Improved protein glycosylation enabled heterologous biosynthesis of monoterpenoid indole alkaloids and their unnatural derivatives in yeast

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ABSTRACT

With over 3000 reported structures, monoterpenoid indole alkaloids (MIAs) constitute one of the largest alkaloid groups in nature, including the clinically important anticancer drug vinblastine and its semi-synthetic derivatives from \textit{Catharanthus roseus} (Madagascar’s periwinkle). With the elucidation of the complete 28-step biosynthesis for anhydrovinblastine, it is possible to investigate the heterologous production of vinblastine and other medicinal MIAs. In this study, we successfully expressed the flavoenzyme O-acetylsystemenmadenin an oxidase in \textit{Saccharomyces cerevisiae} (baker’s yeast) by signal peptide modification, which is a vinblastine biosynthetic gene that has not been functionally expressed in this system. We also reported the simultaneous integration of ~18 kb MIA biosynthetic gene cassettes as single copies into four genomic loci of baker’s yeast by CRISPR-Cas9, which enabled the biosynthesis of vinblastine precursors catharanthine and tabersonine from the feedstocks secologanin and tryptamine. We further demonstrated the biosynthesis of fluorinated and hydroxylated catharanthine and tabersonine derivatives using our yeasts, which showed that the MIA biosynthesis accommodates unnatural substrates, and the system can be further explored to produce other complex MIAs.
1. Results and discussion

Previously, we have shown the production of a key MIA intermediate stemmadenine by feeding 19E-geissoschizine substrate, which was produced in vitro by geissoschizine synthase (GS), to baker’s yeast expressing the CYP geissoschizine oxidase (GO) and two reductases Redox 1 and 2 (strain 0) (Qu et al., 2018)). In this study, we further included the stemmadenine O-acetyltransferase (SAT), O-acetyl-stemmadenine oxide (ASO), dihydroprecondylocarpine acetate synthase (DPAS), hydrolase 1 (HL1, catharanthine synthase), and hydrolase 2 (HL2, tabersonine synthase) in yeast to investigate the biosynthesis of catharanthine and tabersonine in this strain (Fig. 1). ASO is a flavo-protein oxygenase and a member of the enzyme class Berberine Bridge Enzyme (BBE)-like proteins, which require eukaryotic glycosylation pathway to fold and mature into functional proteins (Qu et al., 2018a; Winkler et al., 2008). In addition to BBE itself involved in the benzyli pathway to fold and mature into functional proteins (Qu et al., 2018a; Winkler et al., 2008). In addition to BBE itself involved in the benzyli pathway to fold and mature into functional proteins (Qu et al., 2018a; Winkler et al., 2008). In addition to BBE itself involved in the benzyli.

Previously we have shown that ASO, as either intact proteins with its N-terminal signal peptide (SP) to direct the nascent polypeptide through ER lumen and trans-Golgi network for protein folding and N-glycosylation (Nagashima et al., 2018). In cannabis, THCAS is secreted out of the cells similar to many other apoplastic glycoproteins, whereas BBE and ASO are re-routed to vacuoles and small vesicles, which is similar to the vacuolar glycoprotein carboxypeptidase Y (CPY) and proteinase A in baker’s yeast (Caputi et al., 2018; Sirikantaramas et al., 2005; Bird and Facchini, 2001). Previously we have shown that ASO, as either intact proteins with its own SP or N-terminal truncated proteins after removing the SP, could not be expressed to detectable levels in baker’s yeast, while active proteins could be purified by transient expression in Nicotiana benthamiana (tobacco) leaves (Qu et al., 2018a). The results were consistent with another report on unsuccessful ASO expression in baker’s yeast and other reports on the difficulty to produce functional BBE-like oxygenases without N-terminal SP modification in this system (Caputi et al., 2018; Winkler et al., 2008; Sirikantaramas et al., 2004). Recently, THCAS and a serine carboxypeptidase-like (SCPL) acyltransferase litorrane synthase involved in plant tropane alkaloid biosynthesis that also require N-glycosylation were expressed functionally in baker’s yeast by swapping their native SP with those from yeast CPY or proteinase A (Luo et al., 2019; Srinivasan and Smolke, 2020). The results suggest that plant SPs may not be effectively recognized by the N-glycosylation pathway in baker’s yeast and SP replacement may lead to functional expression of ASO in yeast.

We therefore generated two codon-optimized ASO with the addition of yeast CPY SP (amino acid AA1-34, Fig. 2, Supplementary Fig. 1). The first version (CPY-ASO) included the yeast CPY SP addition directly to the N-terminus of intact ASO, which resulted in two tandem N-terminal SPs. The second version (CPY-dASO) instead swapped ASO SP (AA1-24) with CPY SP. Both versions were expressed as C-terminal myc-tagged proteins under the control of Gal1 promoter in high-copy pESC-Ura vector (2µ origin of replication). The Western blot showed successful protein expression for both CPY-tagged ASO versions, while the none-CPY tagged ASO showed less protein expression. (Fig. 2).

Next, we co-expressed GO, Redox1, Redox2, SAT, CPY-dASO, DPAS, and HL1 (Strain 1) or HL2 (strain 2) encoded in 2µ plasmids with 3 distinct selection markers under inducible Gal1/10 promoters in yeast (Fig. 3). When fed with 19E-geissoschizine, the strains successfully produced catharanthine and tabersonine, respectively (Supplementary Figs. 2 and 3). The yields from geissoschizine were 0.28% for catharanthine and 0.36% for tabersonine (Fig. 3). When we replaced CPY-dASO with non-modified ASO containing native SP (strain 3), the catharanthine levels after geissoschizine feeding dropped by 71.9-fold to only 0.004% (Fig. 3). This result confirmed that SP swapping is necessary for functional expression of ASO in baker’s yeast.

We further compared MIA biosynthesis in yeast between using high-copy plasmids and single genomic gene copies. A CRISPR-Cas9 compatible yeast strain was previously engineered to include identical

Fig. 1. The biosynthetic pathway for monoterpenoid indole alkaloids (MIAs) catharanthine and vindoline in C. roseus, which couple to form the anti-cancer anhydrovinblastine and other derivatives. TDC: tryptophan decarboxylase (Genbank P17770); STR: strictosidine synthase (Genbank CAA43936); SGD: strictosidine β-glucosidase (Genbank AAF28800); GS: geissoschizine synthase (Genbank MF770507); GO: geissoschizine oxidase (Genbank MF770508); Redox1/2: oxidized geissoschizine reductase 1/2 (Genbank MF770509, MF770510); SAT: stemmadenine O-acetyltransferase (Genbank MF770511); ASO: O-acetyl-stemmadenine oxide (Genbank MH136588); DPAS: dihydroprecondylocarpine synthase (Genbank AA1B1FHP3); HL1: hydrolase 1/catharanthine synthase (Genbank MF770512); HL2: hydrolase 2/tabersonine synthase (Genbank MF770513).
Fig. 2. Placing yeast carboxypeptidase Y (CPY) signal peptide (SP) at N-terminus of O-acetylstemmadenine oxidase (ASO) allowed detectable expression of this flavoprotein oxygenase by Western blot. Two codon-optimized ASO were synthesized. The CPY-ASO version contained a N-terminal CPY SP and ASO own SP, whereas the CPY-dASO version instead has ASO SP replaced by CPY SP (left panel). When expressed in yeast, ASO expression from both versions was detected by Western blots using anti-myc antibodies targeting the myc epitope tagged at C-terminus of ASO (upper right panel), while the native ASO and its SP removed version showed less expression. The SDS-PAGE gel (lower right panel) showed protein loading for Western blot. The reduced protein size (~55 kDa) suggested successful removal of SP in both versions as the expected full-length protein was ~63 kDa.

gRNA-binding sequences that are placed adjacent to six high-expressing yeast genes, allowing simultaneous integration of up to six DNA cassettes as single copies in a single transformation (Baek et al., 2021). Using this in-house strain, we integrated the genes GS, GO, CPR, Redox1, Redox2, CPY, CPY-dASO, and DPAS in four yeast genomic loci as single copies (Supplementary Fig. 4). The total length of integrated foreign genes was ~18 kb over four loci, which is one of the largest foreign DNA integrations by multiplex CRISPR/Cas9 in yeast (Utomo et al., 2021). All MIA biosynthetic genes were under the control of galactose-inducible, bi-directional Gal1/10 promoters to avoid potential cytotoxicity from heterologous gene expression. The terminal enzymes HL1 and HL2 were instead expressed from 2μ plasmids, which directed the biosynthesis to either catharanthine or tabersonine by simple plasmid transformation (Fig. 3). When fed with 19E-geissoschizine, the genomic integrated yeast with HL1 on plasmid (strain 4) successfully produced catharanthine at amounts comparable to strain 1 (0.26% geissoschizine conversion, Fig. 3). We further transformed GO and CPR on 2μ plasmid into strain 4 to generate strain 5, which resulted in 4.9-fold increase (1.27% geissoschizine conversion, 85 μg L⁻¹) in catharanthine production and suggested that the CYP GO activity was a limiting factor in strain 4. In strain 5 feeding experiment, 83.6% of 15 mg L⁻¹ 19E-geissoschizine feedstock was consumed by yeast. The only other detectable MIA was a pathway by-product akuammicine, decomposed from GO reaction, comprising 9.6% of feedstock (Supplementary Fig. 5).

Since only small fraction of geissoschizine was converted to catharanthine, we investigated strain 5 using downstream intermediates O-acetylstemmadenine (OAS, product of SAT) and O-acetylprecondylocarpine (OAP, product of ASO, Fig. 1) as feedstocks. These two compounds are the few stable intermediates in this section of pathway as many other downstream intermediates rapidly decompose, which is likely a major limitation for MIA biosynthesis in heterologous systems. When we fed OAS (7.5 mg L⁻¹) to strain 5 overnight, 91.0% of substrate was consumed by yeast, and other detectable MIAs included catharanthine (11.0% substrate, 312 μg L⁻¹), OAP (31.9% substrate), and the deacetylated stemmadenine (14.6% substrate, Fig. 3, Supplementary Fig. 5). When we fed OAP (6 mg L⁻¹) to strain 5, 91.2% substrate was consumed, and other detectable MIAs included catharanthine (16.2% substrate, 366 μg L⁻¹) and the deacetylated precondylocarpine (14.4% substrate, Fig. 3, Supplementary Fig. 5). These low conversion rates suggests that majority of the MIA intermediates were lost due to their instability in the yeast environment.

To complete catharanthine and tabersonine biosynthesis in yeast from simple feedstocks tryptamine and secologenanin, we included upstream genes strictosidine synthase (STR), strictosidine β-glucosidase (SGD) on a 2μ plasmid in strain 5 background to create strain 6 (HL1) and strain 7 (HL2). We opted to use a codon-optimized STR lacking its N-terminal vacuole targeting SP (AA1-18, Supplementary Fig. 1) to localize STR to yeast cytosol, where other downstream enzymes reside. After secologenanin and tryptamine feeding, strain 6 produced detectable levels of catharanthine (Fig. 3, Supplementary Fig. 6). Feeding downstream intermediates strictosidine aglycones (15 mg L⁻¹, product of SGD) and 19E-geissoschizine (15 mg L⁻¹) produced from in vitro reactions (Supplementary Fig. 2), we observed 230-fold increase in catharanthine production and suggeted poor efficiency of STR and SGD in living yeast cells. When using strain 7 with

Fig. 3. Various yeast strains with different alkaloid biosynthetic genes used in this study. The biosynthetic genes are shown in coloured blocks. Yellow colour indicates the expression from high-copy 2μ plasmids; blue colour indicates the expression from single genomic copies; and lavender colour indicates the expression from both high-copy 2μ plasmids and single genomic copies. All genes are under the control of Gal1/10 bi-directional promoters and their expression was induced by galactose in yeast media. The arrows show the points where pathway intermediates were fed to the yeasts, and the yields of catharanthine with HL1 activity and those of tabersonine with HL2 activities were indicated next to the arrow signs. By feeding yeasts with 12-fluorostrictosidine aglycones and 10-hydroxystrictosidine aglycones, respective substituted catharanthine and tabersonine were detected (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
2. Materials and methods

2.1. Yeast strains, cloning, and plasmid combinations

The yeast strain BY4742 (MATa, HIS3Δ1, LEU2Δ0, LYS2Δ0, URA3Δ0) were transformed with various alkylidene biosynthetic genes encodes on pESC-Leu-, His-, Ura-vectors. The primers and gene cloning combinations were found in Supplementary Tables 1 and 2. Codon-optimized CPY-ASO and dSTR were synthesized and subcloned in PET-Duet (BamHI/HindIII) and pUG19 (NotI/Clal) (Bio Basic Inc., Toronto, Canada) and their sequences are listed in Supplementary Fig. 1. The yeast strain BYV104 (MATa, HIS3Δ1, LEU2Δ0, LYS2Δ0, URA3Δ0) was used for CRISPR-Cas9 based genomic gene integration as described (Baek et al., 2021). Briefly, eight MIA genes in four bi-directional cassettes were amplified using primers 31–40 and cloned into four donor DNA plasmids using Gibson Assembly method (Supplementary Fig. 4). The genes are distributed as follows, iADH1 (H1): GO-CPR; iPDC1 (H2): GS-Redox 2, iP GK1 (H5): DPAS-Redox 1, and iCDC19 (H7): CPY-dASO-SAT. The plasmid combinations used to transform strain 0-6 is listed in Supplementary Table 3. The GenBank accession numbers are: X61932(STR), AF112888(SGD), MF770507(GS), MF770508 (GO), MF770509 (Redox1), MF770510 (Redox2), MF770511 (SAT), MH136588 (ASO), KU865331 (DPAS), MF770512 (HL1), and MF770513 (HL2).

2.2. Multiplex CRISPR-Cas9-based integration

From the four donor plasmids, linear donor DNA fragments were amplified using repliQ HiFi ToughMix (QuantaBio, Beverly, MA, USA) and primers 41–48. Yeast transformation was performed using the lithium acetate method as previously described (Baek et al., 2021). Briefly, overnight yeast culture was diluted 100 times into 25 ml YPD media and incubated until OD600 reached 0.4–0.8. The cells were collected by centrifugation and washed with H2O. Approx. 2 × 107 cells were incubated in 200 μl of 60% (w/v) PEG, 1 μg of pESC-URA: Cas9-gRNA plasmid, and 2 μg of each linear donor DNA for 15 min, which was followed by adding 50 μl of 2 mg/ml boiled salmon sperm DNA and 18 μl of 2 M lithium acetate into the mixtures. The mixtures were incubated at 42 °C for 40 min, immediately put on ice, spun down, and incubated in synthetic complex (SC)-Ura with glucose media for 2 h at 30 °C before being plated on SC-Ura plates. The plates were incubated overnight at 37 °C and then incubated at 30 °C for 2–3 days. To confirm the genomic gene integrations, the yeast genomic DNA was extracted with Yeast DNA Extraction Kit (Thermo Fisher, Rockford, IL, USA). Three primers, which would produce an 800 bp product for negative colonies and a 2–3 kb product for positive colonies, were used to genotype each colony in each integration site. The primers used were: loci H1, primer 41, 49, 53; H2, 43, 50, 54; H5, 45, 51, 55; and H7, 47, 52, 56 (Supplementary Table 1).

2.3. Yeast cultivation

The yeast strains were grown in 1 ml SC drop-out media (Sigma Aldrich, St. Louis, MO, USA) with 2% (w/v) glucose in test tubes at 30 °C in a shaking incubator overnight, then the cells were collected by centrifugation, washed once with water, resuspended in 1 ml fresh media with 2% (w/v) galactose, and cultured in test tubes at 30 °C in a shaking incubator for 24 h. The cells were collected by centrifugation and resuspended in 300 μl 20 mM pH 7.5 Tris-HCl buffer supplemented with various MIA substrates overnight. The culture supernatants were mixed with equal volume of methanol for LC-MS/MS analyses.

2.4. LC-MS/MS and alkaloid identification

LC-MS/MS was performed on an Agilent Ultivo Triple Quadrupole LC-MS equipped with an Aventor® ACES® UltraCore™ SuperC18™ column (2.5 μm, 50 × 3mm), which included the solvent systems: solvent A, methanol: acetonitrile: ammonium acetate 1 M: water at 30:70:2:98; solvent B, methanol: acetonitrile: ammonium acetate 1 M: water at 130:320:0.25:49:7. The following linear gradient (8 min, 0.6 ml/min) were used: 0 min 80% A, 20% B; 0.5 min, 80% A, 20%B; 5.5 min 1% A, 99% B; 5.8 min 1% A, 99% B; 6.5 min 80% A, 20% B; 8 min 80% A, 20% B. The photodiode array detector records from 200 to 500 nm. The MS/MS was operated with gas temperature at 300 °C, gas flow of 10 L/min, capillary voltage 4 kV, fragmentor 135 V, collision energy 30 V with positive polarity. Catharanthine, tabersonine, and secologanin standards were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tryptamine, 7-fluorotryptamine, 5-chlorotryptamine, and serotonin were purchased from Cayman Chemicals (Ann Arbor, MI, USA). O-acetylated-stemmadine and O-acetyloconidendrcarpine were purified from a C. roseus mutant described previously (Qu et al., 2018b). The remaining intermediates/standards were produced, purified, and identified by NMR as described (Qu et al., 2017, 2018b).
Author statement

M. S., J. C. U., D-K. R., and Y. Q. conceptualized the research. M.S., J. C. U., R. K., M. P.-G., J. J. O. G.-G., Z. M. conducted the research. M. S., J. C. U., D-K. R., and Y. Q. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2022.e00215.

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