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Abstract

Isoflavonoids are a class of plant natural products gaining attention due to their pharmaceutical properties. These natural compounds constitute a subclass of flavonoids, which belong to a broader class of plant products known as phenylpropanoids.

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Flavonoids have been associated with medicinal properties, while isoflavonoids have shown anticancer, antioxidant, and cardioprotective properties due to their role as inhibitors of estrogen receptors. Isoflavonoids are naturally produced by legumes and, more specifically, organisms belonging to the pea family. Harvesting of these natural products through traditional extraction processes is limited due to the low levels of these phytochemicals in plants, so alternative production platforms are required to reduce cost of production and increase availability. Over the last decade, researchers have engineered artificial flavonoid biosynthesis pathways into *Escherichia coli* and *Saccharomyces cerevisiae* to convert simple, renewable sugars like glucose into flavonoids at high production levels. This chapter outlines the metabolic engineering research that has enabled microbial production of plant flavonoids and further details the ongoing work aimed at producing both natural and non-natural isoflavonoids in microorganisms.

Keywords

Metabolic engineering • mutasynthesis • non-natural isoflavonoids • protein engineering • strain improvement

Abbreviations

3GT	3- <i>O</i> -glucosyltransferase
4CL	4-Coumarate-CoA ligase
ACC	Acetyl-CoA carboxylase
Ala	Alanine
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
API	Active pharmaceutical ingredient
Arg	Arginine
BDO	Biphenyl dioxygenase
BMC	Bacterial microcompartment
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CPR	Cytochrome P450 reductase
CUS	Curcuminoid synthase
DFR	Dihydroflavonol reductase
DH	<i>Salmonella typhimurium</i> LT2 TDP-glucose 4,6-dehydratase
EPI	<i>Streptomyces antibioticus</i> Tu99 TDP-4-keto-6-deoxyglucose 3,5-epimerase
ER	Endoplasmic reticulum
F7GAT	Flavonoid 7- <i>O</i> -glucuronosyltransferase
FHT	Flavanone 3 β -hydroxylase
FLS	Flavonol synthase

FSI	Soluble flavone synthase
FSII	Membrane-bound flavone synthase
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GALU	Glucose-1-phosphate uridylyltransferase
GERF	<i>Streptomyces</i> sp. KCTC 0041BP TDP-hexose 3-epimerase
GERK	<i>Streptomyces</i> sp. KCTC 0041BP TDP-4-keto-6-deoxyglucose reductase
Glu	Glutamic acid
Gly	Glycine
HEK	Human embryonic kidney cells
hER	Human estrogen receptor
HI4'OMT	2,7,4'-Trihydroxyisoflavanone 4'-O-methyltransferase
HID	2-Hydroxyisoflavanone dehydratase
HIDH	2-Hydroxyisoflavanone dehydratase hydroxy type
HIDM	2-Hydroxyisoflavanone dehydratase methoxy type
IFR	Isoflavone reductase
IFS	Isoflavone synthase
Ile	Isoleucine
k_{cat}	Turnover number
K_m	Michaelis constant
KR	<i>Streptomyces antibioticus</i> Tu99 TDP-glucose 4-ketoreductase
LAR	Leucoanthocyanidin reductase
LB	Luria-Bertani medium
LDOX	Leucoanthocyanidin dioxygenase
NADPH	Nicotinamide adenine dinucleotide phosphate
NDK	Nucleoside diphosphate kinase
NDO	Naphthalene dioxygenase
PAL	Phenylalanine ammonia-lyase
PGI	Glucose-6-phosphate isomerase
PGM	Phosphoglucomutase
Phe	Phenylalanine
RCIFS	Red clover isoflavone synthase
RCPR	Rice cytochrome P450 reductase
SaOMT-2	<i>Streptomyces avermitilis</i> MA-4680 7-O-methyltransferase
ScCCL	<i>Streptomyces coelicolor</i> A3 cinnamate/coumarate:CoA ligase
Ser	Serine
SERM	Selective estrogen receptor modulator
STS	Stilbene synthase
TAL	Tyrosine ammonia-lyase
TB	Terrific broth
TDP	Thymidylidiphosphate

TGS	<i>Thermus caldophilus</i> GK24 thymidyldiphosphoglucose synthase
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UDG	Uridine diphosphoglucose dehydrogenase
UDP	Uridine diphosphate
UGT	Uridine diphosphate glycosyltransferase
UTP	Uridine triphosphate
UXS1	Uridine diphosphate glucuronic acid decarboxylase
Val	Valine
V_{max}	Maximum reaction rate

1 Metabolic Engineering

1.1 Background

Metabolic engineering involves the genetic manipulation of metabolism for a specific goal, often high-level production of a secondary metabolite. Secondary metabolites are those not critical to the survival of an organism in its normal environment, and they are thus typically found in far lower quantities than primary metabolites involved in energy maintenance and growth [1, 2]. As secondary metabolites have evolved to serve in important ecological roles – usually through interaction with other organisms – they possess unique properties and are thus the target of many metabolic engineering projects [3–5]. Although metabolic engineering has been a distinct discipline for over two decades, advancing technologies in areas such as DNA sequencing and synthesis, computational modeling and optimization, synthetic biology, and protein engineering are enabling metabolic engineers to create economically viable microbial production platforms for specialty chemicals like pharmaceuticals and biofuels [6].

Throughout the past decade, much work has focused on both plant and microbial metabolic engineering for production of pharmaceutically and nutraceutically important plant isoflavonoids [7–11]. This class of phytochemicals has been shown to possess a diverse array of pharmacological activities and demonstrates potential for treatment of certain cancers, cardiovascular diseases, and other conditions [12–17]. In particular, isoflavonoids have high affinity toward human estrogen receptors (hERs) and are therefore being investigated as estrogen receptor agonists and antagonists to modulate estrogen metabolism [18–20]. The relatively low abundance of these valuable compounds in plants makes microbial metabolic engineering an excellent alternative candidate for large-scale isoflavonoid production.

1.2 Metabolic Engineering Products

The majority of work in the field of metabolic engineering has focused on the production of commodity chemicals and biofuels from renewable, simple carbon sources such as glucose and glycerol, or the production of pharmaceutical chemicals and proteins [21–24]. In general, metabolic engineering can be viewed as the process by which scientists combine genes from different sources to construct a biosynthetic pathway in a host organism to convert an inexpensive feedstock into a valuable product. Classic metabolic engineering projects range from the microbial production of biofuels like ethanol and butanol to the production of commodity chemicals like xylitol. Although these efforts are important for ensuring long-term stability of commodity supply from renewable resources, microbial metabolic engineering of valuable plant natural products and other active pharmaceutical ingredients (APIs) with high overhead has the potential to make a much greater impact on society by lowering cost and ensuring availability and widespread access to medically important compounds [6].

1.3 Microorganisms as a Production Platform for Plant Natural Products

1.3.1 Advantages of Microbial Hosts

Microorganisms serve as excellent hosts for production of phytochemicals. The relatively lower genetic complexity of microbes compared to multicellular eukaryotes allows for more accurate prediction of the effects of genetic manipulations in microbes than in plants. Modulation of gene copy or expression level typically leads to an imbalance in reaction fluxes and, subsequently, the accumulation of pathway intermediates. If a genetic pathway is not decoupled from its native environment, accumulation of intermediates can become toxic or elicit unintended regulatory effects like feedback inhibition. Such uncharacterized genetic interactions in multicellular eukaryotic hosts are currently difficult to predict and can be largely avoided by transplanting genes from evolutionarily distinct organisms into an artificial pathway in a microbial host [25].

Perhaps the strongest argument for utilizing microorganisms for metabolic engineering of plant natural products is the high degree of genetic tractability that currently exists for microbial workhorses like *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus subtilis*. Thanks to decades of research, these hosts have innumerable data sets and molecular biology tools available for facile genetic manipulation, characterization, modeling, and scale-up. This genetic tractability reduces experimental unknowns and allows for faster, more predictable experimentation and data collection. Additionally, the high growth rates and simple media requirements associated with microorganisms enable culturing with limited resources [25, 26].

1.3.2 Alternative Production Platforms

Plant natural products have traditionally been harvested through extraction methods, as evidenced by the preparation of traditional medicines and the steeping of tea leaves and coffee beans for millenia. Since plant natural products are generally found at low levels in plant biomass, extraction is usually not a sustainable mass production avenue. Although extraction is still utilized to harvest APIs like the antimalarial drug artemisinin (from *Artemisia annua*, known as Sweet Wormwood) and the chemotherapeutic paclitaxel (from *Taxus brevifolia*, the Pacific yew tree) when chemical synthesis is difficult or expensive, there is a trend and growing necessity to shift toward alternative production platforms to lower cost and increase availability [27, 28].

Alternative production platforms include organic synthesis, plant cell culture, plant tissue culture, and even mammalian cell culture. The field of organic synthesis of complex plant natural products has advanced significantly but is limited as an industrial-scale flavonoid production platform by frequent use of toxic chemicals and extreme reaction conditions, a high number of required steps, exorbitant costs, relatively low overall yields, and nonspecific catalysts leading to by-products and often difficult-to-separate racemic mixtures of target compounds [29–35]. Semisynthesis, which combines organic synthesis steps with biosynthetic steps, is also limited by similar challenges. It is then reasonable to consider plant cell and tissue culture as a closely related alternative production platform since the metabolites of interest are endogenously produced in undifferentiated plant cells [36].

A well-known example of industrial-scale production in plant cell lines is the induction of paclitaxel production through methyl jasmonate elicitation, yielding 0.5 % of dry weight compared to 0.01 % of dry weight by extraction from the Pacific yew [37, 38]. By contrast, chemical synthesis of paclitaxel requires 35–51 steps, with a yield of only 0.4 % [39]. Plant tissue culture is another option, as many secondary metabolic biosynthetic pathways are only active in specific stages of development or in certain tissues [40, 41]. Thus, elicitation of differentiated plant cell tissues by small molecules or light can also be utilized to produce secondary metabolites. Despite progress in plant cell and tissue culture, the elucidation and characterization of all enzymes involved in plant secondary metabolite biosynthetic pathways are still challenging tasks; moreover, the difficulty in unequivocally discerning all sensitive, multilevel regulatory effects instigated by minimal variations in metabolite concentrations often makes the outcome of metabolic engineering in plant cell and tissue cultures unpredictable.

With advances in metabolic engineering of mammalian cells, it is foreseeable that plant natural products might one day be produced and derivatized using mammalian cell culture to take advantage of mammal-specific biotransformations and glycosylation patterns leading to improved pharmaceutical properties and applications. Therapeutic phytochemical production pathways might even be engineered into specific tissues to enable in situ biosynthesis for disease treatment or prophylaxis. To date this alternative remains relatively unexplored; however, engineering of a resveratrol artificial biosynthetic pathway into human embryonic kidney cells (HEK293) circumvented purported difficulties

associated with yeast expression of tyrosine ammonia-lyase (TAL) and highlighted the opportunity to move plant pathways into mammalian cells for in situ production of phytochemical therapeutics in human tissue [42]. Just as predictable metabolic engineering of plant cell and tissue cultures is currently limited by cellular complexity, metabolic engineering of mammalian cells can be encumbered with the same difficulties.

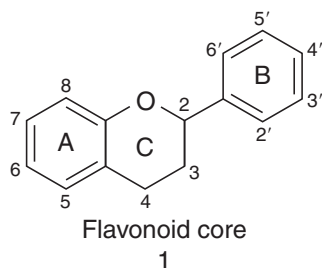
2 Plant Phenylpropanoid Biosynthesis

2.1 Background

Isoflavonoids belong to a broad class of compounds known as phenolics. Any chemical containing one or more phenol group can be classified as a phenolic compound, although the plant phenolics with the most biotechnological relevance are flavonoids and other phenylpropanoids. Phenylpropanoids are secondary plant metabolites that are considered to be beneficial for human health [43]. In particular, a subclass of phenylpropanoids known as flavonoids is typified by bioactive compounds with antioxidant, antiviral and antibacterial, anticancer, antiobesity, and estrogenic properties [9]. The microbial production of flavonoids has attracted much attention due to the prospect of utilizing flavonoids for personal health applications [44]. Flavonoids are currently used as dietary supplements and are the subject of intense investigation as pharmaceutical precursors to treat chronic human pathological conditions like cancer and diabetes [45–51]. Anthocyanins **17**, another class of flavonoids, possess brilliant natural colors and are potential replacements for artificial dyes that have adverse health effects. The antioxidant properties of these glycosylated flavonoids may have a positive health influence and make anthocyanins **17** well suited as natural colorants for the food and beverage industry [52–54]. Anthocyanins **17** are good targets for metabolic engineering since glycosylations remain a challenge from a chemical synthesis perspective. Furthermore, plant extraction of phenolics seldom yields greater than 1 % of the dry weight. Metabolic engineering of flavonoid biosynthesis has already gained traction due to the long-standing interest in phenolic compounds and the corresponding detailed characterization of related genetic pathways and enzymes [43].

As a general classification, phenolics do not contain nitrogen and may contain multiple hydroxyl groups as well as heteroatom substituent groups. Phenolics with greater than 12 phenolic groups are generally considered as polyphenols, lignins, or tannins. Flavonoids are the most well characterized and largest class of natural phenolics, and they are biosynthesized from the aromatic amino acid phenylalanine **2** through the common precursor, chalcone **11**. Further classification draws a distinction between five types of flavonoids that are derived from the common flavanone **12** precursor: flavones **14**, flavonols **15**, isoflavones **13**, flavanols, and anthocyanins **17** [55]. Flavonoids are composed of a C₆-C₃-C₆ skeleton that serves as a 15-carbon phenylpropanoid core **1** for

downstream decorations such as methylations, hydroxylations, reductions, oxidations, glycosylations, acylations, methoxylations, alkylations, and various rearrangements [44, 56–58]. The flavonoid core **1** consists of 3 rings, labeled A, B, and C.



Other phenylpropanoids, so named due to their common phenylalanine **2** precursor, include hydroxycinnamic acids, cinnamic aldehydes and monolignols, coumarins, and stilbenoids **8**.

2.2 Plant Phenylpropanoid Biosynthetic Pathway

Phenylpropanoid biosynthesis is initiated by the conversion of phenylalanine **2** to cinnamic acid **5** as catalyzed by phenylalanine ammonia-lyase (PAL). Cinnamic acid **5** is then converted to flavanone **12** through a series of subsequent enzymatic reactions involving the following steps: the hydroxylation of cinnamic acid **5** to *p*-coumaric acid **6** through cinnamate 4-hydroxylase (C4H); the ligation of *p*-coumaric acid **6** to a CoA group using 4-coumarate-CoA ligase (4CL); the sequential decarboxylative condensation of three acetate units from malonyl-CoA **10** to 4-coumaroyl-CoA **19** by chalcone synthase (CHS), a type III polyketide synthase, to form chalcone **11** in a ring closing step; and the stereospecific isomerization of chalcone **11** to flavanone **12** catalyzed by chalcone isomerase (CHI). Downstream enzymes then catalyze the conversion of flavanones **12** into compounds belonging to the various flavonoid subclasses.

Type III polyketide synthases are particularly relevant to this chapter because they catalyze the formation of phenolic compounds. This group of polyketide synthases consists of CHSs, stilbene synthase (STS), and curcuminoid synthase (CUS), which perform decarboxylative condensations between a starter unit, either *p*-coumaroyl-CoA **19** or cinnamoyl-CoA **18**, and an extender unit, malonyl-CoA **10**. CHS, STS, and CUS convert the substrate molecules into flavonoids (C₆-C₃-C₆), stilbenoids **8** (C₆-C₂-C₆), and curcuminoids **9** (C₆-C₇-C₆), respectively [59]. Stilbenoids **8** and curcuminoids **9** are out of the scope of this chapter but possess medicinal properties as well; resveratrol is a well-known stilbenoid **8** associated with longevity, and curcumin is a common curcuminoid **9** that is responsible for the yellow color in turmeric and can be utilized as a natural pigment possessing antioxidant and anti-inflammatory properties [60–63]. For an in-depth treatment of plant polyketide production in microbes, the reader is directed to a recent comprehensive review by Boghigian et al. [64].

2.3 Plant Flavonoid Pathways

Plant flavanones **12** are enzymatically converted to five major subclasses of flavonoids. Flavanones **12** are oxidized to flavones **14** by the action of either a soluble flavone synthase (FSI) or, as in most cases, a membrane-bound cytochrome P450 monooxygenase flavone synthase (FSII) [65]. Flavone synthases belong to the oxidoreductase family of enzymes and effectively remove the stereocenter from flavanones **12** by oxidation of C3 and introduction of a double bond between C2 and C3. Apigenin, luteolin, and chrysin are common flavones **14** that contribute to human diet as glycosides and are found in large quantities in parsley and celery [66–68].

Alternatively, isoflavone synthase (IFS) catalyzes the 1,2-aryl ring B migration from C2 to C3 on ring C of the phenylpropanoid core **1** and the hydroxylation of C2, converting flavanones **12** to 2-hydroxyisoflavanones [69, 70]. Dehydration of 2-hydroxyisoflavanones into isoflavones **13** occurs spontaneously through the 1,2-elimination of water, but accelerated dehydration is catalyzed by one of two hydro-lyases known as 2-hydroxyisoflavanone dehydratases (HID hydroxy type, HIDH; HID methoxy type, HIDM), depending upon the occurrence of an intermediate 4'-*O*-methylation catalyzed by 2,7,4'-trihydroxyisoflavanone 4'-*O*-methyltransferase (HI4'OMT) [71]. Isoflavonoids are characterized by a 3-phenylchroman skeleton, in contrast to the 2-phenylchroman core **1** possessed by flavonoids, and are incredibly diverse in structure despite being limited to natural existence primarily in leguminous plants [72]. Soy beans and soy bean food products contain high concentrations of isoflavone **13** glycosides such as genistin **31** and daidzin **30** and relatively lower quantities of their respective aglycones, daidzein **27** and genistein **26** [74]. Isoflavones **13** are classified as phytoestrogens because of the structural similarity shared with estrogens, and they are among the most highly studied polyphenols due to their affinities for steroid receptors and demonstrated pharmacological properties [18–20, 74]. These characteristics make isoflavones **13** important metabolic engineering targets.

Flavanones **12** also serve as the substrate for flavanone 3 β -hydroxylase (FHT), which catalyzes the hydroxylation of C3 on the flavanone core **1** into dihydroflavonol **16**, the common precursor to both flavonols **15** and anthocyanins **17**. Dihydroflavonols **16** are subsequently converted to flavonols **15** by reduction of C2 by the oxidoreductase enzyme flavonol synthase (FLS), again removing the stereocenter and introducing a double bond between C2 and C3 [75]. Flavonols **15** such as kaempferol and quercetin exist primarily as glycosides at appreciable levels in onions and kale [67, 68].

Initiating another branch of the flavonoid pathway, C4 of dihydroflavonol **16** can be reduced from a carbonyl group to a hydroxyl group by the oxidoreductase enzyme dihydroflavonol reductase (DFR), producing leucoanthocyanidins, or the colorless precursors to anthocyanins **17**. Leucoanthocyanidins are unstable and are quickly converted to anthocyanidins by anthocyanidin

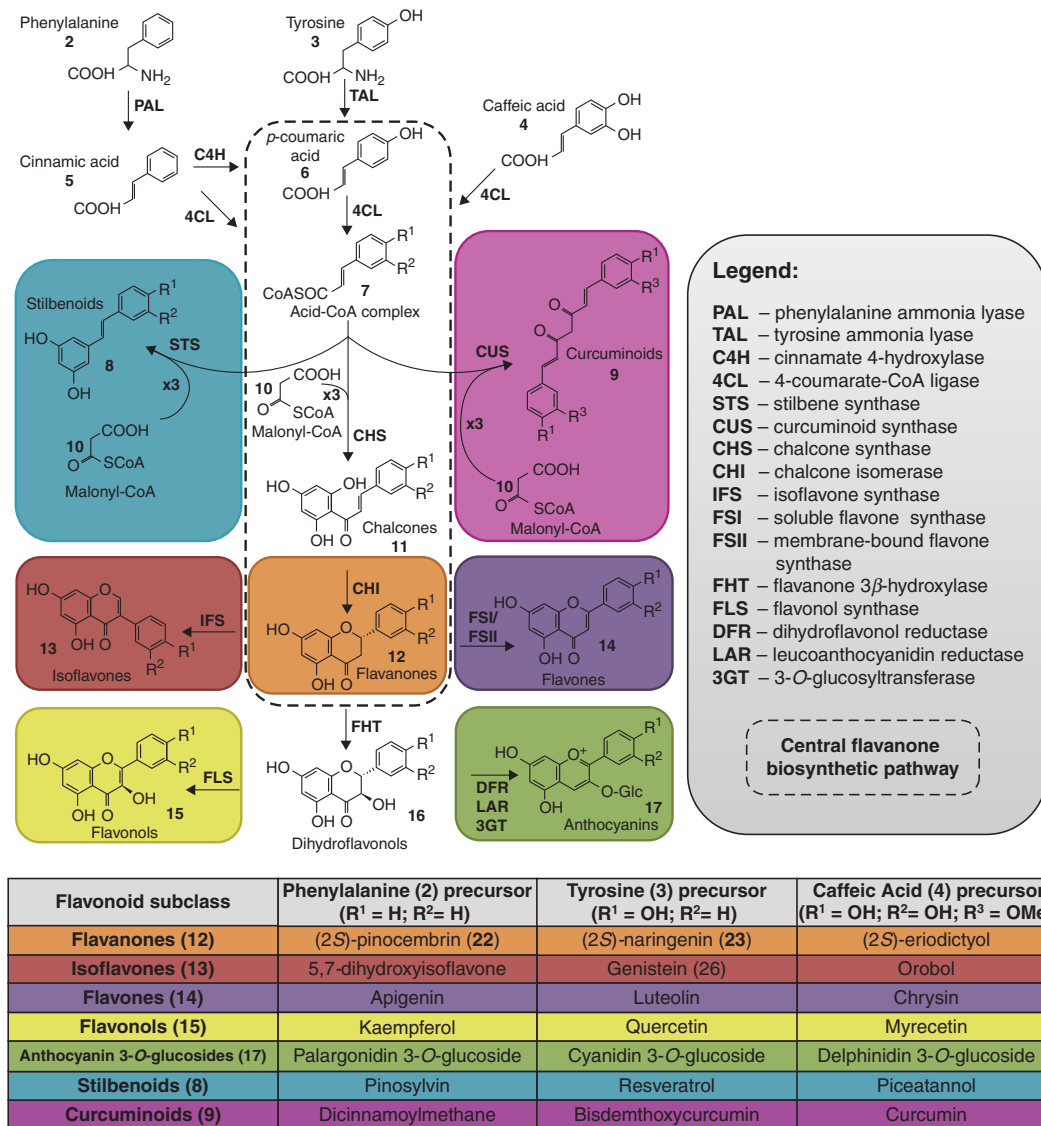


Fig. 54.1 Plant phenylpropanoid and flavonoid biosynthetic pathways; representative compounds from each subclass are named

synthase (ANS), synonymously leucoanthocyanidin dioxygenase (LDOX), working jointly with DFR [76]. Anthocyanidins and leucoanthocyanidins can alternatively be reduced to their corresponding flavan-3-ols (proanthocyanidins, or condensed tannins) by anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), respectively. A flavonoid glycosyltransferase then adds a sugar to the anthocyanidin, enabling pigment storage in the form of stable anthocyanins 17 [77]. Many brilliant red, blue, and purple plant hues arise from anthocyanin-mediated coloration. Figure 54.1 illustrates the alternative pathways for biosynthesis of various plant phenylpropanoid and flavonoid subclasses.

3 Plant Isoflavonoid Production in Microbes

3.1 Construction of an Artificial Biosynthetic Pathway for Flavonoid Production in Microbes

The first construction of an artificial plant flavonoid biosynthetic pathway in microbes involved the transformation of *E. coli* with four heterologous genes. These genes are required for the synthesis of flavanones **12** from phenylalanine **2** and tyrosine **3** (through a promiscuous PAL having the ability to accept both phenylalanine **2** and tyrosine **3** as substrates) [78–80]. This exercise provided a platform for the microbial biosynthesis of a plethora of natural and non-natural flavanone **12** derivatives. It should also be noted here that bacterial TAL catalyzes the conversion of tyrosine **3** to *p*-coumaric acid **6** in one step and can replace the two-step conversion of phenylalanine **2** to *p*-coumaric acid **6** by PAL and C4H in an artificial biosynthetic pathway if so desired [81]. Also, depending upon choice of aromatic amino acid precursor, two parallel biosynthetic paths exist for phenylalanine-based flavonoids in contrast to tyrosine-based flavonoids; other common natural and non-natural aromatic acrylic acids like caffeic acid **4** serve as substrates for 4CL in plants and microbes [82, 83]. The substrate flexibility of all enzymes involved allows for perpetuation of extra hydroxyl side groups throughout the entire pathway, affording flavanones **12** or other flavonoids with divergent hydroxylations. Another key distinction to note while reading this section is whether the project being described utilizes an entirely fermentative process to produce complex compounds from primary microbial metabolites or whether the project takes advantage of intermediate chemical supplementation. Although neither approach is absolutely superior to the other, distinctions can be drawn between them.

For instance, a fermentative approach often suffers from low production due to pathway complexity and increased number of steps, but it allows for production of complex compounds such as phytochemicals from simple, renewable carbon compounds like glucose. Conversely, intermediate supplementation is often utilized to simplify pathway construction and is associated with higher product yields. Although supplementing a microbial culture with an expensive precursor might be feasible for a small-scale experiment, it severely hinders industrial applicability. However, if an inexpensive, readily available intermediate can be utilized as a precursor, an entirely fermentative process with lower titers might not be justifiable. A metabolic engineer must then weigh the impact of generating a complex product entirely from primary metabolites versus the value associated with significantly higher production levels. As will be seen throughout this chapter, research efforts are often initiated with intermediate supplementation in order to limit confounding variables, and full fermentative pathways are constructed after significant breakthroughs are achieved and once distinct metabolic pathways can be connected *in vivo*.

The experiment described in the beginning of this section involved the incorporation of four heterologous genes: *S. cerevisiae* PAL, *Streptomyces coelicolor* A3 cinnamate/coumarate:CoA ligase (ScCCL) with substrate specificity toward both cinnamic acid **5** and *p*-coumaric acid **6**, licorice plant (*Glycyrrhiza echinata*) CHS,

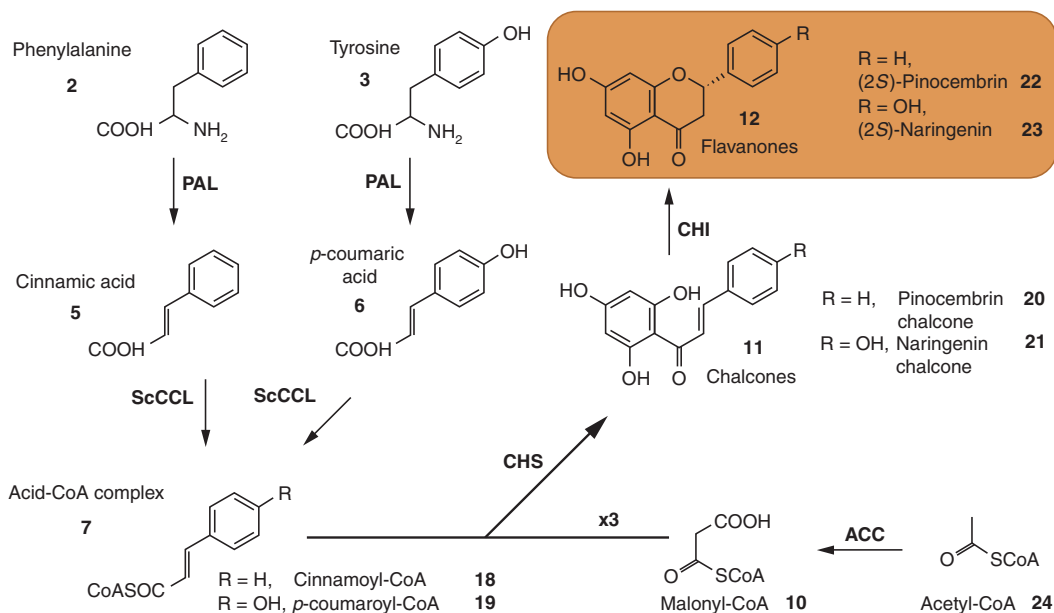


Fig. 54.2 Artificial construction of plant flavanone **12** biosynthetic pathway in microbes

and *Pueraria lobata* CHI. Transformation of *E. coli* with a plasmid harboring these four heterologous genes coupled with overexpression of the *Corynebacterium glutamicum* gene encoding two acetyl-CoA carboxylase subunits, accBC and dtsR1, produced $\sim 60 \text{ mg L}^{-1}$ of the flavanones **12** (2S)-naringenin **23** and (2S)-pinocembrin **22**. The artificial biosynthetic pathway constructed for plant flavanone **12** biosynthesis in microbes is shown in Fig. 54.2. Acetyl-CoA carboxylase (ACC) was selected for overexpression to increase the intracellular pool of malonyl-CoA **10**, which is required for synthesis of flavanones **12** from either 4-coumaroyl-CoA **19** or cinnamoyl-CoA **18**. Further introduction of FSI from *Petroselinum crispum*, FHT from *Citrus sinensis*, and FLS from *Citrus unshiu* produced the flavones **14** apigenin and chrysin, as well as the flavonols **15** kaempferol and galangin in low concentrations [80, 84]. This seminal work has enabled the production in *E. coli* and *S. cerevisiae* of many valuable phenylpropanoid compounds, including natural and non-natural flavones **14**, flavonols **15**, anthocyanins **17**, stilbenoids **8**, and curcuminoids **9** [42, 60, 65, 82, 84–107]. As this chapter focuses on isoflavonoids, however, the reader is directed to a detailed review of microbial biosynthesis of other valuable plant phenylpropanoids by Limem et al. [43].

3.2 Engineering the Plant Isoflavonoid Pathway in Microbes

3.2.1 Production of Isoflavonoid Aglycones in Microbes

The successful construction of an artificial plant flavonoid biosynthetic pathway in microbes, combined with the first report of functional activity of IFS in yeast microsomes by Akashi and coworkers in 1999, paved the way for high-level isoflavonoid production [69]. However, a significant barrier to prokaryotic

expression of IFS hampered progress and precluded taking advantage of the high growth rate of *E. coli* and the abundance of molecular biology tools available for the microbe. IFS is a membrane-bound cytochrome P450 that requires an electron transfer system that is not present in bacterial cells; thus, coexpression of functional IFS with the flavanone **12** pathway in recombinant *E. coli* required creative engineering solutions. Eukaryotic microbes like *S. cerevisiae* and other unicellular fungi possess the requisite machinery for cytochrome P450 enzyme expression; specifically, they constitutively express an endogenous cytochrome P450 reductase (CPR) that is an integral redox partner for IFS and other cytochrome P450s, and they possess an endoplasmic reticulum (ER) on which the N-terminal signal-anchor peptide sequences of cytochrome P450 enzymes can bind [108, 109].

Katsuyama et al. overcame this impediment by coculturing a flavanone-producing *E. coli* strain with recombinant *S. cerevisiae* transformed with a T7-inducible plasmid harboring IFS from *G. echinata*. To demonstrate the production of the isoflavone **13** genistein **26** and the feasibility of cocubation, the yeast strain was first transformed with a pESC vector containing the genes CHS from *G. echinata*, CHI from *P. lobata*, and IFS from *G. echinata*, all under the control of galactose-inducible *GAL* promoters. Growth under supplementation with the precursor, *N*-acetylcysteamine-attached *p*-coumarate (*p*-coumaroyl-NAC), yielded $\sim 342 \mu\text{g L}^{-1}$ genistein **26**. To examine the possibility of production without precursor feeding, a naringenin-producing *E. coli* strain (57 mg L^{-1} of (2*S*)-naringenin **23**) was constructed as described in the previous section and cocultured with a recombinant yeast strain transformed with a vector containing *G. echinata* IFS under control of a galactose-inducible *GAL* promoter [80]. Simultaneous incubation of equal weights of engineered *E. coli* and *S. cerevisiae*, in addition to supplementation of the coculture media with 3 mM tyrosine **3** as a substrate for *E. coli*, yielded $\sim 6 \text{ mg L}^{-1}$ of genistein **26** [110]. This “one-pot synthesis” scheme for production of genistein **26** from tyrosine **3** represented the most valuable microbial isoflavonoid production platform at the time of its publication. Optimization of coculture conditions subsequently improved genistein **26** production up to 100 mg L^{-1} [111].

In order to produce isoflavonoids in a model plant, a native flavonoid pathway must be hijacked by diverting a common precursor away from its natural product and toward the desired isoflavonoid product. Tian and colleagues accomplished production of genistein **26** in the nonleguminous, model plant tobacco through protein engineering of a fusion between IFS and CHI [112]. The spatial proximity between CHI and IFS was engineered to increase the local concentration of the IFS substrate, naringenin **23**, such that the production of nonnative genistein **26** was favored over the dominant, endogenous pink anthocyanin **17** accumulation pathway. Localization of the protein chimera at the ER was maintained by constructing the fusion with IFS at the N-terminus so its innate, hydrophobic N-terminal membrane anchor was free to target the ER as usual [87, 113]. A flexible linker peptide composed of glycine-serine-glycine (Gly-Ser-Gly) residues connected the C-terminus of IFS with the N-terminus of CHI to ensure proper folding of the two independent catalytic domains. Expression of this engineered protein fusion in transgenic tobacco successfully shifted flavonoid

accumulation toward isoflavonoids and enabled production of isoflavonoids in nonleguminous plants. Yeast expression of the protein fusion under precursor supplementation conditions also produced isoflavonoids and highlighted the possibility to utilize protein engineering to improve plant natural product titers in microbes [112].

Although *E. coli* and *S. cerevisiae* have both been utilized as model organisms for plant flavonoid production, it is often beneficial to express entire biosynthetic pathways in a single organism to avoid bidirectional metabolite transport limitations through the cell walls of two organisms simultaneously and to obviate media optimization for two different species at once. Functional expression of IFS in *E. coli* would eliminate the necessity for coculture with yeast. As such, Leonard and colleagues designed and expressed a set of artificial P450 enzymes that enabled robust biosynthesis of the isoflavones **13** genistein **26** and daidzein **27** from the flavanones **12** naringenin **23** and liquiritigenin in *E. coli* for the first time [114]. Two challenges to functional prokaryotic expression of eukaryotic cytochrome P450 enzymes were overcome in this research: the translational fusion of *Catharanthus roseus* CPR to *Glycine max* IFS spatially organized the redox partners for efficient electron shuttling from nicotinamide adenine dinucleotide phosphate (NADPH) to substrate, and rational design of several IFS N-terminal membrane signal sequences modulated activity of the protein fusion, enabling selection of a high-level isoflavone **13** producing chimera [114].

The protein engineering effort began with deletion of 71N-terminal amino acids from CPR to minimize membrane association without hindering catalytic activity. A glycine-serine-threonine (Gly-Ser-Thr) linker sequence was then designed to connect the CPR N-terminus with the IFS C-terminus while thwarting any secondary structure formation that could lead to incorrect folding of the two domains. The protein fusion was then truncated by a varying number of residues from the N-terminus of IFS, and two peptide leader sequences (one mammalian and one endogenous) were independently appended to these constructs in a semicombinatorial manner. Each chimera was separately expressed in *E. coli* and evaluated for production of isoflavone **13** from supplemented precursor. The most prominent fusion produced 10 and 18 mg g⁻¹ (dry cell weight) of genistein **26** and daidzein **27**, respectively, and consisted of the deletion of 6 membrane-anchor residues and the addition of an 8 residue mammalian leader sequence to the N-terminus of IFS. To determine a baseline production level, plant IFS and CPR were coexpressed in *E. coli* and found to yield negligible isoflavonoid concentrations compared to the engineered strain. *S. cerevisiae* coexpressing plant IFS and CPR produced isoflavones **13** at low concentrations approaching those of the poorly performing protein fusion constructs expressed in *E. coli*. After accounting for the significantly higher biomass of yeast versus *E. coli* in minimal media, the specific production level of isoflavones **13** in *E. coli* represented approximately 20-fold increase over yeast [114]. The methodology implemented in this work provides an approach for soluble expression of other eukaryotic membrane-bound cytochrome P450s in prokaryotes. Although not performed in this set of experiments, this research facilitated the impending construction of a complete artificial biosynthetic pathway from aromatic amino acids to isoflavonoids in a single microorganism.

A later report of functional expression of IFS in prokaryotes involved construction of a protein fusion between red clover IFS (RCIFS) and rice CPR (RCPR) in *E. coli* [115]. This work built upon previous results demonstrating that coexpression in yeast of IFS with CPR from rice can convert 100 μM naringenin **23** to 77 μM genistein **26**, research predicated on the hypothesis that a plant CPR, as opposed to a constitutively expressed yeast CPR, would interact more efficiently with a plant IFS [103]. In this project RCIFS was truncated by deletion of the codons for the first 21 amino acids on the N-terminus, a sequence predicted to code for a helical region as indicated by computational secondary structure analysis. Changing the first remaining codon to a start codon (encoded by the nucleotide sequence ATG) enabled functional expression of RCIFS in *E. coli*, while removal of the IFS stop codon and addition of a Gly-Ser-Thr linker sequence followed by the RCPR coding sequence (also with the N-terminal membrane binding domain deleted) enabled expression and proper folding of the two fused domains. It should be noted here that this protein fusion design differs from that constructed previously by Leonard primarily because, in this case, the hydrophobic N-terminal membrane-associated domains were entirely removed from both enzyme constituents in the fusion to enhance solubility of the final construct. The functional expression and spatial proximity afforded by the soluble RCIFS-RCPR protein fusion enabled conversion of 80 μM naringenin **23** into 56 μM genistein **26** in *E. coli*. Difference in conversion between yeast and *E. coli* was not investigated but could be due to disparate expression and growth levels in the two distinct species. Again, it is likely that higher-efficiency electron transfer from NADPH to substrate occurred in *E. coli* due to the conjoined RCPR and RCIFS domains [115].

Coexpression in *S. cerevisiae* of all seven genes in the artificial isoflavone **13** pathway (PAL and CPR from a hybrid poplar, *Populus trichocarpa* \times *Populus deltoides* and C4H, 4CL, CHS, CHI, and IFS from soybean, *G. max*), with phenylalanine **2** supplementation, was ultimately achieved by Trantas et al. and marked the first reported reconstitution of an entire isoflavonoid biosynthetic pathway in microbes. Although yeast contains a chromosomal copy of CPR, coexpression of a heterologous CPR from a the hybrid poplar increased *p*-coumaric acid **6** production fourfold, once again demonstrating the advantage of selecting a plant CPR to improve activity of the other enzymes in the cytochrome P450 metabolon [101, 116]. Only 0.1 mg L^{-1} genistein **26** was produced when the cultures were fed with phenylalanine **2** versus 7.7 mg L^{-1} when fed with naringenin **23**, suggesting the presence of at least one limiting enzyme or that cellular metabolism was burdened by the genes upstream of naringenin **23**. On average, the yeast strains in this work consumed 3.4 mmol L^{-1} phenylalanine **2**, while the wild-type strain consumed 2.8 mmol L^{-1} , a difference in phenylalanine **2** uptake of 0.8 mmol L^{-1} that can be attributed to flux through the heterologous flavonoid pathway. Stoichiometrically, this should lead to 0.8 mmol L^{-1} genistein **26**, but production of only 0.4 $\mu\text{mol L}^{-1}$ indicated approximately 0.05 % efficiency of conversion of phenylalanine **2** to genistein **26** through the artificial biosynthetic pathway. Measurement of some upstream intermediates showed 83 % flux efficiency through PAL and C4H, efficient

conversion through 4CL as deduced from the rapid depletion of *p*-coumaric acid **6**, and approximately 8 % efficiency to naringenin **23**, which suggests that CHS or CHI are rate limiting but could not be confirmed due to the inability to quantify concentrations of the intermediate compounds 4-coumaroyl-CoA **19** and naringenin chalcone **21** [101]. As described by Akashi, coexpression of an HIDH in this engineered *S. cerevisiae* strain could potentially accelerate the spontaneous conversion of naringenin **23** to genistein **26** but was not attempted in this work [71].

The first attempt to coexpress HIDH with IFS and CPR confirmed this speculation. Chemler and coworkers coexpressed IFS, CPR, and HIDH from five various plant sources in yeast in a combinatorial fashion to determine the impact of gene source on individual enzyme activity and coupled enzyme activities [117]. IFS-encoding genes from *G. max*, *Trifolium pratense*, *G. echinata*, *Pisum sativa*, and *Medicago truncatula* were individually cloned into a pYES2.1 vector under control of the *GAL1* promoter and transformed into *S. cerevisiae* strain INVSc1. After growth on minimal medium, the cultures were induced with galactose and supplemented with naringenin **23**. Genistein **26** production was monitored, and *T. pratense* IFS was selected as the best enzyme because it showed significantly higher in vivo activity than the IFS enzymes from other sources. Since it had previously been shown that plant IFS activity in yeast is improved upon coexpression of plant CPR, the researchers coexpressed CPR from *C. roseus* and *G. max* with IFS from either *G. max* or *T. pratense* to determine the enzyme pair with highest coupled activity. Upon comparing genistein **26** production in these engineered strains to yeast expressing IFS with endogenous CPR, the strain coexpressing *T. pratense* IFS with *G. max* CPR was found to be the highest producer at 15 mg L⁻¹ day⁻¹. This illustrates the value in combining different gene sources to determine optimal protein pairing, particularly in the case of enzyme-mediated redox reactions. To assess whether expression of plant HIDH could increase genistein **26** production over its spontaneous generation from its 2-hydroxyisoflavanone precursor in yeast, coexpression of *G. max* or *G. echinata* HIDH was evaluated in the engineered strains. The best triple-enzyme combination was found to include *T. pratense* IFS, *G. max* CPR, and *G. max* HIDH, followed closely by the cognate combination of *G. max* IFS, CPR, and HIDH. Interestingly, *T. pratense* IFS holds some advantage over *G. max* IFS when coexpressed with *G. max* CPR and HIDH, despite presumption that the *G. max* enzymes evolved to work optimally together. Ultimately the three-enzyme combination showed greater than tenfold improvement in production rate over expression of IFS alone, but total production in all strains maximized at around 35 mg L⁻¹ genistein **26**. After further experimentation, it was shown that isoflavones **13** like genistein **26** and biochanin A **29** strongly inhibit conversion of naringenin **23** by IFS in yeast. It was speculated that isoflavone **13** glycosylations, methylations, and other enzymatic biotransformations might ameliorate product inhibition and increase overall isoflavonoid production [117]. The basic artificial biosynthetic pathway for plant isoflavone **13** production from flavanones **12** in microbes is illustrated in Fig. 54.3.

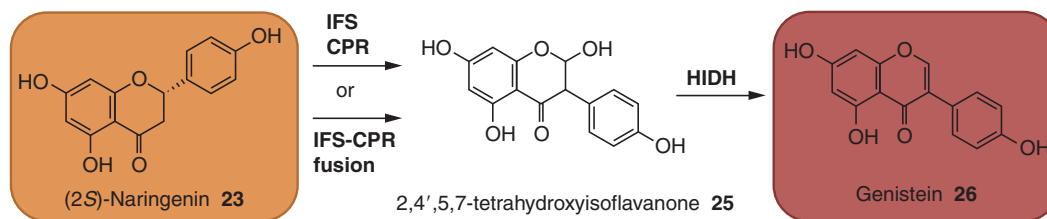


Fig. 54.3 Aggregate artificial biosynthetic pathway for plant isoflavone **13** production from flavanones **12** in microorganisms

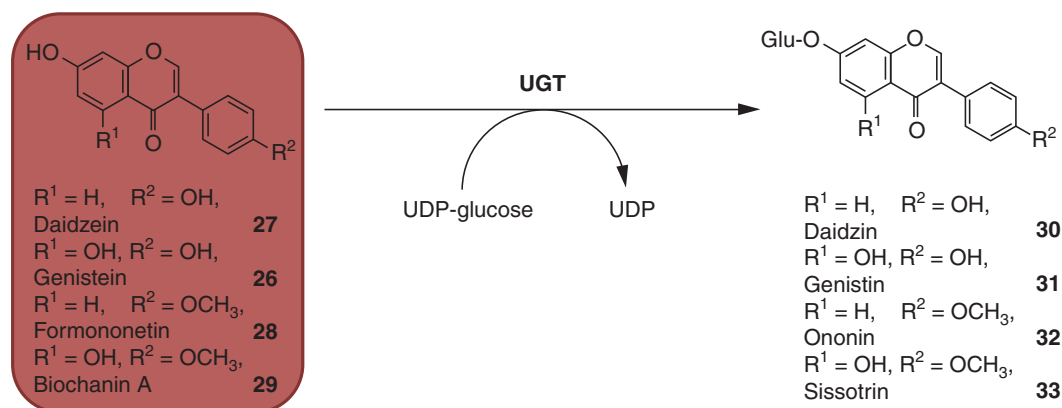


Fig. 54.4 Microbial bioconversion of isoflavone **13** aglycones to isoflavone **13** glycosides

3.2.2 Production of Isoflavonoid Glycosides in Microbes

Many flavonoids and other secondary metabolites exist as glycosides in plants, and examples of engineered microbial glycosylation of various flavonoids like quercetin and anthocyanidins have been reported over the last decade [85, 96, 118, 119]. Glycosylation of flavonoid aglycones is important because it often increases mammalian bioavailability, solubility, and stability [68, 120–129]. In the first example of microbial isoflavonoid glycosylation, expression of UGT71G1, a uridine diphosphate glycosyltransferase (UGT) from the model legume *M. truncatula*, in heterologous *E. coli* with supplementation of genistein **26** and biochanin A **29** yielded mg quantities at greater than 70 % conversion to genistein **26** and biochanin A **29** 7-*O*-glucosides (genistin **31** and sissotrin **33**, respectively) after 24-h incubation (Fig. 54.4). Terrific broth (TB) culture medium supported higher growth than Luria-Bertani (LB) culture medium and thus provided 3.5-fold higher yield of 7-*O*-glucoside. Scale-up to 500 mL culture achieved conversion rates of 30–60 %, about 80 % efficient compared to small scale, yielding up to 20 mg L⁻¹ of isoflavanone glycosides [98]. Of note, 90 % of the glycosylated products were secreted from the cell, enabling facile collection and suggesting that increased solubility or sugar moiety-related signaling affects efflux from the cell. As such, this work suggests that coexpression of *M. truncatula* UGT71G1 in Chemler's engineered yeast strain (*T. pratense* IFS, *G. max* CPR, *G. max* HIDH) could convert naringenin **23** to genistin **31**, the genistein **26** 7-*O*-glucoside, at much higher rates than previously reported because feedback inhibition would

be minimized by the glycosylation and subsequent export from the cell. Whereas extraction of plant flavonoid glycosides is inefficient, and regioselective glycosylation of flavonoids through chemical synthesis methods requires intermittent blocking and deblocking of hydroxyl groups and yields only about 50 % conversion due to the occurrence of nonspecific glycosylations, glycosylation through biotransformation offers a highly efficient and cheap alternative [130–133]. A major barrier to high-level microbial production of any flavonoid glycoside, however, is intracellular supply of uridine diphosphate (UDP) glucose.

As seen in Fig. 54.4, nucleotide-activated sugars are required as donors for glycosylation. In previous work, Yan and colleagues engineered a four-step metabolic pathway for plant anthocyanin **17** biosynthesis in *E. coli*, which involved expression of four heterologous genes including *Malus domestica* FHT and ANS, *Anthurium andraeanum* DFR, and *Petunia hybrida* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT). Anthocyanidins were converted by 3GT to the first stable glycosidic anthocyanins **17** in the flavonoid biosynthetic pathway, pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside [85]. The researchers identified UDP-glucose as the rate-limiting step in anthocyanin **17** biosynthesis in *E. coli* and thereafter optimized UDP-glucose production by supplementing with orotic acid, a cheap uridine triphosphate (UTP) precursor, and performing a gene deletion and a set of gene overexpressions. As synthesis of UDP-glucose interfaces nucleotide biosynthesis, the pentose phosphate pathway, glycolysis, and energy production pathways, engineering its overproduction is a nontrivial task. Episomal overexpression of endogenous phosphoglucumutase (PGM) and glucose-1-phosphate uridylyltransferase (GALU), which convert glucose-6-phosphate (G6P) to glucose-1-phosphate (G1P) and produce UDP-glucose from G1P and UTP, respectively, shunted carbon flux from the pentose phosphate pathway toward UDP-glucose through the G6P branching point [57, 134]. These genetic modifications combined with the overexpression of endogenous nucleoside diphosphate kinase (NDK), the limiting step in the linear UTP synthesis pathway by orotic acid assimilation, and deletion of a gene encoding UDP-glucose dehydrogenase (UDG), which consumes UDP-glucose to form UDP-glucuronic acid, to yield increased UDP-glucose accumulation of 104 mg L⁻¹ [96, 135, 136]. Due to the natural production of UDP-glucose in *E. coli* for cell wall synthesis and the ability to achieve increased production of UDP-glucose, microbial glycosylation of isoflavonoid aglycones with heterologous glycosyltransferases is an economically viable option. To the best of our knowledge, Table 54.1 summarizes the most representative studies of microbial production of plant natural isoflavonoids to date.

4 Mutasynthesis and Protein Engineering for Non-natural Isoflavonoid Production in Microbes

Mutasynthesis is a common semisynthetic tool that hijacks natural product biosynthesis through the feeding of non-natural substrate analogs to produce non-natural analogs to natural products. This methodology takes advantage of the natural allowable range of

Table 54.1 Reports demonstrating microbial production of plant natural isoflavonoids

Isoflavonoid target	Precursor	Host organism	Genes: Donors	Reference
Genistein 26	<i>N</i> -acetylcysteamine-attached <i>p</i> -coumaric acid	<i>E. coli</i>	CHS: <i>G. echinata</i> IFS: <i>G. echinata</i> CHI: <i>P. lobata</i>	[110]
Genistein 26	Tyrosine 3	<i>E. coli</i> and <i>S. cerevisiae</i> coculture	PAL: <i>R. rubra</i> 4CL: <i>S. coelicolor</i> CHS: <i>G. echinata</i> CHI: <i>P. lobata</i> IFS: <i>G. echinata</i> ACC: <i>C. glutamicum</i>	[110]
Genistein 26	Naringenin 23	<i>S. cerevisiae</i>	IFS: <i>T. pratense</i> CPR: <i>O. sativa</i>	[103]
Genistein 26	Phenylalanine 2	<i>S. cerevisiae</i>	PAL: <i>P. trichocarpa</i> × <i>P. deltoides</i> CPR: <i>P. trichocarpa</i> × <i>P. deltoides</i> C4H: <i>G. max</i> 4CL: <i>G. max</i> CHS: <i>G. max</i> CHI: <i>G. max</i> IFS: <i>G. max</i>	[101]
Genistein 26	<i>p</i> -coumaric acid 6	<i>S. cerevisiae</i>	PAL: <i>P. trichocarpa</i> × <i>P. deltoides</i> CPR: <i>P. trichocarpa</i> × <i>P. deltoides</i> C4H: <i>G. max</i> 4CL: <i>G. max</i> CHS: <i>G. max</i> CHI: <i>G. max</i> IFS: <i>G. max</i>	[101]
Genistein 26	Naringenin 23	<i>S. cerevisiae</i>	PAL: <i>P. trichocarpa</i> × <i>P. deltoides</i> CPR: <i>P. trichocarpa</i> × <i>P. deltoides</i> C4H: <i>G. max</i> 4CL: <i>G. max</i> CHS: <i>G. max</i> CHI: <i>G. max</i> IFS: <i>G. max</i>	[101]
Genistein 26 , Daidzein 27	Naringenin 23 , Isoliquiritigenin	<i>S. cerevisiae</i>	CHI ^a : <i>M. sativa</i> IFS ^a : <i>G. max</i>	[112]
Genistein 26	Naringenin 23	<i>S. cerevisiae</i>	IFS: <i>G. max</i>	[137]
Genistein 26 , Daidzein 27	Naringenin 23 , Liquiritigenin	<i>S. cerevisiae</i>	IFS: <i>G. echinata</i>	[69]

(continued)

Table 54.1 (continued)

Isoflavonoid target	Precursor	Host organism	Genes: Donors	Reference
Genistein 26 , Daidzein 27	Naringenin 23 , Liquiritigenin	<i>E. coli</i>	CPR ^a : <i>C. roseus</i> IFS ^a : <i>G. max</i>	[114]
Genistein 26 , Daidzein 27	Naringenin 23 , Liquiritigenin	<i>S. cerevisiae</i>	CPR: <i>C. roseus</i> IFS: <i>G. max</i>	[114]
Genistein 26	Naringenin 23	<i>S. cerevisiae</i>	CPR ^a : <i>O. sativa</i> IFS ^a : <i>T. pratense</i>	[116]
Genistin 31 , Sissotrin 33	Genistein 26 , Biochanin A 29	<i>E. coli</i>	UGT: <i>M. truncatula</i>	[98]
See Table 54.2	See Table 54.2	<i>S. cerevisiae</i>	CPR: <i>G. max</i> IFS: <i>T. pratense</i> HIDH: <i>G. max</i>	[117]

^aProtein fusion

enzyme-substrate specificity and favors highly promiscuous enzymes that can convert non-natural analogs of the natural substrate to novel products. Since many plant natural products possess valuable medicinal properties, it is of significant interest to explore the space of non-natural product analogs that has not yet been evolutionarily surveyed because of the lack of non-natural substrates in the environment. Presumably some of these non-natural analogs could have enhanced or even unique pharmaceutical properties. Production of flavonoids using mutasynthesis or substrate feeding has been accomplished by several groups as reported elsewhere [60, 82, 97, 100].

Structural studies often utilize protein engineering tools such as site-directed mutagenesis to evaluate the roles of various amino acid residues in catalytic mechanisms. While this can furnish indispensable insight on enzyme-substrate interaction, it is of significant interest to metabolic engineers because it also enables construction of tailor-made enzyme mutants with improved kinetic properties, with the ability to accept structurally related substrates, with reaction reversibility for substrate-product interconversion, and with altered substrate and product regiospecificity. Protein engineering tools such as site-directed mutagenesis and directed evolution have been applied to improve production of both natural and non-natural flavonoid, isoflavonoid, and other plant natural product derivatives [87, 138–147]. Plant natural products can also be microbially catalyzed by enzymes native to the microbe to form compounds not known to exist in plants [82, 100, 119, 148–153]. These phytochemical derivatives have the potential to be utilized as human therapeutics, as the microbes catalyzing these novel reactions have been isolated from the human gut and are purported to have beneficial health impacts on their human hosts [152–157].

4.1 Mutasynthesis for Non-natural Isoflavonoid Production

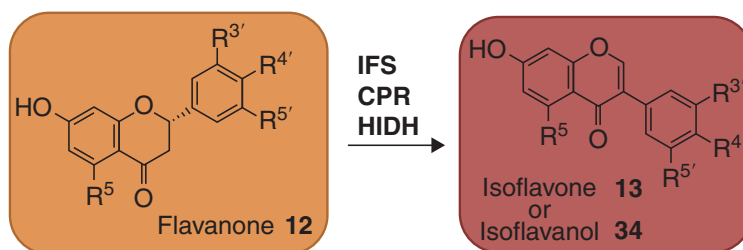
Mutasynthesis involves the chemical synthesis of non-natural substrates that are similar in structure to natural substrates. After a library of non-natural analogs

are chemically synthesized, enzymatic conversion of the non-natural analogs is performed to isolate novel non-natural compounds, and the results can then be assessed to elucidate mechanisms of enzymatic catalysis and to determine substrate specificity requirements. This so-called semisynthetic approach, or the combination of chemical synthesis and biosynthesis, has also been utilized for production of non-natural isoflavonoids.

In a multiplex experiment, Chemler and colleagues evaluated the substrate specificity of IFS enzymes from five different plant species (*G. max*, *T. pratense*, *G. echinata*, *P. sativa*, and *M. truncatula*) [117]. Each enzyme was cloned into yeast and was supplemented with compounds from a library of natural and non-natural flavanones **12**. Non-natural flavanones **12** were synthesized to mimic natural flavanones **12** and isoflavones **13**; specifically, many library constituents were 7-monohydroxylated or 5,7-dihydroxylated. The library also consisted of flavanones **12** with B-ring substituents, such as single or multiple hydroxy, methoxy, ethoxy, and halide side groups. Ultimately 19 non-natural flavanones **12** and 7 natural flavanones **12** were utilized to assess IFS substrate flexibility, resulting in the biosynthesis of 4 natural isoflavones **13** and 14 non-natural isoflavone **13** analogs which are tabulated in Table 54.2. IFS substrate requirements were deduced from the rate of conversion of different flavanones **12**, including the necessity for hydroxylation at C7, the expendability of C5 hydroxylation, the incompatibility of C2' or C6' substitutions, the toleration of small side-group substitutions at C3' or C5', and the absolute requirement of C4' hydroxylation for production of 2-hydroxyisoflavones. Due to the high affinity of genistein **26** for human estrogen receptors α (hER α) and β (hER β), isoflavones **13** are selective estrogen receptor modulator (SERM) drug candidates [158–161]. SERMs can be used to inhibit or stimulate estrogen receptors, thereby enabling their use as hormone replacements and decreasing the risk of diseases such as osteoporosis and breast cancer [17, 50, 53, 160]. In an effort to determine the therapeutic potential of the semisynthetic isoflavones **13** in the previously described library, the interaction of each compound with hER α and hER β was assessed using an in vitro competitive binding assay. As expected, the different isoflavones **13** were found to show variable activity against the human estrogen receptors. Of particular interest, both 3'-bromo-4',5,7-trihydroxyflavone and the natural isoflavone **13** orobol displayed binding capabilities equal to genistein **26**. Structure-activity relationships between isoflavones **13** and hERs were then deduced to yield insight for future design of isoflavone **13** SERMs. Of note, the authors suggest that novel isoflavones **13** with small substituents at the C3' position should elicit improved interactions with estrogen receptors [117].

4.2 Protein Engineering for Non-natural Isoflavonoid Production

Protein engineering has been utilized to study the mechanism by which isoflavonoid aglycones are converted to isoflavonoid glycosides by uridine diphosphate glycosyltransferases, a large protein class catalyzing the transfer of activated

Table 54.2 Mutasyntesis for natural and non-natural isoflavonoid production

Flavanone precursor	Side-group decoration				Primary biotransformation product
	R ⁵	R ^{3'}	R ^{4'}	R ^{5'}	
Naringenin 23	OH	H	OH	H	Genistein 26
Liquiritigenin	H	H	OH	H	Daidzein 27
Eriodictyol	OH	OH	OH	H	Orobol
Butin	H	OH	OH	H	3',4',7-Trihydroxyisoflavone
Homoeriodictyol	OH	OCH ₃	OH	H	3'-Methoxy-4',5,7-trihydroxyisoflavone
4',7-Dihydroxy-3'-methoxyflavanone	H	OCH ₃	OH	H	4',7-Dihydroxy-3'-methoxyisoflavone
3',5'-Dimethoxy-4',5,7-trihydroxyflavanone	OH	OCH ₃	OH	OCH ₃	3',5'-Dimethoxy-4',5,7-trihydroxyisoflavone
4',7-Dihydroxy-3',5'-dimethoxyflavanone	H	OCH ₃	OH	OCH ₃	4',7-Dihydroxy-3',5'-dimethoxyisoflavone
3'-Ethoxy-4',5,7-trihydroxyflavanone	OH	OCH ₂ CH ₃	OH	H	3'-Ethoxy-4',5,7-trihydroxyflavanone
4',7-Dihydroxy-3'-ethoxyflavanone	H	OCH ₂ CH ₃	OH	H	4',7-Dihydroxy-3'-ethoxyflavanone
3'-Methyl-4',5,7-trihydroxyflavanone	OH	CH ₃	OH	H	3'-Methyl-4',5,7-trihydroxyisoflavone
4',7-Dihydroxy-3'-methylflavanone	H	CH ₃	OH	H	4',7-Dihydroxy-3'-methylisoflavone
3',5'-Dimethyl-4',5,7-trihydroxyflavanone	OH	CH ₃	OH	CH ₃	3',5'-Dimethyl-4',5,7-trihydroxyisoflavone
4',7-Dihydroxy-3',5'-dimethylflavanone	H	CH ₃	OH	CH ₃	4',7-Dihydroxy-3',5'-dimethylisoflavone
3'-Chloro-4',5,7-trihydroxyflavanone	OH	Cl	OH	H	3'-Chloro-4',5,7-trihydroxyisoflavone
3'-Chloro-4',7-dihydroxyflavanone	H	Cl	OH	H	3'-Chloro-4',7-dihydroxyisoflavone
3'-Bromo-4',5,7-trihydroxyflavanone	OH	Br	OH	H	3'-Bromo-4',5,7-trihydroxyisoflavone
3'-Bromo-4',7-dihydroxyflavanone	OH	Br	OH	H	3'-Bromo-4',7-dihydroxyisoflavone

sugars to various substrates. These studies yield insight into the interactions between specific amino acid residues and substrate, enabling rational design of enzyme mutants for specific purposes. Structure-guided enzyme engineering is often directed at or around the active site or binding pocket region to alter substrate specificity, enzymatic activity, and product regioselectivity. In the case of UGT71G1 from *M. truncatula*, a point mutation in residue 202 from tyrosine (Tyr) **3** to alanine (Ala), Y202A, enables the conversion of genistein **26** to both 7-*O*-glucoside and 5-*O*-glucoside, whereas the native enzyme only enables conversion of genistein **26** to the 7-*O*-glucoside product. Residue 202 is located at one end of the acceptor (isoflavonoid aglycone) binding pocket, so this mutation from an amino acid with a large aromatic side group to one with a small side group presumably increases the volume of the pocket, providing the acceptor with an increased number of possible docking configurations [140].

Another protein engineering effort for isoflavonoid production focused on *M. truncatula* UGT85H2. A point mutation in residue 305 from isoleucine (Ile) to threonine (Thr), I305T, showed a 19-fold increase in enzyme activity with a 25-fold decrease in the Michaelis constant (K_m) for conversion of biochanin A **29** into sissotrin **33**. Additionally the mutation of residue 200 from valine (Val) to glutamic acid (Glu), V200E, imparted deglycosylation activity in the presence of UDP in the reaction mixture, enabling the removal of the glucose residue from sissotrin **33**, the biochanin A **29** 7-*O*-glucoside, to form biochanin A **29** aglycone. The mutation also decreased K_m by sevenfold, increased maximum velocity (V_{max}) and turnover number (k_{cat}) by sevenfold, and increased catalytic efficiency by 54-fold. Amino acid 200 resides on one end of the acceptor binding pocket, and docking studies indicate that the negatively charged glutamic acid side group might interact with the 7-OH of biochanin A **29**. This novel method utilizing mutagenesis to impart reversibility could be applied to deglycosylation of other flavonoids [141].

The aforementioned UGT mutagenesis studies involved variations in activity and regioselectivity. However, glycosylation of flavonoids with sugars other than glucose occurs in nature and should be possible to engineer in microbes. In addition to UDP-glucose, for instance, UDP-glucuronic acid, UDP-galactose, UDP-xylose, and UDP-rhamnose are all known to act as nucleotide-activated sugar donors in various plant species [162]. In *Bellis perennis* (red daisy) BpUGT94B1, the positively charged guanidinium side group of a single arginine (Arg) residue at position 25 is critical for UDP-glucuronic acid donor activity due to its interaction with the negatively charged carboxylate group on glucuronic acid [139]. Similarly, a family of UGTs known as flavonoid 7-*O*-glucuronosyltransferases (F7GATs) found in plants from the Lamiales order share a conserved arginine residue in the sugar donor binding pocket that is responsible for the specificity toward UDP-glucuronic acid. Site-directed mutagenesis of *Perilla frutescens* UGT88D7 residue 350 containing arginine (which corresponds to tryptophan (Trp) 360 in UGT71G1) to Trp abolished UDP-glucuronic acid specificity and instead invoked UDP-glucose sugar donor specificity. Once again the cationic guanidinium moiety on arginine is crucial for recognition and interaction with the anionic carboxylate group on UDP-glucuronic acid.

4.3 Other Isoflavonoid Biotransformations

In a series of recent reports, a G6P isomerase (PGI, catalyzing the isomerization of G6P to fructose-6-phosphate) knockout strain of *E. coli* was engineered to produce flavonoid glycosides from flavonoid aglycones. Specifically, the strain produced 7-*O*-xylosyl naringenin and 7-*O*-glucuronyl quercetin by overexpressing an *Arabidopsis thaliana* UGT and an artificial UDP-sugar biosynthetic gene cluster (containing *E. coli* K-12 GALU and *Micromonospora echinospora* spp. *calichensis* UDG and UDP-glucuronic acid decarboxylase, known as UXS1) in combination with naringenin **23** and quercetin feeding [163, 164]. Continuing their efforts, Simkhada and coworkers recently engineered *E. coli* for production of 3-*O*-rhamnosyl quercetin, 3-*O*-rhamnosyl kaempferol, and 3-*O*-allosyl quercetin by assembling artificial thymidylidiphosphate (TDP)-sugar biosynthetic pathways for TDP-L-rhamnose and TDP-6-deoxy- β -D-allose and feeding the strain with quercetin and kaempferol aglycones.

TDP-sugar production was enabled by the deletion of PGI to shunt flux toward G1P and overexpression of TDP-glucose synthase (TGS) from *Thermus caldophilus* GK24 to form the activated nucleotide sugar [165]. TDP-L-rhamnose was produced by overexpression of *Salmonella typhimurium* LT2 TDP-glucose 4,6-dehydratase (DH) and *Streptomyces antibioticus* Tu99 TDP-4-keto-6-deoxyglucose 3,5-epimerase (EPI) and TDP-glucose 4-ketoreductase (KR); TDP-6-deoxy- β -D-allose was produced by overexpression of *T. caldophilus* GK24 DH and *Streptomyces* sp. KCTC 0041BP TDP-hexose 3-epimerase (GERF) and TDP-4-keto-6-deoxyglucose reductase (GERK). Overexpression of a 3GT from *A. thaliana* completed the 3-*O*-glycosylation of the flavonoid aglycone precursors with the TDP-sugars [166]. These engineering efforts demonstrate the potential for regiospecific glycosylation of isoflavonoids with tailored sugar moieties that could one day enable design of therapeutics with altered activities and varying degrees of bioavailability; from a microbial production perspective, customizable glycosylations might also mitigate cellular toxicity while improving isoflavonoid solubility, stability, and transport from the cell, ultimately leading to higher product yields [126, 167].

Other flavonoid biotransformations catalyzed by microbial enzymes will also allow for production of novel, nonplant flavonoids from amino acid precursors. Two bacterial nonheme dioxygenases, biphenyl dioxygenase (BDO) and naphthalene dioxygenase (NDO), have recently been shown to regioselectively and stereoselectively convert flavonoids, including isoflavones **13** and isoflavanols **34**, to epoxides and dihydrodiols [151, 168–172]. BDO from *Pseudomonas pseudoalcaligenes* KF707 and NDO from *Pseudomonas* sp. strain NCIB9816-4 are able to accept various flavonoids as substrates due to the presence of biphenyl and naphthalene moieties within the flavonoid core structure **1** [168]. Additionally, expression of *Streptomyces avermitilis* MA-4680 7-*O*-methyltransferase (SaOMT-2) in *E. coli* shows substrate promiscuity and transfers a methyl group to flavones **14** and isoflavones **13** [173]. This is the first example of a methyltransferase known to act upon both flavones **14** and isoflavones **13**, opening up a route for biosynthesis of

non-natural methylated isoflavones **13** by feeding of non-natural precursors. Another example of microbial isoflavonoid biotransformation is the reduction of daidzein **27** to equol. Although several microorganisms isolated from mammalian digestive tracts have been shown to catalyze the nonstereospecific transformation, a recently isolated gram-negative anaerobic species, MRG-1, shares high homology with *Coprobacillus* species and was shown to exhibit stereospecific reductase activity for conversion of several isoflavones **13** to the corresponding isoflavanones. Stereoselective reduction from the highly active MRG-1 isoflavone reductase (IFR) opens new biotechnological routes for production of enantiopure flavanones **12** [153].

5 Conclusions

Metabolic engineering of microbes for isoflavonoid biosynthesis showcases state-of-the-art methodologies for high-level production of pharmaceutically and nutraceutically relevant compounds. Decoupling production of plant secondary metabolites from their native, convoluted regulatory backgrounds enables predictable control and design, while transplanting biosynthetic pathways into fast-growing, well-characterized microorganisms allows utilization of advanced genetic and computational tools and an abundance of biological data. Genetically tractable microbes such as *E. coli* and *S. cerevisiae* provide an unmatched platform for combinatorial biosynthesis of complex plant natural products and their non-natural derivatives by transformation with heterologous genes from different organisms.

Though microbial production of plant natural products is a promising alternative to traditional methods, further research will continue to improve titers and assist in the discovery of novel isoflavonoid biotransformations. A significant challenge that has not yet been accomplished is the expression of the entire isoflavonoid metabolic pathway in *E. coli*, from aromatic amino acid precursors without supplementation of intermediates. Given the propensity for feedback inhibition and host toxicity of many flavonoid and isoflavonoid intermediates, protein engineering efforts will likely be required to enable high-level isoflavonoid production [174–176]. Furthermore, in vivo characterization of all enzymes in the isoflavonoid pathway will help determine rate-limiting steps that require higher relative promotion or expression level. Stoichiometric-based modeling and computational algorithms can also be utilized to predict genetic manipulations for maintaining high growth coupled with high specific production. Several thorough reviews have addressed the relative merits of various algorithms [177–182].

Feedback inhibition can be limited by optimizing both upstream and downstream enzyme expression such that the inhibitor does not significantly accumulate. In instances where a metabolite inhibits an enzyme in the isoflavonoid pathway, enzyme mutagenesis can alter the structural interaction between the enzyme and its inhibitor to block the inhibition mechanism. Recently, allosteric feedback inhibition of a tomato peel 4CL by naringenin **23**, a product several steps downstream, was significantly reduced through directed evolution in *E. coli* [181].

Cellular toxicity can also be ameliorated by various engineering strategies. Toxicity caused by intracellular accumulation of an intermediate can be limited by pathway optimization to ensure that the metabolite is utilized soon after it is produced. Pathway optimization can be achieved by accurate *in vivo* characterization of all enzymes in the pathway. Additionally, spatial localization of the enzymes catalyzing subsequent steps in a pathway serves as a “pipeline” to channel intermediate substrates to their respective catalyzing enzymes [184, 185]. This spatial proximity effectively leads to increased local substrate concentration and can be engineered by creating a protein fusion between adjacent enzymes, by docking multiple enzymes to a protein or RNA scaffold at minimal distance from each other, or by compartmentalizing all of the enzymes in a biosynthetic pathway in an isolated enclosure, such as a bacterial microcompartment (BMC) or an artificial organelle [186–191]. Such methodologies have enabled significant improvement in production levels of other microbial products and are outlined in great detail in a recent review by Agapakis and colleagues [185]. If the final product is toxic to the cell, one conceivable method for reducing the toxicity would be to engineer BMCs or artificial organelles for sequestration of the responsible metabolites. An alternative method is to engineer product transport. Overexpression of a library of efflux pumps and extracellular transporters can pinpoint proteins capable of selective export of a target product, while product glycosylation or deglycosylation could also improve export from the cell [192–194]. It is also important to consider if the product is natively transported into the cell from the extracellular environment; blocking transport of the toxic compound back into the cell can be accomplished by knocking out genes involved in product uptake.

Further work aimed at bioprospecting, culturing hard-to-culture microbes, searching for “unknown” and “orphan” enzymes that have not yet been characterized, and designing promiscuous enzymes capable of decorating and transforming flavonoids and their unnatural analogs will increase the range of isoflavonoid derivatives produced in microbes [195]. The search for enzymes capable of such manipulations should not be limited to plants, however, as many microbes endemic to mammalian guts have evolved to metabolize the plant phenylpropanoids ingested by their hosts. Current research efforts in these areas will lead to economically viable microbial platforms for production of isoflavonoids and products of high medicinal value.

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