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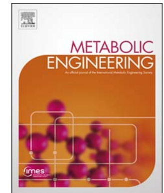

Bioproduction of a betalain color palette in *Saccharomyces cerevisiae*

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ABSTRACT

Betalains are a family of natural pigments found exclusively in the plant order *Caryophyllales*. All members of this chemical family are biosynthesized through the common intermediate betalamic acid, which is capable of spontaneously condensing with various primary and secondary amines to produce betalains. Of particular interest is the red-violet betanin, most commonly obtained from *Beta vulgaris* (beet) as a natural food dye. We demonstrate the first complete microbial production of betanin in *Saccharomyces cerevisiae* from glucose, an early step towards a fermentation process enabling rapid, on-demand production of this natural dye. A titer of 17 mg/L was achieved, corresponding to a color intensity obtained from 10 g/L of beetroot extract. Further, we expanded the spectrum of betalain colors by condensing betalamic acid with various amines fed to an engineered strain of *S. cerevisiae*. Our work establishes a platform for microbial production of betalains of various colors as a potential alternative to land- and resource-intensive agricultural production.

1. Introduction

Dyes improve the desirability of food and provide a visual cue of freshness (Esatbeyoglu et al., 2015). A substantial fraction of the currently approved colorants on the market are chemically synthesized from petroleum (Downham and Collins, 2000; König, 2015); however, there is growing demand for natural pigments as consumers become increasingly concerned with synthetic additives in their diet as well as the sustainability of product supply chains (Downham and Collins, 2000; Esatbeyoglu et al., 2015). Plant cultivation for the extraction of natural dyes has been considered a promising solution; however, the seasonal nature of harvests and the use of arable lands for non-essential foodstuffs is not ideal (Marienhagen and Bott, 2013; Neelwarne, 2012). Plant-cell culture systems have been proposed as an alternative production platform, but these systems will be difficult to scale and can lack genetic tractability (Marienhagen and Bott, 2013; Neelwarne, 2012). Microbial metabolic engineering has the potential to address these concerns and limitations. Specifically, genetically tractable production hosts such as *Saccharomyces cerevisiae* (baker's yeast) can be engineered with heterologous biochemical pathways and rapidly optimized for high production titers (Marienhagen and Bott, 2013).

One clade of useful natural pigments are the betalains, a set of tyrosine-derived compounds exclusively restricted to the *Caryophyllales* order of plants (Brockington et al., 2011). Betalains are divided into the yellow-orange betaxanthins and red-violet betacyanins; of the betalains, betanin is the most utilized in commercial applications (Khan and Giridhar, 2015). Betanin has applications in a variety of short shelf-life foodstuffs, cosmetics, and pharmaceuticals owing to several favorable properties: high water solubility, robust stability and color intensity over a broad range of neutral and acidic conditions (pH 3–7), lack of intrinsic flavor, high extinction coefficient compared to most artificial red dyes, and stability to certain forms of pasteurization in high-sugar solutions (Esatbeyoglu et al., 2015; Hendry and Houghton, 1996; Neelwarne, 2012). Betanin is principally obtained *via* specialized cultivars of *Beta vulgaris* (beet) and sold in the form of "beetroot extract" (E number 162) at price points that can reach $100 per kg of extract (Frost & Sullivan, 2007).

As illustrated in Fig. 1, all betalains share a common betalamic acid chromophore. This central molecule is formed from tyrosine *via* two enzymatic reactions. First, the monophenolase activity of various P450s (of the CYP76AD clade in *B. vulgaris*) generates L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine (Sunnadeniya et al., 2016). Then, DOPA-4,5-dioxygenase (DOD) catalyzes the ring cleavage at the catechol moiety of L-DOPA allowing subsequent spontaneous

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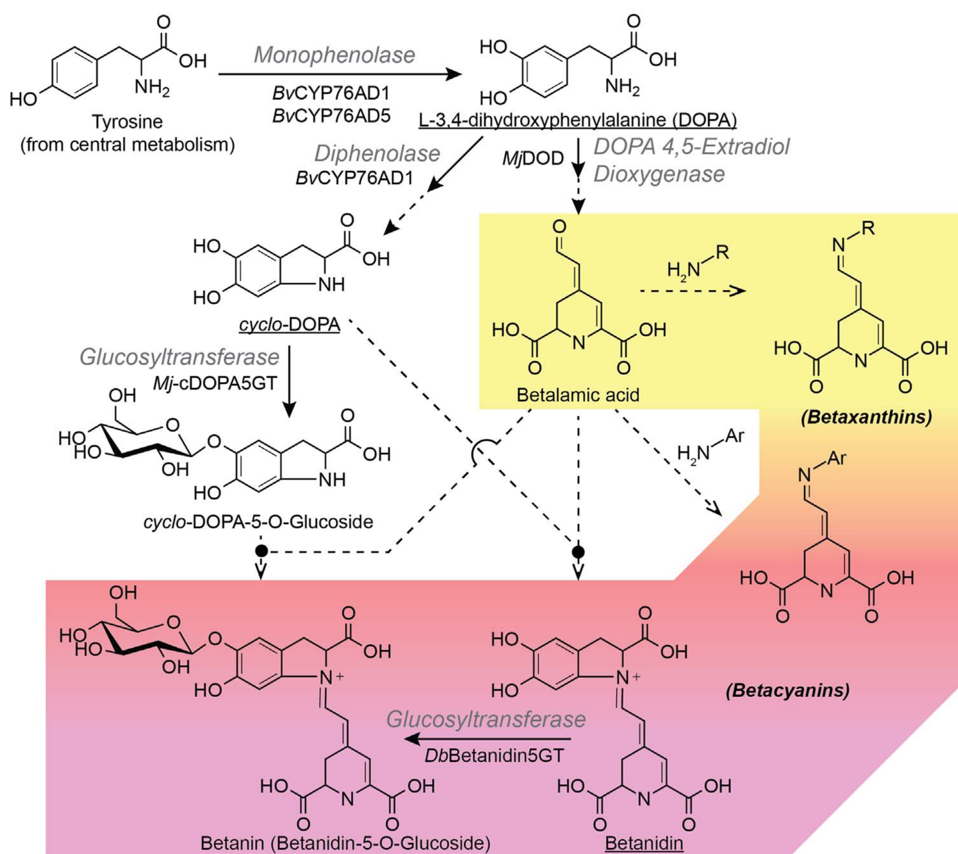

Fig. 1. Betalain biosynthesis. Diagram of the biosynthetic pathway with general enzyme activities in gray and the specific recombinant genes encoding these activities used in this work indicated in black. Solid lines are enzymatic reactions and dashed lines indicate spontaneous reactions. Unstable compounds in absence of reducing agent have their names underlined. Abbreviations: R = any organic chemical; Ar = any organic aromatic chemical.

cyclization with the alpha-amino group to form betalamic acid (Christinet, 2004). Betalamic acid spontaneously undergoes a Schiff-base condensation with free primary or secondary amines *via* its reactive aldehyde group to produce betalains possessing yellow to violet color (Schliemann et al., 1999).

Biosynthesis of the red-violet betanin requires two additional enzymatic activities that supplement the betalamic acid pathway. The first step is an enzymatic oxidation of L-DOPA to form dopaquinone, which spontaneously cyclizes into *cyclo*-DOPA. In *B. vulgaris*, CYP76AD1 is the sole enzyme capable of providing this additional diphenolase activity necessary to produce *cyclo*-DOPA (Hatlestad et al., 2012). Next, the condensation of *cyclo*-DOPA with betalamic acid results in the unstable, red-violet intermediate betanidin. A second enzymatic reaction, glucosylation, produces the dramatically more stable betanin (betanidin 5-*O*-beta-glucoside) pigment. Alternatively, the order of condensation and glucosylation can be reversed: *cyclo*-DOPA can be preemptively glucosylated prior to condensation with betalamic acid (Khan and Giridhar, 2015). The reaction order differs among plant species. For example, *Mirabilis jalapa* (four o′clock flower) produces the *cyclo*-DOPA-5-*O*-glucoside (Sasaki, 2005), whereas *Dorotheanthus bellidiformis* (Livingstone daisy) utilizes a betanidin glucosyltransferase (Vogt et al., 1999). Although specially bred cultivars of *B. vulgaris* are currently the predominant source of betanin (Khan and Giridhar, 2015), the putative glucosyltransferase (*Bv*GT) for betanin biosynthesis in beets (Sepulveda-Jimenez, 2005) has yet to be definitively confirmed by enzymatic assays.

In addition to the food dye betanin, applications of other betalains have been proposed. For example, the yellow betaxanthins have been proposed as replacements for artificial yellow dyes (Martins et al., 2017). Betalains have also shown applicability as photocell sensitizers, spectrofluorometric probes, and medical diagnostic reagents (Gonçalves et al., 2013a, 2013b; Khairy et al., 2016; Zhang et al., 2008). One such chemical is the condensation product of betalamic acid with 7-amino-4-methylcoumarin that has reported utility as a live-cell imaging probe for *Plasmodium*-infected erythrocytes (Gonçalves et al., 2013b). Such results highlight how derivatization with betalamic acid can be used to tune molecular properties and lead to value-added compounds. Although previously studied amines have yielded yellow, orange, and violet pigments upon condensation with betalamic acid (Gandía-Herrero et al., 2010, 2006; Gonçalves et al., 2013b; Khan and Giridhar, 2015), it is unclear what range of spectral and physical properties can be obtained from “designer” betalains.

To our knowledge, we herein provide the first description of betanin production from glucose in a heterologous microbial host. Additionally, we demonstrate that a heterologous system can be used to obtain novel betalain derivatives directly from yeast culture by feeding diverse amines. These results have implications for the fermentative production of natural colorants and further expand the spectrum of betalain colors obtainable *via* amine feeding.

2. Materials and methods

2.1. Chemicals and quantification

Amines used for *in vitro* and *in vivo* feeding were obtained from the following sources: L-DOPA (D9628, Sigma Aldrich), leucine (E811-100G, Amresco), para-aminobenzoic acid (100536-250G, Sigma Aldrich), anthranilic acid (A89855 Sigma Aldrich), 6-aminoindole (018336, Matrix Scientific), and o-dianisidine (01936, Chem-Impex International). Ascorbic acid was obtained from Gibco (13080-023) and iron(II) sulfate heptahydrate was obtained from Sigma Aldrich (215422).

Because pure betanin molecule is not commercially available, we used a > 98% pure beetroot extract (A10132, AdooQ Bioscience) for the majority of experiments, in conjunction with beetroot extract diluted in dextrin (B0397, TCI). Using the Beer-Lambert law and a

betanin extinction coefficient of 65,000 $M^{-1} cm^{-1}$ at 536 nm (Gonçalves et al., 2012; Schwartz and von Elbe, 1980), we determined that 1 g/L of AdooQ beetroot extract contained approximately 1.71 ± 0.14 mg/L of molecular betanin (Fig. S1). This conversion factor was used for the preparation of standards from the AdooQ extract for quantification of betanin titers.

For betanidin, we used the Beer-Lambert law with an extinction coefficient of 54,000 $M^{-1} cm^{-1}$ at 538 nm (Schwartz and von Elbe, 1980) to quantify the amount of betanidin in the fermentation broth of strain yCM420 grown in media with ascorbic acid to prevent betanidin oxidation. We then generated a mass spectrometry calibration curve based on a serial dilution series starting from the yCM420 fermentation broth and used the calibration curve to quantify betanidin titers by mass spectrometry.

Spectroscopic quantification was accomplished using 1-cm optical pathlength plastic cuvettes on a Shimadzu UVmini-1240 spectrophotometer. All other absorbance measurements were obtained on a TECAN M1000 in a Costar 96-well plate (Corning 3904). Normalized absorbance curves were obtained by normalizing against their absorbance maxima after appropriate baseline subtraction. Samples were diluted in phosphate buffered saline (PBS, pH 7.4) as needed in order to obtain a linear signal.

Liquid chromatography/mass spectrometry (LCMS) was performed using a 1260 Infinity LC System connected to a 6120 Quadrupole Mass Spectrometer (Agilent Technologies). All culture supernatant samples, media control samples, and betanin standards were diluted ten-fold in water prior to injection, to achieve a linear calibration curve for betanin with all samples falling within the bounds of the calibration curve. Ten microliters of each ten-fold diluted sample were injected and sample separation was achieved using a Zorbax Eclipse Plus C18 guard column (4.6 cm × 12.5 cm, 5 µm packing, Agilent Technologies) connected to a Zorbax Eclipse Plus C18 column (4.6 mm × 100 mm, 3.5 µm packing, Agilent Technologies) at 20 °C using a 0.5 mL/min flow rate. Water and acetonitrile mobile phases contained 0.1% formic acid as the pH modifier. The elution gradient (water:acetonitrile volume ratio) was as follows: 98:2 (0–2 min), linear ramp from 98:2 to 5:95 (2–17 min), 5:95 (17–22 min), linear ramp from 5:95 to 98:2 (22–23 min), and 98:2 (23–28 min). Absorbance was measured using a diode array detector for UV–Vis analysis. MS was conducted in atmospheric pressure ionization-positive electrospray (API-ES positive) mode at 100-V fragmentor voltage with ion detection set to both full scanning mode (50–1200 *m/z*) and targeted detection of betanidin (389.1 *m/z*) and betanin (551.1 *m/z*).

2.2. Plasmid and strain construction

All plasmids were assembled using the Yeast Tool Kit system (Lee et al., 2015). The final multigene assemblies utilized are described in Table 1.

Strains of *Saccharomyces cerevisiae* were generated following the lithium acetate protocol (Gietz and Schiestl, 2007) and confirmed by colony PCR for proper integrations. Strains are listed in Table 2.

Table 2
List of yeast strains used in this work.

Name	Genotype	Source
BY4741	*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	ATCC
yWCD230	[BY4741] *his3Δ0*	–
yCM208	[yWCD230] *his3Δ::pWCD1934 leu2Δ::pZNR0512 met15Δ::MET15*	This study
yCM240	[yCM208] *ura3Δ::pML1371*	This study
yCM363	[yWCD230] *leu2Δ::pPSG348 ura3Δ::pCMC0756*	This study
yCM371	[yWCD230] *leu2Δ::pPSG350 ura3Δ::pCMC0756*	This study
yCM419	[yWCD230] *leu2Δ::pPSG349 ura3Δ::pCMC0756*	This study
yCM420	[yWCD230] *leu2Δ::pZNR521 ura3Δ::pCMC0756*	This study
yCM421	[yCM208] *ura3Δ::pCMC0759*	This study
yPSG064	[BY4741] *ura3Δ::pPSG165*	This study
yPSG163	[BY4741] *ura3Δ::pPSG331*	This study

2.3. In vitro reactions using purified MjDOD enzyme

Stock solutions of 2 mM L-DOPA and 1 mM iron(II) sulfate with 10 mM ascorbic acid were prepared in PBS (pH 7.4). Stock solutions of amines were prepared at 20 mM in a 1:1 mixture of DMSO:water, except for leucine that was prepared at 20 mM in water, and o-dianisidine that was prepared at 10 mM in a 3:1 mixture of DMSO:water. All stock solutions were made fresh and used within 24 h.

DOPA-4,5-dioxygenase from *Mirabilis jalapa* (*Mj*DOD) was heterologously expressed in *E. coli* and purified to a final concentration of 50 µM. Specifically, the coding sequence for the *Mj*DOD enzyme was cloned into a pET-vector expression cassette including an IPTG-inducible T7 promoter and C-terminal 6x-histidine affinity tag. *E. coli* BL21(DE3) cells carrying the plasmid were grown at 37 °C in Terrific Broth media until an optical density at 600 nm of 3 was reached. After refrigeration at 4 °C for 20 min the culture was induced by the addition of IPTG to a final concentration of 0.2 mM and incubated at 17 °C for approximately 15 h. Cells were harvested by centrifugation and stored at − 20 °C prior to purification. Frozen cell pellets were thawed and resuspended in Lysis Buffer (20 mM HEPES pH 7.6, 500 mM NaCl, 20 mM imidazole, 1 mM DTT) and lysed by sonication. The lysate was clarified by centrifugation and the resulting supernatant was subjected to NiNTA affinity purification using a 5 mL NiNTA FF column (GE) by FPLC. Eluted protein was mixed with TEV protease and allowed to incubate overnight at 4 °C to remove the fusion tag, leaving a glycine-serine scar. Finally, the cleaved protein was further purified by size exclusion on a Superdex S200 column (GE) into a final buffer (20 mM Tris pH 7.6, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT) and concentrated to 50 µM. Aliquots of the purified protein were stored at − 80 °C prior to use.

Enzyme reaction mixtures were prepared similar to Sasaki et al. (2009) using 308 µL of L-DOPA stock solution, 308 µL of iron(II)

Table 1
List of plasmids used in this work.

Name	Description	Source
pDS0835	*Mj*DOD-GSx4-TEV-HISx6 (T7-vector)	This study
pZNR0521	LEU2 marker (LEU2 integration)	–
pWCD1934	HIS3 marker (HIS3 integration)	–
pML1371	URA3 marker (URA3 integration)	–
pCMC0756	pCCW12-*Mj*DOD-tADH1-pTDH3-*Bv*CYP76AD1^{W13L}-tTDH1-pTEF1-*Sc*ARO4^{K229L}-tENO2 (URA3 integration with URA3 marker)	This study
pCMC0759	pCCW12-*Mj*DOD-tADH1-pTDH3-*Bv*CYP76AD5-tTDH1-pTEF1-*Sc*ARO4^{K229L}-tENO2 (URA3 integration with URA3 marker)	This study
pPSG165	pPGK1-ARO4^{K229L}-tADH1-pTDH3-DOD-tTDH1 (URA3 integration with URA3 marker)	This study
pPSG331	pTDH3-Bv_CYP76AD5-tTDH1-pPGK1-ARO4^{K229L}-tPGK1 (URA3 integration with URA3 marker)	This study
pPSG0348	pTEF2-*Mj*cDOPA5GT-tSSA1 (LEU2 integration with LEU2 marker)	This study
pPSG0349	pTEF2-*Db*Betanidin5GT-tSSA1 (LEU2 integration with LEU2 marker)	This study
pPSG0350	pPGK1-*Db*Betanidin5GT-tENO2-pTEF2-*Mj*cDOPA5GT-tSSA1 (LEU2 integration with LEU2 marker)	This study

sulfate/ascorbic acid solution, 70 μL of amine stock solution, and 14 μL of purified *Mj*DOD to achieve final concentrations of 0.88 mM L-DOPA, 0.44 mM iron(II) sulfate, 4.4 mM ascorbic acid, 2 mM amine, and 1 μM *Mj*DOD in 700 μL total volume. Reaction mixtures were incubated at 30 °C for 16 h to allow enzymatic conversion of L-DOPA to betalamic acid and further non-enzymatic condensation of betalamic acid with amines to form betaxanthins.

2.4. In vivo bioproduction and feeding assays

Starter cultures of yeast strains were grown in synthetic complete media with the appropriate dropouts for auxotrophic selection. Saturated cultures after 48 h were then back-diluted by a factor of fifty into 2 mL of minimal media (supplemented with missing auxotrophies) as reported previously (DeLoache et al., 2015) for a 48 h bioproduction experiment. Ascorbic acid (10 mM) was added as indicated. The pilot work comparing glucosyltransferases was performed using 24-well blocks in a Multitron Standard shaker (Infors HT) set to 30 °C and 750 rpm. Flask scale production of betanin from yCM363 was conducted in a similar manner, with cultures instead back-diluted to 50 mL in both non-baffled and baffled Erlenmeyer flasks before incubation in a New Brunswick Scientific Innova 44 shaker set to 30 °C and 220 rpm.

Amine feeding experiments were conducted exactly as the pilot bioproduction experiments, except in media lacking all amino acids and supplemented to 0.5 mM amine using the same stock solutions utilized in the *in vitro* experiments.

2.5. Betalain purification and characterization

In vitro reactions were quenched by addition of two volumes of cold methanol, then stored at − 20 °C for at least ten minutes to precipitate protein. The quenched reactions were then centrifuged and an aliquot of the supernatant was removed for LCMS analysis.

In vivo bioconversions with yeast were centrifuged to remove cells. The supernatant was extracted with ethyl acetate and then chloroform to remove hydrophobic molecules. The betalain pigments remained with the aqueous phase through both extractions. The aqueous phase was then purified using a Sep-Pak Plus C18 cartridge (Waters) using the following procedure: the Sep-Pak cartridge was washed with 5 mL of ethanol and then 10 mL of water; the sample was applied and then washed with 10 mL of water to remove salts; finally, the sample was eluted with methanol. The eluted sample was then subjected to LCMS analysis.

High resolution LCMS was performed using a 6510 Accurate-Mass Q-TOF LCMS instrument (Agilent Technologies) and an Eclipse Plus C18 column (4.6 mm × 100 mm, 3.5 μm packing, Agilent Technologies). Analysis of betanin, PABA-betaxanthin, Ant-betaxanthin, and 6AI-betaxanthin was performed using a linear gradient of water:acetonitrile from 98:2 to 2:98 over 12 min with 0.1% formic acid at a flow rate of 0.5 mL/min. Analysis of oDA-betaxanthin single and double condensates was performed at a flow rate of 0.5 mL/min with 0.1% formic acid using a linear gradient of water:acetonitrile of either 90:10–50:50 over 12 min or 80:20–60:40 over 20 min. High resolution MS/MS analysis was conducted using targeted MS/MS with collision energy of 5–20 V.

3. Results and discussion

3.1. Microbial total synthesis of betanin from glucose

In order to create a *S. cerevisiae* strain capable of producing betanin from central metabolism, we took advantage of a previously codon optimized and engineered version of *B. vulgaris* CYP76AD1, which contains a W13L mutation for increased enzyme expression and activity (DeLoache et al., 2015). We refer to this enzyme as CYP76AD1^{W13L}. For DOPA-4,5-dioxygenase activity, we chose DOD enzyme from *M. jalapa* also optimized for *S. cerevisiae* (DeLoache et al., 2015). Previous work has demonstrated that yeast heterologously expressing various orthologs of P450 and DOD enzymes can produce the betanin precursor molecules *cyclo*-DOPA and betanidin (Hatlestad et al., 2012; Polturak et al., 2016; Sunnadeniya et al., 2016). However, both of these compounds are unstable in the presence of oxygen due to the reactivity of their catechol moieties: *cyclo*-DOPA oxidizes and polymerizes into melanin, while betanidin decomposes into various orange-red and yellow byproducts (DeLoache et al., 2015; Herbach et al., 2006; Wybraniec et al., 2011). These oxidation reactions can be blocked by the addition of a reducing agent such as ascorbic acid (ASC) (DeLoache et al., 2015; Wybraniec et al., 2011). Plants achieve a similar stabilization of the catechol moiety through the enzymatic addition of a glucose molecule, which serves as a biochemical protecting group inhibiting the oxidation pathway (von Elbe and Attoe, 1985). As illustrated in Fig. 1, betanin is produced either through direct glucosylation of betanidin or through glucosylation of the precursor *cyclo*-DOPA followed by condensation with betalamic acid (Sasaki, 2005; Vogt et al., 1999).

In the strain containing CYP76AD1^{W13L} and *Mj*DOD, we tested both the *cyclo*-DOPA glucosyltransferase from *M. jalapa* (*Mj*-cDOPA5GT) and betanidin glucosyltransferase from *D. bellidiformis* (*Db*Betanidin5GT) individually as well as co-expressed for their ability to produce betanin in *S. cerevisiae* (Fig. 2a). *Mj*-cDOPA5GT has been reported to regioselectively glucosylate only at the 5-hydroxyl position on *cyclo*-DOPA (Sasaki, 2005) and *Db*Betanidin5GT has been reported to regioselectively glucosylate at the corresponding position on betanidin (Heuer et al., 1996). Under the industrially-relevant condition of media lacking a reducing agent such as ASC, both glucosyltransferases were able to produce betanin in *S. cerevisiae*. Betanin production was confirmed by comparison of chromatography retention time, high resolution mass spectrometry, and MS/MS fragmentation pattern against a commercial standard (Figs. S2, S3). Expression of *Mj*-cDOPA5GT resulted in 16.8 ± 3.4 mg/L betanin, while expression of *Db*Betanidin5GT resulted in 10.4 ± 2.3 mg/L betanin (average ± one standard deviation). Co-expression of both glucosyltransferases produced 16.5 ± 2.4 mg/L betanin, thus not providing additional benefit over the expression of *Mj*-cDOPA5GT alone (Fig. 2a).

Because the two glucosyltransferases act at different points in the pathway, it is difficult to definitively say whether the placement of the glucosylation reaction or differences in the glucosyltransferases' kinetic parameters account for the observed betanin titers. Glucosylation of *cyclo*-DOPA would prevent oxidation of this earlier intermediate while also bypassing the production of unstable betanidin intermediate. However, the higher accumulation of betanidin in the *Db*Betanidin5GT expressing strain compared to the *Mj*-cDOPA5GT strain under the ASC supplemented conditions (Fig. 2b) suggests *Db*Betanidin5GT might be a less effective enzyme. Moreover, the data suggest that glucosylation is a more effective stabilization strategy than addition of ASC because approximately 17 mg/L of betanin are produced upon expression of *Mj*-cDOPA5GT (in the absence of ASC), whereas only approximately 11 mg/L of betanidin are produced in the presence of ASC (when no glucosyltransferase is expressed).

In addition to oxidative loss of catechol-containing intermediates, a major fermentative challenge for betalain production is the oxygen demand of this pathway. Both the P450 and DOD reactions require oxygen. In order to investigate the effect of oxygenation, we tested the *Mj*-cDOPA5GT-containing strain in both non-baffled and baffled Erlenmeyer flasks at a 50 mL culture volume. The observed betanin titers were, respectively, 4.8 ± 0.1 and 14.2 ± 1.5 mg/L (Fig. S4) with comparable final cell densities, highlighting the importance of oxygenated culture conditions.

S. cerevisiae's ability to export betanin is a key feature as it should simplify purification (Fig. 2). Such efflux activity is particularly attractive when compared to the traditional maceration and extraction protocols utilized on beetroot (Neelwarne, 2012). This advantage in

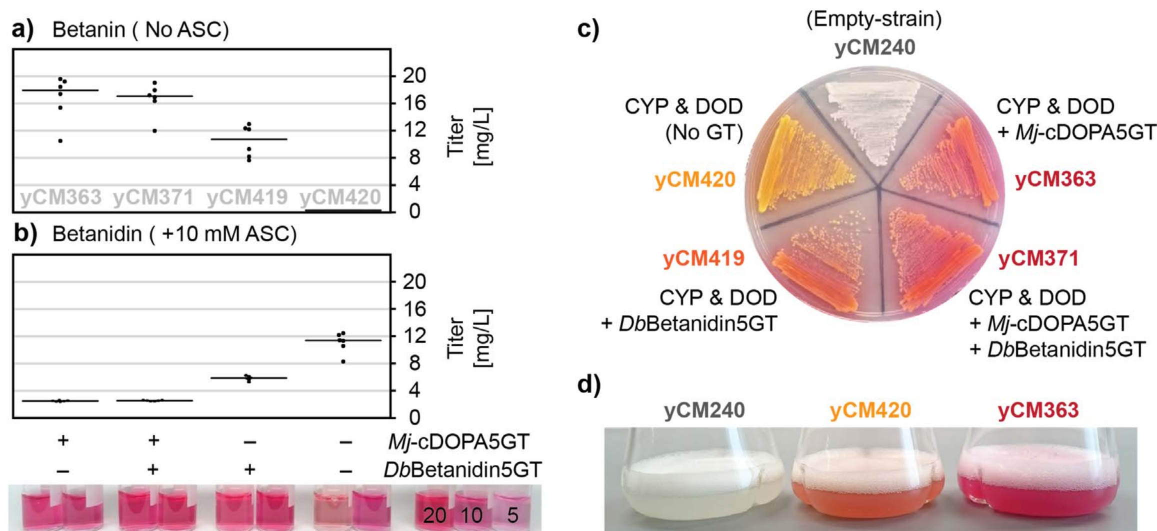

Fig. 2. Production of betanin using various combinations of glucosyltransferases. Titer of **a)** betanin and **b)** betanidin in yeast determined by LCMS for six replicates, cultured in minimal media without and with 10 mM ascorbic acid (ASC), respectively. Data points represent individual measurements and lines indicate median values. Below, vials of supernatant from centrifuged cultures are staged with a dilution series of betanin beetroot extract. Numbers on vials refer to approximate concentrations of molecular betanin as previously calculated, in mg/L. Each pair represents the no ASC and + 10 mM ASC supernatant, respectively. Note that solution color is derived from the total betacyanin (betanin + betanidin) content. **c)** Photograph of various betanin-producing and control strains. CYP denotes CYP76AD1^{W13L}. **d)** Photograph of betanidin and betanin production strains grown in flasks in minimal media.

recovery remains even when yeast is compared to plant cell suspensions. In plants, betalains are primarily stored in the vacuole (Thimmaraju et al., 2003). Stimulating the release of betanin from plant cells grown *in vitro* has required carefully applied stressors, often becoming a balancing act between cell viability and recovery yield (Thimmaraju et al., 2003); in contrast, approximately two thirds of the yeast culture's pigmentation is found in the media without any requisite manipulation (Fig. S5). We anticipate that identification and overexpression of the relevant plasma membrane transporters responsible for export could increase our overall titers.

3.2. Probing of the betalain color palette using structurally diverse amines

Beyond betanin, we investigated the bioproduction of other betalains *via* yeast fermentation. Our inspiration was the *in vitro* work of Gandía-Herrero et al. (2010) examining the effect of amine structure on betalain color, fluorescence, and antiradical activity. In particular, their research elucidated how different structural motifs found in the *cyclo*-DOPA moiety of betanin impact color. In our work, using purified *Mj*DOD enzyme, we tested the condensation of a set of structurally diverse aromatic amines with betalamic acid to determine what other color profiles could be generated *in vitro* (Fig. 3a–b). The selection of amines included metabolites naturally found in yeast, candidates for biosynthesis in future metabolic engineering efforts, and alternative unnatural structural motifs that extend beyond the limited assortment of aromatic amine compounds commonly found in nature. As a known test case, we used the amino acid leucine to produce the betaxanthin vulgaxanthin IV (Khan and Giridhar, 2015). Beyond obtaining the expected yellow from the aliphatic leucine and oranges from aniline-like chemicals (para-aminobenzoic acid and anthranilic acid), we were also able to access the violet color space by producing previously unknown betalains. Previously, all reported violet betalains have been based on the formation of structurally rigid iminium ion adducts best illustrated by the nitrogen-carbon bond between the *cyclo*-DOPA and betalamic acid moieties in betanin (Gandía-Herrero et al., 2010). The synthetic betaxanthins we have generated from condensates of 6-aminoindole (6AI) and o-dianisidine (oDA) with betalamic acid lack this fixed cation and instead contain the imine moiety prevalent in most betaxanthins.

We next tested the ability to accomplish whole-cell semi-synthesis as a platform for potential industrial-scale production of betalain analogs. In order to accomplish high purity semi-synthesis, we created a prototrophic yeast strain heterologously expressing *Mj*DOD with *Bv*CYP76AD5. This strain enabled high purity betalain production for two reasons. First, prototrophy removes the need to supplement the media with high concentrations of amino acids that would function as substrates for a spectrum of undesired betaxanthin side-products. Second, the CYP76AD5's exclusive monophenolase activity (Sunnadeniya et al., 2016) ensures that all produced L-DOPA is focused towards synthesis of betalamic acid. By adding 0.5 mM of each co-substrate amine to the yeast growth medium in individual experiments, we were able to test our previous *in vitro* findings (Fig. 3a–b) in an *in vivo* context (Fig. 3c–d).

Amine feeding experiments with live cells were able to reproduce most of the colors found in the *in vitro* assays (Fig. 3). However, two of the amine substrates – 6AI and oDA – showed unanticipated behavior during the in vivo assays. For 6AI, we were able to identify ascorbic acid as the determining variable. Similar to other recent protocols for *in vitro* reactions with DOD (Sasaki et al., 2009), we used ASC to stabilize the enzyme's Fe^{2+} cofactor; in contrast, our *in vivo* feeding was conducted in media without reducing agent. Supplementation of the 6AI bioconversion media with ASC recovered the color intensity previously observed in the *in vitro* reactions as determined by visual inspection and upon comparison of the absorbance curves (Fig. S6). Experiments with wild-type cells identified that cellular growth was inhibited at 0.5 mM 6AI. Feeding with 6AI also led to the formation of an unknown black precipitate, an undesired side-reaction that was effectively inhibited under reducing conditions (Fig. S7).

The most surprising outcome was the production of a blue-violet color derived from oDA feeding. This coloration was not observed when the condensation was performed *in vitro*. When oDA was fed to strains producing betalamic acid, a blue product was observed as a precipitate that associated with the cell pellet after centrifugation. Recovery of this precipitate required a wash with a salt solution such as phosphate-buffered saline (PBS). We found that the precipitate was soluble in PBS, methanol, and ethanol but not in water. Feeding experiments conducted with yeast strains expressing different parts of the betalamic acid pathway confirmed that the blue precipitate was likely a betalain and not the result of an endogenous metabolite (Fig. S8). When dissolved in PBS, this product exhibited maximum absorbance at approximately 560 nm (Fig. 3d), making it the most red-shifted betalain

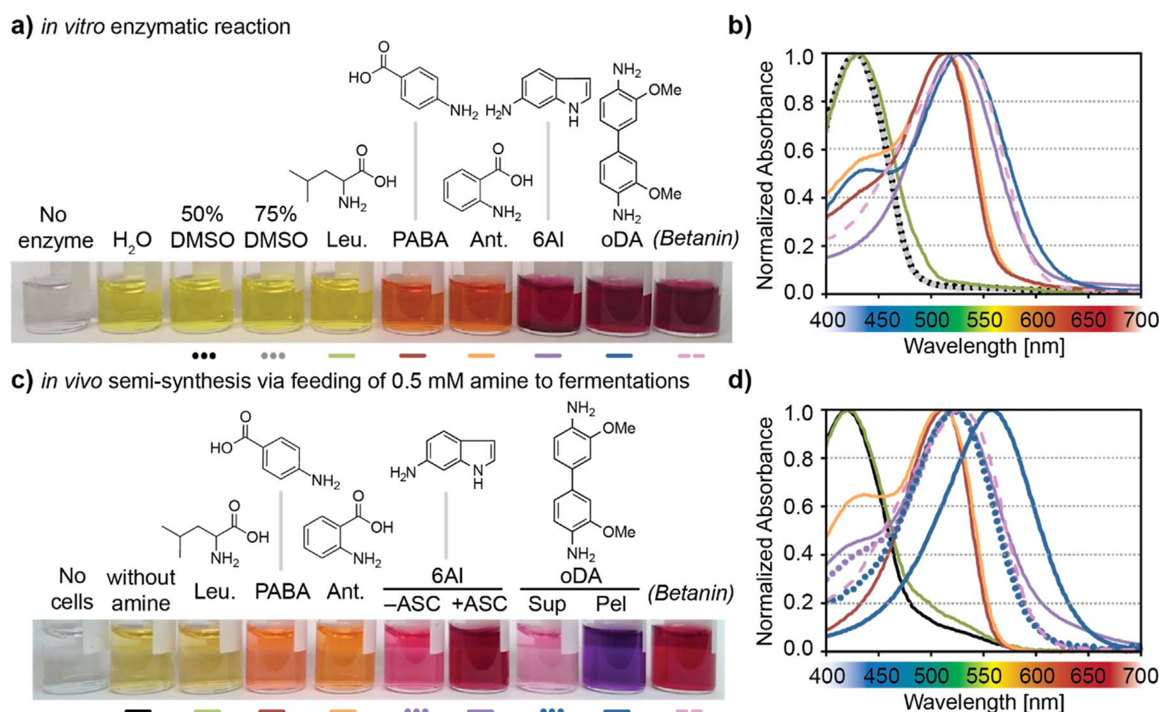

Fig. 3. *In vitro* and *in vivo* condensations of betalamic acid using native metabolites and alternative substrates. a) Resulting solutions from *in vitro* reactions. The structure of each added amine is indicated above the abbreviation. The yellow color seen in the H_2O and DMSO controls is the result of betalamic acid itself as well as dopaxanthin by-product (L-DOPA serving as the amine for conjugation to betalamic acid). All samples were diluted to 0.44 mM betalain (assuming complete amine condensation). Betanin vials contain commercial beetroot extract used as a standard for comparison. **b)** Normalized absorbance curves of betalains generated from *in vitro* experiments, with corresponding colors indicated below each vial. **c)** Vials of supernatant from centrifuged cultures. −/+ refers to the absence or presence of 10 mM ascorbic acid in the case of 6AI. "Sup" refers to the recovered supernatant and "Pel" refers to the washed cell pellet liquor obtained in the case of oDA. All samples were diluted to 0.25 mM betalain (assuming complete amine condensation). **d)** Normalized absorbance curves of betalains generated from *in vivo* experiments, with corresponding colors indicated below each vial. Raw absorbance curves are provided in Fig. S6. **Abbreviations:** Leu. = leucine; PABA = para-aminobenzoic acid; Ant. = anthranilic acid; 6AI = 6-aminoindole; oDA = o-dianisidine.

reported to date. Additionally, betalamic acid producing yeast fed oDA initially displayed red pigmentation 12 h after inoculation but became blue-violet over the next 12 h (Fig. S9). Therefore, we suspected that the red pigment, observed *in vitro* and *in vivo* in the supernatant (Fig. 3), was the condensation product of oDA and one molecule of betalamic acid. Because oDA contains two amine groups, we suspected that the blue precipitate was the result of oDA condensing with two molecules of betalamic acid (a "double condensate"). Indeed, we had initially selected oDA for feeding experiments for its potential ability to conjugate to betalamic acid at both amine positions.

In order to favor production of the double condensate under the *in vitro* reaction conditions, we tested various ratios of L-DOPA to oDA with the hypothesis that a high ratio of betalamic acid to oDA would favor the condensation of oDA with two betalamic acid molecules. However, we were unable to detect production of the double condensate when the reaction was performed *in vitro*. This is likely due to the fact that L-DOPA itself is an amine that can react with betalamic acid. While we tested low concentrations of oDA to favor condensation with two molecules of betalamic acid, we were also favoring the reaction of betalamic acid with L-DOPA to produce dopaxanthin. In the case of *in vivo* production of betalamic acid from glucose, it is likely that L-DOPA does not accumulate to high concentrations as once it is produced by CYP76AD5, it is rapidly converted by the more efficient DOD enzyme to betalamic acid (DeLoache et al., 2015). Thus, dopaxanthin byproduct should be minimized and double condensate formation should be favored. This mechanism is consistent with our observation that the culture initially appears red 12 h after inoculation (indicative of the single condensate) but changes to a blue-violet appearance over the next 12 h (indicative of double condensate formation).

The *in vitro* and *in vivo* reaction products were characterized by high resolution mass spectrometry. Observed masses were within 0.5 ppm of the theoretical masses of the expected betalain products (Fig. 4). Additionally, *m/z* values (Fig. 4) and MS/MS fragmentation patterns (Fig. S10) for reactions performed *in vivo* are the same as those observed *in vitro* when fed PABA, Ant., or 6AI amines, further supporting the production of the expected betalains because the *in vitro* reactions contain only the reactants (L-DOPA and amine), DOD enzyme, $FeSO_4$, ascorbic acid, and PBS. When oDA was used as the amine, reactions performed both *in vitro* and *in vivo* resulted in a product with an *m/z* value consistent with the oDA single condensate. An additional product was observed *in vivo*, with an *m/z* value consistent with the oDA double condensate. The *m/z* of the double condensate was not observed in the supernatant upon centrifugation of the yeast culture (Fig. S11); it was found only in the PBS wash of the cell pellet. The absorbance maxima determined for the single and double condensates when purified and subjected to HPLC UV–Vis analysis (Fig. 4) were approximately 524 nm and 554 nm, respectively, which is consistent with the absorbance curves in Fig. 3d obtained on culture supernatant and cell pellet wash. These results suggest that the observed blue-violet color obtained from a PBS wash of the cell pellet is the result of a mixture of the blue double condensate and the red-violet single condensate. Finally, the supernatants from the amine-fed yeast cultures were tested for stability at room temperature and ambient light conditions. The betalain pigments exhibited variable but significant loss of color one to six days after collection from yeast culture (Fig. S12). Instability of betalains, and betaxanthins in particular, under ambient conditions is well-documented and they are considered more suitable for applications with short shelf-life or for products stored at cold temperatures (Martins et al., 2017). Stability of the oDA double condensate was considerably increased at both 4 °C and − 20 °C (Fig. S13).

4. Conclusion

The completion of the betanin pathway in yeast and production of a

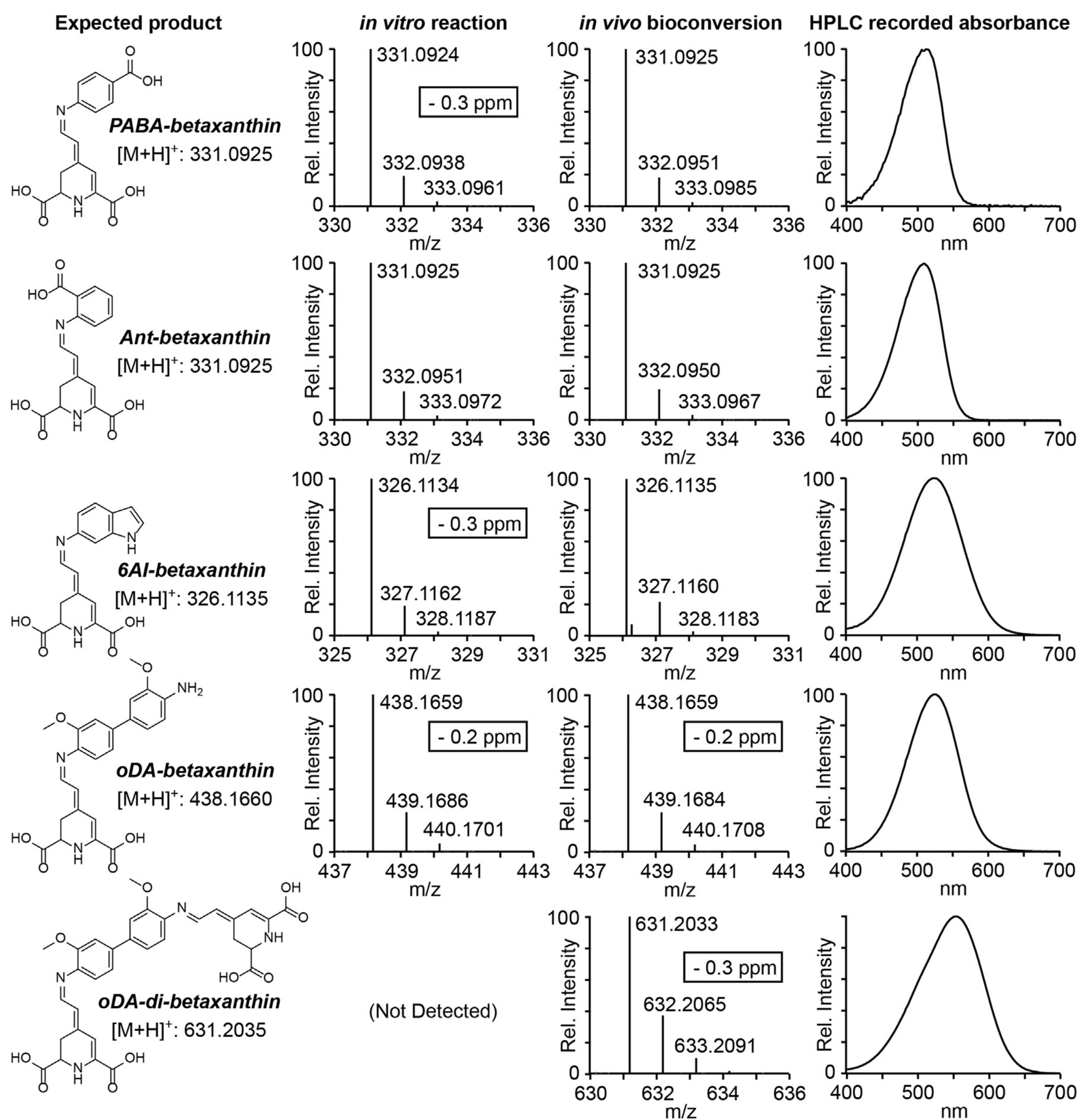

Fig. 4. High resolution mass spectrometry and HPLC UV–Vis analysis of betalains. The predicted structures, theoretical *m/z* values, and betalain names using the generic amine-betaxanthin nomenclature (Khan and Giridhar, 2015) are shown on the left. High resolution mass spectra for *in vitro* and *in vivo* products are provided in the middle, with boxed values referring to differences between theoretical and observed masses in parts per million (ppm). Absorbance curves of the betalains recorded during HPLC analysis are shown on the right. oDA-di-betaxanthin was not detected in the *in vitro* reaction (Fig. S11).

demonstrative suite of both natural and semi-synthetic betalains highlights the potential of *S. cerevisiae* as a heterologous host for the production of betalamic acid derived pigments. For betanin, the ability to shift from plant production to microbial fermentation represents an opportunity to increase the overall market supply and provide a more consistent production level that is not vulnerable to the fluctuations that commonly afflict agricultural processes. Furthermore, a heterologous host could enable the production of alternative forms of betanin where the glucosyl group is further modified (*e.g.*, 3-hydroxy-3-methylglutaryl-betanin or hylocerenin) that may have superior color stability (Herbach et al., 2006) or other improved properties. Currently, our best strain can produce 17 mg of betanin per liter in 48 h. This corresponds to the color intensity of approximately 10 g per liter of purchased beetroot extract powder. In order to economically compete against beetroot for the production of natural betalains and provide the resource-saving advantages often attributed to microbial processes (Marienhagen and Bott, 2013), considerable improvements will be required. To achieve these necessary production gains, we anticipate that metabolic engineering efforts should aim to further improve the P450 activity in *S. cerevisiae* as well as the supply of tyrosine (Gold et al., 2015; Wang et al., 2017).

A major challenge in industrial biosynthesis is ensuring that carbon is not lost to side reactions or degradation (Marienhagen and Bott, 2013). In betanin biosynthesis, unstable metabolites are protected from oxidation *via* glucosylation. Typically, glucosylation as a stabilization strategy is found as a final modification, best exemplified by the anthocyanin pigment pathway (Yonekura-Sakakibara et al., 2008). For betanin, this strategy is seen in the glucosylation of betanidin, which stabilizes the catechol domain and prevents oxidative decomposition. However, the glucosylation of *cyclo*-DOPA presents an alternative route - the glucosylation and stabilization of an unstable intermediate further upstream in the biosynthetic pathway. In our heterologous system, the use of a *cyclo*-DOPA glucosyltransferase is more effective than expression of a betanidin glucosyltransferase. Whether *Beta vulgaris* utilizes a

betanidin and/or *cyclo*-DOPA glucosyltransferase has not yet been determined, but it would be interesting to identify which strategy *B. vulgaris* favors given the commercial use of beetroot extract as a red food dye.

With regard to the larger betalain family, the work here expands the known spectral range and provides a facile bioproduction strategy. Previously, most synthesis methods were based on alkali-catalyzed hydrolysis of betanin (in beetroot extract) followed by re-acidification in the presence of excess amine (Gandía-Herrero et al., 2006). More recent systems have included amine-functionalized solid-phase supports for direct condensation with betalamic acid obtained from base-hydrolysis of betanin (Cabanes et al., 2014). Yeast feeding presents a direct synthesis route with minimal production of unintended betalains. Additionally, the biosynthesized betalains are found in the culture media, which simplifies downstream processing and purification. Future betalain variants could take advantage of tuned spectral properties (color, fluorescence) or decay half-lives. Decay half-life is of potential utility given that color depth or shade has been suggested as an accessible proxy “timer” for determining whether an item or formulation is no longer fit for use (Avent et al., 2008). One particularly interesting avenue towards natural-product derived “unnatural” betalains would be through the rare 4-aminophenylalanine metabolite (Blanc et al., 1997). Previous work has shown that this amino acid can be converted into a phenylpropanoid pathway metabolite (Suvannasara et al., 2014), opening the possibility of linking amino-analogs of flavonoids, stilbenes, and anthocyanins with betalamic acid.

Overall, this work enables future engineering of the unique family of pigments known as betalains by establishing a versatile biosynthetic platform in yeast. With this platform, it is possible to begin the process of pathway optimization that will be required for industrial viability. The results of pathway engineering here also speak to the evolutionary role of glucosyltransferases in enabling the accumulation of otherwise labile small molecules, using glucose as a biochemical protecting group to prevent metabolite oxidation. Lastly, through feeding structurally-diverse amines to yeast strains producing betalamic acid, we produced betalains with expanded spectral properties, including generation of violet and blue-violet molecules containing an uncharged imine group rather than the fixed iminium ion found in betanin.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2017.12.008.

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