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Supplementary information

Biosynthesis of medicinal tropane alkaloids in yeast

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Supplementary Materials for

Biosynthesis of medicinal tropane alkaloids in yeast

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This PDF file includes:

Supplementary Notes 1 to 12
Supplementary Tables 1 to 5
Captions for Supplementary Data 1 to 2

Other Supplementary Materials for this manuscript include the following:

Supplementary Figure 1. Original source images for all Western blots analyzed in this study.
Supplementary Data 1. Oligonucleotide and primer sequences used for plasmid and strain construction
Supplementary Data 2. Plasmids used in this study

Supplementary Text

Supplementary Note 1. Identification and screening of UGT84A27 orthologs from *Solanaceae* transcriptome databases.

We identified orthologs of AbUGT from other TA-producing *Solanaceae* and evaluated their activity on PLA and other phenylpropanoids. We first identified transcripts encoding UGT84A27 from the transcriptomes of *Brugmansia sanguinea* (BsUGT) and *Datura metel* (DmUGT) in the 1000Plants Database using a tBLASTn search. We obtained yeast codon-optimized sequences encoding these orthologous UGTs and screened them for activity by expressing AbUGT, BsUGT, DmUGT, or a BFP negative control from low-copy plasmids in CSY1251. We measured glucoside production in cultures of the transformed strains via LC-MS/MS after 72 h of growth in selective media supplemented with 500 μM PLA, cinnamic acid (CA), or ferulic acid (FA) as glucose acceptors (Extended Data Fig. 2a). All three UGT orthologs exhibited substantial glucosylation of CA (34-65% conversion) and FA (85-90% conversion) and only trace activity on PLA (<3% conversion), with AbUGT showing the greatest conversion of PLA (2.7%) (Extended Data Fig. 2b,c). Although UGT84A27 orthologs from other TA-producing *Solanaceae* might exhibit higher activity on PLA than AbUGT, the small margin of variation between the three tested orthologs suggested that limited improvements, if any, would be achieved via exhaustive screening of other orthologs.

Supplementary Note 2. Structure-guided engineering of the AbUGT active site.

Given the disproportionate variation in activity of AbUGT on the structurally similar substrates cinnamate, ferulate, and PLA, we next applied a structure-guided rational mutagenesis approach to engineer the active site of AbUGT for improved activity on PLA. We first constructed a homology model of AbUGT bound to UDP-glucose based on the crystal structure of *Arabidopsis thaliana* salicylate UDP-glucosyltransferase UGT74F2 (PDB: 5V2K) using the RaptorX web server[1] (Extended Data Fig. 2d) and simulated the docking of D-PLA in the active site using the Maestro/GlideXP software suite. Based on the energy-minimizing binding mode, we determined that the aryl ring of D-PLA is likely stabilized by pi-stacking interactions with F130, while its α-hydroxyl and carboxylate groups are respectively stabilized by hydrogen bonds with Q151 and H24, such that the nucleophilic carboxylate oxygen is within 4 Å of the electrophilic C1 carbon of UDP-glucose (Extended Data Fig. 2e). D-PLA is additionally adjacent to the residues L205 and I292, neither of which appear to interact with either substrate. We therefore hypothesized that mutation of (i) F130 to tyrosine might preserve pi-stacking with the aryl ring of D-PLA while providing an additional hydrogen bond to stabilize the α-hydroxyl oxygen of D-PLA, which is absent from cinnamate and ferulate; (ii) L205 to phenylalanine might increase pi-stacking stabilization of D-PLA with F130Y; and (iii) I292 to glutamine would generate two additional stabilizing hydrogen bonds with D-PLA and UDP-glucose (Extended Data Fig. 2e). We screened the F130Y, L205F, and I292Q point mutants of AbUGT for activity by expressing each mutant, wild-type AbUGT, or a BFP control from a low-copy plasmid in CSY1251 and measured glucoside production by LC-MS/MS after 72 h of growth in selective media supplemented with 500 μm PLA, CA, or FA. All three mutants exhibited comparably low (and statistically indistinguishable) activity on PLA relative to wild-type AbUGT (<3% conversion), although the F130Y and I292Q mutations significantly decreased UGT activity on CA (Extended Data Fig. 2f).

Supplementary Note 3. Structural analysis of *A. belladonna* hyoscyamine dehydrogenase.

We constructed a homology model of AbHDH based on the crystal structure of *Populus tremuloides* sinapyl alcohol dehydrogenase (PtSAD; PDB: 1YQD) (Fig. 2b). AbHDH is a member of the zinc-dependent alcohol dehydrogenase (ZADH) family within the medium-chain dehydrogenase/ reductase (MDR) superfamily. Typical of this family, AbHDH exhibits a bi-lobular structure with a well-

conserved nucleotide-binding domain and a more variable substrate-binding domain. Alignment of residues S216, T217, S218, and K221 within the AbHDH nucleotide-binding domain with the phosphate-stabilizing residues S214, T215, S216, and K219 in PtSAD suggests that AbHDH is an NADPH-dependent oxidoreductase. Also typical of ZADHs, AbHDH appears to bind a structural Zn^{2+} using a tetrad of cysteine residues near the protein surface (C105, C108, C111, and C119) and a catalytic Zn^{2+} within the active site.

To elucidate the catalytic mechanism of AbHDH, we performed molecular docking of the substrate, hyoscyamine aldehyde, into the active site using the Maestro/Glide software package (Fig. 2b). The most favorable binding mode positions the aldehyde group of the substrate within ~5 Å of both the catalytic Zn^{2+} and NADPH hydride donor. Based on this docking result and the general mechanism of ZADHs[2], we propose the following catalytic mechanism for AbHDH. In the absence of substrate, the catalytic Zn^{2+} within the active site is stabilized by C52, H74, C168, and a water molecule, which is positioned via polar interaction with S54 and displaced upon binding of hyoscyamine aldehyde. Nucleophilic attack of the aldehyde carbonyl by a dihydronicotinamide hydride forms an oxyanion intermediate stabilized by interaction with the catalytic Zn^{2+}, and which is likely protonated via a proton shuttle between the ribose group of $NADP^+$ and S54.

To support the role of these residues in the AbHDH catalytic mechanism, we mutated C52, S54, H74, and C168 to alanine or structurally similar residues (C → S, S → C, H → F), then measured relative activities of the mutants via expression from low-copy plasmids in CSY1292 and quantification of scopolamine production by transformants following 72 h of growth in selective media with 1 mM littorine. For C52, H74, and C168, mutation to either alanine or a structurally similar residue resulted in near-complete elimination of HDH activity (Extended Data Fig. 3b). While mutation of S54 to cysteine also eliminated activity, mutation to alanine only decreased activity by ~45%. We suspect that the increased atomic radius of sulfur relative to oxygen (S54C) displaces the water molecule within the active site which participates in coordination of the catalytic Zn^{2+} [2]; although the side chain of alanine cannot form polar interactions with this water molecule (as serine can), its small size does not actually displace the water molecule, resulting in decreased but observable activity for the S54A mutant.

Although these results support the proposed catalytic mechanism involving C52, S54, H74, and C168, we note that the observed disruption of enzyme activity by mutation of these four residues may not be strictly due to perturbation of catalysis, but also due to disruption of enzyme structure and folding, substrate access and binding to the active site, or other non-catalytic factors. Nonetheless, the active site and proposed catalytic mechanism of AbHDH are supported by several shared features with those of other sinapyl and cinnamyl alcohol dehydrogenases (SADs and CADs, respectively). Crystallographic and kinetic characterization of PtSAD[2] revealed a nearly identical catalytic tetrad of C50, S52, H72, and C166; cognates of two residues implicated in PtSAD substrate specificity (W61, L122)[2] also appear to be important for hyoscyamine aldehyde binding in AbHDH (W63, M124; see Supplementary Note 4). This catalytic tetrad and mechanism appear to be conserved in certain CADs, but with some variability in the residue responsible for displacing the Zn^{2+}-interacting water: like PtSAD and AbHDH, the active sites of both *Arabidopsis thaliana* CAD5 and *Sorghum bicolor* CAD4 also include two catalytic cysteines (C47/C163 and C47/C165, respectively) and a catalytic histidine (H69), but use glutamic acid (E70) in place of serine[3,4]. However, other CADs of the short-chain dehydrogenase/reductase (SDR) superfamily, such as *Medicago truncatula* CAD2, are not Zn^{2+}-dependent and appear to use a Ser-Tyr-Lys (or similar) catalytic triad for substrate reduction instead[5].

Computational protein modeling and docking simulations are powerful tools for predicting enzyme function from sequence. However, it should be noted that this approach is subject to systematic biases associated with homology-based searches, as enzyme structure and catalytic features are inferred based on assumed membership of the enzyme of interest in the family or class of the closest sequence

match—for AbHDH, the SAD family within the MDR superfamily. A more conclusive biochemical analysis based on crystallographic and kinetic studies of purified HDH and point mutants is needed to confirm the proposed catalytic mechanism, and the identity of residues involved in cofactor binding and substrate specificity. In particular, future studies may focus on validating HDH dependence on NADPH via mutagenesis of residues 216-221 and further characterizing substrate scope via mutagenesis of W63/M124 and others (see Supplementary Note 4).

Supplementary Note 4. Phylogenetic analysis of *A. belladonna* hyoscyamine dehydrogenase.

We performed phylogenetic analysis of AbHDH, DiHDH, and DsHDH to explore potential pathways for the evolutionary origin of this enzyme. A BLASTp search of the UniProt/SwissProt database indicated that the closest sequence matches for AbHDH from non-TA-producing species were mannitol dehydrogenases, 8-hydroxygeraniol dehydrogenases, and other members of both the classical cinnamyl alcohol dehydrogenase (CAD) and sinapyl alcohol dehydrogenase (SAD)-like enzyme families (Extended Data Fig. 3c). Sequence alignment with selected CAD- and SAD-like enzymes indicated that the identified HDH is a member of the latter family (Extended Data Fig. 5a), which typically catalyzes diverse reactions in lignin biosynthesis and metabolism[2,6]. Surface analysis of the AbHDH substrate binding pocket revealed that whereas the phenylpropanoid aryl ring of hyoscyamine aldehyde is tightly packed by W63, H74, and M124, no residues appear to interact with the tropine moiety which extends out of the substrate-binding pocket (Extended Data Fig. 5b,c). Collectively, these observations suggest that the *Solanaceae* may have co-opted an ancestral enzyme from lignin metabolism for TA biosynthesis, and that HDH might tolerate phenylpropanoid substrates with diverse acyl acceptor groups.

Supplementary Note 5. Analysis and modification of littorine synthase sub-cellular localization in yeast.

In silico sequence analysis of AbLS using the SignalP-5.0 web server[7] indicated that it contains an N-terminal signal peptide (SP) comprising residues 1-32 with a putative cleavage site between E32 and A33, suggesting that it follows the expected SCPL ER-to-vacuole trafficking pathway *in planta*. Based on the co-localization of GFP-AbLS with FM4-64 (Fig. 3a, Extended Data Fig. 6b), we hypothesized that vacuolar sequestration of AbLS in yeast might preclude access to the cytosolic pools of tropine and PLA glucoside, as yeast likely lack the requisite tonoplastic transporters present in plants for exchange of secondary metabolites with the cytosol. To determine whether forced localization of AbLS to other yeast compartments—presumably, with improved access to cytosolic metabolites—would enable activity, we replaced the wild-type N-terminal SP sequence with a panel of N-terminal signal sequences taken from yeast proteins targeted to the vacuole lumen (Prc1p and Pep4p), vacuole membrane facing the lumen (Dap2p), *trans*-Golgi network (Och1p), ER membrane facing the lumen (Mns1p), and mitochondrial matrix (Cit1p). We also removed the wild-type SP entirely, and for another variant appended a canonical peroxisome-targeting sequence (PTS1) to the C-terminus. We expressed these chimeric AbLS variants from high-copy plasmids in CSY1294 and screened transformants by LC-MS/MS after 96 h of growth in selective media, but observed no production of littorine or downstream intermediates with any of the variants. We therefore concluded that some other factor—perhaps in addition to vacuolar sequestration—was impeding AbLS activity.

Supplementary Note 6. Elucidation of littorine synthase N-glycosylation patterns in yeast and tobacco.

As protein N-glycosylation patterns differ between yeast and plants, and previous reports have suggested that correct N-glycosylation of diverse plant enzymes is important for their folding, stability, and/or activity[8–10], we next sought to determine how differences in AbLS glycosylation patterns might impact heterologous expression and activity. Prior studies have established that plants, yeast, insects, and mammals all share a highly conserved set of ancient protein N-glycosylation machinery, and as a result also share a conserved core glycan structure[10]. N-glycosylation patterns have also been found to be well-

conserved across higher plants, and particularly across plants of the same family[10,11]. Accordingly, tobacco has been previously used to study N-glycosylation of heterologous proteins from other *Solanaceae* like tomato[9,12]. Moreover, a prior study which reported the discovery of AbLS also observed that the enzyme retains its activity when expressed in tobacco[13]. Based on these observations, we assumed that N-glycosylation patterns (modification loci and glycan moieties) between *A. belladonna* and tobacco must be sufficiently similar to enable correct folding, processing, and activity in the latter, justifying its use as a substitute for the former to compare N-glycosylation patterns to those of yeast.

In silico analysis of the AbLS polypeptide predicted four N-glycosylation sites: N152, N320, N376, and N416. We additionally confirmed that wild-type AbLS expressed transiently in *N. benthamiana* is not O-glycosylated (Extended Data Fig. 6c). We expressed C-terminally HA-tagged wild-type AbLS, each of the four N→Q mutants (where mutation of N to Q abolishes N-glycosylation[14]), or a quadruple N→Q mutant in CSY1294 and in *N. benthamiana*, and then compared glycosylation profiles by Western blot. Whereas wild-type AbLS, N→Q single mutants, and the quadruple mutant all appeared as single bands in *N. benthamiana*, indicating a single glycosylation state, only the quadruple N→Q mutant produced a single band in yeast; all other variants appeared as either double or triple bands, indicating a combination of multiple glycosylation states (Extended Data Fig. 6d,e). Yeast proteins share the same core glycan structure as those of plants but tend to be more heavily mannosylated, typically existing across a spectrum of mannosylation levels[10]. Based on this core structural conservation, and our observation that the denser of the two wild-type AbLS bands in yeast showed partial overlap with that of wild-type AbLS in tobacco, we inferred that at least some fraction of yeast-expressed AbLS was in a similar glycosylation state to the tobacco-expressed AbLS, and that mis-glycosylation was unlikely to account for the complete lack of AbLS activity in yeast. We use the term 'similar glycosylation state' to indicate that the same sites were occupied by glycosylation moieties comparable in structure and size (i.e., same degree of mannosylation). However, the precision of N-glycosylation structure required for activity of any particular plant enzyme is unclear; for example, once a site has at least 10 mannose residues, it is unknown whether adding extra mannose residues (as might occur during heterologous expression in yeast) will disrupt folding or activity.

Further comparison of N-glycosylation patterns (Extended Data Fig. 6d,e) indicate additional similarities and differences between yeast- and tobacco-expressed AbLS, with unclear ramifications for heterologous expression and activity. Quadruple N → Q mutants produced single bands of the same apparent size in both yeast and tobacco, supporting that any observed size differences for the wild-type and single point mutants are due to differences in N-glycan processing. The incomplete N-glycosylation of wild-type AbLS and point mutants in yeast (indicated by the presence of multiple bands), in contrast to the complete N-glycosylation observed in tobacco (indicated by single bands), suggests key differences in glycosylation machinery. We hypothesize that two primary factors may account for these differences: (i) the throughput of N-glycan processing enzymes relative to AbLS expression in the Golgi, and (ii) the accessibility of N-glycosylation sites. First, high-level overexpression of AbLS in yeast may cause incomplete glycosylation if N-glycan processing enzymes become saturated, particularly as AbLS accumulates in the Golgi without a functional egress pathway to the vacuole (see Supplementary Note 7). In contrast, lower constitutive expression of AbLS from T-DNA in *N. benthamiana*, and/or the presence of a functional Golgi → vacuole egress pathway, may reduce the likelihood of N-glycan enzyme saturation *in planta*, leading to more complete N-glycosylation.

Second, misfolding of the AbLS polypeptide in the yeast Golgi, possibly due to mis-glycosylation or glycosylation of sites in an incorrect order, may impede enzyme access to specific N-glycosylation sites, producing a heterogeneous protein population with a range of discrete glycosylation states. Consistent with this hypothesis, mutating each of the four N-glycosylation sites appears to have different effects on apparent protein sizes. In contrast to N152Q, N376Q, and N416Q, the N320Q point mutant produced bands indistinguishable from those of the wild-type in both yeast and tobacco, suggesting that

N320 may be only weakly glycosylated in both expression systems, if at all. Whereas the primary tobacco-expressed AbLS bands are well-defined, the slight vertical spreading exhibited by the higher of the two bands in yeast-expressed wild-type AbLS is consistent with a gradient of mannosylation levels. Moreover, the N152Q mutant in yeast shows bands of similar size to the wild-type, but with a well-defined upper band, suggesting that the N152 position may be a principal contributor to the heterogeneity in mannosylation. Notably, only the N376Q mutation affected the apparent size of the lower of the two AbLS bands in yeast, indicating that the lower band originates from a sub-population of AbLS that is N-glycosylated at only the N376 site. This suggests that glycosylation of N376 in yeast may affect or even disrupt glycosylation at the other sites, and that the order in which N376 is glycosylated relative to the other sites may contribute to the heterogeneous population and double/triple band patterns.

We observed additional high (~150 kDa) and low (<35 kDa) molecular weight (MW) bands in tobacco-expressed AbLS samples; the high-MW bands were also visible in the yeast protein samples, but with much lower relative intensity (Extended Data Fig. 6c-f). Although we cannot conclusively identify the source of these additional bands, we suggest that the high-MW bands may represent large aggregates of AbLS, as glycoproteins tend to agglomerate during high-temperature steps in the sample preparation workflow for SDS-PAGE, and the apparent sizes of these bands showed similar responses to point mutations as the primary (~54 kDa) bands. Differences in the biochemical properties of the yeast and tobacco expression environments (e.g., pH of cellular compartments), as well as necessary differences in sample preparation for tobacco- and yeast-expressed protein extracts, may influence the degree of aggregation and the relative intensity of the corresponding high-MW bands. We suspect that the low-MW bands may be a result of the degradation of AbLS glycosylation site mutants during expression *in vivo*, sample preparation, electrophoresis, or some combination thereof (refer to Supplementary Note 7 for a specific discussion of the origin of the 37 kDa band in tobacco samples). The variation in the intensity of these lower-MW bands supports this hypothesis; for example, observed variations in the mechanical properties of the tobacco extracts (viscosity, fraction of insoluble matter, etc.) may have caused variations in protein exposure to heat and chemical denaturation. These differences, combined with small variations in gel-to-blot transfer efficiency for low-MW proteins, may have produced the observed differences in the size and abundance of different degradation products between samples. However, we note that these variations did not appear to affect the size or abundance of the primary AbLS bands.

Although our analyses suggested that N-glycosylation was unlikely to be the primary factor limiting AbLS activity in yeast, the structure and function of these post-translational modifications remain open questions. Future efforts to elucidate the precise N-glycosylation requirements for functional expression of AbLS may include activity screening of all single, double, triple, and quadruple N-glycosylation point mutants in both yeast and plants, as well as structural modeling and mass spectrometric analyses to elucidate the structure of the N-glycan moieties added to each of the four sites in both organisms. These approaches may inform novel engineering strategies to optimize the expression and activity of the enzyme in heterologous hosts.

Supplementary Note 7. Analysis of post-translational proteolytic processing requirements for littorine synthase expression.

A subset of SCPL acyltransferases, including sinapoylglucose:choline sinapoyltransferase from *Arabidopsis thaliana* (AtSCT) and an avenacin synthase from *Avena strigosa* (AsSCPL1), have been shown to contain an internal propeptide linker which is proteolytically removed to produce an active heterodimer joined by disulfide bonds[15–17]. Comparison of the AbLS amino acid sequence with those of previously characterized plant serine carboxypeptidases and SCPL acyltransferases revealed the presence of an internal 25- to 30-residue sequence which aligns with the highly variable propeptide of AtSCT, AsSCPL1, and wheat carboxypeptidase 2 (TaCBP2), suggesting that AbLS too undergoes endoproteolytic cleavage to form a heterodimer (Extended Data Fig. 7a). Additionally, a homology model of AbLS

suggested that the predicted internal propeptide blocks the active site, thereby necessitating removal for activity (Extended Data Fig. 7b-e). However, three lines of reasoning led us to conclude that wild-type AbLS expressed in *N. benthamiana* does not undergo proteolytic cleavage, and that the additional 37 kDa band observed in tobacco-expressed AbLS samples (Extended Data Fig. 6c,f) is not evidence of proteolytic processing:

a. If AbLS is indeed processed via propeptide removal (as many other SCPL-ATs are; see Extended Data Fig. 7a), the resultant N- and C-terminal subunits would be ~33 kDa (37 kDa with HA tag) and ~18 kDa (22 kDa with HA tag), respectively. As we used C-terminal HA-tagged AbLS for immunoblotting, we should have observed the ~22 kDa smaller subunit rather than the larger N-terminal 37 kDa fragment, assuming AbLS was indeed processed *in planta*; however, no ~22 kDa subunit was detected. Our quadruple point mutant experiments in Extended Data Fig. 6d,e indicated that the total mass of all AbLS N-glycosylations is ~6-8 kDa, meaning that N-glycosylation cannot account for the ~15 kDa discrepancy between the expected ~22 kDa product and the observed 37 kDa band. This suggests that the 37 kDa band is not a product of AbLS proteolytic processing.
b. We observed the 37 kDa band in samples containing both N- and C-terminal HA-tagged AbLS, as well as AbLS with no HA tag at all (Extended Data Fig. 6f). The observation of identical fragment sizes regardless of which terminus was HA-tagged is inconsistent with the expected N- and C-terminal subunits. Our observation of the 37 kDa band in the non-HA-tagged sample (Extended Data Fig. 6f, lane 1) suggests that this band may represent an AbLS degradation product that is non-specifically recognized by anti-HA antibodies, rather than a product of normal protein processing.
c. In our AbLS deglycosylation experiment (Extended Data Fig. 6c), we observed that the size of the 37 kDa band did not change even as the full-length ~55 kDa AbLS band in the same lane was reduced in apparent molecular weight by N-deglycosylation (lane 2). If the 37 kDa band was indeed a product of AbLS processing, then the deglycosylation of N152 (contained in the predicted 37 kDa subunit) should have produced a visible downward shift on the blot—which we did not observe.

As the putative propeptide does not appear to be cleaved or removed *in planta*, we surmised that AbLS might adopt a native conformation in plants which shifts the propeptide away from the active site, but that differences in the biochemical environment of the yeast secretory pathway and/or vacuole prevents this shift, blocking activity.

To address this failure mode, we constructed split AbLS controls in which the N- and C-terminal domains flanking the putative propeptide linker were expressed independently, with or without separate signal peptides. We additionally constructed AbLS variants in which the putative propeptide was replaced with either a flexible $(GGGGS)_n$ linker, the internal propeptide from AtSCT previously demonstrated to be cleaved in yeast[15], or a synthetic linker containing a poly-arginine site cleaved by the *trans*-Golgi protease Kex2p[18,19] (Extended Data Fig. 6g). We first expressed each of the split AbLS controls and propeptide/linker variants from low-copy plasmids in CSY1294 and screened transformants for LS activity by LC-MS/MS after 96 h of growth in selective media, but observed no production of littorine or downstream TAs. We then expressed each of the above C-terminal HA-tagged AbLS variants from low-copy plasmids in CSY1294 and compared apparent protein sizes to split-AbLS controls by Western blot (Extended Data Fig. 6h). Neither the AtSCT nor the poly-arginine linkers produced the 20-25 kDa C-terminal fragment expected from proteolytic cleavage. Collectively, these observations suggest that failure of the internal propeptide to be removed cannot account for the lack of observed AbLS activity in yeast. Moreover, failure of the poly-arginine AbLS variant to be cleaved suggests that the protein becomes stalled in the secretion pathway upstream of the *trans*-Golgi network (TGN; also referred to as the late Golgi in yeast), which may account for the severe growth defect we observed in CSY1294 expressing wild-type or Golgi-targeted (Och1p SP-fused) AbLS.

Supplementary Note 8. Expression, trafficking, and localization of engineered DsRed-AbLS.

Signal peptide and N-terminal domain fusion:

Although the putative N-terminal SP is expected to enable AbLS trafficking to the vacuole in plants, this role may not be conserved in non-plant cells. For instance, if the vacuole-targeting sequence elements in the SP are plant-specific, they may not be recognized by the yeast trafficking machinery when AbLS is heterologously expressed in our engineered strains. This is consistent with our observation that AbLS (with an unmodified N-terminus) fails to pass the yeast trans-Golgi network (Extended Data Fig. 6g,h), and supports our hypothesis that wild-type AbLS does not successfully reach the yeast vacuole. As the SP presumably enables vacuole targeting in plants, but fails to do so in yeast, we co-opted the SP to develop a novel engineering strategy that allows AbLS to reach the yeast vacuole using an alternative route.

In yeast, proteins can follow two different trafficking routes from the ER via the Golgi to the vacuole: soluble proteins which reach the Golgi require a vacuole-targeting sequence for egress to the vacuole, whereas transmembrane proteins which reach the Golgi appear to be routed to the vacuole by default[20,21]. We hypothesized that masking the SP of AbLS using a fused N-terminal domain (such as GFP or DsRed) converts soluble AbLS into a transmembrane protein wherein the SP serves as the transmembrane anchor, enabling the engineered enzyme to reach the yeast vacuole without a specific vacuole targeting sequence. The microscopy in Fig. 3a and Extended Data Fig. 6b support this hypothesis, showing that GFP fused to the N-terminus of AbLS—now with a medial SP/transmembrane domain—enables localization to the yeast vacuole. The N-terminal fusion may not necessarily disrupt SP activity, as naturally-occurring single-pass transmembrane proteins rely on internal signal peptides for localization to and orientation in the ER[22]. In the context of our engineered fusion enzyme, the hypothesized role of the SP is twofold: (i) to direct the nascent polypeptide to the ER, where it can enter the secretion pathway (a role that is retained from the original N-terminal SP, and for which the SP need not be located at the N-terminus); and (ii) to act as a transmembrane anchor that enables the entire transmembrane fusion protein to be trafficked from the Golgi to the vacuole without needing a vacuole-targeting sequence. We suspect that masking the N-terminus of the SP inhibits SP cleavage and removal in the ER, such that it remains as a transmembrane domain within the fusion protein. As the internal propeptide within the AbLS domain shows no signal sequences or transmembrane character based on *in silico* prediction software[7], is a highly variable region in plant SCPL acyltransferases (Extended Data Fig. 7a), and is removed during protein maturation for some enzymes (e.g., *Arabidopsis* SCT)[15], it is unlikely to contain signal elements important for enzyme localization.

Internal propeptide:

As removal of the internal propeptide sequence appears to be a necessary step for functional expression of other SCPL acyltransferases[15–17], the role of the propeptide in functional DsRed-AbLS expression in our engineered yeast strains remains an open question. Given that wild-type AbLS is functional in tobacco[13] without propeptide removal (as we showed via Western blot in Extended Data Fig. 6), we can infer that propeptide removal is not required for activity *in planta*, and therefore likely does not occur for DsRed-AbLS in yeast either. This suggests that the propeptide is more permeable to substrates and products than suggested by our structural modeling (Extended Data Fig. 7b-e). Future studies may elucidate the role of the internal propeptide in the expression and activity not only of AbLS, but of SCPL acyltransferases in general. As the internal propeptide shows high sequence variability across enzymes of the family, conventional crystallography and modeling studies may be insufficient to study this feature. Instead, we suggest that immunochemical and activity characterization of split and propeptide variants, as performed for AbLS herein, may clarify the propeptide processing requirements in other members of this enzyme family.

N-terminal domain oligomerization:

We observed that AbLS activity in our engineered yeast strains was positively correlated with the degree of oligomerization of the N-terminally fused domain, with the highest activity observed for the homotetrameric DsRed fusion partner. However, it is unclear whether the improvement in activity is directly dependent on N-terminal domain oligomerization, or whether this effect is instead mediated by one or more intermediate or confounding factors. One potential explanation is that oligomerization may inhibit cleavage of the SP between the N-terminal domain and the C-terminal AbLS via steric inhibition of access by signal peptidases, promoting SP retention as a transmembrane domain needed for trafficking from the Golgi to the yeast vacuole. As certain serine carboxypeptidases are known to homodimerize or homooligomerize[23], it is additionally possible that some SCPL acyltransferases like AbLS also require oligomerization for activity—such as for formation of interfacial substrate channels or binding pockets, or for adopting a 3D configuration that enables substrate access to the active site. Future efforts to characterize oligomerization requirements for AbLS and other SCPL acyltransferases *in planta* (e.g., via native immunoblotting) and to identify their native 3D structure (e.g., X-ray crystallography, NMR, etc.) may elucidate the role of quarternary protein interactions in the activity of this enzyme family.

Supplementary Note 9. Stereochemistry of TA intermediates and products in engineered yeast strains.

The stereochemistry of key TA intermediates and products in our engineered strains can be deduced based on prior enzyme characterization studies and biochemical logic. TA stereochemistry is determined by, and primarily relevant for, the biosynthetic steps in Modules III-V. It has been previously demonstrated that the enzymes in Module IV of the pathway are highly stereospecific. Littorine mutase (CYP80F1) has been shown to only catalyze the rearrangement reaction of (R)-littorine to (S)-hyoscyamine aldehyde; if presented with (S)-littorine, a range of hydroxylated intermediates are synthesized instead of the aldehyde[24,25]. Similarly, prior structural and kinetic characterization of the H6H enzyme has demonstrated that it catalyzes the two-step hydroxylation and epoxidation of (S)-hyoscyamine to (S)-scopolamine only, and shows negligible activity on (R)-hyoscyamine[26]. The remaining uncertainty regarding stereochemistry therefore pertains to biosynthesis of littorine in Modules III and V, as the stereospecificity of littorine synthase was not tested or discussed in the paper in which it was initially reported[13]. The stereochemistry of littorine is dictated by that of phenyllactic acid (PLA), with D-PLA (i.e., (R)-PLA) leading to production of (R)-littorine. The stereochemistry of PLA is, in turn, set by the stereospecificity of the phenylpyruvate reductase (PPR), which in our strains was *Wickerhamia fluorescens* PPR—previously demonstrated to be a D-specific PPR[27]. As neither the UDP-glucosyltransferase, the littorine synthase, nor the newly reported hyoscyamine dehydrogenase (HDH) reactions act on the chiral center of the TA intermediate or involve an achiral intermediate, no inversion of stereochemistry occurs in these reactions.

Based on the above biochemical logic and the previously established stereospecificities of the enzymes in Modules III and IV, we can deduce that only (R)-littorine, (S)-hyoscyamine, and (S)-scopolamine were produced *de novo* by our engineered strains. Additionally, as specific stereoisomers were used for substrate feeding experiments ((R)-littorine for screening HDH candidates (Fig. 2a,c) and mutants/orthologs (Extended Data Fig. 3), and (S)-hyoscyamine for screening H6H orthologs (Extended Data Fig. 4)), we can additionally conclude that these same TA stereoisomers were produced in feeding experiments.

Supplementary Note 10. Cellular compartmentalization of TA biosynthesis.

TA biosynthesis in *Solanaceae* exhibits extensive intra- and intercellular compartmentalization, with different enzymes active in specific sub-cellular compartments (cytosol, mitochondrion, chloroplast, peroxisome, ER membrane, vacuole), cell types (root pericycle, endodermis, cortex), and tissues (secondary roots)[28]. This spatial organization poses unique challenges for the reconstitution of plant natural product biosyntheses in microbial hosts like yeast: not only do yeast lack distinct tissues which

can be used to produce and store intermediates and products, they also lack plant-specific subcellular compartments like chloroplasts, and the organelles they do share with plants may differ in fundamental function, biochemical properties, or trafficking. Here, we briefly discuss the involvement of each yeast non-cytosolic compartment in our engineered TA biosynthetic pathway (Fig. 1a), and suggest strategies which may be useful for improving the spatial organization of TA biosynthesis in future work.

Mitochondria: Arg2p

Production of the TA precursor tropine (Module II) begins with biosynthesis of arginine from glutamate. This process involves several early steps in the mitochondrial matrix, which in our strains has been modified via overexpression of glutamate N-acetyltransferase (Arg2p; Module I). The urea cycle, a central nitrogen metabolic pathway highly conserved across eukaryotes[29], enables interconversion of cytosolic arginine and the polyamine precursor ornithine; in our strains, this cycle is modified via overexpression of arginase (Car1p) to increase nitrogen flux into ornithine[30]. In engineering an ornithine-overproducing yeast strain, Qin *et al.*[31] evaluated the effect of moving arginine and ornithine biosynthesis from the mitochondrial matrix to the cytosol (the site of glutamate biosynthesis and the urea cycle), and observed modest (~11%) improvements in ornithine titers. However, this strategy necessitated expression of cytosolic arginine biosynthesis enzymes from bacteria, suggesting that cytosolic 're-localization' of the native yeast homologs may be problematic. Moreover, yeast mitochondria express membrane transporters for efficient glutamate import and ornithine export[31], suggesting that transport constraints associated with mitochondrial compartmentalization of arginine/ornithine biosynthesis may not be significant limiters of polyamine accumulation. For these reasons, we elected to retain early arginine/ornithine biosynthesis and Arg2p overexpression in the mitochondria; however, future optimization efforts may benefit from examining systematic re-localization or reconstruction of this pathway in the cytosol.

Peroxisome: DmMPO1$^{\Delta C\text{-}PTS1}$

Oxidation of N-methylputrescine (NMP) to 4-methylaminobutanal (4MAB) in Module II is carried out by methylputrescine oxidase (MPO) in the plant peroxisome lumen[32], and is conserved across the biosynthetic pathways for TAs and nicotine[33]. MPO produces H_2O_2 as a by-product of polyamine oxidation using O_2. As H_2O_2 plays diverse roles in plant signaling pathways[34], plants may have evolved to compartmentalize MPO in the peroxisome both for peroxide detoxification and to reduce disruption to native signaling pathways. A *Nicotiana tabacum* MPO1 mutant lacking a C-terminal peroxisome-targeting tripeptide (PTS1) did not appear to be active outside the peroxisome in tobacco cells[32]. In contrast, our prior attempts to alter MPO1 localization via truncation of PTS1 showed only a small decrease in accumulation of N-methylpyrrolinium (NMPy)[30], indicating that MPO1 remains active outside the yeast peroxisome—possibly facilitated by a highly active native cytosolic catalase[35]. This observation also suggests that transport of NMP and 4MAB/NMPy across the peroxisomal membrane may not be a significant limiter of DmMPO1$^{\Delta C\text{-}PTS1}$ activity, although the mechanisms of peroxisomal transport are unclear. As our prior work[30] demonstrated maximal MPO1 activity with peroxisomal DmMPO1$^{\Delta C\text{-}PTS1}$, we elected to retain this step in the yeast peroxisome.

ER membrane: AbCYP82M3, AbCYP80F1, AtATR1

The cytochromes P450 (CYP450s) tropinone synthase (AbCYP82M3; Module II), littorine mutase (AbCYP80F1; Module IV), and the heterologous cytochrome P450 reductase partner (CPR; AtATR1) that supports their function are located within the ER membrane with their active sites facing the cytosol. The ER tethering of CYP450s and CPRs is thought to provide a more favorable orientation for electron transfer and increases their effective concentrations relative to one another[36]. However, these spatial constraints also pose challenges for engineering complex biosynthetic pathways, as the expression level of ER-bound enzymes may be lower than those of soluble cytosolic enzymes, and the requisite

diffusion of substrates/products of CYP450-catalyzed reactions to/from the ER membrane may limit pathway flux. Although plant CYP450s have been engineered for soluble expression in microbial cells[37–39], our optimization experiments in this study and prior work indicated that neither AbCYP82M3[30] nor AbCYP80F1 expression (Extended Data Fig. 9d) are primary limiters of TA production. Therefore, we retained both CYP450s and the CPR partner (AtATR1) in the yeast ER membrane.

Vacuole and vacuolar membrane: DsRed-AbLS and NtJAT1/NtMATE2

Functional heterologous expression of AbLS, a SCPL-AT which likely localizes to the vacuole/tonoplast lumen *in planta*, was a significant challenge in this work. The fundamental function of the vacuole differs between plants and yeast, serving as a site for biosynthesis and storage of secondary metabolites in the former, and a site for protein degradation in the latter. Moreover, unlike the majority of heterologous proteins used in our engineered TA pathway, SCPL-ATs like AbLS require complex maturation and multi-compartment trafficking pathways for active expression. Extensive engineering effort was required to develop a strategy for recapitulation of AbLS trafficking to the yeast vacuole using N-terminal fusion of a soluble DsRed domain. Our attempts to re-localize the wild-type enzyme showed no observable activity in any other sub-cellular compartments (Supplementary Note 5), suggesting an important role for the ER → Golgi → vacuole trafficking pathway. Post-translational modifications such as disulfide bond formation and N-glycosylation occur only in the ER and Golgi lumen, and are likely difficult to achieve in other compartments. If these modifications are critical for AbLS, then functional expression may only be possible in the two endpoints of the secretion pathway, the vacuole and the extracellular compartment. As AbLS localization to the latter would require at least one biosynthetic step to occur outside the cell—resulting in undesirable diffusion and dilution losses—the vacuole appears to be the only yeast compartment in which AbLS activity can be reconstituted.

This vacuolar requirement imposes two limitations on DsRed-AbLS activity. First, as the vacuole is the site of protein degradation in yeast, DsRed-AbLS may be subject to faster turnover than other pathway enzymes. Future optimization efforts may attempt to engineer the enzyme for improved vacuolar expression and stability. Second, yeast likely lack the vacuolar membrane transporters used in plants for import of substrates (tropine, PLA glucoside) and export of products (littorine). We demonstrated that heterologous alkaloid transporters from tobacco (NtJAT1, NtMATE2) can be used to improve tropine import across the vacuolar membrane. However, the mechanisms of vacuolar PLA glucoside and littorine transport—both in plants and in yeast—are currently unknown. The identification of tonoplastic glucoside importers and littorine exporters from TA-producing *Solanaceae* may enable further attenuation of transport limitations in our engineered yeast platform.

Supplementary Note 11. Strategies for bioprocess optimization and scale-up of fermentative TA production in engineered yeast.

The titers of hyoscyamine and scopolamine from our engineered yeast strains (~30-80 μg/L) are typical of first implementations of complex plant natural product pathways[38,40,41], but require improvements of approximately five orders of magnitude to reach scales appropriate for commercial production (~5 g/L). Here, we discuss three avenues of optimization that may be implemented in future efforts to improve titers towards industrial TA production. We anticipate that these strategies may be tested and implemented by a professional team within 1-2 years of focused effort.

Enzyme engineering and substrate transport:

Engineering of key enzymes in the TA biosynthetic pathway may improve activity in heterologous hosts and reduce losses of intermediates from the pathway, enabling increased metabolite flux into the final products. Our prior work indicated that a substantial fraction of the 4-(1-methyl-2-pyrrodinyl)-3-oxobutanoic acid (MPOB) intermediate produced by AbPYKS in Module II is lost as

hygrine, an undesirable side product, via spontaneous decarboxylation before it can be oxidized to tropinone by the cytochrome P450 AbCYP82M3[30]. Engineered colocalization of AbPYKS and AbCYP82M3 via direct fusion, scaffolding domains[42], and/or tethering of AbPYKS to the ER membrane may attenuate this side reaction by reducing the latency of substrate diffusion between the enzymes. In this work, we observed that the activity of UGT orthologs on PLA is significantly lower than on other phenylpropanoid substrates (Supplementary Note 1), and may constitute a primary contribution to the severe decrease in titer between PLA (>150 mg/L) and hyoscyamine (30 μg/L) (Extended Data Fig. 10). Although our initial attempts to engineer the AbUGT active site for improved activity on PLA were unsuccessful (Supplementary Note 2), more comprehensive structural studies (e.g., crystallography) coupled with exhaustive mutational scanning and/or high-throughput directed evolution[43] may yield substantial improvements in AbUGT selectivity for PLA. Similarly, further structural characterization of AbLS to elucidate the precise mechanism by which N-terminal domain fusion impacts activity (Supplementary Note 8) may inform novel engineering strategies to further improve activity in non-plant cells, or even to enable active cytosolic expression of this enzyme. Recently, Payne *et al*.[44] identified a nitrate/peptide family (NPF) transporter in *Catharanthus roseus* which exports vacuolar strictosidine to the cytosol for conversion to downstream vinca alkaloids. Identification and expression of a cognate vacuolar littorine exporter from TA-producing *Solanaceae* may further reduce transport flux limitations in combination with vacuolar tropine importers like NtJAT1.

Endogenous metabolic modules:

Once enzymatic and transport limitations are addressed, further improvements in TA production may be achieved by increasing flux of central metabolite precursors and co-substrates into the engineered pathway. For example, prior work suggested that malonyl-CoA availability may limit flux through the AbPYKS-catalyzed step in Module II[30], and we demonstrated here that improving the availability of 2-oxoglutarate—a co-substrate for H6H—increases conversion of hyoscyamine to scopolamine (Fig. 4c). Chromosomal modifications to expand the cellular reservoirs of these central metabolites[45–47] may support increased accumulation of downstream TA products. As five different enzymes in the pathway require reducing power for activity (AbCYP82M3, DsTR1, WfPPR, AbCYP80F1, DsHDH), rewiring cellular redox pathways to improve NADPH availability[48,49] may be used to drive increased intermediate flux through these biosynthetic steps. More broadly, central metabolic modules for production of polyamines (arginine/urea cycle)[31] and phenylpropanoids (shikimate/chorismate pathway)[50] may also be useful targets for overexpression and/or deregulation to increase precursor flux into TA biosynthesis.

Nutrient, media, and fermentation optimization:

Strain engineering may be combined with systematic optimization of media and cultivation conditions to improve TA production. Incorporation of complex biosynthetic modules into heterologous hosts can dramatically change the cellular amino acid profile, and may require amendment via media supplementation. For example, TA production in our engineered strains may benefit from increased availability of glutamate/arginine and phenylalanine to furnish Modules I/II and III, respectively. However, our observations of improved TA production via elimination of leucine auxotrophy (Fig. 4c) suggest that some productivity improvements from amino acid optimization may not be obvious, and that comprehensive amino acid titration studies may be useful for amending media composition. We previously observed that different carbon sources can induce dramatic changes in the production of the precursor tropine[30], suggesting that a similar approach may inform media formulations for increased production of hyoscyamine and scopolamine. Finally, improvements in product titers may be expected when transitioning from simple batch cultures to bioreactors[51], which enable maintenance of favorable growth conditions and environmental parameters (pH, temperature, agitation rate, dissolved oxygen, etc.) throughout the fermentation.

Supplementary Note 12. Estimation of reduction in land use for transitioning plant-based scopolamine production to yeast-based fermentation.

General assumptions:

- Typical scopolamine dose is one ~0.5 mg tablet for adults, based on a survey of pharmaceutical reference websites (drugs.com, medscape.com).

Calculation of space-time requirements for Duboisia *scopolamine extraction*:

- *Duboisia* leaves are ~1-2% scopolamine by dry weight[52,53], so we will conservatively take the higher estimate of 2%;
- Australian *Duboisia* plantations have mean annual productivities of ~1000 metric tons of leaves per 1000 ha [54]. This is quite high for medicinal crops, but *Duboisia* is somewhat unique in that it is a tall shrub/tree, so its height allows for much higher medicinal tissue (leaf) production per unit ground area than, for example, opium poppies or other ground-cover medicinal crops.

Unit conversion: 1000 tonnes leaves / 1000 ha = 1 tonne leaves / ha = 1000 kg leaves / 10,000 m^2
= (1 kg leaves / 10 m^2) * (2 kg scopolamine / 100 kg leaves)
= 2 kg scopolamine / 1000 m^2 = 2 g scopolamine / m^2

Therefore, 1000 doses (500 mg scopolamine) would require 0.25 m^2 (20" x 20") land area. As previously stated, the relative space efficiency is due to (a) *Duboisia*'s accumulation of TAs in leaves (abundant in plant) vs. in specialized tissues (e.g. latex pods for poppies), and (b) *Duboisia*'s vertical height (as a tall shrub/tree) giving a very high leaf yield per ground area.

Calculation of space-time requirements for yeast-based scopolamine production platform:

- Sugarcane land efficiency is ~60 tons plant biomass/ha, with a yield of 135 kg sucrose / ton plant biomass[55] → therefore, ~8000 kg sugar / ha = 800 g sugar/m^2

To supply 1000 doses of scopolamine (0.5 g) from yeast culture, assuming 3% yield of scopolamine from fed sugar:

(0.5 g scopolamine) * (100 g sugar / 3 g scopolamine) = ~17 g sugar * (1 m^2 / 800 g sugar)
= 0.02 m^2 of sugar crop.

Therefore, the reduction in required land area for 1000 doses of scopolamine is (0.25 m^2 / 0.02 m^2) ~ 12-fold, or one order of magnitude.

Supplementary Table 1. Amino acid sequences of complete hyoscyamine dehydrogenase (HDH) candidates identified from *Atropa belladonna* and *Datura* transcriptomes.

Gene ID	Sequence	Transcript ID
HDH1	MLETVKYLLGSAGPSGYGSKSTAEKVTEQSIHLRSITAIITGATSGIGA ETARVLAKRGAKLILPARSLKAAEETKSRILSESPDADIIVMSLDLSSL SSVRKFVAQFEYLNFRLNILINNAGKFAHQHAISEDGIEMTFATNHLGH FLLTKLLLKNMIETANKTGVQGRIVNVSSSIHGWFSGDAIQYLRLITKD KSQYDATRAYALSKLANVLHTKELAQILKKMVANVTVNCVHPGIVRTRL TREREGLVTDLVFFLTSKLLKTIPQAAATTCYVATHPRLADVSGKYFAD CNEISSSKLGSNLTEAARIWSASEIMVAKNSNAN*	aba_locus_3722_iso_1_ len_1302_ver_2
HDH2 (AbHDH)	MASEKSLEEKQAENTFGWAAMDSSGVLSPFTFSRRATGEEDVRLKVLYC GICHSDLGCIKNEWGWCSYPLVPGHEIVGIATEVGSKVTKFKVGDRVGV GCMVGSCGTCQNCTQNQESYCPEVIMTCASAYPDGTPTYGGFSNQMVAN EKFVIRIPNSLPLDAAAPLLCAGSTVYSAMKFYGLCSQGLHLGVVGLGG LGHVAVKFAKAFGMKVTVISTSLGKKEEAINQLGADSFLINTDTEQMQG AMEVMDGIIDTVSALHPIEPLLGLLKSHQGKLIIVGLPNKQPELPVFSL INGRKMIGGSAVGGVKETQEMIDFAAEHNITADIEIVPMDYVNTAMERL EKGDVKFRFVIDVENTLVAAQT*	aba_locus_4635_iso_1_ len_1351_ver_2
HDH3	MSKTTPNHTQAVSGWAALDSSGKITPYIFNRRENGVNDVTIKILYCGIC HTDLHYAKNDWGVTIYPVVPGHEITGIVVEVGSNVTNFKTGDKVGVGCM SASCLQCESCKNSEENYCDKVQFTYNGVFWDGSITYGGYSKMLVADYRF VVAVPENLPMDRAAPLLCAGVTVFVPMKDNNLIGSPRKNIGVIGLGGLG HLAIKFAKAFGHRVTVISTSLSKEKDAKTKLGADDFIVSSNAQQMQSRQ KTLDFILDTVSADHSLGPYLELLKIKGTFVIVGAPDKPMGLPAFPLIFG KRTVKGSMIGSIKETQEMLDICGKYNIMCDIEIVTPDRINEAYERIEKN DIKYRFVIDIDGQSSKL*	aba_locus_5175_iso_1_ len_1282_ver_2
HDH4	MAMEGTKVARIKLGSDGLEVSAQGLGCMGMSAFYGPPKPEPDMIQLIHH AINSGVTFLDTSDIYGPHTNEILLGKALKGGIRERVELATKFGISFADG KREVRGDPAYVRATCVASLKRLDVDCIDLYYQHRIDTRVPIEVTVGELK KLVEEGKVKYIGLSEASASTIRRAHAVHPITAVELEWSLWSRDVEEELV PTCRELGIGIVAYSPLGRGFLSSGSKLLEDMSNEDYRKHLPRFQSENLE HNKKLYERICQTAARMGCTPSQLALAWVHHQGNDVCPIPGTTKIENLNQ NIEALSIKLTSEDMTELESIASANAVQGDRYGSGASTYKDSETPPLSAW KVT*	aba_locus_5694_iso_2_ len_1279_ver_2
HDH5	MEVKNKYVAIKSNINGAPQESHFEIKVENLSLIVEPDSKEVIIKNLFVS IDPYQLNRMKSESSSQAAISYASAITPGKAIDTYGVGRVLVSDRPEFKK DDLVAGLLTWGEYTVVKEGSLLNKLDPLGFPLSNHVGVLGFSGLAAYGG FFEVCKPKPGEKVFVSAASGSVGNLVGQYAKLLGCHVVGSAGSQEKVKL LKETLGFDDAFNYKEETDLKSALKRCFPQGIDVCFDNVGGKMLEAAVAN MNLFGRVAICGVISEYTNASTRAAPEMLDIVYKRITIQGFLAADFMKVY ADFLSETVEYLQDGKLKAVEDVSEGVESIPSAFIGLFNGDNIGKKIVKV ADE*	aba_locus_6801_iso_1_ len_1156_ver_2
HDH6	MLRIRSRIISISRSLILRQTSSNKFSTHSERKLEGKVAVITGAASGIGK ETAAKFISHGAKVIIADIQKQLGQETASELGPNATFVSCDVTKESDISD VVDFAVSKHGQLDIMYNNAGIACRTTFSIVDLDLAQFDRIMAINVRGVV AGIKHAARVMIPQGSGCILCTGSITGVMGGLAQPTYSTTKSCVIGIVKS TTGELCKHGIRINCISPFAIPTAFSLDEMKEYFPGVEPEGLVKILQNAS ELKGAYCEPIDVANAAIFLASEDAKFISGENLMVDGGFTSFKKLNLSHL VQ*	aba_locus_8950_iso_1_ len_1109_ver_2

HDH7	MASNGISHVNGTLAKVITCRAAVAYGPGQPLVVEQVQVDPPQKMEVRIK ILFTSICHTDLSAWKGENEAQRVYPRILGHEASGVVESVGEGVTDMKTG DHVVPIFNGECGECVYCNSSKKTNLCGKFRVNPFKSVMANDGKCRFRNK DGNPIYHFLNTSTFSEYTVVDSACLVNIDPHAPLDKMTLLSCGVSTGLG AAWNTADVQTGETVAVFGLGAVGLAVVEGARTRGASRIIGVDINSEKRI KGQAIGITDFINPKEIDVPVHEKIREMTGGGVHYSFECAGNLEVLREAF SSTHDGWGMTIVLGIHPTPRLLPLHPMELFDGRRIVASVFGDFKGKSQL PFFAKQCMAGVVKLDEFITHELPFEKINEGFQLLVDGKSLRCLLHL*	aba_locus_11748_iso_1 _len_557_ver_2
HDH8	MAEKITSLESTRYAVVTGGNKGIGYETCRQLVSKGVVVVLTARDEKRGI EATERLKEESSFTDDQIMFHQLDVVDPDSISSLVDFINTKFGRLDILVN NAGVGGLMVEGDVVILKDLIEGDFVSVSTENEEEGDTEKSIEGIVTNYE LTKQCVETNFYGAKRMSEAFIPLLQLSNSPTIVNVASFLGKLKLLCNEW AIKVLSNANNLTEDRVDEVVNEFLKDFTEKSIEAKGWPTYFAAYKVSKA AMIAYTRVLATKYPNFRINSVCPGYCKTDLTANTGSLTAEEGAESLVKL ALLPNDGPSGLFFYRKDVAAL*	aba_locus_12989_iso_1 _len_1050_ver_2
HDH9	MLRIASRGGITSRSLQLLQTFNKEFSTHIERKLEGKVALITGAASGIGK ETAAKFINNGAKVIIADVQKQLGQETASQLGPNATFVLCDVTKESDVSN AVDFAVSNHGQLDIMYNNAGIICRTPRNIADLDLDAFDRVMAINVRGMM AGIKHAARVMIPRKAGSILCTASITGTMGGLAQPTYSTTKSCVIGMMRS VTAELCQNGIRINCISPFAIPTPFYIDEMKSYYPGVEPEVLVKMLYRAS ELNGAYCEPVDVANAAVFLASDDAKYVSGQNLVIDGGFTSYKSLNFPMS DQE*	aba_locus_16663_iso_1 _len_466_ver_2
HDH10	MGIPSSVTPIVRRLEGKVAVITGGASGIGEAATRLFVKHGAKVVVADVR DDLGRALCKELGSNDTISFAHCSVTDENDVQNAIDGAVSRYGMLDIMFN NAGITGNMKDPSILATDYKNFKNVFDVNVYGAFLGARIAAKAMIPTKQG SILFTASIASVIGGIASPITYASSKHAVVGLTNHLAVELGQYGIRVNCI SPYTVATPLVREILGKMDKEKAEEVIMETANLKGKILEPEDIAEAAVYL GSDESKYVSGINLVIDGGYSKTNPLASMVMQNYI*	aba_locus_114040_iso_ 1_len_645_ver_2
HDH11	MESKSGEGKIVCVTGASGFIASWLVKLLLHRGYTVNATVRNLKDTSKVA HLLGLDGANERLHLFKAELLEEQSFDAAVDGCEGVFHTASPVSLTAKSK EELVDPAVKGTLNVLRSCAKSPSVLRVVITSSTASVICNKNMSTPGAVA DETWYSDPEFCEEREEWYQLSKTLAEQAAWKFAKENEMDLVTLHPGLVI GPLLQPTLNFSCEAIVNFIKEGKEAWSGGVYRFVDVRDVANAHILAFEV PSANGRYCLVGVNGYSSLVLKIVQKLYPSITLPENFEDGLPLTPHFQVS SERAKGLGVKFTPLELSVKDTVESLMEKNFLHI*	aba_locus_125882_iso_ 1_len_348_ver_2A
HDH12	MESKSGEGKIVCVTGASGFIASWLVKLLLHRGYTVNATVRNLKDTSKVA HLLGLDGANERLHLFKAELLEEQSFDAAVDGCEGVFHTASPVSLTAKSK EELVDPAVKGTLNVLRSCAKSPSVLRVVITSSTASVICNKNMSTPGAVA DETWYSDPEFCEERKEWYQLSKTLAEKAARRFAKENGIDLVTLHPGLVI GPLLQPTLNFSCEAIVNFIKEGKEAWSGGVYRFVDVRDVANAHILAFEV PSANGRYCLVGVNGYSSLVLKIVQKLYPSITLPENFEDGLPLTPHFQVS SERAKGLGVKFTPLELSVKDTVESLMEKNFLHI*	aba_locus_125882_iso_ 1_len_348_ver_2B
DiHDH	MAAEKLSEEEAVKTFGWAAMDSSGVLSPFEFSRRATGAEDVRLKVLYCG ICHSDLGCVKNEWGWCSYPLVPGHEIVGIATEVGSRVTKFKVGDRVGVG CMVGSCGSCQNCSQNLESYCPEVIMTCASAYPDGTPTYGGFSNQMVANE KFVIQIPEKLPLDAAAPLLCAGSTVYSPMKFYGLCSPGLHLGVVGLGGL GHVAVKFAKAFGMKVTVISTSIGKKEEAINQLGADSFLTSTDTEQMQGA METMDGIIDTVSALHPIEPLVGLLKSHQGKLIIVGLPNKQPELPVFSLI NGRKMIGGSAVGGVKETQEMIDFAAKHNITADIEIVRMDYVNTAMERLE KGDVKFRFVIDVENTLVPAQT*	medp_datin_20101112| 6354

DsHDH	MAAEKLEERKRWETFGWAAMDSSGVLSPFEFSRRATGEEDVRLKVLYCG ICHSDLGCIKNEWGWCSYPLVPGHEIVGIATEVGSRVTKFKVGDRVGVG CMVGSCGSCQNCSQNLESYCPEVIMTCASAYPDGTPTYGGFSNQMVANE KFVIQIPEKLPLDAAAPLLCAGSTVYSPMKFYGLCSPGLHLGVVGLGGL GHVAVKFAKAFGMKVTVISTSIGKKEEAINQLGADSFLISTDTEQMQGA METMDGIIDTVSALHPIEPLVGLLKSHRGKLIIVGLPNKQPELPVFSLI NGRKMIGGSAVGGVKETQEMIDFAAKHNITADIEIVGMDYVNTAMERLE KGDVKFRFVIDVENTLVPAQT*	medp_datst_20101112| 10433

Supplementary Table 2. Biosynthetic enzyme-coding genes used in this study.

Gene ID	Description	Source organism	Accession
PGM2	Phosphoglucomutase 2	*Saccharomyces cerevisiae*	P37012
UGP1	UDP-glucose pyrophosphorylase	*Saccharomyces cerevisiae*	P32861
EXG1	Glucan 1,3-β-glucosidase I/II	*Saccharomyces cerevisiae*	P23776
SPR1	Sporulation glucan 1,3-β-glucosidase	*Saccharomyces cerevisiae*	P32603
EGH1	Ergosteryl-β-glucosidase	*Saccharomyces cerevisiae*	P40566
PRC1	Carboxypeptidase Y	*Saccharomyces cerevisiae*	P00729
PEP4	Proteinase A (saccharopepsin)	*Saccharomyces cerevisiae*	P07267
DAP2	Dipeptidyl aminopeptidase B	*Saccharomyces cerevisiae*	P18962
OCH1	Initiation-specific α-1,6-mannosyltransferase	*Saccharomyces cerevisiae*	P31755
MNS1	ER α-1,2-mannosidase	*Saccharomyces cerevisiae*	P32906
CIT1	Mitochondrial citrate synthase	*Saccharomyces cerevisiae*	P00890
AbPPR	3-Phenylpyruvate reductase	*Atropa belladonna*	A0A3S8UWA2
WfPPR	3-Phenylpyruvate reductase	*Wickerhamia fluorescens*	F1T2J9
LpPPR	3-Phenylpyruvate reductase	*Lactobacillus plantarum*	A0A2U9AUW1
hcxB	Hydroxycarboxylate dehydrogenase B	*Escherichia coli (K12)*	P30178
BcLDH	Lactate dehydrogenase	*Bacillus coagulans*	ADN38376
LcLDH	Lactate dehydrogenase	*Lactobacillus casei*	AWO81909
LpLDH	Lactate dehydrogenase	*Lactobacillus plantarum*	WP_072534285
AbUGT	UDP-glucosyltransferase 84A27	*Atropa belladonna*	A0A3B6XRM0
BsUGT	UDP-glucosyltransferase 84A27	*Brugmansia sanguinea*	[a]
DmUGT	UDP-glucosyltransferase 84A27	*Datura metel*	[b]
AbLS	Littorine synthase	*Atropa belladonna*	A0A3B6XRA5
AbCYP80F1	Littorine mutase	*Atropa belladonna*	A0A024CI64
AbHDH	Hyoscyamine dehydrogenase	*Atropa belladonna*	[c]
DiHDH	Hyoscyamine dehydrogenase	*Datura innoxia*	[d]
DsHDH	Hyoscyamine dehydrogenase	*Datura stramonium*	[e]
AaH6H	Hyoscyamine 6β-hydroxylase/dioxygenase	*Anisodus acutangulus*	A2IBI0
BaH6H	Hyoscyamine 6β-hydroxylase/dioxygenase	*Brugmansia arborea*	A0A0M3SG09
DmH6H	Hyoscyamine 6β-hydroxylase/dioxygenase	*Datura metel*	Q6EZB3
DsH6H	Hyoscyamine 6β-hydroxylase/dioxygenase	*Datura stramonium*	A0A0M4K1P1
NtJAT1	Jasmonate-inducible alkaloid transporter 1	*Nicotiana tabacum*	AM991692
NtMATE1	Multidrug and toxic compound extrusion 1	*Nicotiana tabacum*	A3KDM4
NtMATE2	Multidrug and toxic compound extrusion 2	*Nicotiana tabacum*	A3KDM5

[a] 1000Plants (1KP) database: scaffold-AIOU-2012986-Brugmansia_sanguinea
[b] 1000Plants (1KP) database: scaffold-JNVS-2051323-Datura_metel
[c] MSU Medicinal Plant Genomics Resource: aba_locus_4635_iso_1_len_1351_ver_2
[d] Medicinal Plant RNAseq database: medp_datin_20101112|6354
[e] Medicinal Plant RNAseq database: medp_datst_20101112|10433

Supplementary Table 3. Yeast strains used in this study.

Strain	Genotype
CEN.PK2-1D	MATα *ura3-52*; *trp1-289*; *leu2-3/112*; *his3Δ1*; *MAL2-8C*; *SUC2*
CSY1251	CEN.PK2-1D; *meu1-14*; *oaz1-16*; *his2*:: P_{TPI1}-*ARG2*-T_{STE2}, P_{PGK1}-*CAR1*-T_{PHO5}, P_{TDH3}-*FMS1*-T_{ADH1}; *hfd1-45*; *ald4-48*; *ald5-53*; *ald6-55*; *ALD2Δ0-ALD3Δ0*:: *HIS3*; *trp1*:: P_{PGK1}-*AsADC*-T_{ADH1}, P_{TEF1}-*SPE1*-T_{CYC1}, P_{TDH3}-*speB*-T_{PHO5}; *ura3*:: P_{PGK1}-*AbPMT1*-T_{PHO5}, P_{TDH3}-*DmMPO1*$^{\Delta C\text{-}PTS1}$-T_{ADH1}, P_{TEF1}-*DsTR1*-T_{CYC1}; *leu2*:: P_{PGK1}-*AbCYP82M3*-T_{PHO5}, P_{TEF1}-*AtATR1*-T_{CYC1}, P_{HXT7}-*AbPYKS*-T_{PGK1}; *ald6*-55:: *ALD6*; *pad1*:: P_{PGK1}-*AbPMT1*-T_{PHO5}, P_{TDH3}-*DsPMT1*-T_{CYC1}, P_{HXT7}-*AbPYKS*-T_{PGK1}
CSY1287	CSY1251; *ald4*:: P_{TDH3}-*WfPPR*-T_{ADH1}
CSY1288	CSY1251; *ald4*:: P_{TDH3}-*WfPPR*-T_{ADH1}, P_{PGK1}-*AbUGT84A27*-T_{PHO5}
CSY1292	CSY1251; *spe4*:: P_{TEF1}-*DsH6H*-T_{CYC1}, P_{TDH3}-*AbCYP80F1*-T_{ADH1}
CSY1294	CSY1292; *ald4*:: P_{PGK1}-*AbUGT84A27*-T_{PHO5}, P_{TDH3}-*WfPPR*-T_{ADH1}, P_{TEF1}-*DsHDH*-T_{CYC1}, P_{HXT7}-*DsH6H*-T_{PGK1}
CSY1296	CSY1294; *egh1*:: P_{PGK1}-*UGP1*-T_{PHO5}, P_{TDH3}-*DsRed-AbLS*-T_{CYC1}
CSY1297	CSY1296; *exg1*:: P_{PGK1}-*DsH6H*-T_{PHO5}, P_{TDH3}-*WfPPR*-T_{ADH1}, P_{TEF1}-*NtJAT1*-T_{CYC1}
CSY1298	CSY1297; pCS4213

Supplementary Table 4. LC-MS/MS metabolite detection parameters for extracted ion chromatograms (EIC) and multiple reaction monitoring (MRM).

Compound	EIC or MRM transition (m/z^+)	Fragmentor	Collision energy	Reference
Tropine	142 → 98	50	21	[30]
D-3-phenyllactic acid	184[†]	10, 20, 50, 100[‡]	--	This work
1-*O*-β-phenyllactoyl-glucose	346[†]	10, 20, 50, 100[‡]	--	This work
Trans-cinnamic acid	149[a]	10, 20, 50, 100[‡]	--	This work
1-*O*-β-cinnamoyl-glucose	149[a]	10, 20, 50, 100[‡]	--	This work
Trans-ferulic acid	195[b]	10, 20, 50, 100[‡]	--	This work
1-*O*-β-feruoyl-glucose	195[b]	10, 20, 50, 100[‡]	--	This work
Hyoscyamine aldehyde	288 → 124	120	25	This work
Hyoscyamine (littorine)	290 → 124 [c]	100	25	This work
Scopolamine	304 → 138	64	17	This work

[†] NH_4^+ adduct.
[‡] Cycle sum of all fragmentor voltages.
[a] Distinguished by retention time (see Extended Data Fig. 2).
[b] Distinguished by retention time (see Extended Data Fig. 2).
[c] Distinguished by retention time; however, based on comparison to authentic standards, only hyoscyamine (but not littorine) was detected in culture supernatant of yeast strains for all experiments.

Supplementary Table 5. Exact P-values from Student's two-tailed t-tests in Figures 1-4.

Figure	Test	P-value
1b	Control vs. BcLDH	0.0325
1b	Control vs. LpLDH	0.0361
1b	Control vs. LpPPR	3.27×10^{-4}
1b	Control vs. hcxB	0.0167
1b	Control vs. WfPPR	0.00238
1d	Control vs. UGP1	0.0108
1e	Control vs. ΔSPR1	0.0154
1e	Control vs. ΔEGH1	1.68×10^{-4}
2a	Control vs. HDH2, hyoscyamine aldehyde	1.54×10^{-4}
2a	Control vs. HDH2, scopolamine	2.34×10^{-4}
3b	Control vs. EGFP, hyoscyamine	0.00372
3b	Control vs. EGFP, scopolamine	0.00533
3b	tagBFP vs. EGFP, hyoscyamine	0.00533
3b	tagBFP vs. mCherry, hyoscyamine	1.75×10^{-4}
3b	tagBFP vs. AbUGT, scopolamine	4.46×10^{-4}
3b	mCherry vs. AbUGT, hyoscyamine	0.0171
3b	AbUGT vs. DsRed.T3, hyoscyamine	4.89×10^{-4}
3b	AbUGT vs. DsRed.T3, scopolamine	0.00153
4a	Control vs. NtJAT1, tropine	0.0395
4a	Control vs. NtJAT1, hyoscyamine	0.00226
4a	Control vs. NtJAT1, scopolamine	0.0281
4a	Control vs. NtMATE1, tropine	0.0180
4a	Control vs. NtMATE1, hyoscyamine	0.00424
4a	Control vs. NtMATE1, scopolamine	0.00180
4a	Control vs. NtMATE2, hyoscyamine	0.00232
4c	CSY1296 vs. CSY1296 + cofactors, hyoscyamine	0.00147
4c	CSY1296 vs. CSY1296 + cofactors, scopolamine	0.00401
4c	CSY1296 vs. CSY1297, hyoscyamine	0.00133
4c	CSY1296 vs. CSY1297, scopolamine	5.55×10^{-6}
4c	CSY1297 vs. CSY1297 + cofactors, hyoscyamine	6.85×10^{-4}
4c	CSY1297 vs. CSY1297 + cofactors, scopolamine	1.22×10^{-6}
4c	CSY1297 + cofactors vs. CSY1298, hyoscyamine	0.00101
4c	CSY1297 + cofactors vs. CSY1298, scopolamine	2.71×10^{-4}

Supplementary Data 1. (separate file) Oligonucleotide and primer sequences used for plasmid and strain construction.

Sequences are provided for all primers used for construction of Cas9/gRNA plasmids, yeast integration vectors, yeast integration cassettes, enzyme mutants, and fusion proteins, as well as for amplification of genes from yeast genomic DNA/colony PCR. Yeast expression plasmids for non-fusion proteins/enzymes were constructed via restriction/ligation cloning of synthesized DNA fragments, so no primers were required.

Supplementary Data 2. (separate file) Plasmids used in this study.

Dash ('-') indicates linear insertion of DNA sequence (e.g., insertion of promoter-gene-terminator cassette, insertion of in-frame fusion protein domain). Underscore ('_') indicates modification of preceding sequence element (e.g., point mutation).

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