Microbial Cell Factories

REVIEW Open Access

Advances in synthesizing plant-derived isoflavones and their precursors with multiple pharmacological activities using engineered yeasts

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Abstract
Isoflavones such as daidzein and genistein are naturally occurring compounds found in plants such as legumes. They have diverse pharmacological activities, making them valuable in the food, pharmaceutical, and cosmetic industries. Currently, isoflavones are mainly obtained through the extraction of plant biomass. Chemical synthesis is challenging for most isoflavones due to the complexity of their structures. The limited supply of isoflavones cannot meet the market demands. Advances in synthetic biology have provided a sustainable and efficient solution for the production of isoflavones, with yeasts often serving as the microbial chassis for biosynthesis. This review summarizes the pharmacological properties of specific isoflavones, their biosynthetic pathways, and the technical strategies used in engineered yeasts for isoflavone production. In addition, the development of synthetic biology and state-of-the-art biotechnological strategies for the environmentally friendly production of bioactive isoflavones is discussed.

Keywords Isoflavones, Synthetic biology, Engineered yeasts, Metabolic pathways

Introduction

Secondary metabolites in plants typically encompass terpenoids, polyphenols, flavonoids, steroids, alkaloids, quinones, and polysaccharides [1]. These compounds not only serve essential functions within plants, but also exhibit significant pharmacological properties such as anticancer, anti-inflammatory, and antimicrobial effects, making them promising for a wide range of medical applications [2–5]. Among these secondary metabolites, flavonoids, which include isoflavones, are key components of natural products and are prevalent in various angiosperm plants like *Fabaceae*, *Aquifoliaceae*, *Rosaceae*, *Polygonaceae*, *Rutaceae*, *Apiaceae*, *Asteraceae*, *Rhamnaceae*, and *Moraceae* [6]. Flavonoids can be categorized into different groups based on their structural variations, including chalcones, flavanols, anthocyanins,

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flavones, dihydroflavonols, and isoflavones [7]. Isoflavones, as a structurally diverse subgroup of flavonoids, have been identified in more than 2,000 species across over 300 plants [8].

The current methods for obtaining isoflavones and other natural products mainly depend on plant extraction. The low content of bioactive compounds such as isoflavones in leguminous plants typically ranges from 1‰ to 1% [9]. Furthermore, the long growth period and vulnerability to seasonal, climatic, and geographic factors impede consistent production of isoflavones through plant cultivation [10]. The complexity of isoflavone structures presents challenges for chemical synthesis, as certain intricate processes like methylation, hydroxylation, and molecular rearrangement cannot be efficiently carried out [11]. The extensive use of organic solvents and reagents in chemical synthesis exacerbates environmental challenges, including issues with waste disposal due to hazardous substances and safety risks. Given the growing emphasis on green and sustainable development, there is a great interest to develop more environmentally friendly and economically viable production methods [12]. The adoption of eco-friendly and effective synthetic biology techniques for synthesizing and increasing the production of isoflavones can enhance their utilization in various fields, including medicine [11].

Synthetic biology has been applied to the biomanufacturing of plant natural products, leading to the establishment of a cost-effective, efficient, and operationally straightfforward green industrial chain [13–15]. The utilization of plant multi-omics data has facilitated the synthesis of plant bioactive compounds in microbial cell factories, enabling the biosynthesis of various plant natural products, including rare ginsenosides [16–18], stevioside [19], cocoa butter equivalent [20–23], icariin [24], cannabidiolic acid [25], taxol core skeleton [26, 27], and polydatin [28] in engineered *Saccharomyces cerevisiae.* This study provides an overview of the pharmacological activities of isoflavones and their precursors, highlighting the key enzymes and biosynthetic pathways engineered in yeasts for their synthesis. In addition, optimization strategies for the production of isoflavones in yeasts are discussed.

Pharmacological activities of isoflavones and their precursors in plants

Plants, especially leguminous plants, such as *Glycyrrhiza uralensis*, *Pueraria lobata*, *Medicago sativa*, and *Lupinus micranthus*, are commonly utilized as medicinal herbs in traditional Chinese medicine. These isoflavones, commonly found in leguminous plants, are part of the phenylpropanoid metabolite subclass and play critical roles in providing resistance to various biotic and abiotic stresses while demonstrating a wide array of biological activities [29].

Various isoflavones in the plants contribute to a unique set of beneficial pharmacological effects. *G. uralensis*, classified as a secondary protected wild medicinal herb in China, contains flavonoids and dihydroflavones in its roots and rhizomes, exhibiting multiple health effects like the anti-inflammatory, the antiviral, the gastroprotective, and the anti-allergic activities [30]. *P. lobata* has the biological activities such as alcohol detoxification, hepatoprotection, hypoglycemia-reducing, lipid-lowering properties, alleviation of postmenopausal osteoporosis, anti-tumor, anti-inflammatory, and antioxidative effects [31]. *M. sativa* is rich in isoflavones, flavones, and polyphenols, demonstrating pharmacological activities including antibacterial, anti-inflammatory, antioxidative, and wound-healing properties [32]. *L. micranthus*, which is rich in isoflavones and saponins, exhibits diverse pharmacological effects such as anti-inflammatory, antioxidative, anti-tumor, and lipid-lowering effects [33].

The key precursors for isoflavone biosynthesis and their pharmaceutical activities

Isoflavones are among the main bioactive constituents in leguminous plants, exhibiting a wide range of pharmacological activities. One of the primary crucial precursors for isoflavone biosynthesis is *p*-hydroxycinnamic acid (*p*-HCA), derived from either L-phenylalanine (L-Phe) or L-tyrosine (L-Tyr). *p*-HCA, a prevalent dietary polyphenol found in various fruits, vegetables, and grains [34], has been shown to possess antioxidative, anti-inflammatory, anti-tumor, anti-hypertensive, cardiovascular-protective, and melanin-inhibiting functions. Isoliquiritigenin, produced from *p*-HCA, exhibits potent anti-cancer activity by inducing apoptosis and autophagy, and directly inhibiting various malignant tumors such as cervical, liver, colon, breast, and prostate cancers [35, 36]. In addition, isoliquiritigenin is effective in treating respiratory infections like chronic obstructive pulmonary disease by inhibiting inflammatory factors.

Liquiritigenin, derived from isoliquiritigenin through isomerization, is a dihydroflavonoid compound and a highly selective plant-derived β-receptor agonist [37]. Liquiritigenin and its derivatives possess anti-inflammatory, antioxidative, and anti-ulcer effects, and exhibit positive roles in the treatment of various tumors [38, 39]. However, due to the low oral bioavailability of liquiritigenin, further investigation is necessary to reveal its biosynthetic mechanism, in order to enhance its bioavailability and broaden its utility through additional modification of its structure. Similarly, naringenin, a dihydroflavonoid and a precursor to isoflavones like genistein or genistin, has diverse medical properties, including antibacterial, anti-inflammatory, anti-viral,

anti-cancer, anti-diabetic, and neuroprotective effects. Nowadays, these characteristics make naringenin a valuable ingredient in functional foods.

Isoflavones with diverse pharmacological activities

Isoflavones feature a flavone skeleton of C_6-C_3-C_6, wherein the B ring is attached at the C_3 position. Genistein and daidzein represent the two primary scaffolds of isoflavones. Through structural modifications or variations in these scaffolds, a wide array of isoflavone derivatives can be created, each displaying unique pharmacological activities (Fig. 1). The isoflavones include pueraria isoflavones, soybean isoflavones, licorice isoflavones, sakuranetin, and pterocarpan. Isoflavones modulate the NF-kB signaling pathway [40], suppress the expression of inflammatory factors like TNF-α, IL-6 [41], decrease NO production, inhibit cGMP activation, and activate BK Ca^{2+} channels [42], thus demonstrating anti-inflammatory and analgesic effects. Furthermore, isoflavones exhibit strong antibacterial activity against

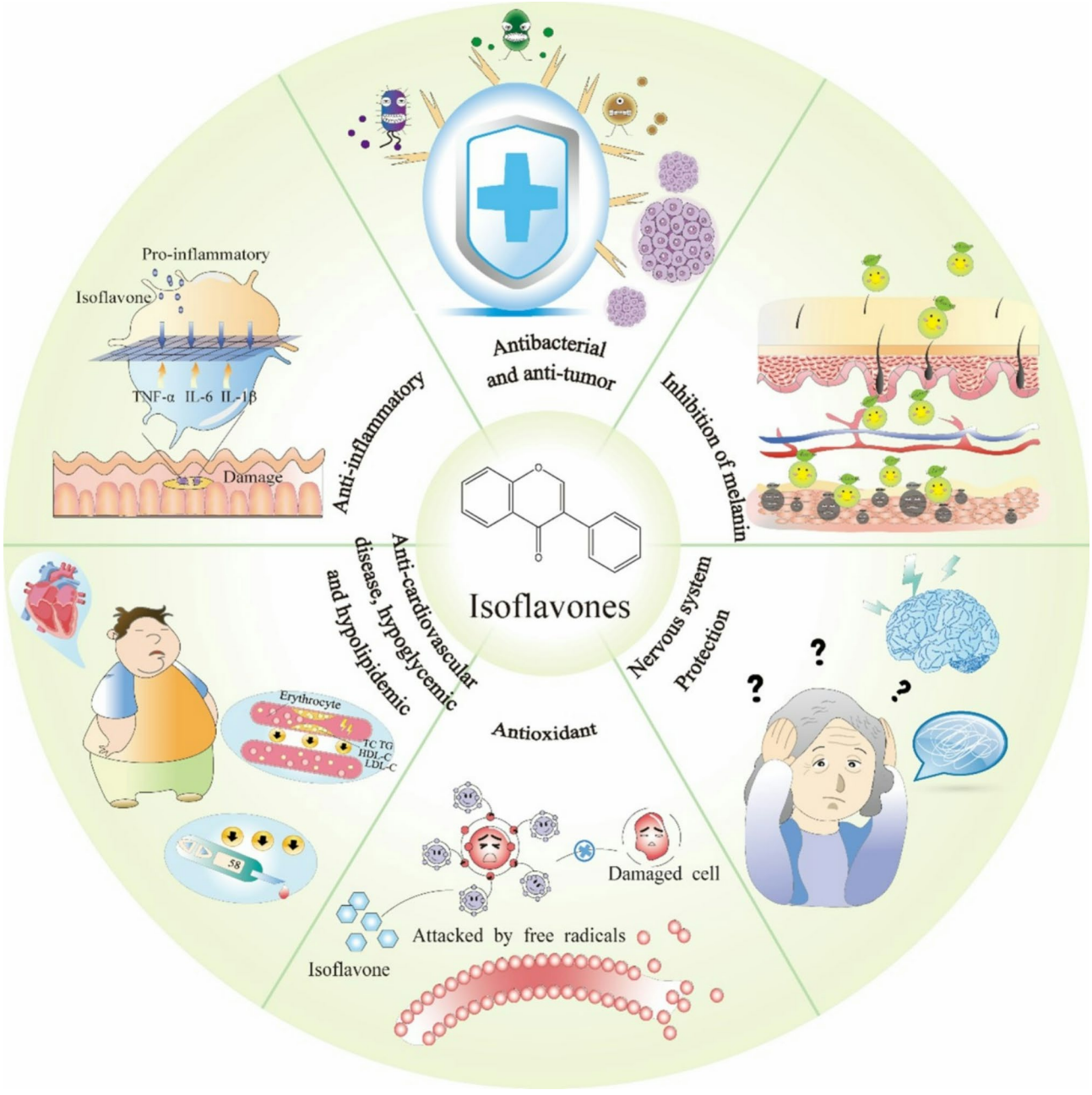

Fig. 1 Isoflavones exhibit diverse pharmacological activities, including antibacterial, anti-tumor, anti-inflammatory, antioxidant, anti-cardiovascular disease, hypoglycemic, hypolipidemic, neuroprotective, and melanin inhibition effects

Staphylococcus aureus, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and certain fungi, with minimum inhibitory concentrations all above 100.0 μg/L [43, 44]. The wide-ranging antibacterial efficacy of compounds like soybean isoflavones, licorice isoflavones, and maackiain suggests potential applications in antimicrobial application.

The antioxidative properties of isoflavones are superior to those of vitamin C, showing strong DPPH free radical scavenging activity. In addition, isoflavones can modulate linoleic acid peroxidation and lipoxygenase activity, helping to prevent the accumulation of free radicals and reduce the risk of various diseases. Isoflavones like pueraria isoflavones, soybean isoflavones, alfalfa isoflavones, licorice isoflavones, and pterocarpan have been shown to enhance cardiac function in rats by ameliorating oxidative stress [45], indicating their significant therapeutic potential in reducing the risk of various diseases, such as those associated with psoriasis.

Isoflavones have been used in cancer treatment. Genistein inhibits COX2 pathway and GLUT receptor expression, thereby, restraining prostate cancer cell proliferation [46]. Daidzein interferes with cancer cell proliferation and triggers apoptosis, combating diseases like breast cancer and endometrial cancer [47]. Calycosin inhibits the c-Myc gene target, leading to reduced breast cancer cell migration and invasion [48]. Puerarin significantly inhibits the growth of human lung cancer cells, demonstrating potential anti-lung cancer activity [49]. Isoflavones display diverse pharmacological effects in combating cardiovascular diseases. Puerarin might be used for atherosclerosis treatment by modulating gut microbiota, especially by inhibiting *Prevotella copri* and its production of trimethylamine [50]. Daidzein could reduce the incidence of cardiovascular diseases in postmenopausal women [47]. Additionally, alfalfa isoflavones can improve lipid level, reduce the risk of heart disease, promote vasodilation, and enhance blood circulation.

Formononetin, one isoflavone, has demonstrated therapeutic effects in traumatic brain injury, spinal cord injury, ischemic stroke, nerve tumors, and Alzheimer's disease [51]. Iridin alleviates oxidative stress reactions in neural cells by enhancing their antioxidative capabilities. Puerarin and calycosin protect the nervous system by alleviating learning and memory impairments and restoring neural function in rats with spinal cord injuries [52]. However, further insights into the impact of isoflavones on central nervous system signaling pathways are needed, in order to establish a theoretical basis for the development of new drugs targeting the central nervous system. Additionally, puerarin is widely recognized for its anti-diabetic effects [53]; it can reduce blood sugar level by increasing glucose tolerance and increasing cellular glucose uptake. Soybean isoflavones play a positive role in controlling blood sugar and lipid levels [54], enhancing low-density lipoprotein (LDL) receptor activity, reducing LDL, preventing LDL oxidation, and thereby lowering LDL deposition on coronary artery walls.

The isoflavones have the ability to inhibit tyrosinase activity and reduce melanin production [55]. Glabridin is a high-end cosmetic ingredient known for its skin-whitening properties. Additionally, isoflavones have demonstrated therapeutic activity in various aspects, including protecting liver and kidney function [56, 57], regulating bone metabolism [58], and improving pulmonary edema. The diverse pharmacological effects of isoflavones offer promising applications in both the medical and cosmetic industries.

The biosynthesis of isoflavones using engineered yeasts

The limitations of both plant extraction and chemical synthesis methods have resulted in a shortage of isoflavones. Yeasts have been widely employed for the heterologous synthesis of bioactive plant natural products. Yeasts possess various endogenous pathways related to isoflavones, such as the mevalonate (MVA) pathway, glycolytic pathway, and shikimate pathway, and it is relatively straightforward to express plant genes in yeasts, making them ideal hosts for isoflavones [59, 60]. To produce isoflavones efficiently, it is necessary to recover isoflavone biosynthetic enzymes, design an efficient isoflavone biosynthetic pathway, and optimize and reprogram yeast metabolism for isoflavones production [61].

Synthetic pathways of isoflavones and their precursors

The biosynthetic pathways of isoflavones and their precursors involve multiple biochemical processes, including central carbon metabolism, shikimate metabolism, aromatic amino acid (AAA) biosynthesis, MVA metabolism, flavonoid metabolism, and downstream product modification (Fig. 2). De novo isoflavone synthesis begins with glycolysis or the pentose phosphate pathway (PPP), leading to the formation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). Next, the catalysis of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (ARO3 and ARO4) results in the formation of DAHP [62]. DAHP then undergoes reactions catalyzed by shikimate dehydrogenase (ARO1) to produce 5-enolpyruvyl-shikimate-3-phosphate (EPSP). The EPSP is further converted to chorismic acid (CHA) through the catalysis of chorismate synthase (ARO2) and chorismate mutase (ARO7), eventually leading to the formation of prephenate (PPA) [62]. In yeasts, PPA can be converted to L-Phe via prephenate dehydratase (PHA2) and aromatic aminotransferase I (ARO8). In *E. coli*, PPA can be simultaneously converted into L-Phe and L-Tyr.

L-Phe can be converted to *p*-HCA with the help of phenylalanine ammonia lyase (PAL), cinnamic acid

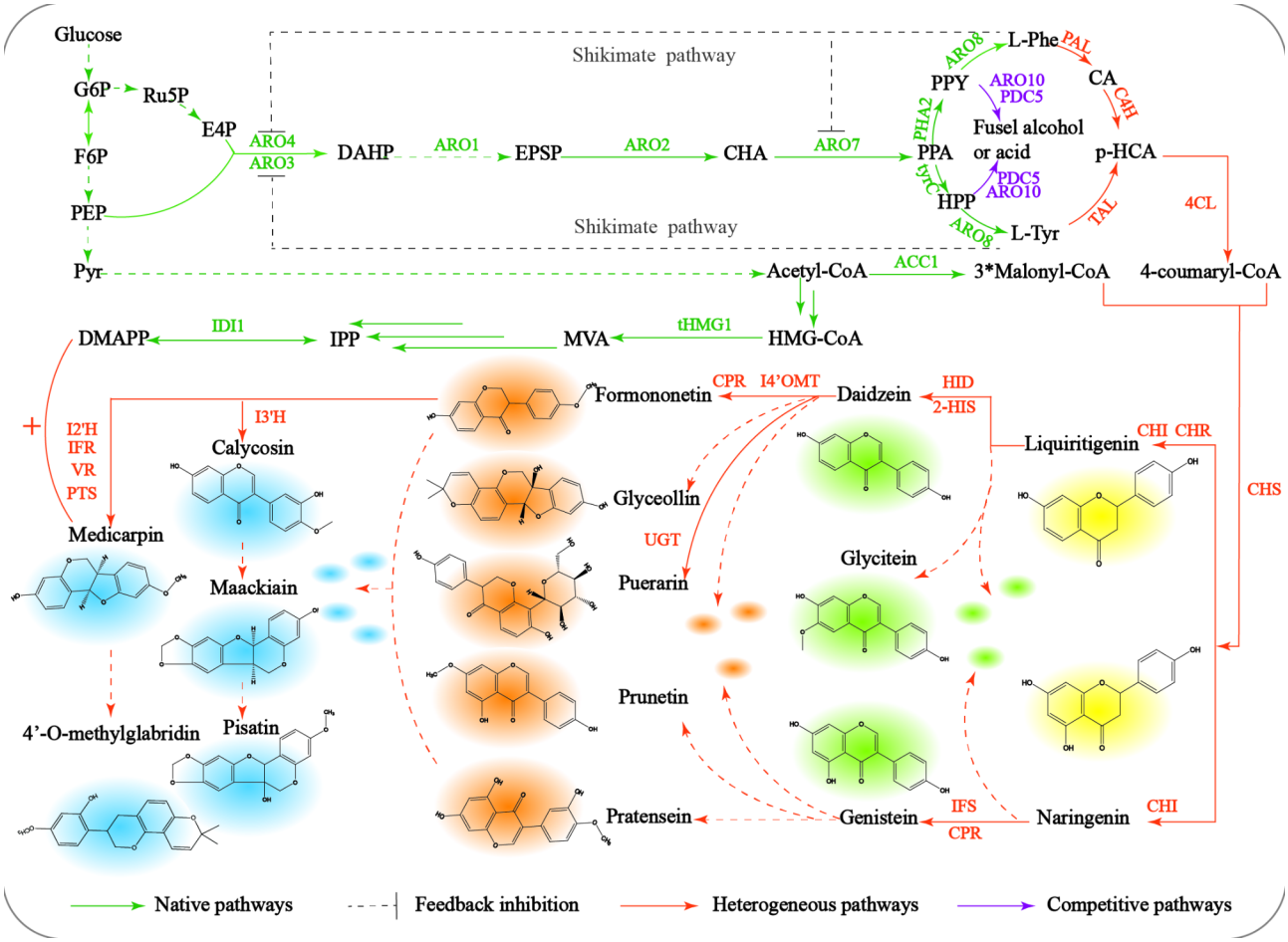

Fig. 2 The isoflavone biosynthetic pathway. Byharnessing glucose as the primary substrate, engineered yeasts can employ theglycolysis, shikimate pathway, and MVA pathway to synthesize compounds likeL-Phe, L-Tyr, acetyl-CoA, and DMAPP. Through the introduction of a set ofheterologous isoflavone biosynthetic enzymes, a diverse array of isoflavonesand their essential precursors can be synthesized. This tailored isoflavonesynthetic pathway can convert yeast into an eco-friendly platform for theefficient production of isoflavones. G6P, glucose-6-phosphate; Ru5P, ribulose5-phosphate; F6P, fructose-6-phosphate; PEP, phosphoenolpyruvate; E4P,erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-2-heptulosonic acid 7-phosphate;EPSP, 5-enolpyruvyl-shikimate-3-phosphate; CHA, chorismic acid; PPA,prephenate; PPY, phenylpyruvate; HPP, *para*-hydroxy-phenylpyruvate;L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; CA, cinnamic acid; *p*-HCA, *p*-coumaricacid; Pyr, pyruvate; Acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP,dimethylallyl diphosphate; ARO1, pentafunctional aromatic protein; ARO2,chorismate synthase; ARO3, DAHP synthase; ARO4, DAHP synthase; ARO7, chorismatemutase; PHA2, prephenate dehydratase; tyrC, L-tyrosine prephenatedehydrogenase; ARO8, aromatic aminotransferase I; ARO10, phenylpyruvatedecarboxylase; PDC5, pyruvate decarboxylase; PAL, phenylalanine ammonia lyase;TAL, tyrosine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroylcoenzyme A ligase; ACC1, acetyl coenzyme A carboxylase 1; CHS, chalconesynthase; CHR, chalcone reductase; CHI, chalcone isomerase; HID,2-hydroxyisoflavone dehydratase; 2-HIS, 2-hydroxyisoflavone synthase; IFS,isoflavone synthase; CPR, cytochrome P450 reductase; I4'OMT, isoflavone 4'-O-methoxytransferase; UGT, UDP-sugar-glycosyltransferase; I2'H, isoflavone 2'-hydroxylase; I3'H, isoflavone 3'-hydroxylase; IFR,isoflavone reductase; VR, vestitone reductase; PTS, pterocarpan synthase

hydroxylase (C4H), and cytochrome P450 reductase (CPR) [63–65]. Tyrosine ammonia lyase (TAL) catalyzes L-Tyr to synthesize *p*-HCA. The conversion of *p*-HCA to 4-coumaroyl-CoA is catalyzed by 4-coumarate-CoA ligase (4CL). Isoliquiritigenin is then synthesized from one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, with the joint catalysis of chalcone synthase (CHS) and chalcone reductase (CHR). Isoliquiritigenin is further transformed into liquiritigenin by chalcone isomerase (CHI) [66]. Another key precursor, naringenin, is directly synthesized from 4-coumaroyl-CoA and malonyl-CoA through CHS and CHI.

Various isoflavones, such as daidzein and genistein, are synthesized by isoflavone synthase (IFS) and CPR. In yeasts, daidzein is generated from liquiritigenin through the synergistic catalysis of 2-hydroxyisoflavone synthase (2-HIS) and 2-hydroxyisoflavone dehydratase (HID) [67]. These isoflavones can be further synthesized to daidzin, genistin, and other isoflavones by UDP-sugar-glycosyltransferase (UGT). The synthesis of glycitein follows a process similar to that of daidzein and involves specific

IFS-catalyzed reaction using liquiritigenin. The bioactive isoflavone in wheat, 5-hydroxy-2',4',7-trimethoxyisoflavone, is synthesized through the CYP71F53 gene cluster-mediated conversion of naringenin [68]. Using daidzein as a substrate, glycinol undergoes C-4 or C-2 isoprenylation through the catalysis of isoflavone 2'-hydroxylase (I2'H) and prenyltransferase (G4DT or G2DT) [69], leading to glyceollin formation. Cytochrome P450 enzymes or related enzymes can catalyze the synthesis of isoflavones such as pratensein and prunetin using genistein as substrate. Formononetin and biochanin A can be synthesized from daidzein under the catalysis of isoflavone 4'-O-methoxy transferase (I4'OMT) [70], and further converted to puerarin with the assistance of UGT [65].

Formononetin can be catalyzed by isoflavone 3'-hydroxylase (I3'H) to synthesize calycosin [71], which serves as a precursor for the synthesis of medicarpin through the coordinated activities of vestitone reductase (VR) and pterocarpan synthase (PTS). Dimethylallyl pyrophosphate (DMAPP) and medicarpin might be the substrates for the synthesis of 4'-O-methylglabridin [70, 72].

Strategies for the synthesis of isoflavones in engineered yeast

The production of plant natural products in engineered yeasts involves a systematic approach that encompasses gene discovery, enzyme characterization, metabolic engineering, and fermentation optimization [73–75] (Fig. 3). By designing and continuous optimization of yeast cell factories, efficient synthesis of isoflavones and other plant natural products can be achieved [12].

The discovery of key enzymes involved elucidation of their biosynthetic pathways is essential for the heterologous biosynthesis of plant bioactive natural products [76]. Transcriptomic data is utilized to analyze and compare with metabolomic data in order to identify key enzymes potentially involved in isoflavone biosynthesis [77]. Additionally, known isoflavone biosynthetic enzyme sequences from databases, along with various omics-based discovery methods and computational tools, can be used to predict candidate enzymes for isoflavone biosynthesis and uncover their potential metabolic pathways [78]. Through the integration of metabolic network analysis and gene family evolution strategies, a comprehensive study of 22 *Scutellaria* species identified 261 flavonoids and delineated five P450 subfamily CYP82D clades with distinct catalytic functions. This work underscores the remarkable catalytic versatility of cytochrome P450 enzymes in plant metabolism, revealing their essential roles in driving the structural diversity of flavonoids [79]. Using multiple omics approaches, a chromosome-level genome of *Callerya speciosa* was de novo assembled, and the key genes involved in the isoflavonoid biosynthetic pathway were identified through gene-to-metabolite correlation analysis, providing genes for future *Callerya* isoflavonoid production using engineered microbes [80]. The recovery of a choromosome-scale *Citrus reticulata* cv. Chachiensis genome enabled the identification of a putative caffeic acid O-methyltransferase, which help reveal the polymethoxylated flavonoid biosynthetic pathway [81].

Following the identification of key enzymes involved in isoflavone biosynthesis, subsequent gene expression and enzymatic characterization in engineered microbes are essential to confirm their catalytic roles in vivo [75, 82]. Metabolome and transcriptomic sequencing were performed on fresh tissue samples of legumes such as *G. uralensis*, soybean, and *P. lobata*, in order to identify candidate enzymes such as IFS, HIS, and HID for isoflavones synthesis [65, 70, 83].

Structural biology methods are employed to reveal the molecular structure and catalytic sites of isoflavone biosynthetic enzymes [67]. Daidzein can be effectively synthesized from (2S)-naringin in one step by assembling a multienzyme system by constructing a protein scaffold [84]. Isoflavone biosynthetic pathways can be designed and optimized using highly efficient key isoflavone enzymes, and metabolic flux can be modulated for isoflavone synthesis in yeasts with the help of yeast genome metabolic models [85].

Overexpression of key genes, increasing isoflavone precursor supply, and knockout of competitive pathway genes have been used to enhance the metabolic flux of isoflavones in engineered yeasts [86]. The key genes involved in daidzein synthesis were overexpressed using a galactose-inducible promoter, resulting in a two to threefold increase in daidzein synthesis efficiency in yeasts. This yield was further enhanced by the addition of glucose and the elimination of inhibitory transcription factors in strain [65]. In the synthesis of medicarpin from liquiritigenin in yeasts, medicarpin was achieved by increasing the availability of the precursor, liquiritigenin [70]. Additionally, by increasing the copy numbers of genistein biosynthetic genes, the yield of de novo genistein synthesis in yeasts was enhanced by approximately 200-fold [83]. Thus, systematic optimization and reprograming yeast metabolism are necessary for efficient synthesis of isoflavones in engineered yeasts.

Synthesis of isoflavones and their precursors in engineered yeasts

A fully understanding of the biosynthetic mechanisms of isoflavones and their precursors is fundamental for the heterologous biosynthesis of isoflavones. To synthesize *p*-HCA, E4P is produced through the glycolysis pathway or PPP, which serves as a limited substrate for yeast synthesis of AAA [87]. The AAA synthesis can be effectively

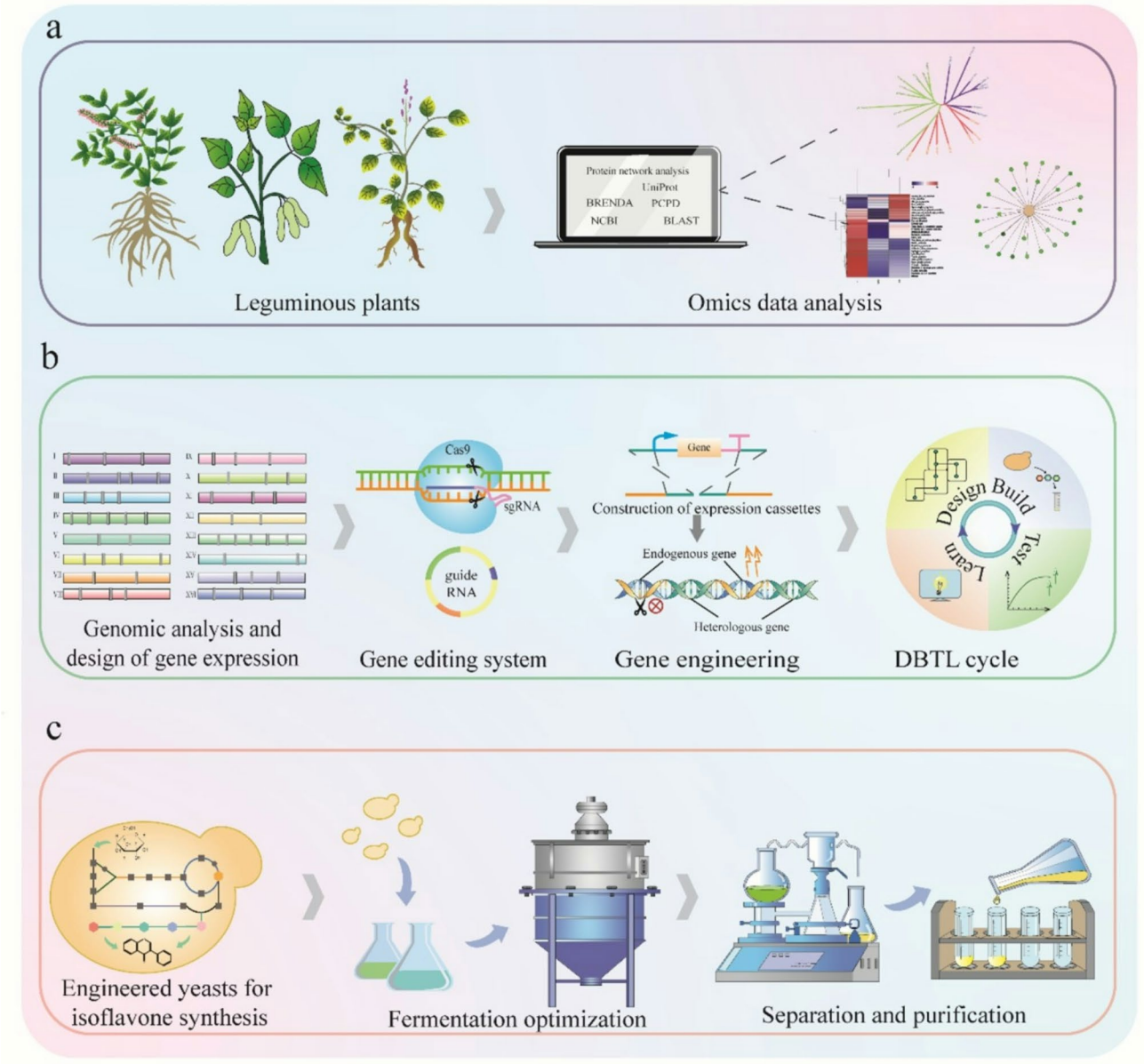

Fig. 3 Building yeast strains for isoflavone production through synthetic biology strategies. **a**, Analyzing omics data and screening isoflavone biosynthetic enzymes. **b**, Designing and building yeast strains for isoflavone production using synthetic biology approaches. **c**, Scaling up fermentation of engineered yeasts for isoflavone production

enhanced by redirecting carbon flux to increase the biosynthesis of E4P. The PHK pathway has been introduced into yeast, including phosphoketolase (Xfpk) and phosphotransacetylase (Pta), enabling the direct conversion of fructose-6-phosphate (F6P) into E4P and acetyl coenzyme A (Acetyl-CoA), thus boosting the production of *p*-HCA and malonyl-CoA [62, 88]. In addition, overexpressing the yeast endogenous genes ARO1, ARO2, ARO3, ARO4, and ARO7 can increase the yield of PPA. However, ARO4, ARO7, and ARO3 are subjected to feedback inhibition by L-Tyr and L-Phe, respectively. Further improvements in *p*-HCA production are achieved by introducing mutations in ARO4 and ARO7 to generate tyrosine-insensitive alleles ($ARO4^{K229L}$ and $ARO7^{G141S}$) or by overexpressing these genes [89, 90]. Overexpression of prephenate dehydrogenase and ARO8 genes can increase the metabolic flux of L-Phe and L-Tyr [62].

In the isoflavone synthetic pathway from L-Phe and L-Tyr to *p*-HCA, introducing heterologous TAL and PAL can enhance the yield of *p*-HCA. Phenylpyruvate decarboxylase (ARO10) and pyruvate decarboxylase (PDC5) might direct the metabolic flux towards the synthesis of other metabolites. By editing these two genes, more metabolic flux can be directed towards L-Phe and L-Tyr,

thereby increasing *p*-HCA synthesis [91]. In *S. cerevisiae*, the *p*-HCA titer of 0.59 g/L and 1.93 g/L was achieved by mutating ARO4^{K229L} and ARO7^{G141S} and knocking out PDC5 and ARO10 genes [91]. Further optimization resulted in a *p*-HCA titer of 12.5 g/L in *S. cerevisiae* [62]. In *Y. lipolytica*, employing strategies such as increasing gene copy number, enhancing flux through the shikimate pathway, and blocking competitive pathways of phenylalanine when utilizing cellulose or hemicellulose as the sole carbon source, led to the *p*-HCA titer of 84.3 ± 2.4 mg/L and 65.3 ± 4.6 mg/L [92]. The efficient synthesis of *p*-HCA lay the foundation for the synthesis of isoflavones using L-Phe and L-Tyr as substrates.

Liquiritigenin and naringenin serve as crucial precursors for various isoflavones, and they are synthesized with one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA [24]. Overexpression of the gene ACC1 or its mutant ACC1$^{S659A/\ S1157A}$ enhances the conversion of Acetyl-CoA into more malonyl-CoA [24, 93–95]. Knocking out gene YPL062W in *S. cerevisiae* boosts Acetyl-CoA accumulation [24], thus promoting malonyl-CoA synthesis. Co-expression of C4H and 4CL in *S. cerevisiae* can significantly promote the synthesis of 4-coumaroyl-CoA [66]. The biosynthesis of liquiritigenin requires CHS, CHR, and CHI [96]. CHS and CHI are crucial for naringenin production, with the CHS efficiency often limiting naringenin synthesis. Thus, enhancing CHS activity is key to increasing naringenin yield [24].

A liquiritigenin synthetic pathway, utilizing GuPAL1, GuC4H1, Gu4CL1, GuCHS1, GuCHR1, and GuCHI1 from *G. uralensis*, was designed, yielding 1.0 μmol/L liquiritigenin when introduced into yeast [66]. Using L-Phe as a substrate is suitable for *p*-HCA synthesis, liquiritigenin, and kaempferol; while L-Tyr favors fisetin synthesis [97]. In *S. cerevisiae*, a titer of 1129.44 mg/L of (2S)-naringenin was achieved through enhanced fatty acid β-oxidation and fed-batch fermentation [98]. Introduction of exogenous naringenin synthetic genes into *S. cerevisiae*, coupled with 2.5 g/L *p*-HCA feeding, yielded 648.6 mg/L naringenin [99]. Further strain optimization, including promoters and fermentation conditions, resulted in a final naringenin titer of 1.2 g/L [100]. By optimizing shikimate and AAA pathways, overexpressing endogenous malonyl-CoA synthetic genes, and using subcellular carbon flux regulation strategies, the naringenin titer reached 3420.6 mg/L [93]. Similarly, by modulating the shikimate and AAA pathways to enhance metabolic flux, the engineered *Y. lipolytica* can produce 898 mg/L of naringenin [101].

Liquiritigenin and naringenin, sharing same substrates and same CHI enzyme, can be simultaneously produced. *S. cerevisiae* strain produced 5.3 mg/L liquiritigenin and 1.3 mg/L naringenin by expressing AmCHR from *Astragalus membranaceus* [97]. In *Y. lipolytica*, ZmPAL from *Zea mays*, Pc4CL from *Petroselinum crispum*, PhCHS from *Petunia hybrida*, and MsCHR and MsCHI from *M. sativa* were introducted for the production of liquiritigenin. Further optimization through promoter engineering using *p*-HCA as a substrate yielded a titer of 62.4 mg/L liquiritigenin [96].

The isoflavone genistein titer reached 31.02 mg/L by enhancing the glucose to *p*-HCA metabolic flux and reconstructing the downstream module with IFS and CPR from different sources [102]. Yeasts engineered with genes including At4CL1 from *Arabidopsis thaliana*, GmCHR5, GmCHS8, GmCHI1B2, Ge2-HIS, GmHID from *Glycine max*, and CrCPR2 from *Catharanthus roseus* produced another isoflavone, daidzein [65]. Further overexpression of genes such as 5-aminolevulinic acid dehydratase (HEM2), 4-porphobilinogen deaminase (HEM3), transcription activator (INO2), NAD^+ kinase (EcysjB) from *E. coli*, transcriptional factor (STB5) genes, and others led to the synthesis of 85.4 mg/L daidzein. Expression of the GmUGT4 gene from *G. max* in the yeasts resulted in a daidzin titer of 73.2 mg/L. Additionally, a novel wheat enzyme, TaCYP71F53, was identified with the ability to catalyze naringenin into various isoflavones, expanding the range of plants capable of synthesizing isoflavones [68].

Formononetin is commonly considered as a daidzein-derived isoflavone compound. The I4'OMT is responsible for methylating 2,7,4'-trihydroxy isoflavone to produce 2,7-dihydroxy-4'-methoxy isoflavone, which then undergoes dehydration to form formononetin. With the introduction of the PlUGT43 gene derived from *P. lobata* into a daidzein-synthesizing yeast strain, the isoflavone compound puerarin titer of 73.8 mg/L was obtained. Additionally, six different glucosyltransferases were identified as highly correlated with puerarin synthesis [79]. Glyceollin is another isoflavone that is synthesized using daidzein as a substrate. Through computer-based homology screening, four genes encoding isopentenyltransferases were identified. Among these, G4DT and G2DT are capable of converting the C-4 and C-2 isoprene units of glycinol to synthesize glyceollin [69]. Furthermore, enzymes involving in the isoflavonoid biosynthetic pathways in *Lotus japonicus* were investigated, revealing the biosynthetic mechanisms of vestitone, medicarpin, and vestitol. The mechanisms of converting 7,2'-dihydroxy-4'-methoxyisoflavanol to medicarpin was elucidated through in vitro validation of the PTS from *G. max*, *Glycyrrhiza echinate*, and *L. japonicus* [72]. The biosynthesis of medicarpin from liquiritigenin in *S. cerevisiae* was achieved by expressing Gg2-HIS, GgHID, GgI2'H5, GgIFR4, GgVR, and GgI4'OMT from *Glycyrrhiza glabra*, GmCPR from *G. max*, and GePTS genes from *G. echinate*, the titer of the product is 0.82 ± 0.18 mg/L. I4'OMT

Table 1 The synthesis of isoflavones in engineered yeast

Target products	Chassis strain	Optimizing strategy	Substrates	Titer (Growth condition)	References
p-HCA	*S. cerevisiae*	Overexpressing genes in the shikimate and AAA pathways; introducing the PHK pathway and ARO4^{K229L}, ARO7^{G141S} mutants; knocking out PDC5, ARO10, GPP1 and GAL7/1/10	Glucose	12.5 g/L (Bioreactor)	62
p-HCA	*Y. lipolytica*	Increasing the copy number of TAL; redesigning the shikimate pathway	Glucose	1035.5 ± 67.8 mg/L (Shake flask)	92
Liquiritigenin	*S. cerevisiae*	Introducing exogenous PAL, C4H, 4CL, CHS, CHR, and CHI	Glucose	1.0 μM/L (Bioreactor)	66
Liquiritigenin	*Y. lipolytica*	Screening the optimal genes for producing five heterologous liquiritigenin compounds: PAL, 4CL, CHS, CHR and CHI; fusing the CHS and CHR; optimizing the length of the promoter	*p*-HCA	62.4 mg/L (Shake flask)	96
Naringenin	*S. cerevisiae*	Introducing the PHK and aldehyde dehydrogenase pathways; overexpressing genes in the shikimate, AAA, and malonyl-CoA synthesis pathways; introducing ARO4^{K229L}, ACC1$^{S659A/S1157A}$ and ARO7^{G141S} mutants; introducing exogenous PAL、TAL、C4H、4CL、CHS、CHI and CPR; knocking out PDC5 and ARO10; regulating subcellular carbon flux	Glucose	3420.6 mg/L (Bioreactor)	93
Naringenin	*Y. lipolytica*	Modulating the shikimate and AAA pathways; introducing a mutant *Y. lipolytica* allele.	Glucose	898 ± 19 mg/L (Bioreactor)	101
Genistein	*S. cerevisiae*	Fusion expression of C4H and 4CL; introducing exogenous CHS, CHI, 2-HIS, HID, and CPR; overexpressing HEM2, HEM3, INO2, STB5, and EcyjfB; knocking out ROX1, OPI1, HMX1, PAH1, GAL1/7/10, and ELP3; supplementing 5-ALA; replacing the FAS1 promoter	*p*-HCA	33.7 mg/L (Shake flask)	65
Daidzein	*S. cerevisiae*	Fusion expression of C4H and 4CL; introducing exogenous CHS, CHR, CHI, 2-HIS, HID, and CPR; overexpressing HEM2, HEM3, INO2, STB5, and EcyjfB; knocking out ROX1, OPI1, HMX1, PAH1, GAL1/7/10, and ELP3; supplementing 5-ALA; replacing the FAS1 promoter	*p*-HCA	85.4 mg/L (Shake flask)	65
Medicarpin	*S. cerevisiae*	Introducing exogenous 2-HIS, CPR, I4′OMT, HID, I2′H, IFR, VR, and PTS; increasing the copy number of VR and PTS	Liquiritigenin	2.05 ± 0.72 mg/L (Shake flask)	70
Sakuranetin	*S. cerevisiae*	Overexpressing genes in the shikimate and AAA pathways; introducing ARO4^{K229L}, ACC1$^{S659A/S1157A}$ and ARO7^{G141S} mutants; introducing exogenous 4CL, CHS, CHI; increasing the copy number of PAL, TAL, and CHS; knocking out ARO10, PDC5, and YPL062W	Glucose	158.65 mg/L (Bioreactor)	94
Xanthohumol	*S. cerevisiae*	Overexpressing genes in the shikimate pathway; introducing exogenous TAL, 4CL and CHS, introducing ARO4^{K229L}, ARO7^{G141S} and ERG20^{N127W} mutants; knocking out ARO10; replacing ACC1 promoter; overexpressing MVA pathway genes	Glucose	142 μg/L (Shake flask)	95
Icariin	*S. cerevisiae*	Overexpressing ACC1 as well as genes in the shikimate, AAA, and MVA pathways; introducing ARO4^{K229L} and ARO7^{G141S} mutants; introducing exogenous 4CL, CHS and CHI	Glucose	130 μg/L (Shake flask)	24
Phloretin	*S. cerevisiae*	Overexpressing genes in the AAA pathway; introducing exogenous 4CL, CHS, ACS, ACC1, and aldehyde dehydrogenase (ADH2, ALD6) genes	Glucose	619.5 mg/L (Bioreactor)	103
Homoeriodictyol	*S. cerevisiae*	Introducing exogenous 4CL, CHS and CHI; fusion expression of CHS and CHI; overexpressing ACC1and acetyl-CoA synthetase (ACS) genes; knocking out phenylacrylic acid decarboxylase (PAD1) and ferulic acid decarboxylase (FDC1) genes	*p*-HCA	3.2 mmol/L (Bioreactor)	104
Eriodictyol	*Y. lipolytica*	Screening efficient heterologous TAL, 4CL, CHS and CHI key enzyme genes; optimizing promoter strategies; increasing the flux of shikimate pathway; promoting fatty acid β-oxidation; increasing the copy number of synthetic pathway genes	Glucose	6.8 g/L (Bioreactor)	108
Scutellarin	*Y. lipolytica*	Screening efficient TAL, 4CL, CHS, and CHI from different sources; introducing feedback-resistant AroG* and TyrA* from *E. coli*; disrupting the key gene MHY1 that controls cell morphology	Glucose	703.01 ± 4.83 mg/L (Shake flask)	109

might be the rate-limiting enzyme in medicarpin biosynthesis, and overexpression of VR and PTS genes can enhance medicarpin synthesis in engineered yeasts, the titer can be increased to 2.05 ± 0.72 mg/L [70]. As a substrate, formononetin might be catalyzed by I3'H derived from *A. membranaceus* to produce calycosin [71]. Medicarpin is proposed as a potential precursor of isoflavone 4'-O-methylglabridin, although further validation of the key enzymes responsible for catalyzing the conversion from medicarpin to glabridin is required [70].

The synthesis of isoflavones like pratensein, maackiain, and pisatin in engineered yeasts remains unrealized due to the absence of isoflavone synthetic genes and optimized biosynthetic pathways. Integrating legume plant omics data, developing new bioinformatic tools for isoflavone gene discovery, functional characterization, and understanding the catalytic mechanisms of isoflavone synthetic enzymes are essential for engineering yeasts capable of isoflavone synthesis. The biosynthetic pathways of flavones such as sakuranetin [94], icariin [24], phloretin [103], homoeriodictyol [104], and xanthohumol [95] share similarities with isoflavone synthetic pathways, offering potential references for isoflavone synthesis.

The isoflavone biosynthetic enzymes would be tested for compatibility with yeast chassis, and a high-efficiency yeast strain for isoflavone production can be obtained using the 'Design-Built-Test-Learn' cycle (DBTL cycle) [105]. Fermentation optimization would enable yeast strains to achieve high titer, rate, and yield of isoflavones, facilitating scale-up production [106]. Furthermore, extraction and purification conditions would be optimized to obtain high-purity isoflavones, providing an alternative solution to the shortage of isoflavones supply [107] (Table 1).

Conclusion and perspective

Currently, the production of isoflavones poses significant challenges, particularly in understanding the catalytic mechanisms of key enzymes involved in their synthesis. To improve the efficiency of isoflavone production in engineered yeasts, it is vital to identify P450 oxidoreductases and other key enzymes and investigate their catalytic mechanisms in isoflavone synthesis. Subsequently, designing and optimizing the isoflavone synthetic pathways to adapt to yeast metabolism, as well as reprogramming and building yeast cell factories to boost their capability in producing isoflavones and their precursors are necessary. Synthetic biology harnesses natural product biosynthetic pathways across various biological systems, offering effective and flexible advantages for building heterologous isoflavone biosynthetic pathways in yeasts. The synthesis of bioactive isoflavones holds great promise for addressing challenges in foods, medicine, cosmetics, and environmental protection, thus promoting sustainable development and environmental conservation in society.

Acknowledgements
During the preparation of this work the authors used ChatGPT in order to polish the language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Author contributions
Y.W. and L.Q. conceived the study; W.N. and Y.W. drafted the manuscript; W.N. prepared the figures and tables; Y.W., J.Z., L.Q., and X.J. revised and polished the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding
This study was supported by the National Natural Science Foundation of China (No. 32371485 and 32111530179), Henan Province Outstanding Youth Foundation (No. 242300421097), and the State Key Laboratory of Materials-Oriented Chemical Engineering (No. SKL-MCE-23B04).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

Received: 16 August 2024 / Accepted: 12 March 2025
Published online: 29 March 2025

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