Supporting Information

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SI Materials and Methods

Cloning. Full-length cDNA sequences of *T3O* (*CYP71D1V2*) and *T3R* (*ADHL1*) were retrieved from the PhytoMetaSyn Project (www.phytometasyn.ca). *T3O* ORF was amplified from leaf total cDNA with primer set (1/2) and cloned in pESC-Leu2d vector previously inserted with *C. roseus CPR* (1) within ApaI/SalI sites. *T3R* ORF was amplified with primer set (3/4) and inserted into pESC-His vector within BamHI/SalI sites. Similarly, the ORFs of *NMT*, *D4H*, and *DAT* were amplified with primer sets (5/6, 7/8, 9/10) and cloned into pESC-His, pESC-Ura, and pESC-Ura vectors within NotI/SpeI, NotI/SpeI, and BamHI/SalI sites, respectively. To simultaneously express the seven-gene pathway in yeast, the cassette including Gal1 promoter, *T3O* ORF, and CYC terminator was amplified from pESC-Leu2d vector with primer sets (11/12) and cloned into pESC-His-T3R/NMT vector within DraIII/NaeI sites. *T16H2* and *16OMT* ORFs were amplified with primer sets (13/14, 15/16) and cloned in pESC-Leu2d vector within ApaI/SalI and NotI/SpeI sites, respectively. The VIGS-*T3O* fragment (Nt 949–1221) was amplified with primer set (17/18) and cloned in pGEM-T-easy vector (Promega Corporation). The fragment was released with EcoRI and cloned into pTRV2 vector. The VIGS-*T3R* fragment (Nt 208–462) was amplified with primer set (19/20) and directly cloned into pTRV2 within the EcoRI site. To express *T3R* in *E. coli*, *T3R* ORF was amplified with primer set (3/21) and cloned into pET30b+ vector within BamHI/XhoI sites. The primers are listed in Table S2. The pESC-Leu2d/His/Ura vectors were mobilized to *S. cerevisiae* strain BY47471 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL154c::kanMX4) for enhanced protein expression. The pTRV2 vectors were mobilized to *A. tumefaciens* strain GV3101. The pET30b+ vector was mobilized to *E. coli* strain BL21-DE3.

RNA Extraction, cDNA Synthesis, and qRT-PCR. Fine powder (10–30 mg) of leaves (LP-1 or LP-4, for young leaves and mature leaves, respectively) ground in liquid nitrogen was immediately mixed with 0.5 mL TRIzol reagent (Invitrogen). Leaf epidermal cells from 5 g young leaves (LP-1) were isolated with the carborundum abrasion method, as described (2). RNA was isolated according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and oligo (dT) 12–18 primer (Invitrogen), using 1–5 μg total RNA according to the manufacturer's protocol. qRT-PCR was performed (CFX96 Real-Time system; Bio-Rad), using iTaq Universal SYBR Green Supermix (Bio-Rad), 5 μM primers, and cDNA template (equivalent to 5 ng total RNA) in a reaction volume of 10 μL. The reaction conditions for qRT-PCR included 1 cycle of 95 °C for 1 min and 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The critical threshold values were used to calculate the relative transcript abundance with 60S ribosome RNA as the internal control (3). The primer efficiency was calculated from qRT-PCR of the serial dilution of total cDNA, and the specificity of the primers was confirmed by the dissociation curve for each primer set. The qRT-PCR primers are listed in Table S2.

Yeast Microsome Isolation. Overnight culture (0.5 mL) of yeast was used to inoculate 50 mL SC-Leucine medium with 1.8% galactose and 0.2% glucose. The yeast was grown at 30 °C for 48 h, and the cells were harvested and broken in TES buffer (10 mM Tris·HCl at pH 7.5, 1 mM EDTA, 0.6 M sorbitol) by shaking at 30 Hz for 5 min at 4 °C (TissueLyser II; Qiagen) with glass beads. The lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4 °C to pellet microsomes. The microsomes were suspended in TEG buffer [10 mM Tris·HCl at pH 7.5, 1 mM EDTA, and 10% (vol/vol) glycerol] and stored at −80 °C.

Recombinant T3R Purification. In LB medium, 400 mL *E. coli* strain BL21-DE3 containing pET30b+T3R was grown to $OD_{600} = 0.4$ and was induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 11 °C overnight. The harvested cells were sonicated in lysis buffer [10 mM Tris·HCl at pH 7.5, 100 mM NaCl, 5 μM $ZnCl_2$, 10 mM imidazole, 1 mM DTT, 1 mM PMSF, and 10% (vol/vol) glycerol]. The supernatant of the lysate collected after centrifugation at 10,000 × *g* was incubated with 2 mL Ni-NTA resin at 4 °C for 1 h. The resin was washed with 50 mL wash buffer [10 mM Tris·HCl at pH 7.5, 100 mM NaCl, 5 μM $ZnCl_2$, 30 mM imidazole, 1 mM DTT, 1 mM PMSF, and 10% (vol/vol) glycerol]. The recombinant protein was eluted with elution buffer (10 mM Tris·HCl at pH 7.5, 100 mM NaCl, 500 mM imidazole, and 10% (vol/vol) glycerol) and stored at −80 °C.

Immunoblot. Yeast microsomal protein (50 μg) or total protein (50 μg) was separated on 12% SDS/PAGE. The gel was transferred to an Amersham Hybond-ECL protein blotting membrane (GE Healthcare Life Sciences), and blotted with anti-c-Myc antibodies (0.5 $\mu g{\cdot}L^{-1}$; Santa Cruz Biotechnology, Inc) and secondary IgG (IRDye800 goat anti rabbit, 0.1 $\mu g{\cdot}L^{-1}$; Rockland-Inc). The membrane was visualized with a scanner (Odyssey 9120, Li-COR).

LC-MS. Samples were analyzed using Acquity UPLC systems (Waters) equipped with BEH C18 column (2.1 × 50 mm; particle size, 1.7 μm), a photodiode array detector, and a mass spectrometer. The solvent systems for alkaloid analysis were as follows: solvent A, methanol: acetonitrile: 5 mM ammonium acetate at 6:14:80; solvent B, methanol: acetonitrile: 5 mM ammonium acetate at 24:64:10. The following linear elution gradient was used: 0–0.5 min, 99% A, 1% B at 0.3 $mL{\cdot}min^{-1}$; 0.5–0.6 min, 99% A, 1% B at 0.4 $mL{\cdot}min^{-1}$; 0.6–8.0 min, 1% A, 99% B at 0.4 $mL{\cdot}min^{-1}$; 8.0–8.3 min, 99% A, 1% B at 0.4 $mL{\cdot}min^{-1}$; 8.3–10.0 min, 99% A, 1% B at 0.3 $mL{\cdot}min^{-1}$. The mass spectrometer was operated as capillary voltage 3.1 kV, cone voltage 48 V, desolvation gas flow 600 $L{\cdot}h^{-1}$, desolvation temperature 350 °C, source temperature 150 °C, and positive ion mode. Alkaloid reference standards and quantification curves were obtained as described (4).

1. Salim V, Wiens B, Masada-Atsumi S, Yu F, De Luca V (2014) 7-deoxyloganetic acid synthase catalyzes a key 3 step oxidation to form 7-deoxyloganetic acid in Catharanthus roseus iridoid biosynthesis. *Phytochemistry* 101:23–31.
2. Murata J, Roepke J, Gordon H, De Luca V (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. *Plant Cell* 20(3):524–542.
3. Salim V, Yu F, Altarejos J, De Luca V (2013) Virus-induced gene silencing identifies *Catharanthus roseus* 7-deoxyloganic acid-7-hydroxylase, a step in iridoid and monoterpene indole alkaloid biosynthesis. *Plant J* 76(5):754–765.
4. Roepke J, et al. (2010) Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. *Proc Natl Acad Sci USA* 107(34):15287–15292.

Fig. S1. Outline of the tabersonine-to-vindoline/vindorosine biosynthetic pathway. Solid arrows indicate the biosynthetic flux, whereas the empty arrows indicate the transportation of intermediates that may take place between cell types. A thicker arrow represents more flux.

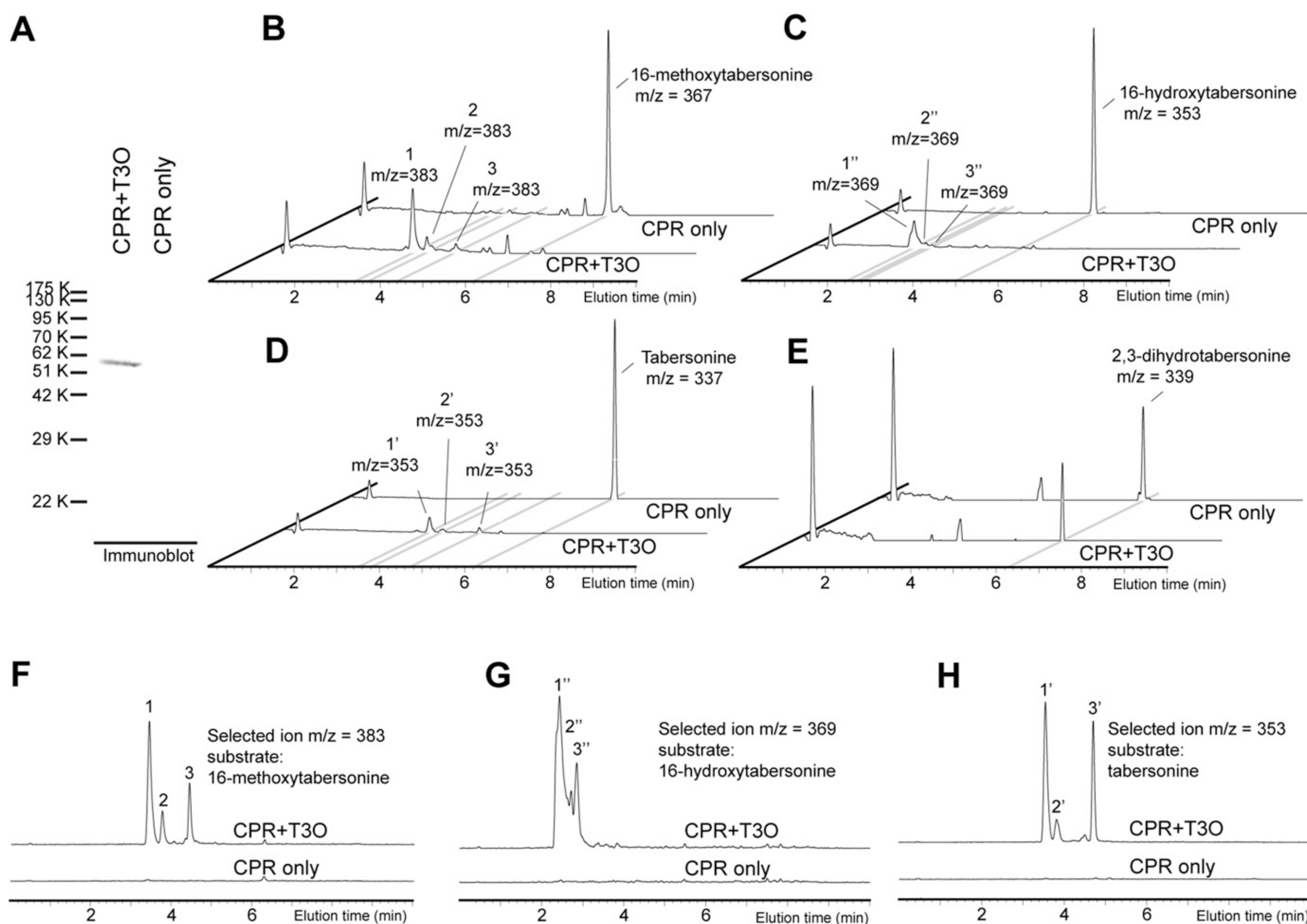

Fig. S2. LC-MS profiles of in vitro oxidation reaction products of different MIAs when incubated with recombinant yeast T3O (CYP71D1V2) expressing microsomes in the presence of NADPH and O_2. Immunoblot of recombinant T3O tagged with a C-terminal c-Myc in *S. cerevisiae*, using anti-c-Myc antibodies (*A*). Enzyme assays were conducted with yeast microsomes expressing CPR only or CPR+T3O with four separate MIA substrates: 16-methoxytabersonine (*B* and *F*), 16-hydroxytabersonine (*C* and *G*), tabersonine (*D* and *H*), or 2,3-dihydrotabersonine (*E*). The reaction products were analyzed by LC-MS at 300 nm (*B*–*E*) or by selected ion mode at *m/z* = 383 (*F*), *m/z* = 353 (*G*), or *m/z* = 369 (*H*). The 3-oxidized isomers of 16-methoxytabersonine (*B* and *F*) could also be detected in leaf surface extract of VIGS-T3R plants in Fig. 2*F*.

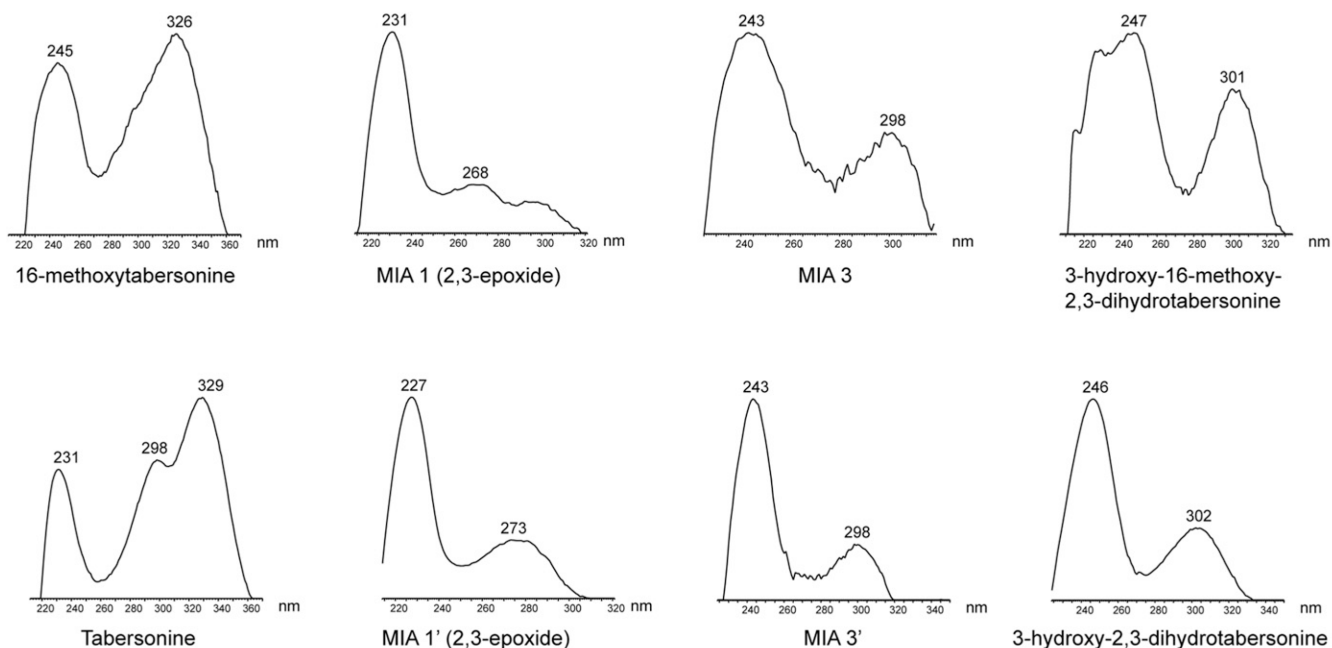

Fig. S3. The UV spectra of T3O substrates and products. Oxidizing the 2,3-double bond to the epoxide dramatically shifted the UV spectra of 16-methoxytabersonine or tabersonine to typical indolenine UV spectra, whereas the unstable MIA 3 or 3′ resembles the UV spectra of the products 3-hydroxy-16-methoxy-2,3-dihydrotabersonine or 3-hydroxy-2,3-dihydrotabersonine.

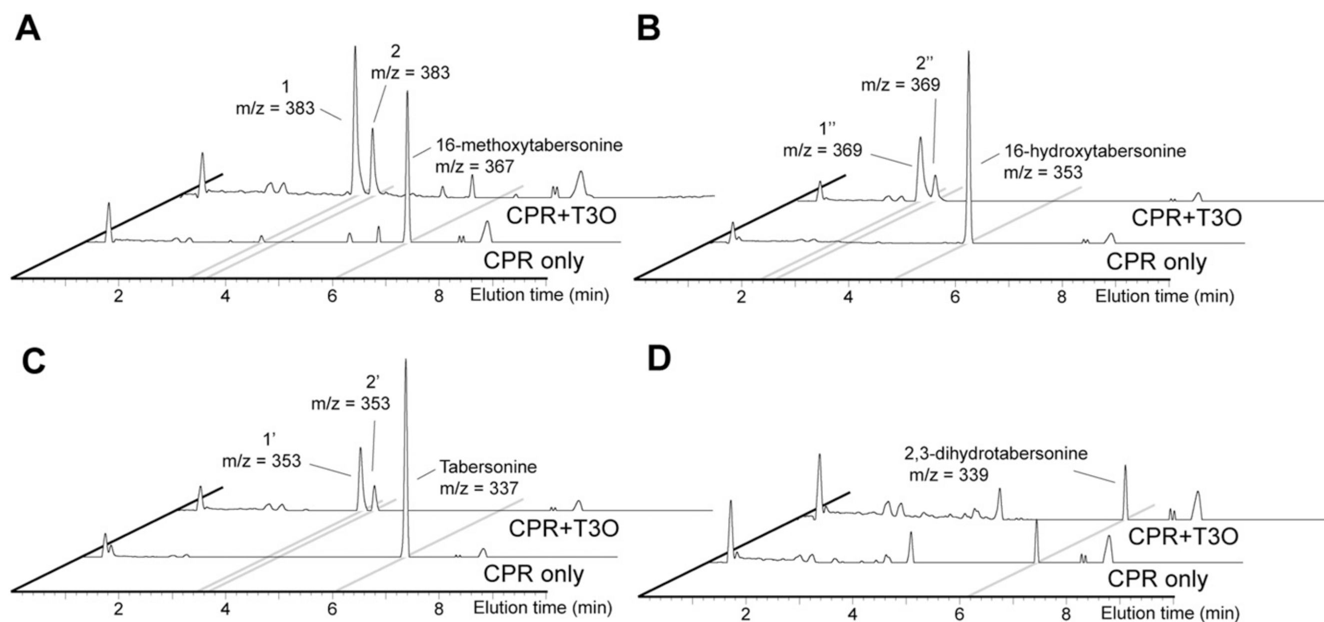

Fig. S4. LC-MS profiles of reaction products from incubations of recombinant CPR or CPR+T3O (CYP71D1V2) expressing strains of yeast with different MIAs. Yeast cells expressing recombinant CPR only or CPR+T3O were fed with 16-methoxytabersonine (*A*), 16-hydroxytabersonine (*B*), tabersonine (*C*), or 2,3-dihydrotabersonine (*D*). MIAs were analyzed by LC-MS at 300 nm. The 3-oxidized isomers of 16- methoxytabersonine (*A*) could also be detected in leaf surface extract of VIGS-T3R plants in Fig. 2*F*.

Fig. S5. ^{1}H NMR analysis of purified product 1′ derived from tabersonine. Results suggest 2,3-epoxytabersonine. ^{1}H NMR (600 MHz, MeOD) δ 7.42 (m, 1H, CH-17), 7.10 (m, 1H, CH-15), 7.07 (m, 1H, CH-14), 7.05 (m, 1H, CH-16), 5.78 (d, J = 9.9, 1H, CH-6), 5.48 (dt, J = 10.3, 3.0, 1H, CH-7), 4.13 (s, 1H, CH-19), 3.81 (s, 3H, OCH_3), 3.43 (m, 1H, CH-10), 3.14 (m, 1H, CH-11), 3.08 (d, J = 2.4, 2H, CH-8), 2.63 (ddd, J = 15.9, 6.6, 1.8, 1H, CH-11), 2.37 (q, J = 14.3, 2H, CH-4), 1.97 (m, 1H, CH-20), 1.64 (m, 1H, CH-11), 1.03 (t, J = 7.6, 3H, CH_3-21); ^{13}C NMR (150 MHz, MeOD) δ 173.0 (C = O), 134.5 (C-18), 130.1 (C-20), 128.8 (CH-6), 123.1 (CH-7), 120.9 (CH-4), 119.6 (CH-16), 117.6 (CH-17), 110.5 (CH-15), 105.0 (C-12), 82.1 (C-3), 57.2 (CH-19), 52.4 (OCH_3), 49.1 (CH-10), 48.5 (C-2), 43.1 (CH-8), 43.0 (CH-4), 36.5 (C-5), 34.2 (CH-20), 16.0 (CH-11), 7.1 (CH-21).

Fig. S6. LC-MS profiles of in vitro reaction products of different MIAs when incubated with NADPH (*A* and *B*) with affinity-purified recombinant T3R (*D*) or with different concentrations of T3R in combination with recombinant yeast T3O (CYP71D1V2)-expressing microsomes (*C*). Recombinant T3R was tested with T3O products 1 and 2 derived from T3O-expressing yeast biotransformations using 16-methoxytabersonine (*Left*) or tabersonine (*Right*) as substrates (*A*). Recombinant T3R was tested with T3O products 1, 2, and 3 derived from T3O-expressing microsome enzyme assays using 16-methoxytabersonine (*Left*) or tabersonine (*Right*) as substrates (*B*). Coupled enzyme assays of T3O expressing yeast microsomes with various amounts of T3R with 16-methoxytabersonine (*Left*) or tabersonine (*Right*) as substrates (*C*). The reaction products in *A*–*C* were analyzed by LC-MS at 300 nm. SDS/PAGE of affinity purification of T3R by Ni-NTA column chromatography (*D*).

Fig. S7. Saturation kinetics of T3O (CYP71D1V2) coupled to the T3R (ADHL1) reaction. Saturating amount of T3R (2 μg) was assayed with T3O (25 μg total microsomal proteins) with various amounts of tabersonine substrate. The reaction velocity was measured as the consumption of NADPH (–ΔOD340 min$-^{1}$), and plotted against tabersonine concentrations. Error bars indicate the SD from three technical replicates.

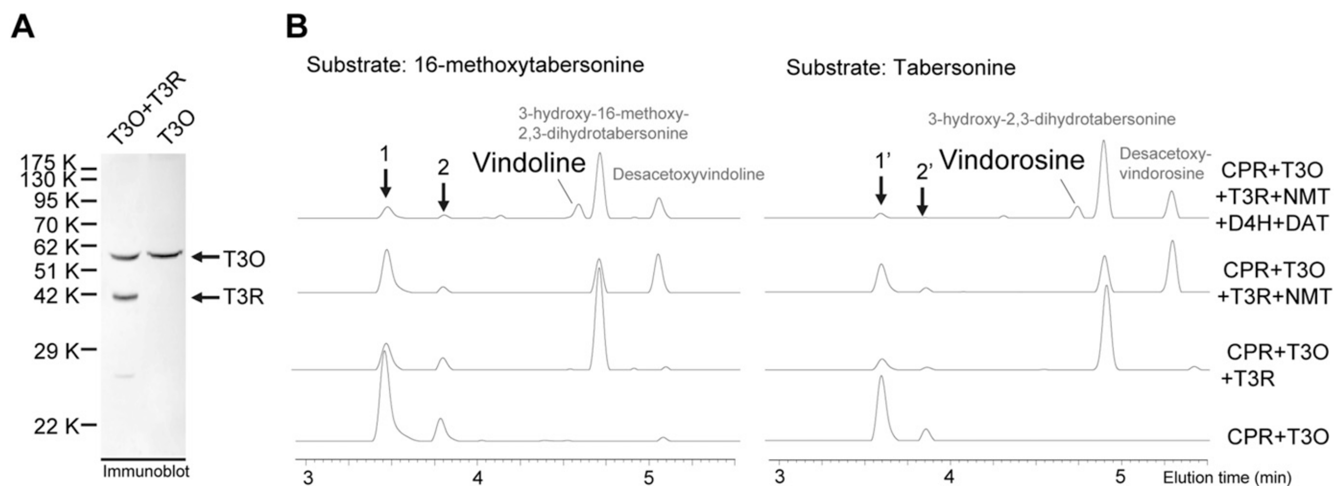

Fig. S8. 3-hydroxylated products are synthesized by T3R and are further modified to form vindoline/vindorosine. Immunoblot of total lysate from yeast expressing CPR+T3O or CPR+T3O+T3R using anti-c-Myc-antibody (*A*). Yeast with recombinant CPR+T3O only, CPR+T3O+T3R, CPR+T3O+T3R+NMT, or CPR+T3O+T3R+NMT+D4H+DAT was fed with either 16-methoxytabersonine (*Left*) or tabersonine (*Right*) (*B*). The biotransformation products were analyzed by LC-MS at 300 nm.

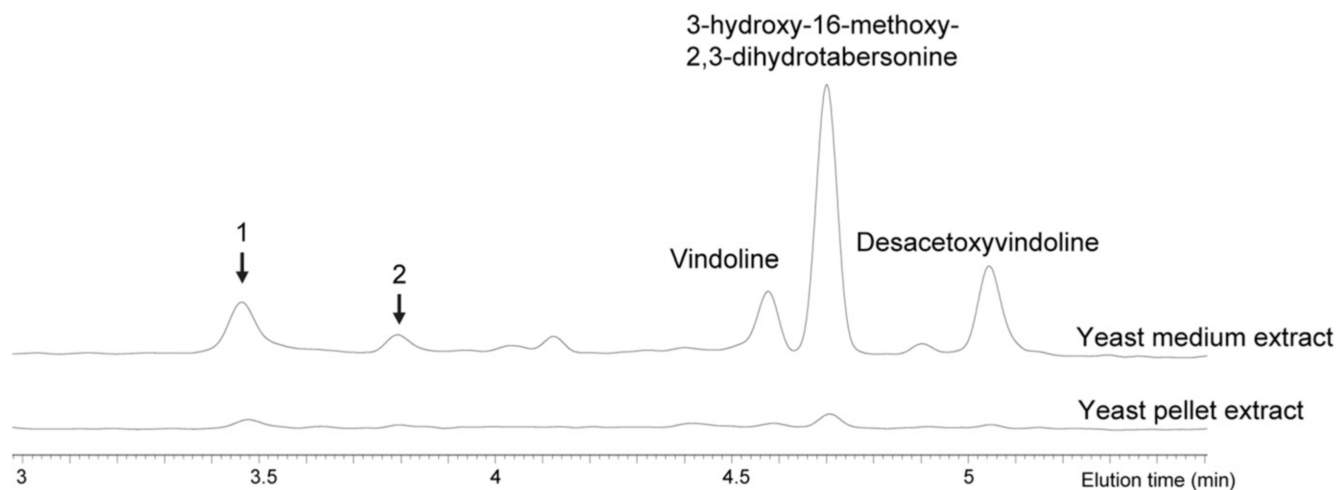

Fig. S9. Yeast secrets vindoline and other intermediates to medium. 16-methoxytabersonine was biotransformed by yeast strain A to vindoline and other intermediates. Both the medium (biotransformation buffer) and broken yeast cells were extracted and diluted to equal volume for LC-MS analysis. The alkaloid profile was analyzed at 300 nm.

Table S1. Enrichment of MIA, iridoid, and triterpene biosynthesis transcripts in *Catharanthus* leaf epidermis

Gene	National Center for Biotechnology Information (NCBI) accession numbers	EST numbers
*Tabersonine 3-reductase (T3R)**	KP122966	59
Secologanin synthase isoform 1 (*SLS*)**	KF415117	54
Loganic acid methyltransferase (*LAMT*)**	EU057974	22
Alpha-amyrin synthase (*AS*)***	JQ027033	19
Tryptophan decarboxylase (*TDC*)*	CAA47898	14
Strictosidine beta-glucosidase (*SGD*)*	AF112888	11
16-hydroxytabersonine O-methyltransferase (*16OMT*)*	EF444544	10
3-hydroxy-16-methoxy-2,3-dihydrotabersonine N-methyltransferase (*NMT*)*	HM584929	9
Tabersonine 3-oxygenase (*T3O*)*	KP122967	8
Tabersonine 16-hydroxylase 2 (*T16H2*)*	JF742645	8
Amyrin oxidase/ursolic acid synthase (*AO*)***	JN613017	8
Strictosidine synthase (*STR*)*	CAA71255	5
10-hydroxygeraniol oxidoreductase (*10HGO*)[†]	AY352047	1
Geraniol-10-hydroxilase (*G10H*)[†]	AJ251269	1
Desacetoxyvidoline 4-hydroxylase (*D4H*)[‡]	U71604	0
Desacetylvindoline O-acyltransferase (*DAT*)[‡]	AF053307	0

*MIA; **late iridoid; ***triterpene. These biosynthesis genes are preferentially expressed in leaf epidermis.
[†]These Early iridoid biosynthesis genes are preferentially expressed in leaf mesophyll IPAP cells.
[‡]These MIA biosynthesis genes are preferentially expressed in leaf mesophyll idioblast or laticifer cells.

Table S2. Primer list

No.	Primer name	Primer sequence (5′-3′)
1	T3O-ApaI-F	ACTCGGGCCCATGGAGTTTCATGAATCTTCTCCCTTCGT
2	T3O-SalI-R	AGTGGTCGACTGCATAGGACGTAGCGATTAATTG
3	T3R-BamHI-F	ATAAGGATCCAATGGCTGCAAAGTCAGTGAAGG
4	T3R-SalI-R	AGAAGTCGACGGGTGATTTGAAAGTGTTTCCAATG
5	NMT-NotI-F	TATAGCGGCCGCATGGAAGAGAAGCAGGAGAAG
6	NMT-SpeI-R	GCGGACTAGTTATTGATTTTCGTCCCGTAACT
7	D4H-NotI-F	ATTAGCGGCCGCTATGAAGGACTTGAACTTTCATGC
8	D4H-SpeI-R	CGGCACTAGTATTGTTTAACCTGAAAGGAGATAAG
9	DAT-BamHI-F	AAGCGGATCCATGGAGTCAGGAAAAATATCGG
10	DAT-SalI-R	GCGGGTCGACATTAGAAACAAATTGAAGTAGCTG
11	Gal1P-DraIII-F	GTCAATCACTACGTGAGTACGGATTAGAAGCCGCCGA
12	CYC-NaeI-R	GTCAATGCCGGCCTTCGAGCGTCCCAAAACCT
13	T16H2-ApaI-F	TACAGCGGGCCCAGGATGGAGTTGTATTATTTTTCCA
14	T16H2-SalI-R	GCGCGTCGACATATTTACCTTTGAGAGAAG
15	16OMT-NotI-F	TATAGCGGCCGCATGGATGTTCAATCTGAGGAGT
16	16OMT-SpeI-R	GCGAACTAGTAGGATAAACCTCAATGAGACT
17	VIGS-T3O-F	GCAGTTGACTGGGCAATGTCAGA
18	VIGS-T3O-R	CCTTCCGATTGCCCAGACATTGAT
19	VIGS-T3R-F	TTGTGAATTCGTGGGTGTTGTAACTGAGGTTGGT
20	VIGS-T3R-R	ATTAGAATTCTGCCCCAGAATCCATAGGAAGGTT
21	T3R-XhoI-R	GATCCTCGAGTTAGGGTGATTTGAAAGTGTTTCCA
22	qPCR-T16H2-F	GATCAACTCACAGTGGCAGTC
23	qPCR-T16H2-R	GACTTGAGGACTTGTGATTGGC
24	qPCR-16OMT-F	GTGGATTCTCCATGACTGGAACGA
25	qPCR-16OMT-R	GATTATCACCTTTCCACCCTTCGC
26	qPCR-T3O-F	TTTGCCATTTGGTGCCGGAAGA
27	qPCR-T3O-R	CTGGGAGTTGCCAGTTGAAATGGT
28	qPCR-T3R-F	CGCGAGTACGGGTGGAAGTATAAA
29	qPCR-T3R-R	CGGGGATAACCTCAACATCTGCAA
30	qPCR-NMT-F	GGCTTCACGTCAATTGTGCTCA
31	qPCR-NMT-R	TCGTCCCGTAACTACAGCGAACT
32	qPCR-D4H-F	GGGTTTCGCCAAGATTGTACGGA
33	qPCR-D4H-R	CGTCAAAGCGTTTGGCAAATCG
34	qPCR-DAT-F	TCCCTCCGGAAGCCATAGAAAAGT
35	qPCR-DAT-R	TACCGTGGCACATCGACTGAGA
36	qRT-PCR-60S-F	TCTTAGTTGGAATGTTCAGCACCTG
37	qRT-PCR-60S-R	CAAGGTTGGAGCCCCTGCTCGTGTT