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De novo biosynthesis of bioactive isoflavonoids by engineered yeast cell factories

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Isoflavonoids comprise a class of plant natural products with great nutraceutical, pharmaceutical and agricultural significance. Their low abundance in nature and structural complexity however hampers access to these phytochemicals through traditional crop-based manufacturing or chemical synthesis. Microbial bioproduction therefore represents an attractive alternative. Here, we engineer the metabolism of Saccharomyces cerevisiae to become a platform for efficient production of daidzein, a core chemical scaffold for isoflavonoid biosynthesis, and demonstrate its application towards producing bioactive glucosides from glucose, following the screening-reconstruction-application engineering framework. First, we rebuild daidzein biosynthesis in yeast and its production is then improved by 94-fold through screening biosynthetic enzymes, identifying rate-limiting steps, implementing dynamic control, engineering substrate trafficking and fine-tuning competing metabolic processes. The optimized strain produces up to 85.4 mg L⁻¹ of daidzein and introducing plant glycosyltransferases in this strain results in production of bioactive puerarin (72.8 mg L⁻¹) and daidzin (73.2 mg L⁻¹). Our work provides a promising step towards developing synthetic yeast cell factories for de novo biosynthesis of value-added isoflavonoids and the multi-phased framework may be extended to engineer pathways of complex natural products in other microbial hosts.

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Isoflavonoids constitute a diverse family of natural products that are primarily synthesized by leguminous plants. In addition to playing significant ecological functions, isoflavonoids exhibit various human health-promoting properties, such as antioxidant activity, cardioprotective activity, osteoporosis reduction, and cancer prevention, all of which have resulted in studies on exploiting these molecules as agents both in the pharmaceutical and nutraceutical industry. The current production of isoflavonoids relies on direct plant extraction. However, the low phytochemical abundance, significant investment of time, energy, and capital, and huge requirement for potentially toxic solvents have excluded this approach from being used as it is neither economical nor environmental-friendly. Moreover, the cultivation of legumes is geographically uneven and the amounts of isoflavonoids vary greatly from cultivars and climatic conditions. All these facts introduce further risk and instability in the supply of these chemicals by means of plant extraction. Developing alternative sources of isoflavonoids is therefore a prominent challenge to be addressed, prior to being able to feasibly produce these chemicals at scale using standardized industrial processes.

With the rapid advance in metabolic engineering and synthetic biology over the last few decades, microbe-based bioproduction has become increasingly pursued as an alternative to traditional chemical production techniques. By re-engineering the cellular metabolism of fast-growing microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, artificial cell platforms have been successfully constructed to produce high levels of chemicals ranging from biofuels to proteins. In addition, grafting and optimizing plant biosynthetic pathways in microbial hosts is becoming a compelling route to supply plant natural products, as demonstrated by substantial biosynthesis of high-value-added alkaloids, stilbenes, and flavonoids, and terpenoids from simple sugar. Based on this growing body of work, we speculated that microbial cell factories may also offer the potential for the production of commercially viable isoflavonoid as well.

Structurally, isoflavonoids contain the common C6-C3-C6 flavonoid skeleton and are characterized by having the B-ring connected at C3 rather than C2 position of the C-ring, compared to other flavonoid subclasses (Supplementary Fig. 1). The isoflavones genistein (GEIN) and daidzein (DEIN) constitute two basic scaffolds from which over thousands of isoflavonoids are derived as a result of diverse structural modifications, including hydroxylation, methylation, glycosylation, and molecular rearrangement. Reconstruction of the isoflavone pathway for biosynthesis of these molecules, therefore, represents the entry point to microbial production of a large variety of different biologically active isoflavonoids. Previously, heterologous biosynthesis of GEIN and DEIN was demonstrated by introducing plant enzymes alongside feeding precursors, such as L-tyrosine, naringenin, or liquiritigenin, in both *E. coli* and *S. cerevisiae*. Moreover, the expression of specific glucosyltransferase in *E. coli* also enabled the bioconversion of GEIN and DEIN to corresponding glucosides genistin (GIN) and daidzin (DIN), the primary form of stored isoflavones in leguminous plants. While the reported low titers necessitate further improvement to support industrial-scale production, there have been rare efforts to engineer and optimize de novo microbial biosynthesis of isoflavones.

Here we present the establishment of a de novo DEIN-producing yeast platform and its application for the biosynthesis of glycosylated isoflavonoids using a multi-phased metabolic engineering strategy (Fig. 1). In screening phase I, we first evaluated diverse plant enzymes to rebuild a functional DEIN pathway and extensively diagnosed exogenous and endogenous metabolic factors affecting the activity of key biosynthetic enzymes. Through pathway reconstruction in phase II, we improved the metabolic flux towards the DEIN pathway by implementing: (1) gene amplification to promote the expression of selected pathway genes; (2) protein fusion strategy to facilitate substrate trafficking; (3) additional genetic manipulations to increase the supply of metabolic cofactors (identified during phase I to be potential bottlenecks in pathway flux); (4) process development; and (5) fine-tuning of gene expression in the competing metabolic pathways. The systematic engineering enabled the production of 85.4 mg L\(^{-1}\) DEIN from glucose in shake flask cultivations. Finally, during application phase III, we demonstrated the efficient conversion of DEIN to bioactive glycosylated isoflavonoids by introducing plant glycosyltransferases. Supplementary Fig. 2 provides an overview of all strains constructed in the different phases of the development process.

### Results

#### Phase I—Establishing the biosynthesis of scaffold isoflavone DEIN

In plants, the general phenylpropanoid pathway uses the aromatic amino acid (AAA) L-phenylalanine as a precursor for the biosynthesis of isoflavonoids as well as other flavonoids. The initial steps engage phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate-coenzyme A ligase (4CL), resulting in the conversion of L-phenylalanine to *p*-coumaroyl shooester. Subsequently, the chalcone precursors, naringenin chalcone (NCO) and deoxychalcone liquiritigenin (ISOLIG), are synthesized from the condensation of *p*-coumaroyl CoA and three molecules of malonyl-CoA by chalcone synthase (CHS) alone or with the co-action of NADPH-dependent chalcone reductase (CHR), respectively. Chalcone isomerase (CHI) is responsible for the further isomerization of chalcone to flavanone. While naringenin (NAG) acts as the shared structural core in isoflavone GEIN and flavonoid pathways, the flavanone liquiritigenin (LIG) is used for the biosynthesis of isoflavone DEIN. The efficient generation of LIG represents therefore the first step towards developing a yeast platform for producing DEIN.

To facilitate the screening of biosynthetic enzymes for LIG production, we used a yeast platform strain (QL11) that has previously been reported to produce a moderate level of *p*-coumaric acid (*p*-HCA) (exceeding 300 mg L\(^{-1}\)) from glucose without notable growth deficiency. The plant candidate genes have been selected according to their source and enzymatic specificity/activity. We first evaluated the combinations of candidate CHS, CHR, and CHI homologs, alongside the well-characterized *At4CL1* from *Arabidopsis thaliana*, for the biosynthesis of LIG (Fig. 2a). Specifically, three CHS-coding genes, including leguminous *GmCHS8* (*Glycine max*) and *PlCHS* (*Pueraria lobata*) as well as non-leguminous *RsCHS* (*Rhododendron simsii*), were selected (Supplementary Fig. 3a). CHR activity has been mostly demonstrated in leguminous species; thus *GmCHR5*, *PcCHR*, and *MsCHR* (*Medicago sativa*) were screened (Supplementary Fig. 3a). Plant CHIs can be categorized into distinct isoflavone groups according to their evolutionary path and enzymatic profiles. Whereas type I CHIs, common to all vascular plants, convert only NCO to NAG, legume-specific type II CHIs are capable of yielding both NAG and LIG. Correspondingly, type II CHI-coding genes *PcCHI1* and *GmCHI1B2* were evaluated, together with a type I CHI-coding gene *PsCHI* (*Paeonia suffruticosa*) being used as a control for enzymatic activity. All biosynthetic genes were chromosomally integrated and transcriptionally controlled by strong constitutive promoters. Co-overexpression of *At4CL1*, *GmCHR5*, *GmCHS8*, and *GmCHI1B2* resulted in the best LIG production at a level of 9.8 mg L\(^{-1}\) (strain
C09) among all resultant strains (C01–C11, Fig. 2b). In addition, strains C01, C04–05 and C10 generated no detectable amounts of isoflavonoid intermediates, which could be attributed to the narrow substrate specificity of type I PsCHI1 (strain C01) and low enzymatic activity of PICH (strain C04-05 and C10), respectively (Fig. 2b and Supplementary Fig. 3b). Subsequently, individual gene replacement in strain C09 allowed the efficient screening of additional 4CL, CHS, and CHI variants. Five plant 4CL-coding genes, including Pl4CL1, Gm4CL3, At4CL2, Ph4CL1 (Petunia hybrida), Ps4CL2 (Petroselinum crispum) and a mutant At4CL1m (1250L, N404K, I461V)29, were overexpressed to generate LIG (strains C12–C17) (Supplementary Fig. 3a); however, none of these genes outperformed the wild-type At4CL1 (C09) (Fig. 2c). Also, no improvement on LIG production was observed for strains expressing alternative variants of CHS (strains C18–C20) or CHI (strains C21–C22) (Fig. 2c and Supplementary Fig. 3c). Strain C09 was therefore chosen as the starting strain for further engineering to produce DEIN.
The entry point enzyme in the isoflavonoid biosynthetic pathway is 2-hydroxysisoflavanone synthase (2-HIS), which belongs to the cytochrome P450 family and catalyzes the intramolecular arylation of the B-ring yielding the intermediate 2-hydroxyisoflavanones. Subsequently, dehydrogenation of the resultant intermediate products, catalyzed by 2-hydroxyisoflavanone dehydratase (HID), gives rise to corresponding isoflavones (Fig. 2a). The 2-HIS and HID-coding genes were mainly identified in legumes that have been confirmed to produce isoflavonoids. To identify efficient biosynthetic enzymes for DEIN formation, a group of leguminous 2-HIS and HID homologs were screened. Specifically, five 2-HIS-coding genes, including Pl2-HIS, Gm2-HIS1, Mt2-HIS1 (Medicago truncatula), Tp2-HIS (Trifolium pretense), and Ge2-HIS (Glycerrhiza echinata), and three HID-coding genes, including PlHID, GmHID, and GeHID, were combined and overexpressed in strain C09 (Fig. 2d). While most engineered strains generated detectable amounts of DEIN, strain C28, harboring the gene combination of...
Ge2-HIS and GmHID, accumulated the highest level of DEIN to 0.9 mg L⁻¹ (Fig. 2d). These results show the feasibility to build de novo biosynthesis of DEIN in yeast by harnessing the diversity of plant pathway enzymes.

**Phase I—Engineering the redox partner of the key enzyme P450 2-HIS.** Though strain C28 produced DEIN, only a low titer was observed with a concomitant build-up of the biosynthetic intermediate LIG (Supplementary Fig. 4), suggesting inefficiencies in product formation in the late stage of the DEIN pathway. We, therefore, moved on to engineering the activity of Ge2-HIS, considering that the P450-mediated reactions are irreversible and often rate-limiting, whereas HID is believed to facilitate the spontaneous dehydration of 2-hydroxyisovalvamones.

The redox partner (RP) is an integral part of canonical P450 systems that shuttles the electrons derived from NAD(P)H to the heme iron-center to enable oxygen cleavage and substrate monooxygenation. The endoplasmic reticulum (ER)-anchored plant P450s recruit a single RP protein, the membrane-attached flavin adenine dinucleotide (FAD)/flavin mononucleotide (FMN)-containing cytochrome P450 reductase (CPR), to transfer electrons to the heme iron center to enable oxygen cleavage and substrate monooxygenation. The endoplasmic reticulum (ER)-anchored plant P450s recruit a single RP protein, the membrane-attached flavin adenine dinucleotide (FAD)/flavin mononucleotide (FMN)-containing cytochrome P450 reductase (CPR), to transfer electrons to the heme iron center to enable oxygen cleavage and substrate monooxygenation.

Co-expression of the cognate CPRs is a common practice for reconstituting P450-involved plant biosynthetic pathways in yeast, such as the production of terpenoids and alkaloids, which has been believed to permit more efficient P450-CPR coupling compared to the native yeast CPR. In addition, plant CPRs exhibit a certain degree of versatility, due to the high level of conservation represented by the amino acid residues that mediate P450 interactions. Accordingly, in our DEIN-producing strains, Ge2-HIS might receive electrons from AtATR2, a CPR homolog of *A. thaliana* introduced for optimizing the activity of P450 AtIC4H to produce p-HCA (Fig. 3a).

The selected CPR-coding genes were chromosomally integrated in combination with a second copy of Ge2-HIS to strain C28. Using this approach, DEIN production of both *GmCPR1*-expressing strain C34 and *CrCPR2* (*Catharanthus roseus*) (Fig. 3b) was significantly enhanced to a titer of 5.9 and 9.9 mg L⁻¹, respectively, accounting for a 284 and 544% increase compared with that of strain C33 only harboring a second copy of Ge2-HIS (Fig. 3c). This strongly indicates that there may be an improved coupling of the alternative CPRs to Ge2-HIS. Among the diverse range of P450-dependent electron transport systems, an intramolecular electron transfer mechanism has been employed by the so-called self-sufficient P450s, in which the RP domain is naturally fused with the catalytic domain of P450 BM3 from *Bacillus megaterium*; RhFRED, the FMN/Fe₂S₂-containing reductase domain of P450RhF from *Rhodococcus* sp. strain NCIMB 9784; RhF-fdx, a hybrid reductase by substituting Fe₂S₂ domain of RhFRED with ferredoxin (Fdx) from spinach. See Fig. 1 and its legend regarding abbreviations of metabolites and gene details.
P450RhF from *Rhodococcus* sp. strain NCIMB 9784; and (3) RhF-fdx, a hybrid reductase by substituting Fe_{2}S_{2} domain of RhFRED with ferredoxin (Fdx) from spinach, were C-terminally fused to Ge2-HIS and investigated for DEIN biosynthesis in strain C39, yielding a DEIN titer of 12.8 mg L^{-1}. We, therefore, deleted *ROX1* in strain C39, which encodes heme oxygenase, to inactivate the transcriptional repressor Rox1 and thereby render an elevated heme level. The production of active P450s requires sufficient incorporation of cofactor heme, which may deplete the intracellular pool of heme and thereby incur a cellular stress response that in turn damages the net enzymatic activity. To mitigate this potential adverse effect on the activity of Ge2-HIS, we tested different approaches to regulate heme metabolism of yeast. In strain C39, *HMX1* deletion significantly enhanced the production of DEIN to 8.4 mg L^{-1}. In addition, a previous study illustrated that inactivation of the transcriptional repressor Rox1 could render an elevated cellular heme level, resulting from the derepression of the heme biosynthetic gene *HEM13*. We, therefore, deleted *ROX1* in strain C35, yielding a DEIN titer of 12.8 mg L^{-1} by the resultant strain C40 (Fig. 4b), a 46% increase compared with that of the parental strain. Besides reinforcing the biosynthetic pathway, reducing degradation of heme also contributes to its intracellular accumulation and improves the P450s activity. Accordingly, we, therefore, deleted *ROX1* in strain C35, yielding a DEIN titer of 12.8 mg L^{-1} by the resultant strain C40 (Fig. 4b), a 46% increase compared with that of the parental strain. Besides reinforcing the biosynthetic pathway, reducing degradation of heme also contributes to its intracellular accumulation and improves the P450s activity. Accordingly, we, therefore, deleted *ROX1* in strain C35, yielding a DEIN titer of 12.8 mg L^{-1} by the resultant strain C40 (Fig. 4b), a 46% increase compared with that of the parental strain.
resultant strain C41 (10.6 mg L$^{-1}$) was increased by 21% relative to strain C35 (Fig. 4b).

Most plant-derived P450s and CPRs are independently tethered onto the ER via hydrophobic transmembrane anchors$^{42}$. Modulating the biogenesis and size of the ER has previously been shown to enhance P450-involved biosynthesis of terpenoids in S. cerevisiae$^{43,44}$, a result which is likely due to a higher protein folding capacity enabled by ER expansion. To evaluate the possible beneficial effect of ER expansion for DEIN biosynthesis, we therefore evaluated the intracellular level of phospholipids for ER assembly by implementing (1) the deletion of PAH1-encoded phosphatidate phosphatase that competes for the phospholipid precursor$^{45,46}$, (2) the deletion of the transcription factor Opi1 and (3) overexpression of the transcription factor Ino2 that negatively and positively control the expression of the phosphorylated form of this cofactor without impact on the metabolic barriers.

To reduce the metabolic loss due to an excessive supply of p-HCA in background strain QL11, we instead turned to reconstructing the DEIN biosynthesis in a “clean” background without an engineered AAA pathway. With this strain, we used the galactose-induced dynamic transcription mechanism$^{35}$, which is mediated by GAL promoters (GALps) and has been successfully used for high-level production of value-added chemicals$^{42,47}$, to enhance the expression of DEIN pathway genes. A previously described strain QL179$^{27}$, derived from IMX8S1 by disrupting galactose utilization genes GAL11/10, was thus selected to confer galactose as the gratuitous inducer to activate GALps (Fig. 5a). As expected, simultaneous introduction of the GALps-controlled p-HCA and LIG pathways greatly boosted LIG titer to 37.6 mg L$^{-1}$ in strain I01 (Fig. 5b), a 284% increase relative to strain C09, in which a constitutive gene expression pattern was used. Interestingly, a markedly low level of p-HCA was detected in strain I01, indicating that to a certain extent the metabolic flux between the redesigned p-HCA producing and consuming pathways was balanced (Supplementary Fig. 8).

The spatial organization of catalytic enzymes is increasingly recognized to be critical for optimizing the metabolic flow through multi-step biosynthetic pathways$^{54}$. Coordinating the physical distance of adjacent enzymes, mediated by peptide linkers or synthetic scaffolds, could elevate local concentrations of enzymes and metabolites thereby speeding up the turnover of intermediates, minimizing metabolic crosstalk, and improving reaction flux$^{55}$. To test this, we implemented a synthetic fusion enzyme strategy to optimize the substrate trafficking through the LIG pathway. Specifically, two distinct oligopeptide linkers possessing flexible (GGGS, L1) or rigid (VDEAAAKSGR, L2) conformations$^{56}$ were applied to fuse the coding sequences of neighboring enzymes (Fig. 5a). Fused AtC4H (E1) with At4CL1 (E2) in both tandem orientations together with other biosynthetic genes were chromosomally integrated into strain QL179, creating strains I02–I05 (Fig. 5b). A significantly enhanced LIG production was observed for these strains; and fusion enzyme in the E1-L1-E2 orientation provided the highest LIG titer of 77.7 mg L$^{-1}$ (strain I02), representing a 107% improvement compared with strain I01 (Fig. 5b). This result indicates that the physical fusion of two enzymes responsible for the formation and consumption of p-HCA, respectively, could greatly drive the metabolic flux towards LIG biosynthesis. Encouraged by this, we further introduced fusion enzymes consisting of GmCHS8 (E3)-Linker-GmCRTR5 (E4) or GmCHR5 (E4)-Linker-GmCH11B2 (E5) to strain I02 (Fig. 5a). In contrast, here we found the titer of LIG to be decreased in the resultant strains I06-I13 (Supplementary Fig. 9), which may be attributed to a reduced CHR activity of fusion enzymes, considering the lower variation we observed for byproduct formation. We, therefore, selected strain I02 as the platform for incorporating the best downstream DEIN-forming biosynthetic and auxiliary enzymes (Gez-HIS, GmHID, and GCRCP2). The DEIN titer of resultant strain I14 (20.7 mg L$^{-1}$, Fig. 5c) doubled relative to strain C35 harboring the constitutive promoter-driven DEIN pathway (9.9 mg L$^{-1}$, Fig. 3c).

Phase II—Gene amplification and engineering of substrate trafficking improve DEIN biosynthesis. In screening phase I, through performing combinatorial gene screening in parallel with multiple genetic modifications, we achieved substantial de novo DEIN biosynthesis and identified important metabolic factors affecting its overproduction in yeast. However, the resultant strains exhibited two major unfavorable phenotypes, including a large amount of non-consumed precursor p-HCA (Supplementary Fig. 6) and the formation of several metabolic intermediates and byproducts (Supplementary Fig. 7). This may result from (1) metabolic imbalance between upstream p-HCA producing and the downstream pathways, (2) insufficient activity and (3) substrate promiscuity of some of the plant enzymes, and (4) inefficient cytosolic substrate transfer. We therefore next aimed to improve the production of DEIN via relieving these potential metabolic barriers.

Redox cofactors NAD(P)H, the ultimate electron source in cellular metabolism, are indispensable for the catalytic cycle of plant P450s$^{33}$. Lack of NAD(P)H could reduce the P450 activity due to inefficient electron transfer. A recent report indicated that improved cellular NADPH level could enhance the P450-mediated protopanaxadiol production$^{48}$. Thus, we decided to reroute the redox metabolism to fuel the activity of Gez2-HIS. In the first strategy, genetic modifications engaged to boost the direct generation of NADPH were devised and individually implemented, including (M1a) overexpression of the transcription factor Sbt5 that activates the expression of genes involved in the pentose phosphate pathway (PPP)$^{49}$, the major source of NADPH for anabolic processes in yeast; (M1b) overexpression of the ADL6-encoded cytoplasmic NADP$^+$-dependent aldehyde dehydrogenase that converts acetaldehyde to acetate; (M2) introduction of E. coli pntAB genes encoding a membrane-bound transhydrogenase capable of reducing NADP$^+$ at the expense of NADH$^{50}$, and (M3) overexpression of yeast YEF1-encoded ATP-NADH kinase that directly phosphorylates NADH to NADPH$^{51}$, resulting in an elevated concentration of the phosphorylated form of this cofactor without impact on the NADPH/NADP$^+$ ratio (Fig. 4a-II). The resultant strains C49 (M1b) and C51 (M3) produced 9.9 and 9.7 mg L$^{-1}$ of DEIN, representing a 14% and 11% increase, respectively, compared with the parental strain C35 (Fig. 4c). Moreover, strain C46 harboring PAH1 deletion and INO2 overexpression also had a 20% increase in DEIN formation (10.8 mg L$^{-1}$) compared with that of strain C35, whereas other combinatorial modifications led to a reduction in DEIN titers of strains C45 and C47 (Fig. 4c). This difference could be attributed to the markedly impaired cell growth of the latter two strains (Supplementary Fig. 5).

Evaluating the possible benefi
cial effect of ER expansion for DEIN biosynthesis, we therefore evaluated the intracellular level of phospholipids for ER assembly by implementing (1) the deletion of PAH1-encoded phosphatidate phosphatase that competes for the phospholipid precursor$^{45}$, (2) the deletion of the transcription factor Opi1 and (3) overexpression of the transcription factor Ino2 that negatively and positively control the expression of the phosphorylated form of this cofactor without impact on the metabolic barriers.
Phase II—Combinatorial strategies to increase DEIN production. Improving the expression of biosynthetic genes and the cellular substrate transfer greatly enhanced the DEIN titer of strain I14. However, we also observed considerable accumulation of both intermediates (15.8 mg L$^{-1}$ of ISOLIG and 42.3 mg L$^{-1}$ of LIG, Fig. 5c) as well as byproducts (10.0 mg L$^{-1}$ of NAG and 1.3 mg L$^{-1}$ of GEIN, Fig. 5c), showing a need for strengthening the later stage of DEIN biosynthesis. To solve this, we first aimed to improve the activity of Ge2-HIS by combining effective P450-centered genetic targets identified in phase I engineering (Fig. 4a). Expectedly, the removal of heme degradation by disrupting HMX1 gene resulted in a 19% increase in DEIN titer of strain I15 (23.3 mg L$^{-1}$) compared with that of strain I14 (Fig. 6a), whereas ROX1 deletion negatively affected DEIN production (strain I16, Fig. 6a), this potentially being caused by the resulting loss of its regulatory role in stress resistance of S. cerevisiae. Subsequently, the deletion of OP11 or overexpression of INO2 genes was individually carried out to stimulate ER expansion in strain I15; however, both resultant strains gave a lower DEIN titer (Supplementary Fig. 10a). While compromised cell growth associated with these strains (Supplementary Fig. 10b) could have weakened their DEIN generation, a shortage of intracellular heme may also be limiting the functional P450 folding and thereby blunting the effect of ER adjustment. Previous studies showed that feeding 5-aminolevulinic acid (5-ALA), the direct precursor of heme biosynthesis, could significantly increase the cellular heme level of yeast. Indeed, we found exogenous supplementation of 1 mM 5-ALA resulted in 45% (34.3 mg L$^{-1}$, strain I15 $+$ A), 65% (17.3 mg L$^{-1}$, strain I17 $+$ A), and 42% (27.1 mg L$^{-1}$, strain I18 $+$ A), respectively, further increases in DEIN production for the strains tested (Fig. 6b and Supplementary Fig. 10a). ER-targeting modifications however exhibited no beneficial effects on DEIN production, which could be ascribed to the distinct engineering context of strains C35 and I15, implying a need for fine-tuning the interplay between ER biogenesis and P450 anchoring. Thus, strain I15 was subject to the integration of NADPH generation systems. Among selected targets, co-overexpression of native STB5 and bacterial EcYP1B genes (M1a + M4) led to the highest DEIN titer of 40.2 mg L$^{-1}$, a 12% improvement relative to strain I15 (strain I21, Supplementary Fig. 11).

Based on established results of cofactor refinement, we speculated that the availability of biosynthetic enzymes could emerge as a limiting factor for the conversion of LIG to DEIN. Especially, previous reports indicated that the 2-HIS enzyme in microsomal preparation from soybean cells is labile and the catalytic characteristics of 2-HIS have evolved by sacrificing protein stability. We, therefore, introduced extra copies of the best DEIN-forming gene combination, Ge2-HIS with GmHID, to strain I21. Interestingly, while there was a 17% increase in DEIN production in strain I24 containing the second copy of selected genes, the introduction of the third copy of this gene combination further enhanced DEIN production to 53.5 mg L$^{-1}$ (strain I25), representing a 38% increase compared with strain I21 (Fig. 6c). Compared with batch (glucose excess) cultivations, yeast cells grown under glucose-limited cultivation are known to have a higher biomass yield and an enhanced PPP flux, the latter being anticipated to favor AAA biosynthesis by increasing the availability of the precursor erythrose 4-phosphate. We, therefore, grew DEIN-producing strains under a mimicked glucose-limited fed-batch cultivation by using FeedBeads (FB) (Supplementary Fig. 12), a slow-release system for glucose. Expectedly, under FB conditions, strain I25 produced 62.1 mg L$^{-1}$ of DEIN, representing an 18% increase relative to the same strain under batch conditions (Fig. 6d). Moreover, the application of this FB strategy led to observable growth improvements and a striking increase in byproduct formation of strain I25 (Supplementary Fig. 13). These results agree also with our previous work wherein significant improvements on cellular biomass formation and p-HCA production could be achieved by growing yeast cells under glucose-limited conditions.

For the biosynthesis of one molecule of DEIN, one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA are consumed (Fig. 6c). Following our optimization of metabolic flux using the p-HCA pathway and reinforcement of the DEIN biosynthetic pathway, we speculated that the supply of malonyl-CoA had become the next limiting factor in DEIN production. In S. cerevisiae, the majority of cytosolic malonyl-CoA pool is invested in the synthesis of fatty acids (FAs), which are essential for multiple cellular functions and cell growth. The FAS...
**Fig. 6 Combinatorial optimization to increase the production of DEIN.** a Effect of deleting genes involved in the regulation of heme metabolism on DEIN biosynthesis. Production of DEIN by strains fed with the heme biosynthetic precursor 5-ALA (b) or expressing different copies of Gez-HIS and GmHID genes (c). d Process optimization for DEIN production. Cells were grown in a defined minimal medium with 30 g L\(^{-1}\) glucose (batch) or with six tablets of FeedBeads (FB) as the sole carbon source and 10 g L\(^{-1}\) galactose as the inducer. Cultures were sampled after 72 h (batch) or 90 h (FB) of growth for metabolite analysis. e Schematic view of the interplay between isoalloxazine biosynthesis and yeast cellular metabolism connected by the branchpoint malonyl-CoA. See Fig. 1 and its legend regarding abbreviations of metabolites and gene details. f Fine-tuning the expression of gene FAS1 via promoter engineering improves DEIN formation under optimized cultivation conditions. g Effect of genetic modifications altering the regulation of GAL induction on DEIN production under optimized cultivation conditions. The constitutive mutant of galactose sensor Gal3 (GAL3S509P) was overexpressed from a multi-copy plasmid (2 µm) under the control of GAL10p and gene ELP3, encoding a histone acetyltransferase, was deleted. Cells were grown in a defined minimal medium with six tablets of FB as the sole carbon source and 10 g L\(^{-1}\) galactose as the inducer. Cultures were sampled after 90 h of growth for metabolite detection. Statistical analysis was performed by using Student’s t test (two-tailed; two-sample unequal variance; *p < 0.05, **p < 0.01, ***p < 0.001). All data represent the mean of n = 3 biologically independent samples and error bars show standard deviation. The source data underlying panels (a – d) and (f, g) are provided in a Source Data file.

The complex, composed of Fas1 and Fas2, is responsible for FAs generation in yeast with the FASI gene product known to impose positive autoregulation on FAS2 expression to coordinate the activity of the FAS complex\(^{62}\). Hence, we set out to fine-tune the expression of the FASI gene to divert malonyl-CoA towards DEIN biosynthesis (Fig. 6e). A group of yeast promoters, exhibiting differential transcriptional activities in response to glucose\(^{63}\) (Supplementary Table 1), were used to substitute the native FASI promoter. Among seven evaluated promoters, replacement with BGL2p brought about the greatest DEIN titer of 76.3 mg L\(^{-1}\) (strain I27), a 20% increase compared with strain I25 (Fig. 6f). Additionally, the production of intermediates and byproducts was also notably elevated (Supplementary Fig. 14), further reflecting that promoter replacement of FASI has boosted the overall metabolic flux towards isoalloxazines.

The galactose-inducible transcriptional response (the GAL induction) of *S. cerevisiae* initiates with the association of the galactose sensor Gal3 with the regulatory inhibitor Gal80, leading to dissociation of the latter from the transcription activator Gal4, thereby allowing rapid expression of *GAL* genes\(^{53}\). Constitutive *GAL3* mutants (*GAL3C*) have been demonstrated to confer galactose-independent activation of Gal4\(^{64}\). This trait was recently engineered to build a positive feedback genetic circuit in which expressed Gal3\(^{C}\) provokes greater expression of Gal3\(^C\) and thereby enhances GAL induction\(^{65}\). We speculated that DEIN production may benefit from overexpression of such a Gal3\(^C\) mutant as a result of further induction of the GAL1p-controlled biosynthetic pathway. However, when expressed from a high-copy vector under the control of GAL10p, the introduction of constitutive Gal3\(^{S509P}\) mutant led to a significant decrease in both DEIN and GEIN titers (Fig. 6g and Supplementary Fig. 15). On the other hand, by deleting gene ELP3, encoding a histone acetyltransferase, that is part of elongator and RNAPII elongation complexes, a final DEIN titer of 85.4 mg L\(^{-1}\) was achieved in the resultant strain I34 (Fig. 6g), representing a 12% improvement relative to strain I27. The production of GEIN was also slightly increased to 33.7 mg L\(^{-1}\) (Fig. 6g and Supplementary Fig. 15). These results also show to be consistent with a published study wherein ELP3 deletion was found to enhance the GAL10p-mediated beta-galactosidase activity in the presence of galactose\(^{67}\). The high-level accumulation of DEIN could exert cellular toxicity in *S. cerevisiae* and thereby impede the further improvement of its titer. We, therefore, evaluated the growth profiles of the background strain IMX581 under different concentrations of DEIN within its solubility limit. The results revealed that yeast could tolerate up to 150 mg L\(^{-1}\) of DEIN without significant loss of growth capacity (Supplementary Fig. 16). Hence, it is reasonable to assume that the production of DEIN is non-toxic to yeast at the levels produced here.

**Phase III—Production of DEIN-derived glucosides.** Glycosylation represents a prevalent tailoring modification of plant flavonoids that modulates their biochemical properties, including
solubility, stability, and toxicity. In soybean, enzymatic 7-O-glucosylation of DEIN leads to the biosynthesis of DIN, one of the key ingredients found in soybean-derived functional foods and nutraceuticals. Moreover, puerarin (PIN), an 8-C-glucoside of DEIN, is ascribed as the major bioactive chemical of P. lobata roots extract, which has long been used in Chinese traditional medicine for the prevention of cardiovascular diseases. Recent studies also show that PIN exhibits diverse pharmacological properties including antioxidant, anticancer, vasodilation, and neuroprotection-related activity. With the establishment of efficient DEIN-producing yeast platform during reconstitution phase II (Fig. 6g), we explored its application potential in the production of PIN and DIN.

The biosynthesis of flavonoid glycosides is mediated by UDP-sugar-glycosyltransferases (UGTs), which catalyze the formation of O−C or C−C bond linkages between the glucosyl group from uridine diphosphate (UDP)-activated donor sugars and the acceptor molecules. While a soybean isoflavone 7-O-glucosyltransferase exhibiting broad substrate scope was first described over 50 years ago, only recently Funaki et al. revealed that its homolog GmUGT4 enables highly specific 7-O-glucosylation of isoflavones. On the other hand, the complete PIN pathway was fully elucidated when Wang et al. successfully cloned and functionally characterized a P. lobata glucosyltransferase, encoded by PIUGT43, which displays strict in vitro 8-C-glucosylation activity towards isoflavones and enables PIN production in PIUGT43-expressed soybean hairy roots. We, therefore, tested the feasibility of using these UGTs in generating DEIN glycosides (Fig. 7a). Different copies of PIUGT43 and GmUGT4 under the control of constitutive promoters were integrated into the basic DEIN producer C28, but the resultant yeast strains (E01−E03 for PIN and E04−E06 for DIN, Supplementary Fig. 2) generated no detectable level of glycosides for HPLC analysis. However, through further analysis with high-resolution LC-MS, we validated that strains E03 and E06 could generate trace amount of PIN and DIN, respectively (Fig. 7b and Supplementary Fig. 17), demonstrating that both UGTs were functional in yeast.

Besides the selection of active UGTs, the supply of glycosyl group donor UDP-glucose also plays a pivotal role in regulating glucoside production. With the efficient DEIN producer I34 in hand, we moved to enhance its capacity for biosynthesizing UDP-glucose. In S. cerevisiae, metabolic enzymes phosphoglucomutase (encoded by PGM1 and PGM2) and UDP-glucose pyrophosphorylase (encoded by UGP1) catalyze the formation of UDP-glucose branching from glucose-6-phosphate (Supplementary Fig. 18a). Through chromosomally integrated expression of UGP1 with PGM1 or PGM2 in strain I34, strains E07 and E08 were created. Additionally, to ensure adequate UGT activity, two multi-copy plasmids, harboring genes PlUGT43 (pQC229) and GmUGT4 (pQC230) under the control of GAL1p, were constructed and individually introduced into the high-level producers of DEIN (strains I34, E07, and E08). In doing so, we found the resultant strains E09 and E10, derived from the I34 background, to produce 45.2 mg L−1 of PIN and 73.2 mg L−1 of DIN, respectively (Fig. 7c). Interestingly, compared with strain E10, the PlUGT43-expressing strain E09 still accumulated a considerable amount of DEIN (28.9 mg L−1, Fig. 7c). This discrepancy may be attributed to the insufficient activity of PIUGT43, whose determined kinetic parameters for DEIN (Kcat = 0.35 s−1, Km = 32.8 μM, and Kcat/Km = 1.1 × 104 M−1 s−1)71 show to be considerably less optimal compared to GmUGT4 (Kcat = 5.89 s−1, Km = 20.3 μM, and Kcat/Km = 2.91 × 105 M−1 s−1)74. Furthermore, the conversion of GEIN to 25.9 mg L−1 of C-glycoside genistein 8-C-glucoside (GBG) and 26.5 mg L−1 of O-glycoside genistin (GIN) was observed for strains E09 and E10 (Supplementary Fig. 18b and c), respectively, since the selected UGTs exhibit comparable glucosyltransferase activity towards GEIN. Moreover, the overexpression of UDP-glucose-forming genes resulted in full consumption of DEIN and enhanced PIN production to 72.8 mg L−1 in E07-derived strain E11 and 65.4 mg L−1 in E08-derived strain E12, representing a 61% and 45% increase respectively compared with strain E09 (Fig. 7c). On the other hand, such modifications resulted in no significant increase in the production of DIN (Fig. 7c) and byproduct glucosides (Supplementary Fig. 18b and c), reflecting a shortage of precursor isoflavones. Similarly, we analyzed the growth-inhibitory effects of the two glucosides on strain IMX581. Compared with their aglycon DEIN, an increased level of PIN (500 mg L−1) and DIN (250 mg L−1) can be tolerated by yeast to retain normal cell growth (Supplementary Fig. 19); both concentrations are much higher than the best titer achieved for the two glucosides in our study. Specifically, supplementation of DIN improved growth of yeast, which could result from the uptake of DIN and then release of glucose catalyzed by native glucosidases, such as the steryl-beta-glucosidase Eghlp75.

Discussion

Isoflavonoids play important roles in the plant defense system and have many human health-related benefits. They, therefore, represent promising candidates in the development and engineering of agents for agricultural, nutraceutical, and pharmaceutical applications. Here we established a yeast-based de novo production platform for the efficient production of the isoflavonoid carbon skeleton DEIN as well as the high-value glucosides PIN and DIN. This was achieved by first identifying functional biosynthetic enzymes to generate DEIN (screening phase I), then by optimizing metabolic flux at enzyme and pathway levels to further increase DEIN titer (reconstruction phase II) and finally by introducing plant UGTs to convert DEIN to corresponding glycosides (application phase III).

Gene duplication and diversification occur in the evolution of plant secondary metabolism to tackle the changing environment, creating a rich variability and complexity of plant products as a result. However, this functional divergence poses an obstacle to identifying ideal candidate enzymes for reconstructing heterologous biosynthetic pathways for plant metabolite production. Most of the structural genes involved in isoflavonoid pathways have been characterized, and here we exploited their genetic diversity, by performing a combinatorial evaluation of biosynthetic genes from both leguminous and non-leguminous plants, to enable DEIN production (Fig. 2b–d). The P450s constitute the most versatile tailoring enzymes that catalyze irreversible and often rate-limiting reactions in the biosynthesis of plant-specialized products. Though S. cerevisiae is generally identified as a superior host for the functional expression of membrane-bound plant P450s over its prokaryotic counterparts, extra efforts are required to maximize their catalytic efficiency. Two distinct P450s, the upstream C4H hydroxylating cinamic acid and the downstream 2-HIS mediating the migration of aryl moiety of LIG, are involved in the biosynthesis of DEIN (Fig. 3a). While the activity of AtC4H has been enhanced by co-expressing RP in our screening strains providing excess precursor p-HCA (QL11 background), the selected Ge2-HIS still exhibited sub-optimal performance in converting LIG to DEIN (Supplementary Fig. 4). Starting with evaluating plant CPRs and artificial RP surrogates, which could impact the transfer of electrons required for P450 activity, we therefore proceeded with the optimization of Ge2-HIS activity by exploring other endogenous metabolic factors, including heme metabolism, ER homeostasis, and NADPH generation. These modifications increased DEIN titer to a level exceeding...
increased UDP-glucose supply. Combined overexpression of genes coming the intrinsically low catalytic ef

compartmentalization via the formation of metabolons, which

Another challenge for isoflavanoid production lies in over-

complexes of enzymes participating in sequential

characterization of metabolic enzymes responsible for glucoside biosynthesis. Three copies of PIUGT43 and GmUGT4 under the control of constitutive promoters were integrated into the DEIN producer C28, resulting in strains E03 and E06, respectively. Cells were grown in a defined minimal medium with 30 g L\(^{-1}\) glucose as the sole carbon source, and cultures were sampled after 72 h of growth for LC-MS analysis. e Production profiles of PIN and DIN in DEIN hyper-producing strain 134 background with or without increased UDP-glucose supply. Combined overexpression of genes PGMI/2 with UPG1 was implemented to enhance the generation of glycosyl group donor UDP-glucose. See Fig. 1 legend for gene details. Cells were grown in a defined minimal medium with six tablets of FB as the sole carbon source and 10 g L\(^{-1}\) galactose as the inducer. Cultures were sampled after 90 h of growth for metabolite detection. Statistical analysis was performed by using Student’s t test (two-tailed; two-sample unequal variance; *p < 0.05, **p < 0.01, ***p < 0.001). All data represent the mean of n = 3 biologically independent samples and error bars show standard deviation. The source data underlying figure c are provided in a Source Data file.

12 mg L\(^{-1}\) (Fig. 4b), accounting for a seven-fold improvement compared with the parental strain C33.

Another challenge for isoflavanoid production lies in over-

improving the metabolic flux through the LIG pathway and increasing its titer by 107% (Fig. 5b). Besides the AAA-derived p-HCA, de novo isoflavanoid biosynthesis consumes malonyl-CoA, whose formation is predominately invested in FAs synthesis in S. cerevisiae\(^{61}\). By fine-tuning the expression of key enzymes involved in FAs synthesis, we were able to redistribute the cellular malonyl-CoA pool, resulting in a 20% further increase in DEIN titer (Fig. 6f).

In conclusion, as a proof-of-concept study, a final DEIN titer of 85.4 mg L\(^{-1}\) was achieved using glucose as the sole carbon source in shake flask cultivations (Fig. 6g). This production level is comparable and, in some cases, higher than isoflavanoid levels produced by previous studies, which have additionally been aided with precursor feeding (Supplementary Table 2). Via further expression of different glycosyltransferases, approximately 80 mg L\(^{-1}\) of C- or O-glycosylated bioactive compounds PIN or DIN were produced (Fig. 7c), showing the application potential of our platform strain. Moreover, our work sheds light on the complete microbial biosynthesis of value-added isoflavanoids such as DEIN-derived legume phytoalexins\(^{61}\) and may be applied in characterizing novel metabolic enzymes for the
production of isoflavonoid derivatives. Additional improvements on the catalytic efficiency and specificity of key isoflavonoid biosynthetic enzymes through protein engineering, directed pathway evolution facilitated by biosensor-mediated high-throughput screening as well as engineering of extra-cellular transport of isoflavonoids[25,33], may further optimize the phenotypes of our platform strains, including higher titer/productivity and reduction/elimination of byproducts, to meet industrial-scale production requirements in the future. Finally, the multi-faceted framework we herein present also offers the potential to be applied for engineering the biosynthetic pathways in other microbial host cells as well, for the production of complex natural products.

Methods

Strains, plasmids, and reagents. Escherichia coli DH5α strain was used for the construction and amplification of plasmids. All plasmids and S. cerevisiae strains used in this study were derived from the Genetic Background (SC-URA) consisting of 6.7 g L−1 yeast nitrogen base (YNB) without amino acids (Formedium), 0.77 g L−1 (NH4)2SO4, 14.4 g L−1 KH2PO4, 0.5 g L−1 MgSO4·7H2O, 4.5 g L−1 CoCl2·6H2O, 4.5 g L−1 CaCl2·2H2O, 18.4 g L−1 MnCl2·2H2O, 0.3 g L−1 CoCl2·6H2O, 0.3 g L−1 CuSO4·5H2O, 0.4 g L−1 Na2MoO4·2H2O, 1.0 g L−1 H3BO3, 0.1 g L−1 I, and 19.0 g L−1 Na2EDTA·2H2O, and 1 mL L−1 vitamin solutions (0.05 g L−1 D-Biotin, 1.0 g L−1 D-Pantothenic acid, 0.1 g L−1 HCl, 1.0 g L−1 Thiamine-HCl, 1.0 g L−1 Pyridoxine-HCl, 1.0 g L−1 Niacin, 0.2 g L−1 Niacin-amide, and 25.0 mL L−1 5-methyl-inosinic acid) supplemented with 60 mg L−1 uracil and 1 mL 5-methylaminolevulinic acid (5-ALA) if required. Single colonies, with PCR-verified genetic modifications, were inoculated into 14 mL tubes with 1.5 mL fresh minimal medium and incubated at 30°C with 220 rpm agitation overnight. Precultures were then diluted and transferred to a 125 mL non-baffled flask containing 15 mL minimal medium at an initial optical density measured at 600 nm (OD600) of 0.05 and cultivated at 220 rpm, 30°C for an additional 72 h. For mimicked fed-batch shake cultivation, six tablets of FeedBeads® (SMF80001, Kuhnner Shaker, Basel, Switzerland) were used as the sole carbon source and cultivated for 90 h at 30°C with 220 rpm agitation (corresponding to 30 g L−1 glucose). To induce the transcription of genes under the control of Data 1, 10 g L−1 galactose was added into the medium as the inducer. Analysis of the growth-inhibitory effect of DEIN and its glucosides on yeast was performed using Growth Profiler 960 (EnzyScreen). Overnight precultures were diluted and transferred to 96-well microplates containing 230 μL defined minimal medium with the initial OD600 of 0.1. DEIN was added to the wells in final concentrations of 25, 100 and 150 mg L−1. PIN was added in a final concentration of 50, 500, 1000 and 2000 mg L−1. DIN was added in a final concentration of 25, 50 and 250 mg L−1. The cultures were grown at 30°C with 250 rpm shaking, and the OD600 was measured with an interval of 30 min.

Genetic manipulation. All constructed yeast strains were derived from the genetic background S. cerevisiae CEN.PK113-5D derivative IMX581 (MATa ura3-52 can1Δ::cas9-natNT2 TRP1 LEU2 HIS3). For gene overexpression, in-vitro-assembled DNA constructs were integrated at target genomic loci by means of the CRISPR/cas9 system. For the amplification of native promoters, genes, and terminators, IMX581 genomic DNA served as the template. Plasmids or synthetic fragments were utilized as the DNA template to amplify optimized heterologous genes or genes generated by means of in vitro extension PCR using Smart Technology. The DNA polymerase was used for routine DNA fragment amplifications, except for the construction of plasmids. The isolation of plasmid DNA was performed by using the PureLink Quick Plasmid Mini Preparation Kit (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Discovery HS ES 15 cm × 4.6 mm column (particle size 5 μm, Sigma-Aldrich, St. Louis, MO, USA) connected to a photodiode array (PDA) detector (250, 270, 290, 304 and 370 nm). The column was kept at 30°C, and metabolites from 10 μL of supernatants were separated. Samples were analyzed using a gradient method with two standard solutions: with 0.1% formic acid (A) and acetonitrile (B). NAG, GEIN, ISOL, LIG, DEIN, PIN, DIN, and G8G detection, a flow rate of 1.2 ml min−1 was used. The program started with 5% of solvent B (0–0.5 min), after which the fraction was increased linearly from 5 to 60% (0.5–18.5 min), then the fraction was maintained at 60% (18.5–19 min), after that the fraction was decreased from 60% to 5% (19–20 min), finally, the fraction was maintained at 5% (19.5–20 min). p-HCA was detected at 93 min (304 nm), NAG at 14.8 min (290 nm), GEIN at 14.5 min (270 nm), ISOL at 16.3 min (370 nm), LIG at 12.8 min (270 nm), DEIN at 12.0 min (250 nm), DIN at 8.1 min (230 nm), PIN at 7.1 min (250 nm), GIN at 9.7 min (230 nm) and G8G at 8.7 min (230 nm). Isoliquiritigenin, 5-ethylcaffeylquinic acid (5-ECQA) and 5-ethylcoumaroylquinic acid (5-ECQA) were identified by comparing the UV absorbance spectra and retention times of the unknown with authentic samples. Metabolite extraction and quantification. Isoflavonoids and aromatic metabolite production were quantified by high-performance liquid chromatography (HPLC)[37]. In detail, 0.5 mL of cell culture was mixed with an equal volume of absolute ethanol (100% v/v), vortexed thoroughly and centrifuged at 13,000 × g for 5 min. The supernatant was stored at −20°C until HPLC analysis. Quantification of isoflavonoids and aromatics was performed on a Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Discovery HS ES 15 cm × 4.6 mm column (particle size 5 μm, Sigma-Aldrich, St. Louis, MO, USA) connected to a photodiode array (PDA) detector (250, 270, 290, 304 and 370 nm). The column was kept at 30°C, and metabolites from 10 μL of supernatants were separated. Samples were analyzed using a gradient method with two standard solutions: with 0.1% formic acid (A) and acetonitrile (B). NAG, GEIN, ISOL, LIG, DEIN, PIN, DIN, and G8G detection, a flow rate of 1.2 ml min−1 was used. The program started with 5% of solvent B (0–0.5 min), after which the fraction was increased linearly from 5 to 60% (0.5–18.5 min), then the fraction was maintained at 60% (18.5–19 min), after that the fraction was decreased from 60% to 5% (19–20 min), finally, the fraction was maintained at 5% (19.5–20 min). p-HCA was detected at 93 min (304 nm), NAG at 14.8 min (290 nm), GEIN at 14.5 min (270 nm), ISOL at 16.3 min (370 nm), LIG at 12.8 min (270 nm), DEIN at 12.0 min (250 nm), DIN at 8.1 min (230 nm), PIN at 7.1 min (250 nm), GIN at 9.7 min (230 nm) and G8G at 8.7 min (230 nm). Metabolite extraction and quantification were confirmed by comparing the UV absorbance spectra and retention times of the samples with authentic standards. A six-point calibration curve, ranging from 6.25 mg L−1 to 200 mg L−1 (p-HCA), 3.125 mg L−1 to 100 mg L−1 (NAG), and 1.5625 mg L−1 to 50 mg L−1 (GEIN, ISOL, LIG, DEIN, PIN, DIN and G8G) was prepared from authentic samples. The correlation coefficient for the resulting calibration curve was 0.99. Quantitative analysis was carried out using Microsoft Excel.

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The glucose release kinetic of the FeedBeads was determined in a minimal medium without a carbon source. Briefly, six tablets of FeedBeads were placed in a 125 mL non-baffled flask containing 15 mL minimal medium and incubated at 30 °C with an agitation rate of 220 rpm. 50 µL cultures were removed from the flask at multiple time points and centrifuged at 13,000 × g for 5 min. The supernatant was then stored at −20 °C until further analysis. The concentration of glucose was quantified by HPLC analysis on an Aminex HPX-87/G column (Bio-Rad) on an Ultimate 3000 HPLC with a refractive index detector. The column was eluted with 5 mM H₂SO₄ at a flow rate of 0.6 mL min⁻¹ at 45 °C for 35 min. Chromeleon was used for HPLC data collection and Microsoft Excel for further quantitative analysis.

Identification of glycosylated products. Liquid chromatography-mass spectrometry (LC-MS) analysis was performed to verify the production of PIN and DIN by engineered yeast cells. Specifically, strains C28, E63, and E66 were cultivated in 15 mL minimal medium with 30 g L⁻¹ glucose for 72 h. For the LC-MS sample preparation, 2 mL resultant cell culture was collected and freeze-dried in a Christ Alpha 2-4LSC for 48 h. Then, 1 mL of absolute ethanol was added, vigorously vortexed for 10 min, and centrifuged at 13,000 × g for 5 min. The supernatant was collected, fully dried under vacuum, and resuspended with 200 µL absolute ethanol. Ten microdrops of each sample was injected and analyzed on an Agilent Infinity 1290 UHPLC connected to an Agilent 6520 high-resolution mass spectrometer. The UHPLC used a Waters UPLC HSS T3 100 × 2.1 mm column (particle size 1.8 µm). The column temperature was set to 45 °C and the flow rate was 0.4 mL min⁻¹ with a solvent system containing 0.04% formic acid (solvent A) and methanol with 0.04% formic acid (solvent B). The gradient started at 5% solvent B and ramped to 100% solvent B over 6 min and held for 4.5 min. The LC eluent was directed to the MS system for high-resolution mass spectrometry. The GenBank (https://www.ncbi.nlm.nih.gov/Genbank/) accession numbers and codon-optimized nucleotide sequences of the genes referenced in this study are provided in this paper. All other data that support the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in Saccharomyces cerevisiae. Meta. Biol. 11, 355–366 (2009).

Kim, D. H., Kim, B. G., Jung, N. R. & Ahn, J. H. Production of genistein from naringenin using Escherichia coli containing isoflavone synthase-cytochrome P450 reductase fusion protein. J. Microbiol. Biotechnol. 19, 1612–1616 (2009).

Katsuyama, Y., Miyahisa, I., Funa, N. & Horinouchi, S. One-pot synthesis of genistein from tyrosine by coinfection of genetically engineered Escherichia coli and Saccharomyces cerevisiae cells. Appl. Microbiol. Biotechnol. 73, 1143–1149 (2007).

Leonard, E. & Koffas, M. A. Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by Escherichia coli. Appl. Environ. Microbiol. 73, 7246–7251 (2007).

Koirala, N., Pandey, R. P., Thang, D. V., Jung, H. J. & Sohng, J. K. Glycosylation and subsequent malonylation of isoflavonoids in E. coli: strain development, production and insights into future metabolic perspectives. J. Ind. Microbiol. Biotechnol. 41, 1647–1658 (2014).

He, X. Z., Li, W. S., Blount, J. W. & Dixon, R. A. Regioselective synthesis of plant (iso)flavonol glycosides in Escherichia coli. Appl. Microbiol. Biotechnol. 80, 253–260 (2008).

Ferrer, J. L., Austin, M. B., Stewart, C. J. & Noel, J. P. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol. Biochem. 46, 356–370 (2008).

Du, H., Huang, Y. & Tang, Y. Genetic and metabolic engineering of isoflavone biosynthesis. Appl. Microbiol. Biotechnol. 86, 1293–1312 (2010).

Daftarmilch, M. & Dhaubhadel, S. Soybean chalcone isomerase: evolution of the fold, and the differential expression and localization of the gene family. Planta 241, 507–523 (2015).

Liu, Q. et al. Rewiring carbon metabolism in yeast for high level production of aromatic chemicals. Nat. Commun. 10, 4976 (2019).

Bomati, E. K., Austin, M. B., Bowman, M. E., Dixon, R. A. & Noel, J. P. Structural elucidation of chalcone reductase and implications for deoxychalcone biosynthesis. J. Biol. Chem. 280, 30496–30503 (2005).

Xiong, D. et al. Improving key enzyme activity in phenylpropanoid pathway with a designed biosensor. Metab. Eng. 40, 115–123 (2017).

Hashim, M. F., Hakamatsuka, T., Ebizuka, Y. & Sankawa, U. Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis. FEBS Lett. 271, 219–222 (1990).

Shang, Y. & Huang, S. Engineering plant cytochrome P450s for enhanced biosynthesis of natural products: past achievements and future perspectives. Plant Commun. 1, 100012 (2020).

Hannemann, F., Bichet, A., Ewen, K. M. & Bernhardt, R. Cytochrome P450 systems-biological variations of electron transport chains. Biochim. Biophys. Acta 1770, 330–344 (2007).

Jensen, K. & Møller, B. L. Plant NADPH-cytochrome P450 oxidoreductases. Phytochemistry 71, 132–141 (2010).

Caramella, A., Minieri, D. & Gilardi, G. Catalytically self-sufficient cytochromes P450 for green production of fine chemicals. Rendiconti Lincei 28, 169–181 (2016).

Narhi, L. O. & Fuko, A. J. Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in Bacillus megaterium. J. Biol. Chem. 261, 7160–7169 (1986).

Hunter, D. J. et al. Analysis of the domain properties of the novel cytochrome P450 RfH: FEBS Lett. 579, 2215–2220 (2005).

Zhang, W. et al. New reactions and products resulting from alternative interactions between the P450 enzyme and redox partners. J. Am. Chem. Soc. 136, 3640–3646 (2014).

Michener, J. K., Nielsen, J. & Smolke, C. D. Identification and treatment of heme deletion attributed to overexpression of a lineage of evolved P450 monooxygenases. Proc. Natl Acad. Sci. USA 109, 19504–19509 (2012).

Hoffman, M., Gora, M. & Rytkä, J. Identification of rate-limiting steps in yeast heme biosynthesis. Biochem. Biophys. Res. Commun. 310, 1247–1253 (2003).
Zhang, T., Bu, P., Zeng, J. & Vancura, A. Increased heme synthesis in yeast induces a metabolic switch from fermentation to respiration even under conditions of glucose repression. J. Biol. Chem. 292, 16942–16954 (2017).

Savitskaya, J., Protzko, R. J., Li, F. Z., Arkin, A. P. & Dueber, J. E. Iterative screening methodology enables isolation of strains with improved properties for a FACS-based screen and increased L-DOPA production. Sci. Rep. 9, 5815 (2019).

Werck-Reichhart, D. & Feyereisen, R. Cytochromes P450: a success story. Genome Biol. 1, REVIEWS3003 (2000).

Kim, J. E. et al. Tailoring the Saccharomyces cerevisiae endoplasmic reticulum for functional assembly of terpene synthesis pathway. Metab. Eng. 56, 30–59 (2019).

Arendt, P. et al. An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metab. Eng. 40, 165–175 (2017).

Liu, Y., Liu, Q., Krivoruchko, A., Khoomrung, S. & Nielsen, J. Engineering yeast phospholipid metabolism for de novo oleoylthanolamide production. Nat. Chem. Biol. 16, 197–205 (2020).

Adeyoye, O. et al. The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J. Cell Biol. 192, 1043–1055 (2011).

Carman, G. M. & Han, G. S. Regulation of phospholipid synthesis in the yeast Saccharomyces cerevisiae. Annu. Rev. Biochem. 80, 859–883 (2011).

Kim, J. E., Jang, I. S., Sung, B. H., Kim, S. C. & Lee, J. Y. Rerouting of NADPH is there a room for enzyme improvements? Curr. Opin. Biotechnol. 24, 310–319 (2013).

Blazek, J., Garg, R., Reed, B. & Alper, H. S. Controlling promoter strength and regulation in Saccharomyces cerevisiae using synthetic hybrid promoters. Biotechnol. Bioeng. 109, 2831–2848 (2012).

Jorgensen, K. et al. Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr. Opin. Plant Biol. 8, 280–291 (2005).

Ahmed, S. & Kvinovich, N. Regulation of phytoalexin biosynthesis for agriculture and human health. Phytochem. Rev. 20, 483–503 (2021).

Liu, Q., Liu, Y., Chen, Y. & Nielsen, J. Current state of aromatics production using yeast: achievements and challenges. Curr. Opin. Biotechnol. 65, 65–74 (2020).

Verduyn, C., Postma, E., Scheffers, W. A. & Van Dijken, J. P. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517 (1992).

Mans, R. et al. CRISPR/Cas9: a molecular Swiss army knife for simultaneous gene cloning, gene editing, and cotranscriptional mRNA processing. Curr. Opin. Biotechnol. 90, 887–899 (2020).

Egriboz, O. et al. Self-association of the Gal4 inhibitor protein Gal80 is a major regulator of phenylpropanoid availability and biological activity in plants. Front. Plant Sci. 7, 735 (2016).

Le Roy, J., Huss, B., Creach, A., Hawkins, S. & Neutelings, G. Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. Front. Plant Sci. 7, 735 (2016).

Noguchi, A. et al. A UDP-glucoselisoflavone 7-O-glycosyltransferase from the roots of soybean (glycine max) seedlings. Purification, gene cloning, phylogenetics, and an implication for an alternative strategy of enzyme catalysis. J. Biol. Chem. 282, 23581–23590 (2007).

Wittschieben, B. O. et al. A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol. Cell 4, 123–128 (1999).

Muratani, M., Kung, C., Shokat, K. M. & Tansey, W. P. The F box protein Dgsl/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnove and cotranscriptional mRNA processing. Cell 129, 887–899 (2007).

Ahmed, S. & Kovinich, N. Regulation of phytoalexin biosynthesis for agriculture and human health. Phytochem. Rev. 20, 483–503 (2021).

Mans, R. et al. CRISPR/Cas9: a molecular Swiss army knife for simultaneous gene cloning, gene editing, and cotranscriptional mRNA processing. Curr. Opin. Biotechnol. 90, 887–899 (2020).

Verduyn, C., Postma, E., Scheffers, W. A. & Van Dijken, J. P. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517 (1992).

Mans, R. et al. CRISPR/Cas9: a molecular Swiss army knife for simultaneous gene cloning, gene editing, and cotranscriptional mRNA processing. Curr. Opin. Biotechnol. 90, 887–899 (2020).

Egriboz, O. et al. Self-association of the Gal4 inhibitor protein Gal80 is impaired by Gal3: evidence for a new mechanism in the GAL gene switch. Mol. Cell 33, 3667–3674 (2013).

Sui, K. H. et al. Synthetic scaffolds for pathway enhancement. Curr. Opin. Biotechnol. 56, 98–106 (2015).

Albertsen, L. et al. Diversion of flux toward sesquiterpene production in Saccharomyces cerevisiae by fusion of host and heterologous enzymes. Appl. Environ. Microbiol. 77, 1033–1040 (2011).

Lee, D., Lloyd, N. D., Pretorius, I. S. & Borneman, A. R. Heterologous production of raspberry ketone in the wine yeast Saccharomyces cerevisiae via pathway engineering and synthetic enzyme fusion. Micro. Cell Fact. 15, 49 (2016).

Kochs, G. & Grisebach, H. Enzymic synthesis of isoflavones. Eur. J. Biochem. 155, 311–318 (1986).

Sawada, Y. & Ayabe, S. Multiple mutagenesis of P450 isoflavonoid synthase reveals a key active-site residue. Biochem. Biophys. Res. Commun. 330, 907–913 (2005).

Gomber, A. K., Moreira dos Santos, M., Christensen, B. & Nielsen, J. Network identification and flux quantification in the central metabolism of Saccharomyces cerevisiae under different conditions of glucose repression. J. Bacteriol. 183, 1441–1451 (2001).

Jeude, M. et al. Fed-batch mode in shake flasks by slow-release technique. Biotechnol. Bioeng. 95, 433–445 (2006).

Koštálková, O., Schreiner, K. & Kohlwein, S. D. Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta 1771, 235–270 (2007).

Wenz, P., Schwank, S., Hoja, U. & Schuller, H. J. A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene FAS2 by the FAS1 gene product. Nucleic Acids Res. 29, 4625–4632 (2001).

Keren, L. et al. Promoters maintain their relative activity levels under different growth conditions. Mol. Syst. Biol. 9, 701 (2013).

Blank, T. E., Woods, M. P., Lebo, C. M., Xin, P. & Hopper, J. E. Novel Gal3 proteins showing altered Gal3p binding cause constitutive transcription of Gal3p-activated genes in Saccharomyces cerevisiae. Mol. Cell Biol. 17, 2566–2575 (1997).

Ryo, S. et al. Positive feedback genetic circuit incorporating a constitutively active mutant Gal3 into yeast GAL induction system. ACS Synth. Biol. 6, 928–935 (2017).

Wittschieben, B. O. et al. A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol. Cell 4, 123–128 (1999).
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