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Optimizing Oleaginous Yeast Cell Factories for Flavonoids and Hydroxylated Flavonoids Biosynthesis

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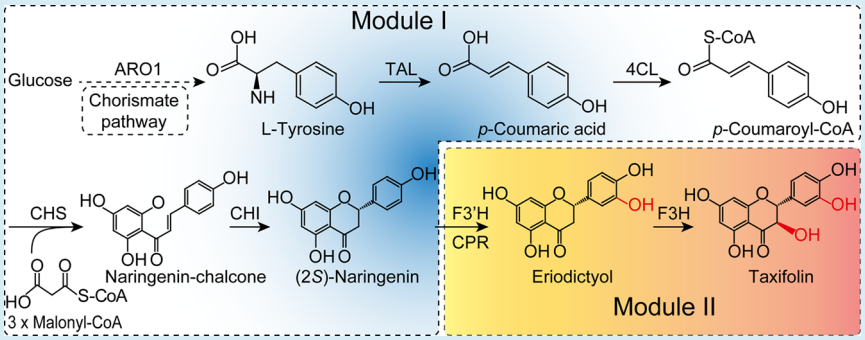
Supporting Information



ABSTRACT: Plants possess myriads of secondary metabolites with a broad spectrum of health-promoting benefits. To date, plant extraction is still the primary route to produce high-value natural products which inherently suffers from economics and scalability issues. Heterologous expression of plant biosynthetic gene clusters in microbial host is considered as a feasible approach to overcoming these limitations. Oleaginous yeast produces a large amount of lipid bodies, the abundant membrane structure and the lipophilic environment provide the ideal environment for the regioselectivity and stereoselectivity of many plant-derived P450 enzymes. In this work, we used modular method to construct, characterize, and optimize the flavonoid pathways in *Yarrowia lipolytica*. We also evaluated various precursor biosynthetic routes and unleashed the metabolic potential of *Y. lipolytica* to produce flavonoids and hydroxylated flavonoids. Specifically, we have identified that chalcone synthase (CHS) and cytochrome P450 reductases (CPR) were the bottlenecks of hydroxylated flavonoid production. We determined the optimal gene copy number of CHS and CPR to be 5 and 2, respectively. We further removed precursor pathway limitations by expressing genes associated with chorismate and malonyl-CoA supply. With pH and carbon−nitrogen ratio (C/N) optimization, our engineered strain produced 252.4 mg/L naringenin, 134.2 mg/L eriodictyol, and 110.5 mg/L taxifolin from glucose in shake flasks. Flavonoid and its hydroxylated derivatives are most prominently known as antioxidant and antiaging agents. These findings demonstrate our ability to harness the oleaginous yeast as the microbial workhorse to expand nature's biosynthetic potential, enabling us to bridge the gap between drug discovery and natural product manufacturing.

KEYWORDS: *natural products, flavonoids, hydroxylation, metabolic engineering, oleaginous yeast, P450*

Flavonoids represent a diversified family of phenylpropanoid-derived plant secondary metabolites with an estimated 10 000 unique structures.[1,2] They are widely found in fruits, vegetables, and medicinal herbs. Pharmaceutical studies and animal tests have demonstrated their antiobesity, anticancer, anti-inflammatory, and antidiabetic activities.[3,4] Flavonoids are among the phytochemicals with proven activity toward the prevention of aging-related diseases, including the treatment of nervous and cardiovascular diseases, Parkinson's and Alzheimer's disease, and so forth.[5] In the last decades, various flavonoid pathways have been reconstituted in microbial species including *Escherichia coli* and *Saccharomyces cerevisiae*, resulting in the production of naringenin, eriodyctiol, resveratrol, pinocembrin, anthocyanins, quercetin, kaempferol, silybin, isosilybin, baicalein, scutellarein, and so forth. Structural activity relationship (SAR) studies demonstrated the side chain modifications are highly correlated with flavonoid biological activities.[2,6] The hydroxylation of

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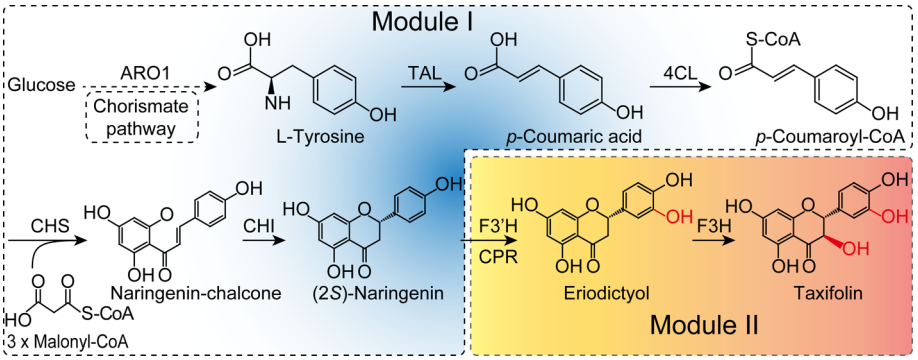

Figure 1. Modular strategy to optimize naringenin, eriodictyol, and taxifolin pathways. On the basis of the reaction cascades, flavonoid pathway was partitioned into 2 modules, naringenin synthetic module (Module I) and hydroxylation module (Module II). Module I contains chorismate pathway and malonyl-CoA utilizing step and Module II contains flavonoid 3'-hydroxylase and cytochrome P450 reductase.

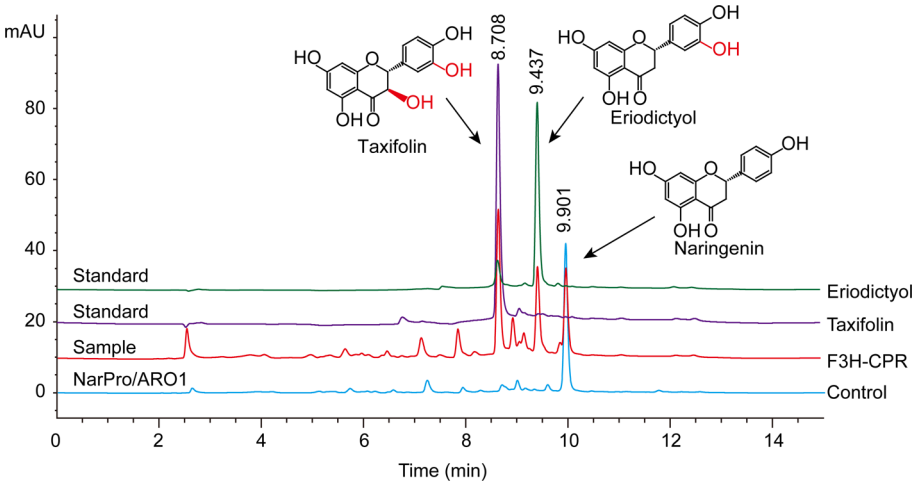

Figure 2. HPLC profiles of naringenin, eriodictyol, and taxifolin. Two hydroxylated flavonoid standards (taxifolin, purple; eriodictyol, green) were injected to HPLC. One naringenin-producing sample (blue) and one taxifolin-producing sample (red) are shown in the chromatogram.

flavonoids enhances the solubility and antioxidant property.[7] To date, there remains a significant challenge to produce hydroxylated flavonoids due to our inability to efficiently express plant P450-related enzymes (hydroxylases and the cytochrome P450 reductases).[8]

Oleaginous yeast is rich in membrane structure and subcellular compartments (i.e., peroxisome, ER, and oleosome), which provide the hydrophobic environment that is ideal for regioselectivity and stereoselectivity in hydroxylation and prenylation of flavonoids.[9−12] *Yarrowia lipolytica* is known to internalize a substantial portion of carbon feedstock as lipids and fatty acids.[13,14] The high precursor acetyl-CoA and malonyl-CoA flux make *Y. lipolytica* a promising host to produce various natural products with complex structures. It has been recognized as a "generally regarded as safe" (GRAS) organism for the production of organic acids, polyunsaturated fatty acids (PUFAs),[15,16] and carotenoids[17−20] in the food and nutraceutical industry. Compared to *S. cerevisiae*, *Y. lipolytica* lacks Crabtree effects, which does not produce ethanol under high-glucose or respiration-limited conditions. The low pH tolerance,[21] strictly aerobic nature,[22,23] and versatile substrate-degradation profile[23−25] enable its robust growth from a wide range of renewable feedstocks. A collection of genetic toolbox, including protein expression,[26−28] promoter characterization,[29−31] YaliBrick-based cloning,[32,33] Golden gate cloning,[19,34] *piggyBac* transposon,[35] genome-editing,[32,36,37] and iterative gene integration,[38] have enabled us to streamline and accelerate pathway engineering in oleaginous yeast species.

To bridge this gap, we tested and assessed various plant-derived polyketide synthases, P450 monooxygenase/hydroxylases and cytochrome P450 reductases in *Y. lipolytica*, to diversify the structure of flavonoids. Systematic pathway debottlenecking indicated that chalcone synthase (CHS), acetyl-CoA carboxylase (ACC), and cytochrome P450 reductases (CPR) are the rate-limiting steps for hydroxylated flavonoid production, specifically, optimizing precursor pathway, increasing *PhCHS* copy number, and controlling culture pH elevated naringenin production up to 252.4 mg/L. Screening three CPRs led us to identify that CrCPR derived from *Catharanthus roseus* is the most efficient electron shuttle to complete the hydroxylation reaction, despite that endogenous YlCPR (YALI0D04422g) displays similar function with relatively low efficiency. Further expression of the *Gerbera hybrid* flavonoid3′-hydroxylase (GhF3′H) led the engineered strain to produce about 110.5 mg/L of taxifolin and 134.2 mg/L of eriodictyol. This work set the foundation to use oleaginous yeast as the chassis for cost-efficient production of flavonoids and hydroxylated flavonoids, which expands our capability to access nature's biosynthetic potential for drug discovery and natural product manufacturing.

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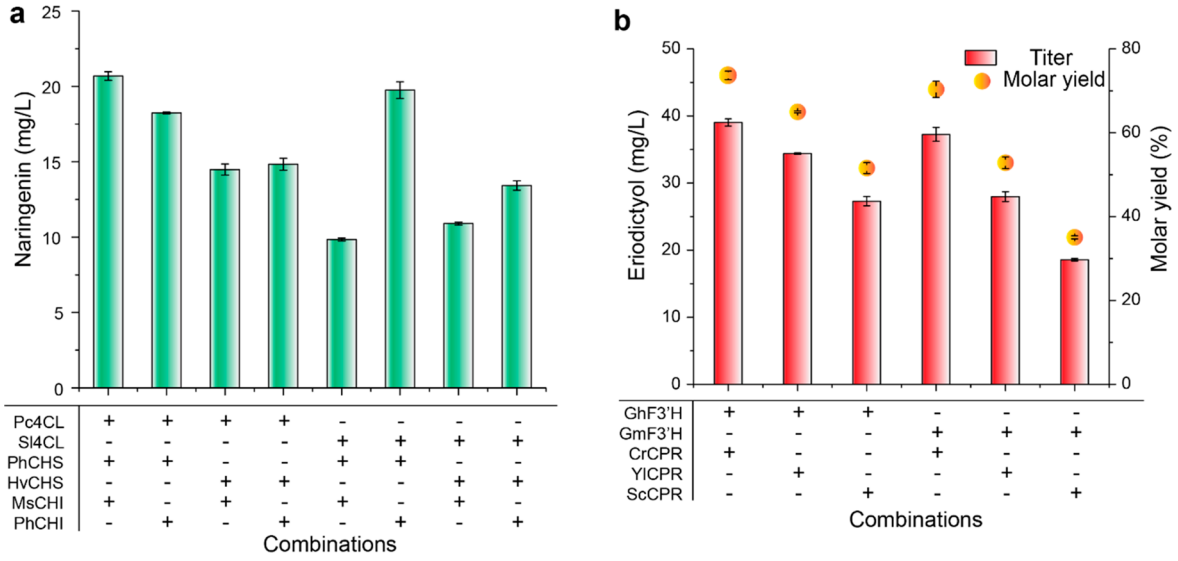

Figure 3. Screening of gene combinations for improving flavonoid production. (a) Screening of *4CL*, *CHS*, and *CHI* genes from different pants for naringenin production. (b) Screening of *F3′H* and *CPR* genes from different organisms for eriodictyol production. The acronyms refer to the organism names. The organism name and gene sources can be found in SI Table S1.

RESULTS AND DISCUSSION

Modular Construction and Characterization of Hydroxylated Flavonoid Pathway in *Y. lipolytica*. We first reconstructed the synthetic pathway and validated the feasibility of using *Y. lipolytica* as the chassis to produce flavonoids. In addition, the cytochrome P450 (CYP) flavonoid 3′-hydroxylase (F3′H) plays a critical role in oxidizing the phenyl ring and generating hydroxylated flavonoids.[39] On the basis of the gene organization of flavonoid biosynthetic gene clusters, we partitioned the flavonoid pathway into two modules, the naringenin synthesis module (Module I) and the hydroxylation module (Module II) (Figure 1). Module I is the essential precursor pathway to provide chorismate, malonyl-CoA, and chalcone precursors; whereas Module II will hydroxylate chalcone by the cytochrome P450 flavonoid 3′-hydroxylase (F3′H) and CPR. As a direct assessment of the module efficiency, we have established a high-performance liquid chromatography (HPLC) method to simultaneously analyze naringenin, eriodyctiol, and taxifolin (Figure 2) with putative chemicals as standards.

Naringenin is the starting point for many flavonoid functionalization chemistries. We first constructed Module I in *Y. lipolytica* Po1f to synthesize chalcone and naringenin. Because genes from different plants have different specificity and activity, we selected two genes for each of the first three steps in Module I based on the sequence similarity of closely related plant species. Pathways containing 4CL, CHS, and CHI were assembled in monocistronic form by YaliBricks cloning platform.[32] We observed that all eight constructs resulted in the synthesis of naringenin from *p*-coumaric acid with production ranging from 10 to 21.5 mg/L (Figure 3a). Interestingly, the three top producers (Figure 3a) share the same source of chalcone synthase from *Petunia x hybrid*, indicating that chalcone synthase dictates the efficiency of Module I. To achieve *de novo* synthesis of naringenin, we coexpressed a codon-optimized tyrosine ammonia-lyase from *Rhodotorula toruloides* (RtTAL).[40,41] With RtTAL, we detected *p*-coumaric acid as the direct deamination product of tyrosine (Supporting Information (SI) Figure S1). By complementing the 4CL-CHS-CHI pathway, the resulted strain *Y. lipolytica* Po1f/T4SI produced 14.9 mg/L naringenin from glucose. These results demonstrated the feasibility of using *Y. lipolytica* as the chassis for *de novo* synthesis of naringenin.

There has been a number of reports that *Y. lipolytica* could selectively hydroxylate limonene to perillyl alcohol, perillaldehyde, and perillic acids,[42,43] indicating the endogenous cytochrome P450 reductase (CPR) is active enough to perform hydroxylation reaction. Similarly, a greenish P450-derived violacein derivative has been produced in *Y. lipolytica*.[44] For these reasons, we argue that *Y. lipolytic* could be an excellent platform for expression of plant P450 enzymes.

F3′H is the critical enzyme involved in the hydroxylation of flavonoids. CPR is required for electron transfer from NADPH to CYP.[45] We chose two plant-derived F3′Hs and three CPRs to evaluate which F3′H−CPR pair could perform hydroxylation chemistry (Figure 3b). All six combinations of F3′H−CPR pairs produced eriodictyol. We observed that strain Po1f/HR with overexpression of CrCPR (derived from *Catharanthus roseus*)[46] coupled with GhF3′H (derived from *Gerbera hybrid*) or GmF3′H (derived from *Glycine max*), led to the highest eriodictyol production around 39 mg/L with molar conversion yield up to 73.7% from naringenin (Figure 3b). Interestingly, the two yeast-sourced CPRs, YlCPR from *Y. lipolytica* and ScCPR from *S. cerevisiae*, also produce eriodictyol. This result reconfirmed that the endogenous CPR is sufficient to complete the oxidation reaction. We further constructed strain *Y. lipolytica* Po1f/HRH expressing F3H from *Solanum lycopersicum* (SlF3H) and detected about 26.0 mg/L taxifolin with a molar yield of 46.5% from naringenin. To achieve *de novo* synthesis of eriodictyol and taxifolin, we next complemented the naringenin pathway with GhF3′H−CrCPR and GhF3′H−CrCPR−SlF3H, resulting in strains Po1f/T4SIHR and Po1f/T4SIHRH, respectively. When testing in shake flasks, we obtained 17.2 mg/L eriodictyol and 11.3 mg/L taxifolin. These results validated that *Y. lipolytica* is an ideal chassis to express plant P450 enzymes and produce hydroxylated flavonoids.

Tuning Gene-Copy Number To Remove Pathway Bottlenecks. The balance of metabolic flux and mitigation of

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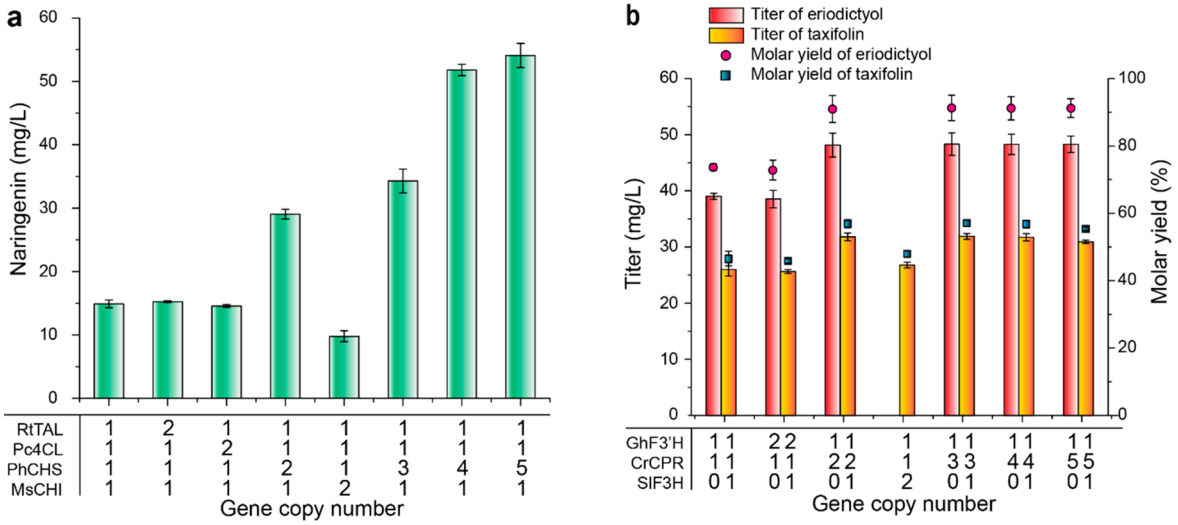

Figure 4. Overcoming rate-limiting steps by tuning gene copy numbers. Rate-limiting steps were determined by gradually increasing the gene copy number of each step. Numbers refer to gene copy numbers. (a) Rate-limiting step analysis and optimization of module I to improve naringenin production. (b) Rate-limiting step analysis and optimization of module II to improve eriodictyol and taxifolin production. The molar yield was calculated using 50 mg/L naringenin as the feeding substrate. The number 0 refers to that the module does not contain the respective gene.

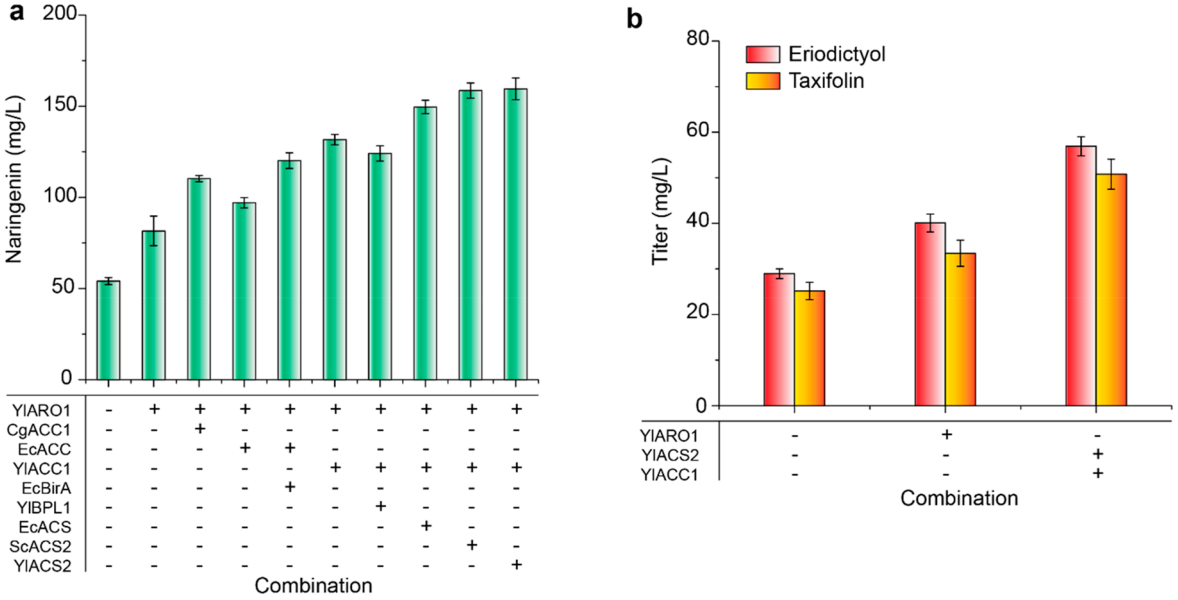

Figure 5. Improving naringenin, eriodictyol, and taxifolin production by enhancing precursor synthesis. (a) Identification of possible rate-limiting steps by overexpression of chorismate pathway (ARO1), malonyl-CoA pathway (ACC), and acetyl-CoA pathway (ACS). The related genes were overexpressed in strain Po1f/T4S$_{x5}$I. (b) Effects of improving malonyl-CoA and chorismate synthesis on eriodictyol and taxifolin production. For eriodictyol production, the related genes were overexpressed in Po1f/T4S$_{x5}$IHR$_{x2}$. For taxifolin production, the related genes were overexpressed in Po1f/T4S$_{x5}$IHR$_{x2}$H. +: Referred to the presence of gene overexpression. −: Referred to the absence of gene overexpression.

metabolic burden is an essential part for metabolic engineering. Metabolic flux improvement by overexpression of upstream pathways may not be accommodated by downstream pathways;[47] intermediate accumulation or depletion may reduce cell viability,[48] and overexpressed gene clusters may overload the cell and elicit cellular stress response.[49,50] We next attempted to probe the rate-limiting steps in Module I and Module II by tuning the gene copy number with the YaliBrick assembly platform.[32] Naringenin production increased by 2.64-fold when the gene copy number for chalcone synthase (PhCHS) increased from one to five (Figure 4a), indicating that CHS is the rate-limiting step in Module I. Increasing the copy number of other metabolic genes (*RtTAL* and *Pc4CL*) did not have an obvious effect on naringenin titer, whereas increasing the copy number of *MsCHI* decreased naringenin titer by 34.4% (Figure 4a). The optimal gene copy number for *PhCHS* was found to be five. As larger plasmid size may cause genetic instability, we did not further increase the copy number of PhCHS.

In Module II, increasing one copy number of *CrCPR* resulted in eriodictyol and taxifolin titers increased by 26.8% and 22.3%, reaching 48.1 mg/L and 31.8 mg/L (Figure 4b), respectively. Increasing the copy number of *SlF3H* and *GhF3′H* did not improve eriodictyol or taxifolin production (Figure 4b), indicating that CPR is the rate-limiting step in Module II. Eriodictyol and taxifolin titers remained stable,

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when the gene copy number of *CrCPR* was increased from 2 to 5, suggesting that the optimal ratio of F3′H to its reductase CrCPR is 1:2, which is consistent with a previous report.[51] The naringenin-to-eriodictyol and taxifolin conversion ratio reached 90.5% and 56.8%, respectively (Figure 4b), under the optimal F3′H−CPR ratios. To achieve *de novo* synthesis of eriodictyol and taxifolin, we complemented the hydroxylation module with the naringenin pathway. The resulting strains Po1f/$T4S_{x5}IHR_{x2}$ and Po1f/$T4S_{x5}IHR_{x2}$H produced 28.9 mg/L eriodictyol and 25.2 mg/L taxifolin from glucose, respectively, which are 68.0% and 123.0% higher than the control strains Po1f/T4SIHR and Po1f/T4SIHRH. These results suggest that tuning gene copy number is a critical step to remove pathway bottlenecks and achieve metabolic balance in genetically engineered cell factories.

Improving Flavonoid Production by Enhancing Precursor Synthesis. We next sought to investigate the upstream chorismate and malonyl-CoA pathways to further improve flavonoid production. By supplementing 100 mg/L L-tyrosine with the strain Po1f/T4SI, we observed that naringenin production was increased by 33.6%, indicating that upstream chorismate pathway is a bottleneck for naringenin synthesis in *Y. lipolytica*. We then overexpressed the pentafunctional arom protein YlARO1 (YALI0F12639g), which catalyzes steps 2 through 6 in the biosynthesis of chorismate, to boost the precursor for L-tyrosine.[52] To mitigate unintended mRNA splicing and transcriptional regulation, we removed the internal intron for *YlARO1* and *YlACC1*. When this *YlARO1* gene was overexpressed in strain Po1f/$T4S_{x5}I$ with optimal Module I settings, naringenin production was increased to 81.6 mg/L, a 50.9% increase over the parental strain (Figure 5a). When we combined Module I with Module II, the resulting strains Po1f/$AT4S_{x5}IHR_{x2}$ and Po1f/$AT4S_{x5}IHR_{x2}$H produced 40.1 mg/L eriodictyol and 33.4 mg/L taxifolin, which is 38.8% and 32.5% higher than that of the control strains Po1f/$T4S_{x5}IHR_{x2}$ and Po1f/$T4S_{x5}IHR_{x2}$H, respectively (Figure 5b).

Acetyl-CoA and malonyl-CoA are shared precursors for both lipid and flavonoid pathways.[53,54] Malonyl-CoA was reported to be a rate-limiting step of flavonoid synthesis in many microorganisms.[55−57] To mitigate this competition, it is desirable to redirect the acetyl/malonyl-CoA flux from lipid pathway to flavonoid pathway. Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, which is the source pathway for malonyl-CoA.[55] To enhance malonyl-CoA level, we screened and tested three ACCs, including Gram-positive bacteria *Corynebacterium glutamicum* ATCC 13032 (CgACC), Gram-negative bacteria *Escherichia coli* MG1655 (Ec_accABCD), and *Y. lipolytica* (YlACC1, GRYC ID: YALI0C11407g).[58,59] Biotinylation of ACC was found to be essential for ACC activity.[60] Genes encoding CgACC, Ec_accABCD, and YlACC1, together with their biotin-apoprotein ligases, EcBirA and YlBPL1 (YALI0E30591g), were introduced to the naringenin-producing strain. All three ACCs could lead to substantial improvement in naringenin production (Figure 5a), with YlACC1 demonstrating the most obvious effect. For example, overexpression of YlACC1 in Po1f/$AT4S_{x5}I$ improved naringenin titer by 61.4%, reaching 131.7 mg/L (Figure 5a). Coexpression of Ec_accABCD with EcBirA also resulted in naringenin production increasing by 22% compared with the strain without EcBirA overexpression, indicating the essential role of biotinylation in bacterial ACC activity. This is the first report that EcACC could be functionally expressed in oleaginous species. Unlike the bacterial ACC, coexpression of YlACC1 and YlBPL1 resulted in decreased naringenin production (Figure 5a). This might indicate the endogenous biotin-apoprotein ligase (YlBPL1) is sufficient to biotinylate YlACC1 in *Y. lipolytica*.

We observed that pH dropped dramatically during the fermentation process (i.e., pH below 3.5 at the end of cultivation), and this possibly due to respiration and the formation of organic acids. It was recently discovered that acetate secretion could be resulted from the CoA-transfer activity between acetyl-CoA and succinate in *Y. lipolytica* by a mitochondrial enzyme YlACH1 (YALI0E30965).[21] We next sought to overexpress acetyl-CoA synthetases and recycle acetate to acetyl-CoA. We tested three acetyl-CoA synthetases from *E. coli* (EcACS), *S. cerevisiae* (ScACS2), and *Y. lipolytica* (YlACS2) (Figure 5a). The native YlACS2 was found to be most efficient to recycle acetate (Figure 5a). To boost both chorismate and acetyl/malonyl-CoA precursors, we overexpressed YlARO1 along with YlACS2-YlACC1 in strain Po1f/$T4S_{x5}I$. The resulting strain produced 149.5 mg/L naringenin, which was 176.3% higher than the titer of the parental strain (Po1f/$T4S_{x5}I$). We next translated this knowledge to Module II and tested whether overexpression of ARO1, ACC1, and ACS would benefit the accumulation of hydroxylated flavonoids. Overexpressing YlARO1 increased eriodictyol and taxifolin production by 38.8% and 32.5%, yielding 40.1 and 33.4 mg/L (Figure 5b), respectively. Overexpressing YlACS2 and YlACC1 further increased eriodictyol and taxifolin titers by 41.9% and 52.1%, reaching 56.9 mg/L and 50.8 mg/L, respectively (Figure 5b). These results indicated that acetyl-CoA, malonyl-CoA, and chorismate pathways were important steps to improve flavonoid production in *Y. lipolytica*.

Boosting Flavonoid Production by Bioprocess Optimization. The C/N ratio is an important factor for regulating the acetyl-CoA and NADPH fluxes in *Y. lipolytica*.[31] It was recently found that C/N ratio regulates lipogenic promoter activity in *Y. lipolytica*.[31] To improve flavonoid production, we investigated the C/N ratio by either adjusting the amount of nitrogen source (ammonia sulfate) or carbon source (glucose). Simply altering $(NH_4)_2SO_4$ content did not improve naringenin titer. Slightly higher naringenin titer was achieved at higher C/N ratio (C/N = 120) (SI Figure S2a). On the contrary, increasing glucose content was advantageous to naringenin accumulation. Specifically, naringenin titer was increased about 56% when the C/N was altered from 40 to 160 (SI Figure S2b) by increasing the level of glucose.

To produce flavonoids with inexpensive YPD (yeast extract, peptone, and dextrose) medium, we next integrated the optimized pathways into *Y. lipolytica* Po1f genome with our recently developed integration method.[61] The best-performing strains NarPro/ASC, ErioPro, and TaxiPro produced 71.2 mg/L naringenin, 54.2 mg/L eriodictyol, and 48.1 mg/L taxifolin in YPD medium, respectively.[38] We observed that the pH dropped to 3.2 at the end of the fermentation in YPD, possibly due to the accumulation of organic acids.[18] We next sought to control the medium pH by using either phosphate buffer saline (PBS) or calcium carbonate ($CaCO_3$). Supplementation of 40 g/L $CaCO_3$ maintained stable pH and improved naringenin titer by 31.2%, reaching 138.1 mg/L at 144 h, whereas PBS buffer did not improve naringenin production (SI Figure S3). We also attempted to inhibit fatty acid synthesis by using cerulenin,[62] maintain medium pH, and feed sodium acetate (SI Figure S4). Specifically, supplementation of 1 mg/L cerulenin

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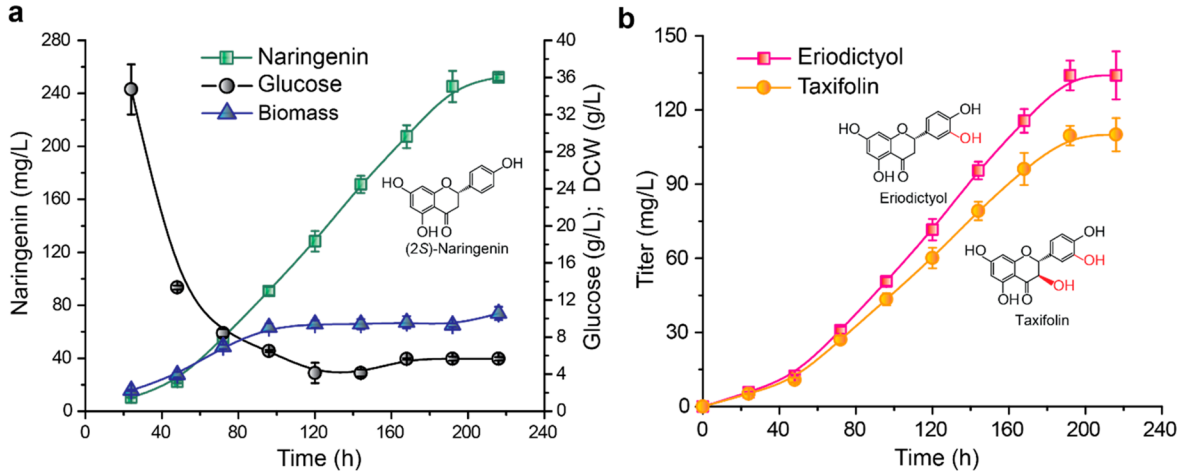

Figure 6. Naringenin, eriodictyol and taxifolin production under the optimal conditions. (a) Naringenin production; (b) eriodictyol and taxifolin production. The engineered strains were cultivated in fed-batch fermentation and buffered with 40 g/L $CaCO_3$. A final concentration of 1 mg/L cerulenin was supplemented at 48 h to inhibit fatty acid synthesis.

with 40 g/L $CaCO_3$ improved naringenin titer by 31.2%, (SI Figure S5c). On the other hand, feeding 5 mM NaAc did not result in increase in naringenin production (SI Figure S5d). By intermittently feeding glucose after 48 h, the chromosomally integrated strain produced 252.4 mg/L naringenin under optimal conditions (Figure 6a). Likewise, we tested the eriodictyol and taxifolin production in YPD medium by controlling medium pH with 40 g/L $CaCO_3$ and inhibiting fatty acid synthesis with 1 mg/L cerulenin. Strains ErioPro and TaxiPro produced 95.5 mg/L eriodictyol and 79.1 mg/L taxifolin in 144 h, which were 76.2% and 64.4% higher than that without $CaCO_3$ and cerulenin. ErioPro and TaxiPro produced 134.2 mg/L eriodictyol and 110.5 mg/L taxifolin at the end of the fermentation cultivation (Figure 6b). Our experiments demonstrated that *Y. lipolytica* is an ideal platform to efficiently express plant-derived P450 enzymes and produce hydroxylated flavonoids. By optimizing C/N ratio and pH, we further improved the titer of naringenin, eriodictyol, and taxifolin in *Y. lipolytica*.

CONCLUSIONS

The heterologous production of hydroxylated flavonoids remains a challenging task; only limited successful engineering endeavors have been reported to date. Oleaginous yeast is abundant in lipid and internal membrane structures, which provide the hydrophobic environment that is critical for plant P450 enzyme functionality. In this report, we validated that *Y. lipolytica* is a superior platform for heterologous production of high value flavonoids and hydroxylated flavonoids. By modular construction and characterization of various flavonoid biosynthetic genes, we determined that chalcone synthase (CHS) and cytochrome P450 reductase (CPR) are the critical steps to engineer flavonoid production in *Y. lipolytica*. Coupling with tyrosine ammonia lyase, for the first time we achieved *de novo* production of naringenin, eriodictyol, and taxifolin from glucose in *Y. lipolytica*. Taking advantage of a modular cloning platform to assemble multiple genetic constructs, we further determined the optimal gene copy number for CHS, F3H, and CPR to cooperatively improve flavonoid and hydroxylated flavonoid production. We then unleashed the metabolic potential of *Y. lipolytica* by screening and testing a number of precursor pathways, including the acetyl-CoA synthetase, acetyl-CoA carboxylase, and chorismate pathway (the pentafunctional AROM polypeptide ARO1). With the optimized chalcone synthase module and the hydroxylation module, our engineering strategies synergistically removed pathway bottlenecks and led to a 15.8-fold, 6.9-fold, and 8.8-fold improvement in naringenin, eriodictyol, and taxifolin production, respectively. Collectively, these findings demonstrated our abilities to harness oleaginous yeast as microbial workhorse to expand nature's biosynthetic potential, which allows us to produce complex natural products from cheap feedstocks.

MATERIALS AND METHODS

Genes, Plasmids, and Strains. Genes encoding *Rhodotorula toruloides* tyrosine ammonia lyase (RtTAL), *Petroselinum crispum* (parsley) 4-coumarate-CoA ligase (Pc4CL), *Petunia x hybrid* chalcone synthase (PhCHS), *Medicago sativa* chalcone isomerase (MsCHI), *Escherichia coli* acetyl-CoA synthetase (EcACS), and *Corynebacterium glutamicum* ATCC 13032 acetyl-CoA carboxylase (CgACC) were from our laboratory.[58] Genes encoding *Solanum lycopersicum* 4-coumarate-CoA ligase (Sl4CL), *Hordeum vulgare* chalcone synthase (HvCHS2), *Petunia x hybrid* chalcone isomerase (PhCHI), *Gerbera hybrid* flavonoid 3′-hydroxylase (GhF3′H), *Glycine max* flavonoid 3′-hydroxylase (GmF3′H), *Catharanthus roseus* cytochrome P450 reductase (CrCPR), and *Solanum lycopersicum* flavanone 3-hydroxylase (SlF3H) were codon-optimized and synthesized by GenScript (Nanjing, China). Genes encoding *Yarrowia lipolytica* pentafunctional arom protein (YlARO1), *Yarrowia lipolytica* cytochrome P450 reductase (YlCPR1), and *Yarrowia lipolytica* acetyl-CoA synthetase (YlACS2) were amplified from *Yarrowia lipolytica* Po1f genomic DNA by PCR. *Saccharomyces cerevisiae* cytochrome P450 reductase (ScCPR1) and *Saccharomyces cerevisiae* acetyl-CoA synthetase (ScACS2) were amplified from *Saccharomyces cerevisiae* S288c genomic DNA by PCR. Genes used in this project are listed in SI Table S1.

Plasmid pYLXP′ was a stock in our laboratory.[53] Plasmid pYLXP′2 was constructed by replacing *LEU2* marker with *URA3* marker. Both pYLXP′ and pYLXP′2 were YaliBrick plasmids and used for flavonoid pathway construction.[32] *E. coli* NEB 5*α* was used for plasmid construction, propagation, and maintenance. *Y. lipolytica* Po1f (ATCC MYA-2613, MATA

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ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2) was used as the chassis to construct flavonoid pathways.

To achieve *de novo* synthesis of eriodictyol and taxifolin, we transformed pYLXP′2-GhF3′H-CrCPR and pYLXP′2-GhF3′H-CrCPR-SlF3H into Po1f/T4SI, resulting in Po1f/T4SIHR and Po1f/T4SIHRH, respectively. The strain containing five copies of PhCHS was named as Po1f/T4S_{x5}I. We chose to use the plasmids pYLXP′2-HR_{x2} and pYLXP′2-HR_{x2}H, which contain two copies of CrCPR, to construct eriodictyol and taxifolin pathways. To achieve *de novo* synthesis of eriodictyol and taxifolin, strains Po1f/T4S_{x5}IHR_{x2} and Po1f/T4S_{x5}IHR_{x2}H were constructed by transforming plasmids pYLXP′2-HR_{x2} and pYLXP′2-HR_{x2}H into strain Po1f/T4S_{x5}I, respectively. We overexpressed YlARO1 along with YlACS2-YlACC1 in strain Po1f/T4S_{x5}I and named the new strain as Po1f/AT4S_{x5}I-YlACS2-YlACC1. By introducing Module II into strains Po1f/AT4S_{x5}I and Po1f/AT4S_{x5}I-YlACS2-YlACC1, we obtained eriodictyol-producing strains Po1f/AT4S_{x5}IHR_{x2} and Po1f/AT4S_{x5}IHR_{x2}-YlACS2-YlACC1 and taxifolin-producing strains Po1f/AT4S_{x5}IHR_{x2}H and Po1f/AT4S_{x5}IHR_{x2}H-YlACS2-YlACC1. Strains constructed in this project are listed in SI Table S2.

Pathway Construction. Genes *RtTAL*, *Pc4CL*, *PhCHS*, *MsCHI*, *EcACS*, *CgACC*, *YlCPR*, *YlACS2*, *ScCPR1*, and *ScACS2* were amplified using respective primers listed in SI Table S3. The PCR product was assembled with *Sna*BI digested pYLXP′ or pYLXP′2 using Gibson Assembly method. *YlARO1* is composed of two exons and one intron. The exons were amplified by using primer pairs ARO1_up F/ARO1_up R and ARO1_down F/ARO1_down R, respectively. The resulting PCR products were assembled with *Sna*BI digested pYLXP′ to yield pYLXP′-ARO1, removing the intron sequence. Similarly, the two introns in *YlACC1* (YALI0C11407) were also removed and only the exons (coding sequence) were used for gene overexpression. To comply with the YaliBrick cloning platform, the start codon of the expressed gene was removed and a nucleic acid sequence "TAACCGCAG" was added at the upstream of coding gene to complete the intron.[32]

The YaliBrick method was used to assemble the synthetic pathways.[32] pYLXP′-derived plasmids were used to assemble the pathways of Module I, whereas pYLXP′2-derived plasmids were used to assemble the pathways of Module II and ACS and ACC. Generally, the donor plasmids were digested with *Avr*II/*Sal*I, and the destination plasmids were digested with *Nhe*I/*Sal*I. The resulting plasmids containing monocistronic configurations were obtained by T4 ligation. For the assembly of genes containing any of these isocaudomers, other isocaudomers were used. Specifically, the donor plasmid pYLXP′-YlARO1 was digested with *Hpa*I/NheI, and the destination plasmid pYLXP′-T4S_{x5}I was digested with *Hpa*I/*Avr*II. The resulting plasmid pYLXP′-AT4S_{x5}I was obtained by inserting *YlARO1* into pYLXP′-T4S_{x5}I using T4 ligation. The donor plasmid pYLXP′2-HR_{x2}H was digested with *Cla*I/*Nhe*I, and the destination plasmid pYLXP′2-ScACS2-YlACC1 was digested with *Cla*I/*Avr*II. The resulting plasmid pYLXP′2-HR_{x2}H-ScACS2-YlACC1 was obtained by inserting genes GhF3′H-CrCPR_{x2}-SlF3H into pYLXP′2-ScACS2-YlACC1 using T4 ligation. Plasmids pYLXP′2-YlACC1, pYLXP′2-EcACCABCD-EcBirA, and pYLXP′2-YlBPL1 were from our laboratory.[32] Plasmids used in this paper are listed in SI Table S4.

Yeast Transformation and Screening. The lithium acetate (LiAc) method was used for the transformation. *Y. lipolytica* was cultured on YPD plate at 30 °C for 16−22 h. The transformation solution was prepared as follows: 90 μL of 50% PEG4000, 5 μL of 2 M LiAc, 5 μL of boiled single-strand DNA (salmon sperm, denatured), and 200−500 ng of plasmid DNA. The transformation solution was mixed well by vortexing before use. Next, the yeast was transferred to the transformation solution and mixed well by vortexing for at least 10 s. The transformation mixtures were then incubated at 30°C for 30−45 min. The transformation mixture was then vortexed for 15 s every 10 min, followed by an additional 10 min heat shock at 39 °C to increase transformation efficiency. For the transformation of pYLXP′ and derivative plasmids, the mixture was plated on leucine drop-out complete synthetic media (CSM-Leu). For the transformation of pYLXP′2 and derivative plasmids, the mixture was plated on uracil drop-out complete synthetic media (CSM-Ura). For the transformation of both plasmids, the mixture was plated on leucine and uracil drop-out complete synthetic media (CSM-Leu-Ura). Strains NarPro/ASC, ErioPro, and TaxiPro were constructed in previous work.[38] Strains constructed in this project are listed in SI Table S2.

Cultivation and pH control. The seed was cultured in regular leucine, or uracil, or leucine and uracil drop-out complete synthetic media (CSM-Leu, or CSM-Ura, or CSM-Leu-Ura) at 30 °C for 2 days. The seed culture was inoculated to 25 mL nitrogen-limited media (C/N = 80) to a final concentration of 2% (v/v). The fermentation was carried out in 250 mL shake flask at 30 °C 220 rpm. C/N ratio was optimized by two patterns: (i) fixing glucose content (40 g/L) and altering $(NH_4)_2SO_4$ content; (ii) fixing $(NH_4)_2SO_4$ content (0.73348 g/L) and altering glucose content. To analyze the effect of cerulenin, oleic acid, and sodium acetate (NaAc) on flavonoid synthesis, a final concentration of 5 g/L oleic acid or 1 mM NaAc was added at the starting point, whereas a final concentration of 1 mg/L cerulenin was added at 48 h. To control the pH, 20 mM phosphate buffer saline (PBS, Na_2HPO_4−NaH_2PO_4) or 40 g/L $CaCO_3$ was used, respectively. In the fed-batch fermentation, the starting glucose concentration was 40 g/L, and a final concentration of 10 g/L glucose was added every 24 h from 48 h.

Analytical Methods. Samples were taken at 144 h. In the fed-batch fermentation, samples were taken every 24 h. For naringenin, eriodictyol, and taxifolin analysis, samples were diluted in equal volume of pure methanol; for glucose analysis, samples were diluted in H_2O to final concentrations of 0.5−5 g/L. Flavonoid samples were shaken (Vortex Genie 2, Scientific Industries, NY) with 0.25 mm glass beads for at least 2 min to release the metabolites for analysis. The debris and glass beads were removed by centrifugation at 14 000 rpm for 5 min and filtration with 0.2 μm membrane. Naringenin, eriodictyol, taxifolin, and glucose were analyzed using Agilent HPLC 1220 as previously described.[38]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00193.

Four SI tables and five SI figures. (SI Table S1) Genes used in this paper. (SI Table S2) Strains constructed in this paper. (SI Table S3) Primers used in this paper. (SI Table S4) Plasmids used in this paper. (SI Figure S1)

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HPLC profile of strains producing both naringenin and *p*-coumaric acid. (SI Figure S2) C/N ratio optimization using different patterns. (SI Figure S3) Effects of PBS and $CaCO_3$ on the pH and naringenin titer. (SI Figure S4) Effects of cerulenin, oleic acid, and sodium acetate on naringenin titer. (SI Figure S5) Time course of fed-batch fermentation in YPD media (PDF)

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P.X. and J.Z. conceived the topic. Y.L. performed genetic engineering and fermentation experiments. Y.L. and P.X. wrote the manuscript. J.Z. and M.K. revised the manuscript.

Notes

The authors declare no competing financial interest.

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