants via bioinformatic analysis of Pbr.89405-like sequences. Bioinformatic query was Pbr.89405 amino acid sequence and subject sequences were the complete PhytoMetaSyn and 1000 Plants Project transcriptomes. Sequences included in this tree contained both cytochrome P450 and reductase domains. The scale bar indicates amount of genetic change in amino acid substitutions per site. Branches highlighted in red indicate sequences for which data is included in this work. Phylogenetic tree was generated using ClustalX bootstrap NJ tree with 1000 trials and visualized with FigTree.

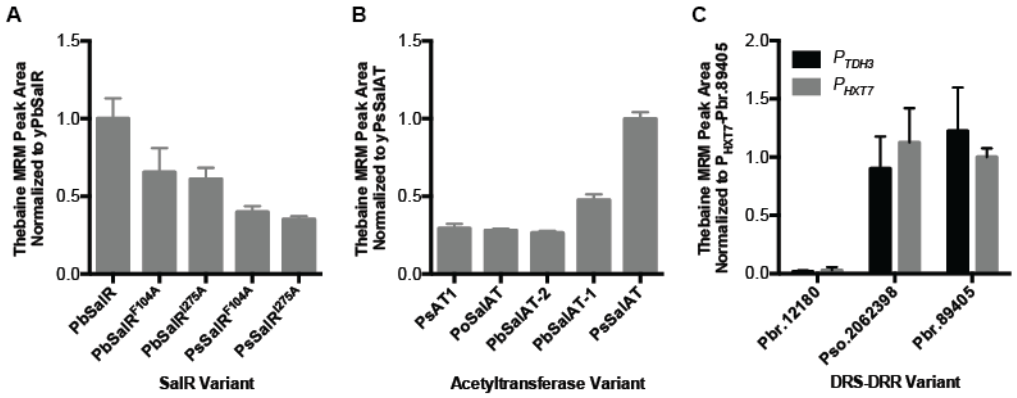

Figure S7. Characterization of SalR, SalAT, and DRS-DRR variants in the context of the engineered biosynthetic pathway in yeast.

(A) Thebaine production from SalR variants and mutants. **(B)** Thebaine production from SalAT variants and mutants. SalR (pCS3271, 3343-3346) and SalAT (pCS3272, 3347-3350) variants and mutants were expressed from YACs assembled in CSY3. Yeast strains were fed 10 μM salutaridine, and the growth media was analyzed for thebaine by LC-MS/MS MRM. **(C)** Comparison of thebaine production from DRS-DRR variants expressed from glycolytic (P_{TDH3}) and late-stage (P_{HXT7}) promoters. DRS-DRR variants were expressed from low-copy plasmids (pCS3300-3305) in CSY1071. Yeast strains were fed 10 μM (*S*)-reticuline, and the growth media was analyzed for thebaine by LC-MS/MS MRM. Error bars represent the standard deviation of three biological replicates.

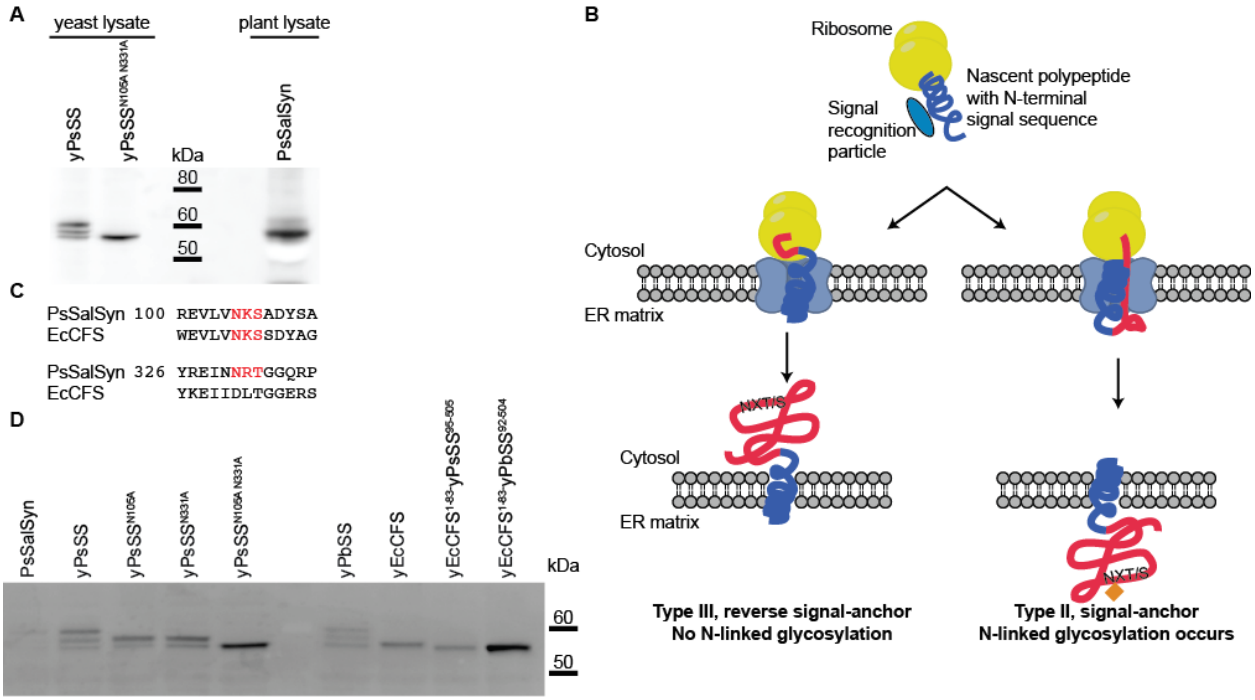

Figure S8. SalSyn is mis-processed in yeast and N-glycosylated.

(A) Western blot analysis of the C-terminally HA-tagged SalSyn protein expressed in yeast and plants. In plants the protein runs primarily as a single band and in yeast the protein runs as three bands, indicative of N-linked glycosylation. When mutations are introduced into the protein to remove the N-glycosylated sites (yPsSS$^{N105A\ N331A}$), the yeast-expressed protein runs as a single band. yPsSS, yeast codon-optimized SalSyn; PsSalSyn, wild-type *P. somniferum* SalSyn. The expected molecular weight of SalSyn was 62.5 kDa. **(B)** Schematic showing hypothesized correct and mis-processing of the nascent SalSyn peptide when heterologously expressed in yeast. **(C)** Amino acid sequence alignment from ClustalX of EcCFS (*E. californica* cheilanthifoline synthase) and PsSalSyn in the regions of SalSyn that are N-glycosylated. NXT/S sites indicated in red. **(D)** Western blot analysis of the engineered SalSyn fusion proteins expressed in yeast. The fusions run as a single band on the gel, indicating that they are processed correctly when heterologously expressed in yeast. yPsSS^{N105A}, yPsSS^{N331A}, single mutants to remove one of the two glycosylated sites in yeast codon-optimized SalSyn; yPbSS, yeast codon-

optimized *P. bracteatum* salutaridine synthase; yEcCFS, yeast codon-optimized *E. californica* CFS; yEcCFS$^{1\text{-}83}$yPsSS$^{95\text{-}505}$, yEcCFS$^{1\text{-}83}$yPsSS$^{92\text{-}504}$, engineered SalSyn fusions. Blots are representative of two biological replicates.