

# De novo production of the plant-derived alkaloid strictosidine in yeast

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The monoterpene indole alkaloids are a large group of plant-derived specialized metabolites, many of which have valuable pharmaceutical or biological activity. There are ~3,000 monoterpene indole alkaloids produced by thousands of plant species in numerous families. The diverse chemical structures found in this metabolite class originate from strictosidine, which is the last common biosynthetic intermediate for all monoterpene indole alkaloid enzymatic pathways. Reconstitution of biosynthetic pathways in a heterologous host is a promising strategy for rapid and inexpensive production of complex molecules that are found in plants. Here, we demonstrate how strictosidine can be produced de novo in a *Saccharomyces cerevisiae* host from 14 known monoterpene indole alkaloid pathway genes, along with an additional seven genes and three gene deletions that enhance secondary metabolism. This system provides an important resource for developing the production of more complex plant-derived alkaloids, engineering of nonnatural derivatives, identification of bottlenecks in monoterpene indole alkaloid biosynthesis, and discovery of new pathway genes in a convenient yeast host.

monoterpene indole alkaloid | strictosidine | secologanin | *Catharanthus roseus* | *Saccharomyces cerevisiae*

Monoterpene indole alkaloids (MIAs) are a diverse family of complex nitrogen-containing plant-derived metabolites (1, 2). This metabolite class is found in thousands of plant species from the Apocynaceae, Loganiaceae, Rubiaceae, Icacinaceae, Nyssaceae, and Alangiaceae plant families (2, 3). Many MIAs and MIA derivatives have medicinal properties; for example, vinblastine, vincristine, and vinflunine are approved anticancer therapeutics (4, 5). These structurally complex compounds can be difficult to chemically synthesize (6, 7). Consequently, industrial production relies on extraction from the plant, but these compounds are often produced in small quantities as complex mixtures, making isolation challenging, laborious, and expensive (8–10). Reconstitution of plant pathways in microbial hosts is proving to be a promising approach to access plant-derived compounds as evidenced by the successful production of terpenes, flavonoids, and benzyloisoquinoline alkaloids in microorganisms (11–19). Microbial hosts can also be used to construct hybrid biosynthetic pathways to generate modified natural products with potentially enhanced bioactivities (8, 20, 21). Across numerous plant species, strictosidine is believed to be the core scaffold from which all 3,000 known MIAs are derived (1, 2). Strictosidine undergoes a variety of redox reactions and rearrangements to form the thousands of compounds that comprise the MIA natural product family (Fig. 1) (1, 2). Due to the importance of strictosidine, the last common biosynthetic intermediate for all known MIAs, we chose to focus on heterologous production of this complex molecule (1). Therefore, strictosidine reconstitution represents the necessary first step for heterologous production of high-value MIAs.

## Results

*Escherichia coli* and *Saccharomyces cerevisiae* are the most common choices for a heterologous microbial host for plant metabolite production because of the short generation times and the

abundance of available genetic tools for these organisms (21, 22). We chose to use *S. cerevisiae* as a host because functional expression of microsomal plant P450s has more precedence in yeast (23). Additionally, plants exhibit extensive intracellular compartmentalization of their metabolic pathways (24), and the impact that this compartmentalization has on alkaloid biosynthesis can only be explored further in a eukaryotic host (25). To enhance genetic stability, we used homologous recombination to integrate the necessary biosynthetic genes under the control of strong constitutive promoters (*TDH3*, *ADH1*, *TEF1*, *PGK1*, *TPI1*) into the *S. cerevisiae* genome (Fig. S1).

Strictosidine, like all monoterpenes, is derived from geranyl pyrophosphate (GPP) (Fig. 2) (24). In *S. cerevisiae* GPP is synthesized via the mevalonate pathway by the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). To increase flux through the IPP/DMAPP pathway in the yeast host, we combined several previously reported approaches (Fig. 2): (i) we integrated a truncated HMG-CoA reductase gene (*tHMGR*), which encodes a non-feedback-regulated rate-limiting enzyme of the mevalonate pathway (16); (ii) we installed a second copy of *IDII*, the gene encoding IPP isomerase to increase DMAPP formation (26); (iii) because IPP is a common substrate for both GPP and tRNA synthesis, we integrated a second copy of *MAF1*, a negative regulator of tRNA synthesis, to direct IPP away from tRNA synthesis and into GPP production (27). However, although farnesyl pyrophosphate (FPP) production for sesquiterpene biosynthesis has been extensively optimized in *S. cerevisiae*, GPP production requires additional metabolic engineering because *S. cerevisiae* does not naturally produce GPP. The *S. cerevisiae* *ERG20* gene encodes a farnesyl

## Significance

Plants make a wide variety of complex molecules with potent biological activities including several anticancer therapeutics. Unfortunately, plants often produce these molecules in low amounts, making them expensive to obtain. Engineering simpler organisms, such as yeast, to produce these plant-derived compounds provides one solution to production challenges. One group of plant-derived molecules, the monoterpene indole alkaloids, is synthesized from a common intermediate, strictosidine. Here, we report how we developed a yeast strain that produces strictosidine. This required introducing 21 new genes and three gene deletions into the yeast genome. This yeast strain provides an important resource for the production of expensive, complex molecules that plants normally produce in small amounts.

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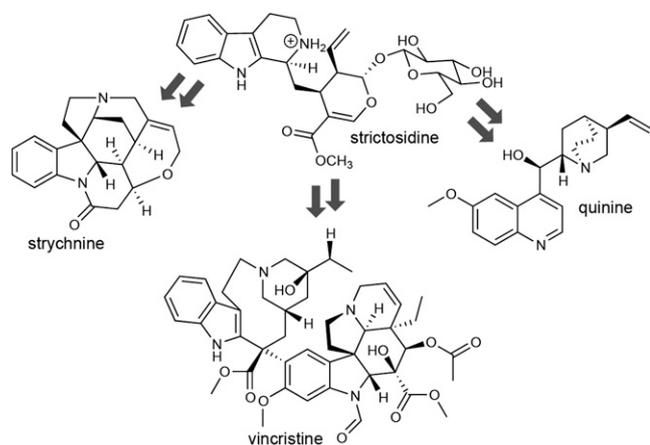
The authors declare no conflict of interest.

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**Fig. 1.** Strictosidine, the central intermediate in monoterpene indole alkaloid (MIA) biosynthesis, undergoes a series of reactions to produce over 3,000 known MIAs such as vincristine, quinine, and strychnine.

pyrophosphate synthase (FPPS) that, although having both GPP and FPP synthase activity, does not release GPP from its catalytic site (28). Therefore, to synthesize free GPP we incorporated *AgGPPS2*, a GPP-specific synthase from grand fir (*Abies grandis*), into this *S. cerevisiae* strain (29). To further enhance GPP yields, we replaced the native *ERG20* gene with *mFPS144*, a previously reported mutant FPPS (N144W mutation) from *Gallus gallus*, which has a much greater GPP synthase activity relative to FPP synthase activity (30).

In contrast to canonical monoterpenes that are synthesized in a single enzymatic step from GPP, MIA/strictosidine biosynthesis requires that GPP is shuttled into a nine-step pathway to produce the highly derivatized monoterpene secologanin (Fig. 2) (3, 24). Secologanin is then enzymatically condensed with tryptamine, derived from tryptophan by the action of a decarboxylase, to produce strictosidine, the central building block for all known MIAs. All required biosynthetic genes for strictosidine production (*GES*, *G8H*, *GOR*, *ISY*, *IO*, *7-DLGT*, *7-DLH*, *LAMT*, *SLS*, *STR*, *TDC*) have been elucidated in the MIA-producing plant *Catharanthus roseus* and thus were stably incorporated into this enhanced yeast strain (Fig. 2) (1, 3, 31). To allow for ample production of cofactors, which several of these enzymes require, we added an extra copy of *SAM2* for production of *S*-adenosyl-L-methionine (SAM) (*LAMT* is a SAM-dependent methyltransferase) and *ZWF1* to increase NADPH availability (*ISY*, *IO*, *7-DLH*, *G8H*, and *SLS* require NADPH) (32, 33). The secologanin biosynthetic pathway includes four cytochrome P450 (CYP) enzymes (*G8H*, *IO*, *7-DLH*, *SLS*) whose catalysis relies on electrons shuttling to the CYP from cytochrome P450 reductase (CPR) and in some instances also from cytochrome *b*<sub>5</sub> (*CYB5*) (34). Both *C. roseus* *CPR* and *CYB5* were integrated into the genome to enhance the reaction rates of the P450s (34, 35). The intracellular and extracellular metabolites of this strain (strain 0) were extracted and analyzed by liquid chromatography-mass spectrometry (LC-MS). However, we observed no production of strictosidine or downstream pathway intermediates loganic acid, loganin, and secologanin in this strain.

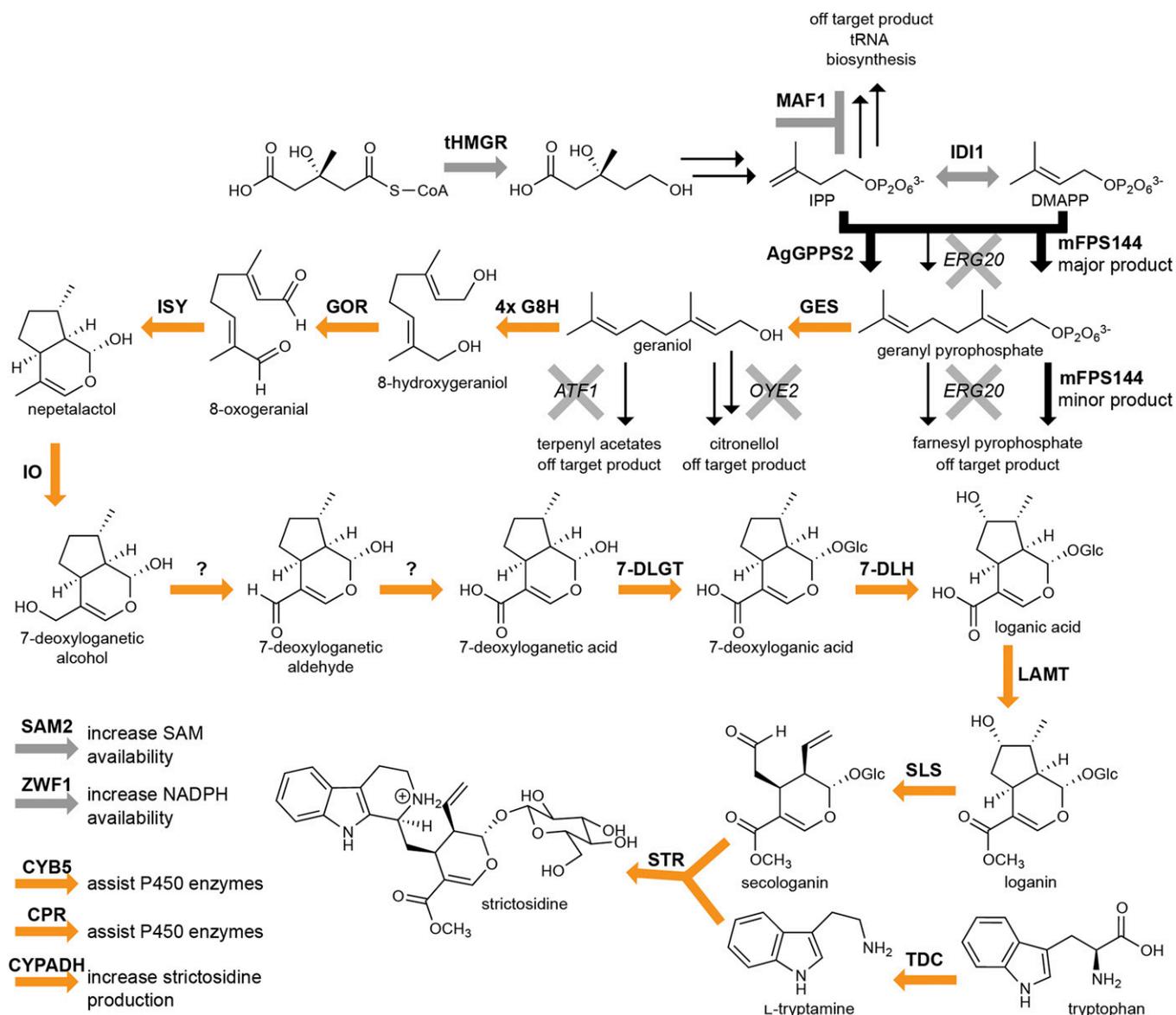
To determine why strictosidine was not observed, we analyzed the activity of the strictosidine biosynthesis enzymes by feeding strain 0 with upstream pathway intermediates (geraniol, 8-hydroxygeraniol, 8-oxogeraniol, and nepetalactol; Fig. 2), then extracting extracellular metabolites, and monitoring strictosidine production by LC-MS (Fig. 3A). Feeding with 8-hydroxygeraniol (the product of the *G8H* enzyme), 8-oxogeraniol (product of *GOR*), and nepetalactol (product of *ISY*) each result in production of a peak corresponding to strictosidine. In stark contrast,

feeding with geraniol (the *G8H* enzyme substrate) resulted in no observable strictosidine product peak. These results suggest that low *G8H* activity or poor *G8H* expression is the bottleneck to strictosidine production in strain 0. Indeed, upon addition of a high-copy number plasmid encoding a codon-optimized *G8H* into strain 0, we observed de novo production of strictosidine (Fig. 3B). Notably, only one diastereomer of the secologanin-tryptamine condensation product, strictosidine, was observed, whereas a 1:1 mixture of strictosidine and its diastereomer, vincoside, is observed for the nonenzymatic reaction that occurs under acidic conditions (36). Based on these findings, to create strain 1, we integrated a copy of the codon-optimized *G8H* into strain 0, but this did not result in detectable strictosidine production, suggesting that two copies of *G8H* are not sufficient to overcome this bottleneck. However, when strain 1 is supplemented with codon-optimized *G8H* expressed on a high-copy plasmid, without exogenous geraniol, 0.011 mg/L strictosidine is produced in the extracellular fraction, suggesting that additional copies of *G8H* improve strictosidine production titers (Table 1 and Fig. 3C).

To further alleviate the bottleneck at the *G8H* step, we attempted to minimize metabolism of geraniol, the *G8H* substrate, by the yeast host. Geraniol is metabolized by yeast primarily through esterification to form geranyl acetate and through reduction to form citronellol (37). Deletions of *ATF1*, encoding an alcohol acetyltransferase, and *OYE2*, encoding an NADPH oxidoreductase, are reported to attenuate these reactions (37). Thus, we deleted *OYE2* and *ATF1* in strain 1 to create strain 2. Trace amounts of strictosidine production in strain 2 can be observed, but is increased to 0.06 mg/L when supplemented with codon-optimized *G8H* expressed on a high-copy plasmid (Table 1). The 0.06 mg/L produced is a sixfold improvement over a strain without the *OYE2-ATF1* deletion (strain 1 plus *G8H* plasmid), suggesting attenuating yeast geraniol metabolism can boost strictosidine production and highlighting anew the critical requirement of enhanced *G8H* expression.

Salim et al. (38) propose that the CYP *IO* converts nepetalactol to 7-deoxyloganic acid via a three-step oxidation mechanism where nepetalactol is converted to an alcohol, then an aldehyde, and finally to a carboxylic acid (Fig. 4A). Similarly, the three-step oxidation of amorphadiene to artemisinic acid in *Artemisia annua* can be catalyzed by one CYP, but a recently discovered alcohol dehydrogenase and an aldehyde dehydrogenase have been found to improve the rate of this oxidation substantially (Fig. 4A) (35). Analogously, additional reductases may assist *IO* or other CYP-catalyzed reactions involved in strictosidine biosynthesis. Based on similar coexpression profiles with other MIA biosynthetic enzymes (24), we identified two putative alcohol dehydrogenases *ADH1* and *ADH2* and one aldehyde dehydrogenase *ALDH1*. To test whether these candidate genes assist in any of the CYP-catalyzed reactions, the genes were cloned into a high-copy number replicative plasmid and overexpressed in *S. cerevisiae* strain 0. Using LC-MS, we analyzed strictosidine production in these strains 48 h after feeding the cultures with several biosynthetic intermediates. Expression of *ADH2*, now named *CYPADH*, resulted in a 2- to 15-fold increase in strictosidine production compared with an empty vector control depending upon the biosynthetic intermediate that was fed (Fig. 4B). These feeding studies do not definitively show which CYP(s) this *CYPADH* may assist, but the presence of this enzyme clearly improved the yield of strictosidine. For the other two candidates, *ALDH1* and *ADH1*, no substantial improvement of strictosidine production was noted regardless of which biosynthetic intermediate was fed to the strain.

To further increase de novo strictosidine production, we created strain 3 by integrating *CYPADH* into strain 2, which is capable of producing 0.017 mg/L strictosidine (Table 1). Additionally, we incorporated two additional copies of codon-optimized *G8H* into strain 3 to produce strain 4 (Fig. S1). After 6 d at 28 °C, strain 4



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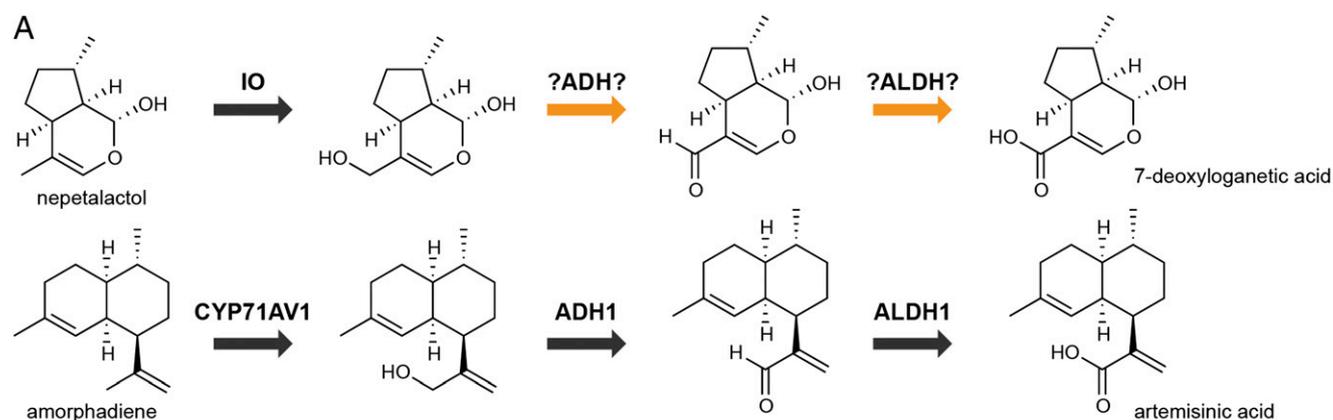
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**Fig. 2.** The reconstituted *S. cerevisiae* strictosidine biosynthetic pathway. *C. roseus* genes (orange) were integrated into the *S. cerevisiae* genome to allow for strictosidine production. To increase flux through the pathway, second copies of native *S. cerevisiae* genes (gray) were integrated into the genome. The gray line indicates *MAF1* is a repressor of tRNA biosynthesis. The gray X represents genes that were deleted to reduce the consumption of pathway intermediates involved in other biosynthetic pathways. *ERG20* was deleted and replaced by *AgGPPS2* (*A. grandis*), which exclusively produces geranyl pyrophosphate, and *mFPS144* (*G. gallus*), which favors the production of geranyl pyrophosphate over farnesyl pyrophosphate, an essential *S. cerevisiae* off-pathway compound. The arrows with question marks are catalyzed by IO, but may also require helper enzymes. National Center for Biotechnology Information accession numbers for all integrated genes are located in [Table S1](#). Abbreviations: AgGPPS2, *Abies grandis* geranyl pyrophosphate synthase; CPR, cytochrome P450 reductase; CYB5, cytochrome *b*<sub>5</sub>; 7-DLGT, 7-deoxyloganic acid glucosyl transferase; 7-DLH, 7-deoxyloganic acid hydroxylase; DMAPP, dimethylallyl pyrophosphate; G8H, geraniol 8-hydroxylase; GES, geraniol synthase; GOR, 8-hydroxygeraniol oxidoreductase; IDI1, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase; IO, 7-deoxyloganic acid synthase/iridoid oxidase; IPP, isopentenyl pyrophosphate; ISY, iridoid synthase; LAMT, loganic acid *O*-methyltransferase; mFPS144, *Gallus gallus* mutant farnesyl pyrophosphate synthase N144W; SAM2, *S*-adenosylmethionine synthetase; SLS, secologanin synthase; STR, strictosidine synthase; TDC, tryptophan decarboxylase; tHMGR, truncated 3-hydroxy-3-methyl-glutaryl-CoA reductase; ZWF1, glucose-6-phosphate dehydrogenase.

produced 0.03 mg/L strictosidine in both rich media and minimal media (Table 1). In an attempt to further increase titers, we incorporated into strain 4 high-copy number plasmids containing one of the upstream pathway genes (Fig. S2). Only introduction of codon-optimized *G8H* dramatically increased strictosidine production [0.5 mg/L compared with 0.03 mg/L for strain 4 without plasmid (Table 1 and Fig. S3)]. Despite the four integrated copies of the *G8H* gene (three codon optimized, one wild type) in strain 4, hydroxylation of geraniol by *G8H* still limits strictosidine production.

Using LC-MS, we subsequently monitored accumulation of all biosynthetic intermediates downstream of 7-deoxyloganic acid in strain 4 containing *G8H* plasmid to identify additional potential downstream pathway bottlenecks. Loganin was the only detected accumulated intermediate (Fig. S4). After 6 d at 28 °C, this optimized yeast strain produces 0.8 mg/L loganin in the media, levels that surpass those of strictosidine (0.5 mg/L). These data suggest that, in addition to *G8H*, *SLS* may also be a rate-limiting step. However, introducing *SLS* on a high-copy number plasmid into strain 4, as well as testing independently four identified





**B**

Normalized Strictosidine Peak Area Relative to Empty Plasmid Control

substrate added \ plasmid	nepetalactol	7-deoxyloganetic acid	7-deoxyloganetic acid	loganetic acid	loganin
empty	1	1	1	1	1
ALDH1	1.3 ± 0.2	1.8 ± 0.7	1.8 ± 0.5	1.7 ± 0.3	1.8 ± 0.6
ADH1	1.4 ± 0.5	1.9 ± 1.3	1.8 ± 1.1	1.7 ± 0.6	1.8 ± 0.7
ADH2 (CYPADH)	12.2 ± 3.5	14.1 ± 4.0	15.1 ± 5.0	2.6 ± 1.4	2.4 ± 1.4

**Fig. 4.** CYPADH increases strictosidine production. (A) The proposed three-step mechanism to convert nepetalactol to 7-deoxyloganetic acid (Top) compared with the similar three step mechanism to convert amorphadiene to artemisinic acid. (B) Amount of strictosidine produced in strain 0 containing plasmids encoding ALDH1, ADH1, or ADH2 relative to strain 0 harboring empty plasmid when incubated with the indicated pathway intermediates. Data are mean ± SD of three independent experiments.

reconstituted in yeast at yields of 20–40 mg/L (12). Reconstitution of alkaloids, which yeast does not naturally produce, has thus far been limited to the later steps of the benzylisoquinoline pathway (19, 25).

Here, we report a *S. cerevisiae* strain capable of producing strictosidine, the central intermediate for thousands of monoterpene indole alkaloids. This is the first report (to our knowledge) of de novo production of a plant-derived alkaloid in yeast. Many of these alkaloids are expressed in small amounts in the host plant, which makes isolation from natural sources challenging. The best of our engineered strains (strain 4 containing *G8H* plasmid) harbored three gene deletions ( $\Delta\text{ERG20}$ ,  $\Delta\text{ATF1}$ ,  $\Delta\text{OYE2}$ ), 15 plant-derived genes (*AgGPPS2*, *GES*, *G8H*, *GOR*, *ISY*, *IO*, *7-DLGT*, *7-DLH*, *LAMT*, *SLS*, *TDC*, *STR*, *CYB5*, *CYPADH*), one animal-derived gene (avian mutant *FPPS*: *mFPPS144W*), and five additional copies of yeast genes (*tHMG*, *MAF1*, *ID11*, *SAM2*, *ZWF1*) to allow de novo production of strictosidine in the extracellular fraction at yields of ~0.5 mg/L.

Although the titers of strictosidine are low, development of this strain is a crucial first step toward the heterologous production of MIAs such as vincristine, quinine, and strychnine, all of which are derived from the strictosidine backbone (Fig. 1). Furthermore, this strain can be used as a starting point for incorporation of new enzymes to produce nonnatural products. This strain can also serve as a platform to identify undiscovered pathway enzymes, as demonstrated by the discovery of *CYPADH*, which assists secologanin biosynthesis. Finally, we can use this host to explore how the differential intracellular compartmentalization of MIA biosynthetic enzymes impacts production levels. For example, although *STR* is located in the vacuole and *GES* is located in the chloroplast of plant cells, in these experiments we used truncated versions of these genes that lacked the localization signal sequence to avoid potential enzyme mislocalization or bottlenecks related to transport across intracellular membranes.

This yeast platform will allow us to further explore the impact that localization has on product yield, which may provide further opportunities for enhancing small-molecule production (25). In short, this strain provides an important resource for further study of plant monoterpene-derived alkaloids.

## Materials and Methods

**Materials.** The *S. cerevisiae* parental strain BY4741 was purchased from EUROSCARF. The pXP plasmid set (43) used to construct DNA fragments for homologous recombination is available from Addgene. PCR for construction of plasmids and linear fragments was performed using KOD Hot Start DNA Polymerase (Merck Millipore), and AmpliTaq DNA polymerase (Life Technologies) was used to confirm chromosomal integration. All compounds were purchased from Sigma except nepetalactol (44), secologanin (45), strictosidine, and d4-strictosidine (46), which were prepared as described previously. *S. cerevisiae* codon-optimized *G8H* was synthesized by DNA 2.0. The *mFPPS144W* gene (strain ZXM144) was a gift from Joe Chappell (University of Kentucky, Lexington, KY). *AgGPPS2* was a gift from Reuben Peters (Iowa State University, Ames, IA).

**Strains and Growth Conditions.** Yeast strains were cultured in YPD or synthetic complete drop-out (SC) media with 2% (wt/vol) glucose lacking specific amino acids (Formedium) at 30 °C unless otherwise indicated. Yeast-competent cells were prepared and transformed as described by Gietz and Schiestl (47). During transformation, the cells were incubated at 30 °C for 15 min (vortexing briefly every 5 min), and then 42 °C for 15 min before plating on selective media.

**Construction of *S. cerevisiae* Strains.** All primers, plasmids, and yeast strains used are listed in Tables S2–S4. Detailed construction of various pXP plasmids (43) can be found in *SI Materials and Methods*. Constructed pXP plasmids containing genes to be integrated were used as template to amplify by PCR a linear fragment containing the desired gene (and marker when appropriate) flanked by 50 bp homologous to either the *S. cerevisiae* genomic DNA integration site or the adjoining linear DNA fragment. The fragments were cotransformed into yeast for selection on SC media lacking the appropriate amino acid. Several of the resulting transformed colonies were randomly picked to confirm correct strain construction by PCR analysis. The

*loxP* flanked markers were recycled out using the pBR3060 plasmid, which was later cured from the strain using 5-fluorouracil (1 mg/mL).

**Alcohol/Aldehyde Dehydrogenase Screening.** Strain 0 containing pXP plasmids harboring either *CYPADH* (*ADH2*), *ADH1*, *ALDH1*, or empty plasmid (to serve as wildtype control) were diluted to an initial OD<sub>600</sub> of 0.1 into 2 mL of SD-ura media containing 3  $\mu$ L of 10 mg/mL loganin or loganic acid or 22.5  $\mu$ L of chemoenzymatic reactions that produced 7-deoxyloganic acid or 7-deoxyloganic acid as described in *SI Materials and Methods*. Chemoenzymatic reactions with microsomes generated with empty plasmid served as nepetalactol controls. The cultures were left shaking at 30 °C for 2 d, and then extracted as described below using MeOH:acetone.

**Extraction of Strictosidine Pathway Products.** The supernatant and pellet of the cultures were separated by centrifugation. The pellet (intracellular material)

was resuspended in 50:50 MeOH:acetone and lysed with glass beads. After centrifugation, the soluble portion was removed, evaporated to dryness, and resuspended in MeOH. The original supernatant fraction (extracellular material) was added to 50:50 MeOH:acetone, vortexed, and left at –20 °C for 20 min. The sample was centrifuged, and the remaining soluble portion was evaporated to dryness and resuspended in MeOH. Both fractions were then analyzed by LC-MS. Further details can be found in *SI Materials and Methods*.

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