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Microbial biosynthesis of medicinally important plant secondary metabolites

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Secondary metabolites derived from plants are a valuable source of pharmaceuticals, nutraceuticals, and cosmetics. To harness the potential of these natural products, reliable methods must be developed for their rapid and sustainable resupply. Microbial production of plant secondary metabolites through the heterologous expression of plant biosynthetic genes represents one such solution. This highlight focuses on recent advances in the microbial biosynthesis of plant secondary metabolites including terpenoids, flavonoids, and alkaloids as well as providing a brief insight into the current limitations and future prospects.

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1 Introduction

Plant secondary metabolites possess diverse chemical structures and biological activities and have been an extraordinarily rich source of valuable compounds for use in the pharmaceutical, cosmetic, fine chemical, and nutraceutical industries. For instance, artemisinin, paclitaxel, ginsenoside, lycopene, and resveratrol are well-known valuable plant natural products used as drugs or dietary supplements. However, whilst 25% of the molecules used in the pharmaceutical industry are of natural plant origin,[1] a general impediment to the industrial application of many of these exciting natural products is the limited quantities that can be obtained directly from the plants. Moreover, the total chemical syntheses of these structurally complex secondary metabolites are economically impractical in many cases. Therefore, an attractive alternative is to express the plant biosynthetic pathway genes in microbial hosts such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Streptomyces* species and to engineer the metabolic pathway/host to improve the production of these metabolites. Microbial production offers several advantages over both field and plant cell cultivation due to the rapid growth of microbes compared to plants, the ease of genetic manipulation, and the well-established metabolic engineering tools developed for use in microbes. In addition, microbial biosynthesis is more environmentally friendly than chemical synthesis. However, the functional reconstruction of plant biosynthetic pathways in microbes and the application of microbial biosynthesis for the industrial production of important compounds is still challenging. Herein, we highlight the recent efforts in the microbial production of representative compounds of the terpenoid, flavonoid, and alkaloid families. The current challenges in and the potential of these approaches are also briefly discussed.

2 Microbial biosynthesis of terpenoids

2.1 Biosynthetic pathway and structural diversity of terpenoids

Terpenoids (also known as isoprenoids) are the largest and the most diverse group of natural products.[2] All terpenoids are synthesized from the two universal isoprene units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors are produced by two distinct pathways: the mevalonate (MVA) pathway and the non-mevalonate (2-*C*-methyl-D-erythritol-4-phosphate; MEP) pathway (Fig. 1).[3] The MVA pathway is prevalent in bacteria and in the cytosol and mitochondria of plants, animals and fungi, whereas the MEP pathway is found mainly in eubacteria, cyanobacteria, and green algae.[4,5] The MVA pathway consists of six steps to convert the precursor acetyl-CoA into the two basic building blocks IPP and DMAPP, while the MEP pathway utilizes seven enzymes beginning with the condensation of pyruvate and glyceraldehyde-3-phosphate (G3P) to obtain these two building blocks (Fig. 1; see reviews[5,6] for the detailed biosynthetic mechanism). IPP and DMAPP are then condensed to form geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and geranylgeranyl diphosphate (GGPP, C_{20}), which respectively are the precursors to monoterpenes, sesquiterpenes, and diterpenes. Two molecules of GGPP are typically condensed to phytoene, an intermediate in the biosynthesis of the carotenoids.[5] Since the

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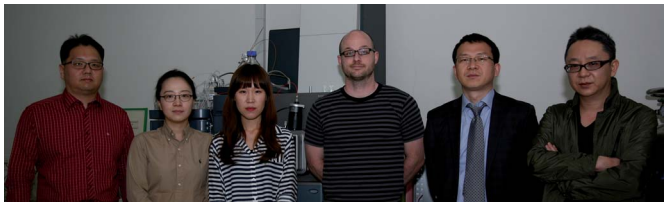
2000s, the focus on engineering terpenoid production in microbes has shifted from carotenoids to high value therapeutic compounds such as sesquiterpenes and diterpenes.

2.2 Sesquiterpenes: artemisinin

Artemisinin, a sesquiterpene lactone endoperoxide isolated from *Artemisia annua* L. plants, has strong antimalarial activity against the multi-drug-resistant parasite *Plasmodium falciparum*.[7] However, artemisinin can only be produced naturally in small quantities and low yields from the natural plant source. In addition, the total synthesis of artemisinin is too costly and inefficient.[8] Thus, the semi-synthesis of artemisinin from a readily available source is an effective way to provide sufficient quantities of artemisinin for medicinal purposes.

Heterologous production of artemisinin precursors was first described in *E. coli*. Keasling and co-workers reported the synthesis of approximately 24 mg L^{-1} of amorpha-4,11-diene (amorphadiene) by the expression of a codon-optimized synthetic amorphadiene synthase (ADS) gene and the MVA pathway from *S. cerevisiae* in *E. coli*.[9] Development of a two-phase partitioning bioreactor with a dodecane organic phase to trap the amorphadiene lost to air-stripping of the fermentation broth improved the production of amorphadiene 20-fold (500 mg L^{-1}) in the same strain.[10] The identification of two rate-limiting enzymes, mevalonate kinase (MVK) and ADS, and increasing the expression of these genes by optimizing the promoter strength and modifying the plasmid copy number, increased amorphadiene production to a specific productivity of ~300 mg per L per OD_{600}.[11] Replacement of the *S. cerevisiae* genes for hydroxymethylglutaryl (HMG)-CoA synthase (HMGS) and HMG-CoA reductase (HMGR) with the equivalent genes from *Staphylococcus aureus* and cultivating the resultant strain under carbon and nitrogen restriction yielded 27.4 g L^{-1} of amorphadiene.[12] Furthermore, when the codon optimized and N-terminal transmembrane engineered plant cytochrome P450 monooxygenase (AMO) was expressed in the amorphadiene-producing *E. coli* strain, and the expression vector, host strain, and culture conditions were optimized, the production of 105 mg L^{-1} of artemisinic acid, which can be converted into artemisinin in two chemical steps, was observed.[13] Because constructing the engineered metabolic pathways using



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Kris Rathwell grew up in Coronation, Alberta, Canada. He was educated at the University of Alberta (B.Sc. (Honors Chemistry), 2005) and at the University of Auckland (Ph.D. (Organic Chemistry), 2010). His doctoral research focused on the synthesis of analogues of the nanaomycin family of antibiotics. He is currently a post-doctoral researcher at Ewha Womans University working on the synthesis of polyketide-N-acetylcysteamine thioesters pertaining to polyketide natural products.

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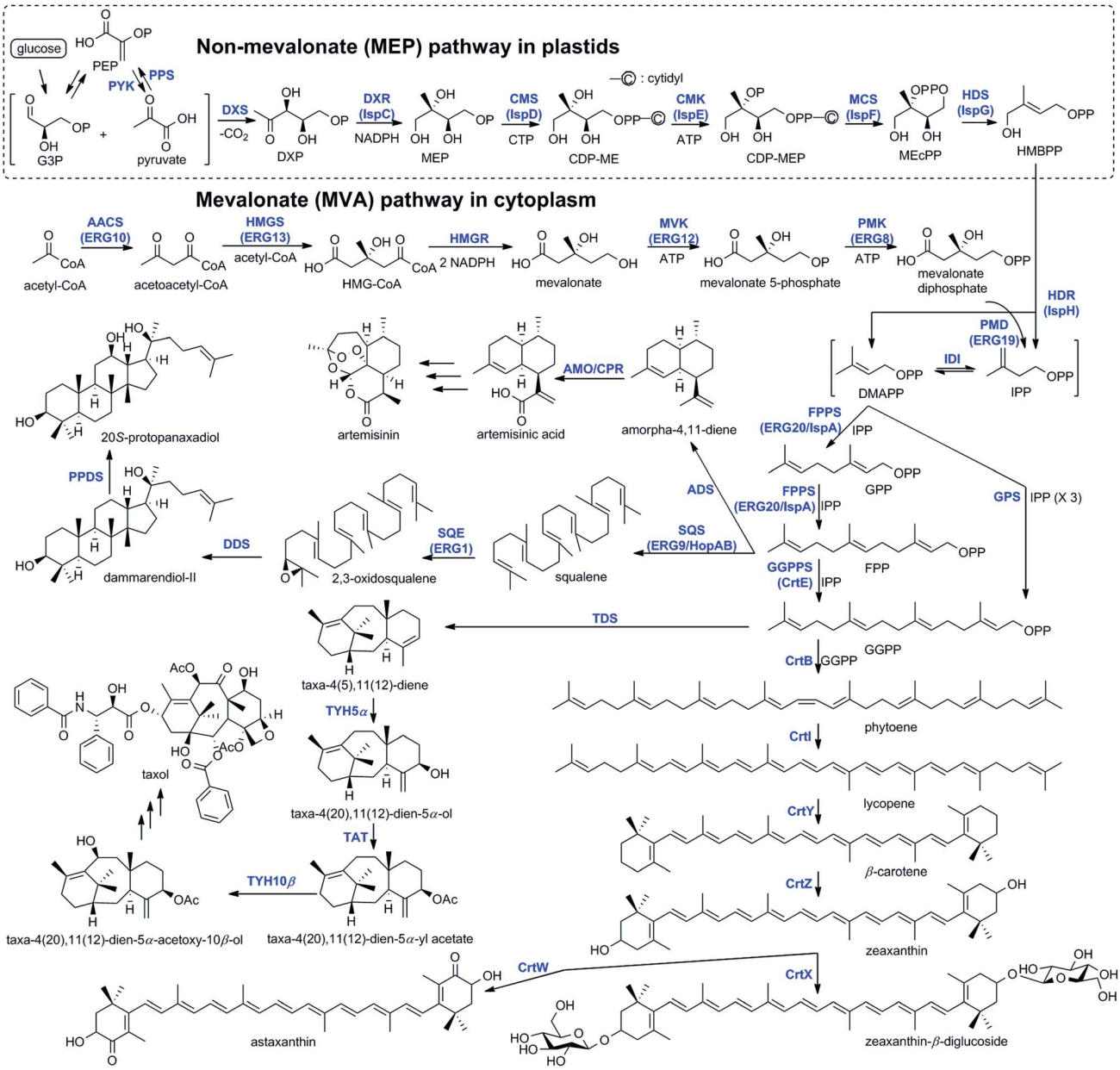

Fig. 1 Biosynthetic pathways of the representative terpenoid compounds. Abbreviations are described as follows: PEP, phosphoenolpyruvate; PYK, pyruvate kinase; PPS, phosphoenolphosphate synthase; G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DXP synthase; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; DXR (IspC), DXP reductase; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CMS (IspD), CDP-ME synthase; CDP-MEP, CDP-ME 2-phosphate; CMK (IspE), CDP-ME kinase; MEcPP, 2-*C*-methyl-D-erythritol-2,4-cyclo-diphosphate; MCS (IspF), MEcPP synthase; HMBPP, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate; HDS (IspG), HMBPP synthase; HDR (IspH), HMBPP reductase; AACS, acetoacetyl-CoA synthase; HMGS, hydroxymethylglutaryl (HMG)-CoA synthase; HMGR, HMG-CoA reductase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate diphosphate decarboxylase; IPP, isopentenyl diphosphate; IDI, IPP isomerase; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FPPS (EGR20, IspA), FPP synthase; GGPP, geranylgeranyl diphosphate; CrtE (GGPPS), GGPP synthase; GPS, multifunctional GGPPS; ADS, amorphadiene synthase; AMO, amorphadiene oxidase; CPR, NADPH:cytochrome P450 oxidoreductase; SQS (ERG9, HopAB), squalene synthase; SQE (ERG1), squalene epimerase; DDS, dammarendiol-II synthase; PPDS, protopanaxadiol synthase; TDS, taxadiene synthase; TYH5α, cytochrome p450-taxadiene-5α-hydroxylase; TAT, taxadienol-5α-*O*-acetyl transferase; THY10β, taxane-10β-hydroxylase; CrtB, phytoene synthase; CrtI, lycopene synthase; CrtY, lycopene cyclase; CrtZ, β-carotene hydroxylase; CrtX, zeaxanthine glucosyltransferase; CrtW, β-carotene ketolase.

heterologous enzymes induced a pathway flux imbalance in *E. coli*, Keasling and co-workers built artificial protein scaffolds to co-localize the three MVA biosynthetic enzymes (acetoacetyl-CoA synthase (AACS), HMGS and HMGR). When a scaffold containing one AACS, two HMGSs, and two HMGRs was used *in vivo* in *E. coli*, a maximum titer of 1.7 g L^{-1} of mevalonate was observed. This constituted a 77-fold improved production over the non-scaffolded control.[14]

The production of artemisinic acid (115 mg L^{-1}) in yeast was first achieved by expressing an engineered MVA pathway (where the expression of several genes responsible for FPP synthesis were upregulated, and one gene responsible for FPP conversion to sterols was downregulated) along with the ADS and AMO (CYP71AV1) from *A. annua* in a *S. cerevisiae* strain.[15] Overexpression of acetaldehyde dehydrogenase (ALD6) and the introduction of AACS from *Salmonella enterica* enhanced the flux of acetyl-CoA to the MVA pathway and produced 120 mg L^{-1} of amorphadiene in *S. cerevisiae*.[16] The development of a defined medium fed-batch process, using galactose as both the carbon source and the inducer combined with the use of a dissolved oxygen-stat process using a strain in which sterol biosynthesis was repressed in a manner similar to the strain described above, improved the production of artemisinic acid to 2.5 g L^{-1} in bioreactors.[17] The overexpression of every MVA pathway enzyme as far as FPP synthase (FPPS, also known as ERG20 or IspA) using codon-optimized genes in *S. cerevisiae*, and the development of a fermentation process with ethanol feed, resulted in the production of 41 g L^{-1} of amorphadiene.[18] It was thought that ethanol might increase the supply of cytosolic acetyl-CoA to the MVA pathway. Expression of CYP71AV1 and its cognate reductase, NADPH:cytochrome P450 oxidoreductase (CPR), was not sufficient for the high-level conversion of amorphadiene to artemisinic acid. Therefore, the complete biosynthetic pathway for artemisinic acid was elucidated, and a novel cytochrome b5 (CYB5) that enhances the reaction rate of cytochrome P450, an alcohol dehydrogenase (ADH1), and an artemisinic aldehyde dehydrogenase (ALDH1), were discovered. The *A. annua* genes for this pathway were co-expressed in *S. cerevisiae*, and a novel extractive fermentation process utilizing isopropyl myristate oil was developed, resulting in the production of 25 g L^{-1} of artemisinic acid.[19]

In *Streptomyces avermitilis*, expression of the optimized synthetic amorphadiene pathway for *Streptomyces* codon usage resulted in the production of only small amounts of amorphadiene (30 μg L^{-1}).[20] *Bacillus subtilis*, a promising microbial host due to its fast growth rate and GRAS (generally regarded as safe) status, was also engineered to produce amorphadiene. Increasing the efficiency of translation by employing N-terminal fusion tags, systematically optimizing the media, and independently controlling the expression levels of 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS), IPP isomerase (IDI), and ADS resulted in the production of ~20 mg L^{-1} amorphadiene in *B. subtilis*.[21]

2.3 Diterpenes: taxol and taxane skeleton derivatives

Paclitaxel (trade name Taxol), a complex diterpenoid from yew (*Taxus*) trees, and its derivative docetaxel (trade name Taxotere) are very important anticancer agents for the treatment of ovarian and breast cancers. Unfortunately, the total synthesis of Taxol is impractical, and the semi-synthesis from the advanced taxoid-10-deacetylbaccatin III, isolated from the needles of the yew tree or from plant cell cultures, still has limitations in terms of productivity and scalability.[22] The biosynthesis of paclitaxel starts with the cyclisation of GGPP giving rise to taxa-4(5),11(12)-diene (taxadiene) (Fig. 1).[23]

The genetic engineering of *S. cerevisiae* for the production of taxadien-5α-acetoxy-10β-ol through the installation of GGPP synthase (GGPPS), taxadiene synthase (TDS), cytochrome p450-taxadiene-5α-hydroxylase (TYH5α), taxadienol-5α-*O*-acetyl transferase (TAT), and taxane-10β-hydroxylase (THY10β) genes from *Taxus* species provided 1.0 mg L^{-1} of taxadiene and 25 μg L^{-1} of taxadiene-5α-ol.[24] A codon-optimized TDS, an archaeal (*Sulfolobus acidocaldarius*) GGPPS, and the truncated HMGR genes from *Taxus chinensis*, which are not subject to feedback inhibition, were expressed in the aforementioned *S. cerevisiae* producing 8.7 mg L^{-1} of taxadiene.[25]

In *E. coli*, 1.3 mg L^{-1} of taxadiene was produced by co-expression of the TDS from *Taxus brevifolia* with a GGPP synthase (GGPPS) isolated from *Erwinia herbicola*, an IspA from *Schizosaccharomyces pombe*, and the endogenous *E. coli* DXS.[26] Recently, combined optimization of the promoter strength and the plasmid copy number to optimally balance the MEP and terpenoid-forming pathways increased the production of taxadien-5α-ol to approximately 1 g L^{-1} in *E. coli*.[27]

2.4 Triterpenes: dammarane skeleton and ginsenoside

Ginsenosides are a group of active triterpenoids found in the plant genus *Panax*, such as *P. ginseng* C.A. Meyer and *P. quinquefolius* L. They exhibit diverse pharmacological effects on the central nervous, cardiovascular, endocrine and immune systems.[28] The linear triterpene squalene is derived from the reductive coupling of two molecules of FPP (Fig. 1). Squalene synthases (SQS/ERG9, HopAB) and IspA from *Streptomyces peucetius* along with DXS and IDI from *E. coli* were overexpressed in *E. coli* to produce 11.8 mg L^{-1} of squalene.[29] A dammarenediol-producing *S. cerevisiae* was constructed *via* the heterologous expression of the dammarenediol-II synthase (DDS) from the hairy roots of *Panax ginseng*. When cultured under aerobic conditions, the engineered *S. cerevisiae* produced 251 μg L^{-1} of dammarenediol-II. When an anaerobic shift strategy was employed, up to 493 μg L^{-1} dammarenediol-II was synthesized. This increase could be explained by the fact that, under anaerobic conditions, the *S. cerevisiae* strain could accumulate high levels of squalene, which in turn could be converted to 2,3-oxidosqualene when exposed to aerobic conditions, resulting in an increased metabolic flux towards dammarenediol.[30] Recently, the DDS and protopanaxadiol synthase (PPDS) of *P. ginseng* and the CPR of *Arabidopsis thaliana* were introduced into a *S. cerevisiae* that had been engineered to increase squalene and 2,3-oxidosqualene supplies. When combined with the optimization of a two-phase extractive fermentation process, the production of 20*S*-protopanaxadiol (1189 mg L^{-1}) and dammarenediol-II (1548 mg L^{-1}) was observed.[28] These results could be applied to the microbial production of the ginsenosides, the bioactive compounds of ginseng, instead of relying on their extraction from plants.

2.5 Tetraterpenes: carotenoids and derivatives

Carotenoids are tetraterpenoids (C40 compounds) produced in many plants and microorganisms, and the simplest carotenoid, phytoene, arises from the head to head condensation of two

GGPP molecules (Fig. 1). These structurally diverse pigments have potential beneficial effects on human health. Notably, lycopene has antioxidant and potential anticancer properties.[31] The production of carotenoids in microorganisms was first achieved in *E. coli*. The carotenoid biosynthetic genes *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtX* were cloned from a phytopathogenic bacterium, *Erwinia uredovora*, and expressed in *E. coli* to produce 2.0 mg per g dry cell weight (DW) of lycopene, 2.0 mg per g DW of β-carotene, and 2.2 mg per g DW of zeaxanthin.[32] The functions of GGPPS (CrtE), phytoene synthase (CrtB), lycopene synthase (CrtI), lycopene cyclase (CrtY), β-carotene hydroxylase (CrtZ), and zeaxanthine glucosyltransferase (CrtX) were characterized through several independent studies.[33] Co-expression of CrtE, CrtB, CrtI, CrtY, and CrtZ from *Agrobacterium aurantiacum* along with CrtZ from *E. uredovora* in *E. coli* resulted in the production of 289 μg per g DW of zeaxanthin.[34] By co-expressing CrtE, CrtB, CrtI, CrtY, and CrtZ from *E. uredovora* with either the carotenoid 3′,4′-desaturase (CrtD) from *Rhodobacter spharoides* or the 1,2-hydratase (CrtC) from *Rhodobacter capsulatus* in *E. coli*, two linear carotenoids, 1-hydroxyneurosporene (176 μg per g DW), demethylspheroidene (46 μg per g DW), and two cyclic carotenoids, 1′-hydroxy-γ-carotene (40 μg per g DW) and 7,8-dihydrozeaxanthin (122 μg per g DW), were produced.[35] Three different IDIs from *Phaffia rhodozyma*, *Haematococcus pluvialis* and *S. cerevisiae* were tested for enhancing isoprenoid biosynthesis in *E. coli*. Among them, *H. pluvialis* IDI showed the best productivity when expressed in *E. coli* harboring CrtE, CrtB, CrtI and CrtY from *E. uredovora*. The engineered strain produced lycopene (1.0 mg per g DW), β-carotene (1.3 mg g per DW) and phytoene (0.5 mg per g DW), respectively.[36]

Liao and coworkers cloned and overexpressed an IDI from *E. coli*, a multifunctional GGPPS (GPS) from *Archaeoglobus fulgidus* that directly converts DMAPP and 3 IPP to GGPP, and CrtB, CrtI, CrtY, CrtZ and β-carotene ketolase (CrtW) from *A. aurantiacum* in *E. coli* to synthesize astaxanthin (1.3 mg per g DW).[37] Because the expression and activity of a GPS derived from an extreme thermophile may be suboptimal in *E. coli*, directed evolution was applied to this GPS. Overexpression of DXS and the mutated GPS in the aforementioned *E. coli* increased the production of lycopene to 45 mg per g DW.[38] Farmer and Liao introduced a *glnAp2* promoter-based artificial regulon to control the expression of the two rate-limiting enzymes, IDI and phosphoenolphosphate synthase (PPS), in lycopene biosynthesis. This strategy significantly increased lycopene production while alleviating the metabolic imbalance and growth retardation. The lycopene-producing strain containing an artificial regulon controlling IDI and PPS (*glnAp2-idi* + *glnAp2-pps*) in addition to the lycopene pathway (DXS, GPS, CrtB and CrtI) produced more than 150 mg L^{-1} of lycopene.[39] They also redirected the flux from pyruvate to G3P by deleting pyruvate kinase (PYK) and the overexpressing PPS, phosphoenolpyruvate (PEP) carboxylase (PPC), and PEP carboxykinase (PCK), to increase PEP through the TCA cycle. The production of lycopene was increased to 25 mg per g DW.[40] These results showed that the flux distribution between pyruvate and G3P plays a major role in isoprenoid synthesis in *E. coli*.

Furthermore, DXS was found to control the flux through the MEP pathway, and could be manipulated to increase isoprenoid production in *E. coli*. The expression of DXS from *B. subtilis* and *Synechocystis* sp. 6803 in *E. coli* harbouring CrtE, CrtB, and CrtI from *E. uredovora* enabled the synthesis of lycopene (1.0 mg per g DW).[41] The overexpression of DXS from *E. uredovora* led to the production of lycopene (1.3 mg per g DW) and zeaxanthin (0.6 mg per g DW) in *E. coli*,[42] and promoter optimization for the overexpression of DXS and IDI led to an increase in lycopene biosynthesis (5.7 mg per g DW).[43]

Based on the results of a stoichiometric flux balance analysis, Stephanopoulos and co-workers constructed a triple knockout mutant in which *gdhA*, *aceE*, and *fdhF* genes encoding glutamate dehydrogenase, pyruvate dehydrogenase, and formate dehydrogenase, respectively, were inactivated to overproduce lycopene in *E. coli*. This triple knockout mutant showed an approximately 40% increase in lycopene biosynthesis (6.6 mg per g DW) compared with that of a parental strain in which only the MEP pathway was expressed.[44] High cell density fermentations with this triple knockout Δ*gdhA*Δ*aceE*Δ*fdhF* strain, along with the optimization of growth conditions, such as high oxygen levels and increased pH values, improved the production of lycopene (8.15 mg per g DW).[45]

Kim and co-workers expressed IDI from *H. pluvialis* with CrtE, CrtB, and CrtI from *E. herbicola* in *E. coli* to synthesize 17 mg L^{-1} of lycopene. Introducing an additional MVA pathway to increase the levels of IPP and DMAPP and adding surfactant to prevent clump formation increased lycopene production to 102 mg L^{-1} (22 mg per g DW).[46] Replacement of CrtE, CrtB, and CrtI with those from *Pantoea agglomerans* in the same *E. coli* resulted in the production of 60 mg L^{-1} of lycopene without surfactant.[31]

In addition, Lee and co-workers developed the 'flux scanning based on enforced objective flux (FSEOF)' method, a metabolic model to identify gene amplification targets *in silico*, and successfully employed this strategy for the construction of an *E. coli* strain in which several target genes predicted by FSEOF were amplified or inactivated. Batch-fed fermentation of the resulting strain produced 283 mg L^{-1} of lycopene.[47]

There are a few reports of carotenoid production in yeast. Carotenoid biosynthetic pathways including CrtE, CrtB, CrtI, and CrtY from *E. uredovora* were introduced in *S. cerevisiae* to synthesize 113 μg per g DW of lycopene and 103 μg per g DW of β-carotene.[48] The carotenoid biosynthetic genes were individually modified based on the codon usage of *Candida utilis* and expressed in *C. utilis*. The resulting yeast strain produced 1.1 mg per g DW of lycopene.[49] In this strain, the combined action of disrupting the ERG9 gene encoding SQS and overexpressing HMGR further improved lycopene production to 7.8 mg per g DW.[50]

3 Microbial biosynthesis of flavonoids and stilbenoids

3.1 Biosynthetic pathways and structural diversity of flavonoids and stilbenoids

Flavonoids and stilbenes are a class of plant-specific polyketides that have attracted much attention as pharmaceuticals and

nutraceuticals due to their antioxidant and radical scavenging activities showing protective effects against cancer, age-related diseases, and cardiovascular diseases.[51,52] As is the case for many natural products, the isolation of flavonoids from plants is limited by low productivity and the complexity of the mixtures recovered from plants. The biosynthesis of flavonoids and stilbenes begins with the phenylpropanoid pathway,[53] in which phenylalanine is converted to cinnamic acid by phenylalanine ammonia lyase (PAL). Cinnamate-4-hydroxylase (C4H) catalyzes the hydroxylation of cinnamic acid to form 4-coumaric acid (4CA). 4CA is then converted to 4-coumaroyl-CoA by 4-coumarate:CoA ligase (4CL). Next, chalcone synthase (CHS), the archetypal plant-specific type III polyketide synthase, catalyzes the sequential condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone, the precursor of a large number of flavonoids. Naringenin chalcone is subsequently converted to the flavanone by chalcone isomerase (CHI). While stilbene synthase (STS) acts on the same substrates as CHS, the ring folding in this reaction differs, leading to the production of the stilbenes (Fig. 2).[54] The major flavonoid skeletons are further modified by various reactions, such as those involving flavanone 3β-hydroxylase (F3H), flavone synthase I and II (FNSI and FNSII), flavonol synthase (FLS),[55] and flavonoid 3′ and 3′5′ hydroxylase (F3′H and F3′5′H), as well as various methylations and glycosylations (Fig. 2). In the isoflavonoid biosynthetic pathway, isoflavone synthase (IFS), a cytochrome P450 enzyme CYP93C that catalyzes a unique aryl migration reaction of the B-ring from the C-2 to the C-3 position of the C-ring, yields 2-hydroxyisoflavanone.[56] This intermediate product is then dehydrated to give the corresponding isoflavonoids (daidzein and genistein) *via* 2-hydroxyisoflavanone dehydratase (HID) (Fig. 2).[57]

3.2 Metabolic engineering of flavonoid/stilbenoid biosynthetic pathways in microbes

E. coli was the first microbe engineered to produce flavonoids. The heterologous expression of an artificial gene cluster including PAL from the yeast *Rhodotorula rubra*, ScCCL (a 4-coumarate/cinnamate:CoA ligase (CCL)) from the actinomycete *Streptomyces coelicolor* A3(2), and CHS from a licorice plant *Glycyrrhiza echinata*, resulted in the production of flavanones.[58] The PAL from *R. rubra* can convert both phenylalanine and tyrosine to cinnamic acid and 4CA, respectively.[59] Moreover, bacterial ScCCL can attach CoA to both cinnamic acid and 4CA. Plant C4H is a membrane-bound cytochrome P450 hydroxylase that requires a specific electron transfer system to be active in bacteria,[60] limiting its use. By using the aforementioned PAL and ScCCL, 0.75 mg L^{-1} of pinocembrin and 0.45 mg L^{-1} of naringenin were produced from fed phenylalanine and tyrosine, bypassing the C4H step.[58] By using a tyrosine ammonia lyase (TAL) from *R. sphaeroides*, which is able to convert tyrosine to 4CA together with 4CL and CHS in the same strain, naringenin was produced in a considerable amount (20.8 mg L^{-1}).[61] For high-level production of flavonoids in *E. coli*, increasing the intracellular malonyl-CoA pool was attempted. Overexpressing the acetyl-CoA carboxylase (ACC) from *Photorhabdus luminescens* in combination with the expression of acetate assimilation pathways increased the intracellular malonyl-CoA pool in an *E. coli* expressing 4CL from *Petroselinum crispum*, CHS from *Petunia hybrida*, and CHI from *Medicago sativa* that was grown in acetate supplemented media. This resulted in an improved production of pinocembrin (429 mg L^{-1}), naringenin (119 mg L^{-1}), and eriodictyol (52 mg L^{-1}) from the corresponding phenylpropanoic acids.[62] Another strategy employed to increase flavanone production was the inhibition of a competitive reaction pathway. Minimizing the malonyl-CoA lost to fatty acid synthesis by repressing FabB and FabF with optimized concentrations of cerulenin increased the production of pinocembrin (710 mg L^{-1}) and naringenin (186 mg L^{-1}) in *E. coli* expressing 4CL, CHS, and CHI.[63] An integrated computational and experimental approach was used to improve the intracellular availability of malonyl-CoA in *E. coli*.[64] The use of OptForce, which both contrasts the metabolic flux patterns of an initial strain with that of a strain that overproduces the target compound and identifies the necessary changes required in the network for overproduction, resulted in the prediction of several combinatorial recombinant strains.[65] Knockout of the *fumC* gene encoding fumarase and the *sucC* gene encoding succinyl-CoA synthetase combined with the overexpression of ACC, phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphatedehydrogenase (GAPD), and pyruvate dehydrogenase (PDH) forced the carbon flux towards malonyl-CoA and produced 474 mg L^{-1} of naringenin in *E. coli* harboring 4CL, CHS, and CHI.[64] These fermentation strategies required supplementation of the media with expensive phenylpropanoic acid precursors. An *E. coli* host system was developed to bypass this problem. 3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS), chorismate mutase (CM), and prephenate dehydratase (PDT), which convert glucose to phenylalanine, were coexpressed with PAL, 4CL, CHS, CHI, malonyl-CoA synthetase (MatB), and malonate carrier protein (MatC) resulting in a 40 mg L^{-1} yield of pinocembrin directly from glucose without any precursor supplementation.[66] Although the reported yield was low, this strategy could be further developed to afford a more economical process than those which use expensive chemical precursors.

S. cerevisiae co-expressing 4CL from *A. thaliana*, CHS from *Hypericum androsaemum* and PAL from *Rhodosporidium toruloides*, which has TAL activity, produced naringenin (7 mg L^{-1}) and pinocembrin (0.8 mg L^{-1}) from cinnamic acid and 4CA, respectively.[67] By expressing the four-step flavanone biosynthetic pathway including C4H (*A. thaliana*), 4CL (*P. crispum*), CHS and CHI (*P. hybrida*) in *S. cerevisiae*, pinocembrin (16.3 mg L^{-1}), naringenin (28.3 mg L^{-1}), and eriodictyol (6.5 mg L^{-1}) were produced from the fed cinnamic acid, 4CA, and caffeic acid, respectively.[68] *S. cerevisiae* was further engineered to produce flavonoids directly from glucose. Deregulation of aromatic amino acid synthesis by alleviating feedback inhibition and the reduction of byproduct formation improved tyrosine availability for the production of naringenin. In this recombinant *S. cerevisiae*, co-expression of an increased copy number of CHS with TAL, PAL, C4H and its associated CPR as well as 4CL and CHI resulted in the production of 58 mg L^{-1} of naringenin directly from glucose.[69]

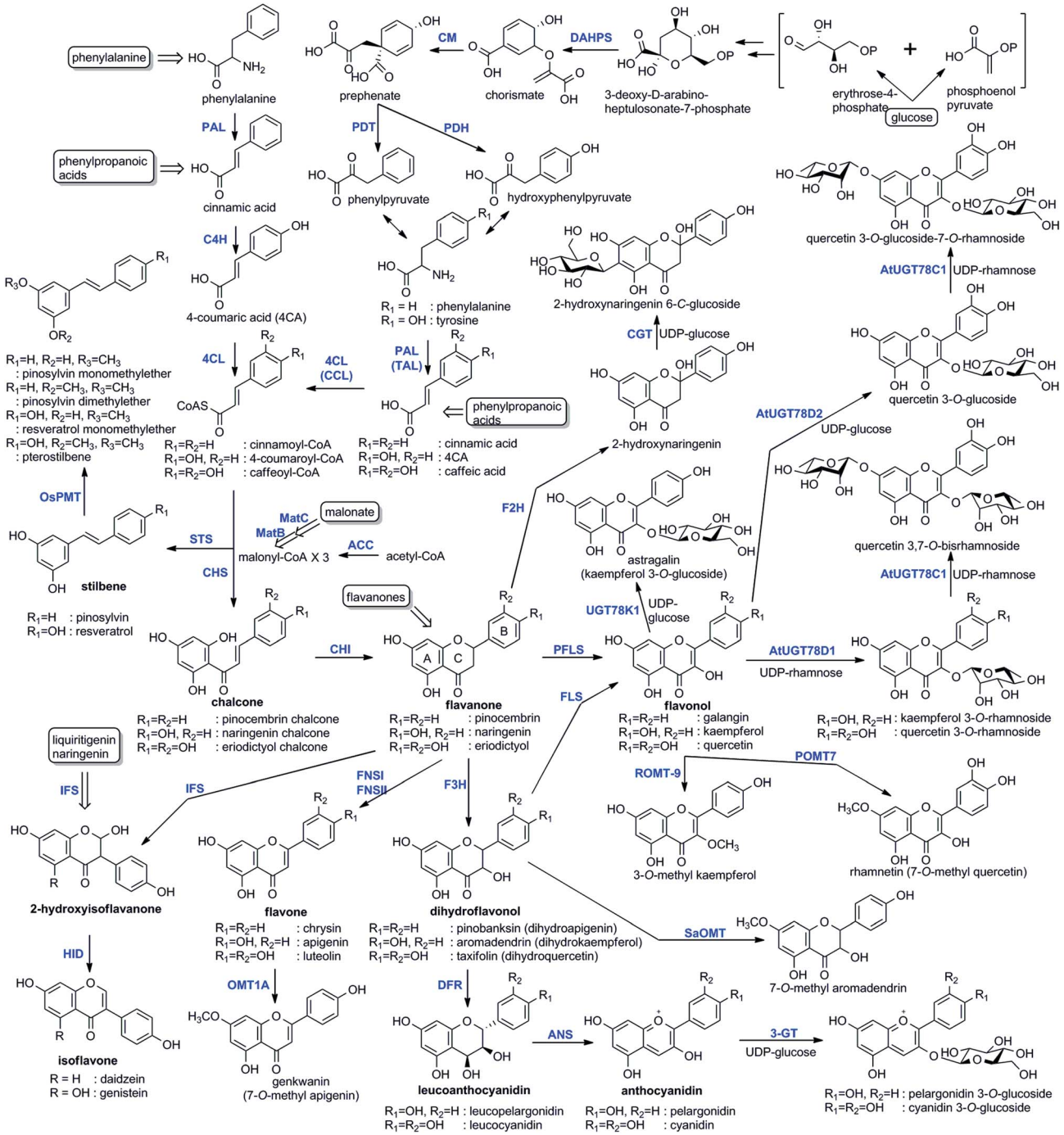

Fig. 2 Biosynthetic pathways of the representative flavonoid compounds. Enzyme names are abbreviated as follows: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; STS, stilbene synthase; F3H, flavanone-3β-hydroxylase; FNSI(II), flavone synthase I(II); FLS, flavonol synthase; IFS, isoflavone synthase; HID, 2-hydroxyisoflavanone dehydratase; CCL, 4-coumarate/cinnamate:CoA ligase; TAL, tyrosine ammonia lyase; ACC, acetyl-CoA carboxylase; DAHPS, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase; CM, chorismate mutase; PDT, prephenate dehydratase; PDH, pyruvate dehydrogenase; MatB, malonyl-CoA synthetase; MatC, malonate carrier protein; PFLS, flavonol synthase from *Populus deltoids*; ROMT-9, 3-OMT from rice; POMT7, 7-OMT from poplar; SaOMT, 7-OMT from *Streptomyces avermitilis*; OMT1A, 7-OMT from *Mentha* × *piperita*; OsPMT, pinosylvin methyltransferase from *Oryza sativa*; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; 3-GT, UDP-glucose:flavonoid-3-*O*-glucosyltransferase (GT); UGT78K1, 3-GT from *Glycine max*; AtUGT78D1, UDP-rhamnose flavonol GT; AtUGT78D2, UDP-glucose flavonol GT; AtUGT78C1, UDP-rhamnose flavonol GT; F2H, flavanone-2-hydroxylase; CGT, 6-*C*-GT from *Oryza sativa*. Double-lined arrows indicate exogenously fed substrates.

By co-expressing 4CL from *Populus trichocarpa* × *Populus deltoides* and STS from *Vitis vinifera* in *S. cerevisiae*, 1.45 μg L^{-1} of resveratrol was produced from 4CA.[70] The yield was improved in *S. cerevisiae* by fusing 4CL from *A. thaliana* with STS from *V. vinifera*. The 4CL::STS fusion protein increased resveratrol production by up to 15-fold (5.25 mg L^{-1}) compared to the co-expression of 4CL and STS.[71] Resveratrol production was further increased using an industrial *S. cerevisiae* strain that was isolated from a Brazilian sugar cane plantation and expressed 4CL and STS. When grown in a rich medium, 391 mg L^{-1} of resveratrol was produced.[72] PAL, CPR, and C4H were added to the stilbene pathway, but the yield of resveratrol from fed phenylalanine was less than 1 mg L^{-1}.[73]

Two FNSs, a soluble FNSI from parsley and a membrane-bound FNSII from snapdragon, were introduced into *S. cerevisiae* expressing genes encoding C4H, 4CL, CHS, and CHI to produce 2–3 mg L^{-1} of apigenin and luteolin from phenylpropanoic acid precursors.[74] By feeding various aromatic acrylic acids, cultures of *S. cerevisiae* expressing recombinant flavonoid enzymes, including 4CL, CHS, CHI, and F3H from *Malus domestica*, produced various novel flavanones and dihydroflavonols.[75] A novel strategy for the *in vivo* synthesis of diverse flavonoids was demonstrated. Genes encoding the enzymes for the flavonoid pathway were individually cloned into yeast expression cassettes, and these cassettes were randomly combined on yeast artificial chromosomes. Recombinant clones of these new combinatorial chromosomes created a variety of flavonoid producing pathways, and feeding various precursors resulted in the production of diverse flavonoids.[76]

The gene encoding IFS from soybean was expressed together with CPR in *S. cerevisiae*. The resultant strain converted 7,4′-dihydroxyflavanone (liquiritigenin) and naringenin to daidzein and genistein, respectively, *via* a probable 2-hydroxyisoflavanone intermediate.[77] In addition, when IFS and CPR were co-expressed with CHI from *Glycine max* in *S. cerevisiae*, chalcone substrates were converted first into 2-hydroxyisoflavanones by CHI and IFS and then into isoflavanones and isoflavones nonenzymatically.[78] To improve the turnover rate of the plant P450 IFS in *E. coli*, IFS and CPR enzymes were fused to mimic the architecture inherent in bacterial P450BM-3. The membrane recognition signal of the fusion enzyme was also fine-tuned. As a result, 18 mg per g DW of daidzein and 10 mg per g DW of genistein were produced.[79] This production was 20-fold higher than that of the wild-type enzyme expression. When the CHS from *G. echinata*, CHI from *Pueraria lobata*, and IFS from *G. echinata* were expressed in *S. cerevisiae*, 340 μg L^{-1} of genistein was produced. Another strategy for the production of genistein employed the co-incubation of an *E. coli* strain harboring PAL, ScCCL, CHS, CHI, and ACC with an *S. cerevisiae* harboring IFS. This co-incubation yielded 6 mg L^{-1} of genistein from fed tyrosine.[80]

Streptomyces venezuelae was also used as a heterologous host to produce flavonoids and stilbenoids. When ScCCL and codon-optimized CHS from *A. thaliana* were expressed in *S. venezuelae*, relatively small amounts of racemic naringenin (4 mg L^{-1}) and pinocembrin (6 mg L^{-1}) were produced from the exogenously fed 4CA and cinnamic acid, respectively. Expression of codon-optimized CHI from *M. sativa*, together with ScCCL and codon-optimized CHS, led to the production of (2*S*)-flavanones, but the yield was reduced. Also, the co-expression of ScCCL and codon-optimized STS from *Arachis hypogaea* resulted in the production of resveratrol (0.4 mg L^{-1}) and pinosylvin (0.6 mg L^{-1}).[81] An *S. venezuelae* system was expanded for the production of flavones and flavonols. Codon-optimized FNS from *P. crispum* was introduced to *S. venezuelae*, and the resulting strain generated flavones (1.4 mg L^{-1} of apigenin and 2.9 mg L^{-1} of chrysin) when fed with flavanones. In addition, *S. venezuelae* expressing both codon-optimized F3H from *Citrus sinensis* and FLS from *Citrus unshiu* produced flavonols (0.2 mg L^{-1} of kaempferol and 1.0 mg L^{-1} of galangin) from fed flavanones.[82] Genes involved in malonate assimilation were further engineered to improve flavonoid production. The introduction of MatB and MatC from *S. coelicolor* into *S. venezuelae* expressing flavanone and flavone pathways combined with the supplementation of exogenous malonate led to approximately 9-fold (35.6 mg L^{-1}) and 7-fold (44.1 mg L^{-1}) enhanced production of naringenin and pinocembrin, respectively. Likewise, approximately 15.3 mg L^{-1} of apigenin and 30.9 mg L^{-1} of chrysin were produced, showing a 9-fold enhanced production compared to the flavone producing strains without MatB/C expression and malonate feeding.[83] Among *Streptomyces*, the pikromycin-producing *S. venezuelae* has certain advantages as a heterologous host for the production of plant secondary metabolites due to its rapid growth and relative ease of genetic manipulation. Nevertheless, the heterologous expression system based on *S. venezuelae* has not been fully optimized and shows relatively low productivity compared to *E. coli* and *S. cerevisiae*.

3.3 Derivatization *via* methyltransferases

Modifications such as methylations, glycosylations, and hydroxylations can be combined with the biosynthesis of flavonoid backbones to create greater structural diversity (Fig. 2). For example, the *O*-methylation of flavonoids by *O*-methyltransferases (OMTs) has an effect on solubility and increases their antimicrobial activity.[52] 3-*O*-Methyl kaempferol was obtained from naringenin by using two *E. coli* transformants harbouring either PFLS or ROMT-9.[84] PFLS from *P. deltoides* is a flavonol synthase that converts naringenin to kaempferol. ROMT-9 from rice is able to transfer the methyl group to the 3′-hydroxyl group of flavonoids.[85] Independently grown cells were mixed and incubated with naringenin, which resulted in the production of 3-*O*-methyl kaempferol (22.5 mg) along with kaempferol (11.5 mg).[84] POMT7 from poplar, a 7-OMT that transfers a methyl group to the 7-hydroxyl, was shown to have a higher affinity towards quercetin producing rhamnetin (7-*O*-methyl quercetin). By the optimization of both the copy number of the expression vectors containing POMT7 and the overexpression of *S*-adenosyl methionine synthetase in *E. coli*, 111 mg L^{-1} of rhamnetin was synthesized from quercetin.[86] 30 mg L^{-1} of 7-*O*-methyl aromadendrin (7-OMA) was produced from fed naringenin in *E. coli* by using SaOMT, which has 7-OMT activity, from *S. avermitilis* and F3H.[87] Overexpression of 4CL, CHS, CHI, F3H, and SaOMT together with the addition of

malonyl-CoA supply pathways resulted in the production of 2.7 mg L^{-1} of 7-OMA from fed 4CA.[87] Methylated flavone-producing *E. coli* was developed by using 4CL, CHS, CHI, FNS and OMT1A (7-OMT from *Mentha* × *piperita*). The recombinant *E. coli* produced apigenin (415 μg L^{-1}) and genkwanin (7-*O*-methyl apigenin, 208 μg L^{-1}) from fed 4CA and luteolin (10 μg L^{-1}) from fed caffeic acid.[88] Co-expression of pinosylvin methyltransferase (OsPMT) from *Oryza sativa* with PAL, 4CL, STS and ACC in *E. coli* resulted in the production of mono- and dimethylated stilbenes (18–27 mg L^{-1}), including a potent chemopreventive agent pterostilbene (resveratrol-3,5-dimethyl ether) (5.8 mg L^{-1}). This OMT showed potential for the production of unnatural stilbene methyl ethers due to its substrate flexibility.[89]

3.4 Derivatization *via* glycosyltransferases

Glycosylation of flavonoids affects their biological activity, stability, and solubility.[52] By employing glycosyltransferases (GTs), several glycosylated flavonoids were synthesized. Glycosylated anthocyanin was synthesized in *E. coli*. F3H from *M. domestica*, dihydroflavonol-4-reductase (DFR) from *Anthurium andraeanum*, anthocyanidin synthase (ANS) from *M. domestica* and UDP-glucose:flavonoid-3-*O*-glucosyltransferase (3-GT) from *P. hybrida* were expressed in *E. coli*, and this strain converted exogenously fed naringenin and eriodictyol to pelargonidin-3-*O*-glucoside (5.6 μg L^{-1}) and cyanidin-3-*O*-glucoside (6.0 μg L^{-1}), respectively.[90] By the co-expression of flavanone modification pathways (F3H and FLS) and UGT78K1 (3-GT from *G. max*), astragalin (AST, kaempferol-3-*O*-glucoside) was synthesized from the exogenous feeding of naringenin and endogenous UDP-glucose in *E. coli*. To increase AST production, *E. coli* was engineered to enhance the intracellular UDP-glucose concentration by deleting the chromosomal glucose phosphate isomerase, D-glucose-6-phosphate dehydrogenase, and UDP-glucose hydrolase. In addition, phosphoglucomutase from *Nocardia farcinia* and glucose-1-phophate uridylyltransferase from *E. coli* K12, two enzymes involved in UDP-glucose synthesis, were overexpressed, and 109.3 mg L^{-1} of AST was produced by this strategy.[91] Using an *E. coli* harboring UDP-rhamnose flavonol GT (AtUGT78D1) and rhamnose synthase (RHM2), both from *A. thaliana*, 150 mg L^{-1} of quercetin-3-*O*-rhamnoside and 200 mg L^{-1} of kaempferol-3-*O*-rhamnoside were produced from fed quercetin and kaempferol, respectively.[92] Moreover, sequential glycosylation of flavonoids was achieved by three GTs from *A. thaliana*: AtUGT78D1 and AtUGT78D2, which transfer rhamnose and glucose to the 3-hydroxy group of quercetin, respectively, and AtUGT78C1 which transfers rhamnose to the 7-hydroxyl group of quercetin-3-*O*-glycoside. A combination of AtUGT78D2 and AtUGT78C1 resulted in the production of 67 mg L^{-1} of quercetin-3-*O*-glucoside-7-*O*-rhamnoside, and another combination of AtUGT78D1 and AtUGT78C1 produced 67.4 mg L^{-1} of quercetin-3,7-*O*-bisrhamnoside from quercetin.[93] A flavone-*C*-glucoside production system was developed in yeast. A fusion protein of flavanone 2-hydroxylase (F2H) and 6-*C*-GT (CGT) from *O. sativa* was able to convert dihydrochalcone and flavanone precursors directly to *C*-glycosides. When the yeast harboring a F2H-CGT fusion protein was fed naringenin, 8 mg L^{-1} of 2-hydroxynaringenen-*C*-glucoside was produced.[94]

4 Microbial biosynthesis of alkaloids

4.1 Biosynthetic pathways and diversity of alkaloids

Alkaloids are found in 20% of plant species and are compounds containing a basic nitrogen atom derived from the decarboxylation of amino acids. Alkaloids can be classified into several major groups depending on the amino acid of origin in their biosynthesis: tropane-, pyrrolidine- and pyrrolizide-alkaloids (originated from ornithine), benzylisoquinoline (tyrosine), indolequinoline (tryptophane), and quinolizidine- and piperidine-alkaloids (lysine).[95] The benzylisoquinoline alkaloids (BIAs) are a large and structurally diverse family of pharmaceutical alkaloids, which includes the narcotic analgesic morphine, and the antibacterial agents berberine, scoulerine, and magnoflorine. Briefly, BIAs are produced in plants from L-tyrosine *via* (*S*)-reticuline. In the biosynthetic pathway of (*S*)-reticuline, L-tyrosine is converted to both dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) by tyrosine hydroxylase and tyrosine/dopa decarboxylase, and these are combined to form (*S*)-norcoclaurine by norcoclaurine synthase (NCS) (see the review[96] for the detailed biosynthetic mechanism). (*S*)-Norcoclaurine is then converted to (*S*)-reticuline in four steps; 6-*O*-methylation of norcoclaurine to give coclaurine by the action of norcoclaurine-6-*O*-methyltransferase (6OMT), *N*-methylation of coclaurine by coclaurine-*N*-methyltransferase (CNMT), hydroxylation of the 3′-C in *N*-methylcoclaurine by P450 hydroxylase (CYP80B), and a final 4′-*O*-methylation by 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′OMT) (Fig. 3).[97] BIAs have great medicinal applications, but their supply in sufficient quantities is difficult, due to the low level of production in plants and their complicated and costly total synthesis.

4.2 Metabolic engineering of the benzylisoquinoline biosynthetic pathway in microbes

The microbial production of (*S*)-reticuline, the key intermediate in BIA biosynthesis, was accomplished in *E. coli*.[98] To synthesize reticuline from dopamine, five enzymes were utilized: monoamine oxidase (MAO) from the microbe *Micrococcus luteus*,[99] and NCS, 6OMT, CNMT, and 4′OMT from the plant *Coptis japonica*. In the first step, dopamine is deaminated to give 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) by the microbial MAO from *M. luteus*. Then, dopamine and 3,4-DHPAA are combined giving rise to norlaudanosoline by NCS. Bypassing CYP80B was enabled by coupling dopamine and 3,4-DHPAA in *E. coli*. Of the two types of NCS from *C. japonica*, CjPR10A is expressed better in *E. coli* than is CjNCS1. Accordingly, CjPR10A was used for reticuline production.[100] The MAO and NCS selected were expressed in *E. coli* with 6OMT, CNMT, and 4′OMT. The engineered *E. coli* strain yielded racemic reticuline (11 mg L^{-1}) when supplemented with dopamine due to NCS not functioning efficiently in *E. coli*, and, consequently, a spontaneous condensation reaction occurred forming norlaudanosoline. Furthermore, to produce BIAs, two more BIA biosynthetic

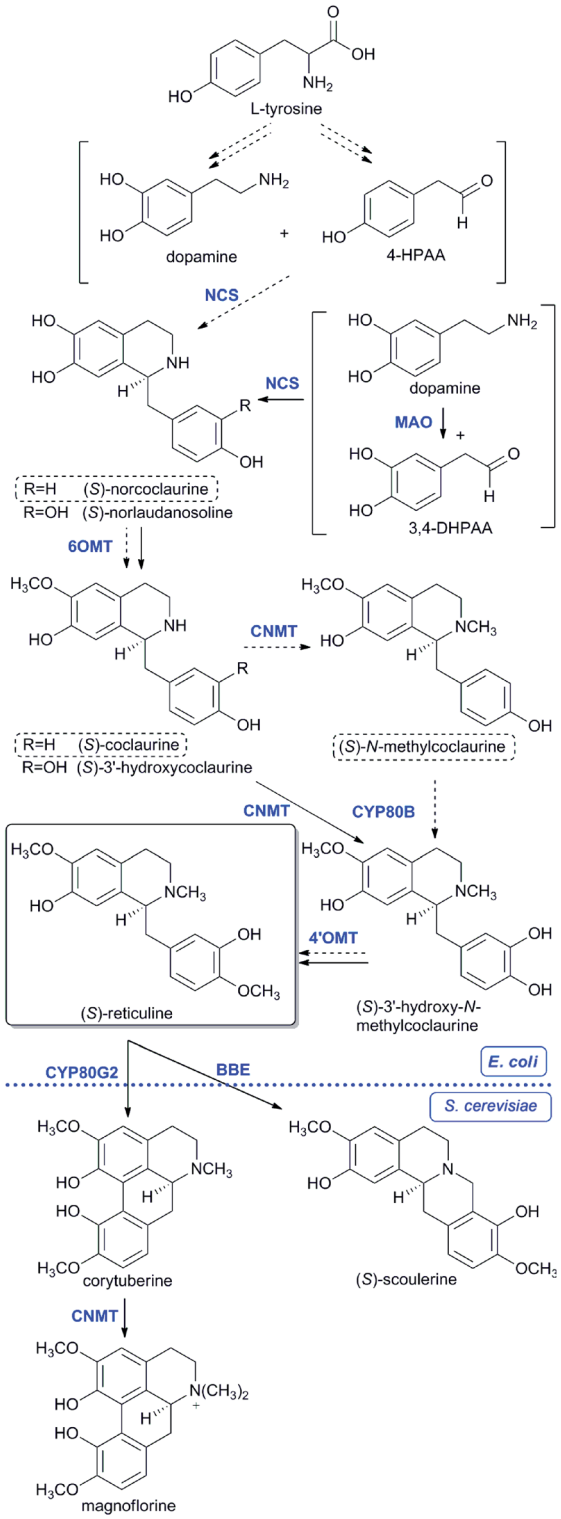

Fig. 3 Plant BIA biosynthetic pathway (dashed lines) and the microbial biosynthetic pathway reconstructed in *E. coli* and *S. cerevisiae* (solid lines). Abbreviations are as follows: NCS, norcoclaurine synthase; 4-HPAA, 4-hydroxyphenylacetaldehyde; MAO, monoamine oxidase; 3,4-DHPAA, 3,4-dihydroxy-phenylacetaldehyde; 6OMT, norcoclaurine-6-*O*-methyltransferase; CNMT, coclaurine-*N*-methyltransferase; CYP80B, *N*-methylcoclaurine-3′-hydroxylase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase; BBE, berberine bridge enzyme; CYP80G2, corytuberine synthase.

enzymes were introduced: corytuberine synthase (P450 enzyme, CYP80G2) to synthesize magnoflorine with CNMT and berberine bridge enzyme (BBE) for the synthesis of (*S*)-scoulerine. These enzymes were expressed in *S. cerevisiae* instead of *E. coli* because the functional expression of P450 is difficult in *E. coli*. Co-cultures of *E. coli* harboring reticuline biosynthetic enzymes and *S. cerevisiae* containing downstream enzymes yielded 7.2 mg L^{-1} of magnoflorine and 8.3 mg L^{-1} of (*S*)-scoulerine. Application of this system can produce many other alkaloids (Fig. 3).[98]

An *E. coli* was developed to produce plant alkaloids from a simple carbon source without any additional substrates. Firstly, an *E. coli* strain overproducing L-tyrosine was developed by the knockout of *tyrR*, whose product represses the expression of genes included in aromatic amino acid biosynthesis, and by the overexpression of feedback-inhibition-resistant (fbr)-DAHPS and fbr-CM/PDH in the shikimic acid pathway (see Fig. 2). To increase the metabolic flow into the shikimic acid pathway, phosphoenolpyruvate synthase and transketolase were also overexpressed. This engineered *E. coli* produced L-tyrosine at a yield of ~4.37 g L^{-1} from glycerol as the carbon source. Secondly, to convert L-tyrosine to dopamine, the tyrosinase from *Ralstonia solanacearum* and the L-DOPA-specific carboxylase from *Pseudomonas putida* were expressed in the L-tyrosine overproducing *E. coli* strain, resulting in the production of 1.05 g L^{-1} of dopamine. Lastly, co-expression of the artificial BIA synthetic pathway (MAO, NCS, 6OMT, CNMT, and 4′OMT) yielded a maximum of 46.0 mg L^{-1} of (*S*)-reticuline from glycerol. This fermentation platform provides opportunities for the low-cost production of many diverse alkaloids.[101]

5 Conclusions

The microbial heterologous production systems of plant secondary metabolites highlighted in this article show the potential of the rapid, economical, and environmentally-friendly production of these valuable molecules, in contrast to their production by natural plant sources or *via* chemical synthesis. Heterologous production systems also enable the creation of non-natural analogues of plant derived natural products. Even though some cases, such as artemisinin, have been successful in producing large amounts of the target compounds, there are still limitations in the production of sufficient amounts of the desired compounds for industrial applications. The functional expression of plant genes in a microbial host is a challenging task. Although using codon-optimized synthetic genes and various protein engineering approaches do not always solve this problem, they do improve the functionality of the heterologous biosynthetic pathway in part. The discovery of new pathways to bypass the natural plant pathways that are difficult to express functionally in microbes is also another approach. The availability of precursors also significantly affects the productivity of pathways engineered in microbes, and precursor supply can be bolstered by conventional or newly developed metabolic engineering technologies, as was the case for the malonyl-CoA or tyrosine/phenylalanine precursors. In addition, there are still many biosynthetic

pathways, enzymes and their regulation mechanisms remaining to be characterized for the biosynthesis of useful plant secondary metabolites such as the ginsenosides. Nevertheless, the development of new metabolic engineering tools (*e.g.* protein scaffolds used to improve mevalonate biosynthesis in *E. coli*,[14] employing synthetic small regulatory RNAs to regulate gene expression in microbes,[102] and *in silico* genome-scale metabolic models and associated simulation strategies[103]) and expanding the current knowledge regarding plant biosynthetic pathways will eventually allow us to develop efficient microbial production systems for a wide range of plant secondary metabolites.

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7 References

1 Y. Liu and M.-W. Wang, *Life Sci.*, 2008, **82**, 445.
2 A. Demain and A. Fang, *Adv. Biochem. Eng./Biotechnol.*, 2000, **69**, 1.
3 H. Harada and N. Misawa, *Appl. Microbiol. Biotechnol.*, 2009, **84**, 1021.
4 J. L. Goldstein and M. S. Brown, *Nature*, 1990, **343**, 425.
5 P. K. Ajikumar, K. Tyo, S. Carlsen, O. Mucha, T. H. Phon and G. Stephanopoulos, *Mol. Pharmaceutics*, 2008, **5**, 167.
6 M. Rohmer, *Nat. Prod. Rep.*, 1999, **16**, 565.
7 M. Enserink, *Science*, 2005, **307**, 33.
8 G. Schmid and W. Hofheinz, *J. Am. Chem. Soc.*, 1983, **105**, 624.
9 V. J. Martin, D. J. Pitera, S. T. Withers, J. D. Newman and J. D. Keasling, *Nat. Biotechnol.*, 2003, **21**, 796.
10 J. D. Newman, J. Marshall, M. Chang, F. Nowroozi, E. Paradise, D. Pitera, K. L. Newman and J. D. Keasling, *Biotechnol. Bioeng.*, 2006, **95**, 684.
11 J. R. Anthony, L. C. Anthony, F. Nowroozi, G. Kwon, J. D. Newman and J. D. Keasling, *Metab. Eng.*, 2009, **11**, 13.
12 H. Tsuruta, C. J. Paddon, D. Eng, J. R. Lenihan, T. Horning, L. C. Anthony, R. Regentin, J. D. Keasling, N. S. Renninger and J. D. Newman, *PLoS One*, 2009, **4**, e4489.
13 M. C. Chang, R. A. Eachus, W. Trieu, D. K. Ro and J. D. Keasling, *Nat. Chem. Biol.*, 2007, **3**, 274.
14 J. E. Dueber, G. C. Wu, G. R. Malmirchegini, T. S. Moon, C. J. Petzold, A. V. Ullal, K. L. J. Prather and J. D. Keasling, *Nat. Biotechnol.*, 2009, **27**, 753.
15 D. K. Ro, E. M. Paradise, M. Ouellet, K. J. Fisher, K. L. Newman, J. M. Ndungu, K. A. Ho, R. A. Eachus, T. S. Ham, J. Kirby, M. C. Chang, S. T. Withers, Y. Shiba, R. Sarpong and J. D. Keasling, *Nature*, 2006, **440**, 940.
16 Y. Shiba, E. M. Paradise, J. Kirby, D. K. Ro and J. D. Keasling, *Metab. Eng.*, 2007, **9**, 160.
17 J. R. Lenihan, H. Tsuruta, D. Diola, N. S. Renninger and R. Regentin, *Biotechnol. Prog.*, 2008, **24**, 1026.
18 P. J. Westfall, D. J. Pitera, J. R. Lenihan, D. Eng, F. X. Woolard, R. Regentin, T. Horning, H. Tsuruta, D. J. Melis, A. Owens, S. Fickes, D. Diola, K. R. Benjamin, J. D. Keasling, M. D. Leavell, D. J. McPhee, N. S. Renninger, J. D. Newman and C. J. Paddon, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **17**, E111.
19 C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, D. R. Polichuk, K. H. Teoh, D. W. Reed, T. Treynor, J. Lenihan, M. Fleck, S. Bajad, G. Dang, D. Diola, G. Dorin, K. W. Ellens, S. Fickes, J. Galazzo, S. P. Gaucher, T. Geistlinger, R. Henry, M. Hepp, T. Horning, T. Iqbal, H. Jiang, L. Kizer, B. Lieu, D. Melis, N. Moss, R. Regentin, S. Secrest, H. Tsuruta, R. Vazquez, L. F. Westblade, L. Xu, M. Yu, Y. Zhang, L. Zhao, J. Lievense, P. S. Covello, J. D. Keasling, K. K. Reiling, N. S. Renninger and J. D. Newman, *Nature*, 2013, **496**, 528.
20 M. Komatsu, T. Uchiyama, S. Omura, D. E. Cane and H. Ikeda, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 2646.
21 K. Zhou, R. Zou, C. Zhang, G. Stephanopoulos and H.-P. Too, *Biotechnol. Bioeng.*, 2013, **110**, 2556.
22 D. Frense, *Appl. Microbiol. Biotechnol.*, 2007, **73**, 1233.
23 S. Jennewein and R. Croteau, *Appl. Microbiol. Biotechnol.*, 2001, **57**, 13; K. Walker and R. Croteau, *Phytochemistry*, 2001, **58**, 1.
24 J. M. Dejong, Y. Liu, A. P. Bollon, R. M. Long, S. Jennewein, D. Williams and R. B. Croteau, *Biotechnol. Bioeng.*, 2006, **35**, 212.
25 B. Engels, P. Dahm and S. Jennewein, *Metab. Eng.*, 2008, **10**, 201.
26 Q. Huang, C. A. Roessner, R. Croteau and A. I. Scott, *Bioorg. Med. Chem.*, 2001, **9**, 2237.
27 P. K. Ajikumar, W. H. Xiao, K. E. Tyo, Y. Wang, F. Simeon, E. Leonard, O. Mucha, T. H. Phon, B. Pfeifer and G. Stephanopoulos, *Science*, 2010, **330**, 70.
28 Z. Dai, Y. Liu, X. Zhang, M. Shi, B. Wang, D. Wang, L. Huang and X. Zhang, *Metab. Eng.*, 2013, **20**, 146.
29 G. P. Ghimire, H. C. Lee and J. K. Sohng, *Appl. Environ. Microbiol.*, 2009, **75**, 7291.
30 Y.-L. Liang, S.-J. Zhao, L.-X. Xu and X.-Y. Zhang, *Lett. Appl. Microbiol.*, 2012, **55**, 323.
31 S. H. Yoon, J. E. Kim, S. H. Lee, H. M. Park, M. S. Choi, J. Y. Kim, S. H. Lee, Y. C. Shin, J. D. Keasling and S. W. Kim, *Appl. Microbiol. Biotechnol.*, 2007, **74**, 131.
32 N. Misawa, M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura and K. Harashima, *J. Bacteriol.*, 1990, **172**, 6704.
33 G. Sandmann and N. Misawa, *FEMS Microbiol. Lett.*, 1992, **69**, 253; U. Windhovel, B. Geiges, G. Sandmann and P. Boger, *Plant Physiol.*, 1994, **104**, 119; N. Misawa, Y. Satomi, K. Kondo, A. Yokoyama, S. Kajiwara, T. Saito, T. Ohtani and W. Miki, *J. Bacteriol.*, 1995, **177**, 6575.
34 A. Ruther, N. Misawa, P. Böger and G. Sandmann, *Appl. Microbiol. Biotechnol.*, 1997, **48**, 162; N. Misawa, Y. Satomi,

K. Kondo, A. Yokoyama, S. Kajiwara, T. Saito, T. Ohtani and W. Miki, *J. Bacteriol.*, 1995, **177**, 6575.
35 M. Albrecht, S. Takaichi, N. Misawa, G. Schnurr, P. Boger and G. Sandmann, *J. Biotechnol.*, 1997, **58**, 177.
36 S. Kajiwara, P. D. Fraser, K. Kondo and N. Misawa, *Biochem. J.*, 1997, **324**, 421.
37 C. W. Wang, M. K. Oh and J. C. Liao, *Biotechnol. Bioeng.*, 1999, **62**, 235.
38 C. W. Wang, M. K. Oh and J. C. Liao, *Biotechnol. Prog.*, 2000, **16**, 922.
39 W. R. Farmer and J. C. Liao, *Nat. Biotechnol.*, 2000, **18**, 533.
40 W. R. Farmer and J. C. Liao, *Biotechnol. Prog.*, 2001, **17**, 57.
41 M. Harker and P. M. Bramley, *FEBS Lett.*, 1999, **448**, 115.
42 P. D. Matthews and E. T. Wurtzel, *Appl. Microbiol. Biotechnol.*, 2000, **53**, 396.
43 S. W. Kim and J. D. Keasling, *Biotechnol. Bioeng.*, 2001, **72**, 408.
44 H. Alper, Y. S. Jin, J. F. Moxley and G. Stephanopoulos, *Metab. Eng.*, 2005, **7**, 155.
45 H. Alper, K. Miyaoku and G. Stephanopoulos, *Appl. Microbiol. Biotechnol.*, 2006, **72**, 968.
46 S. H. Yoon, Y. M. Lee, J. E. Kim, S. H. Lee, J. H. Lee, J. Y. Kim, K. H. Jung, Y. C. Shin, J. D. Keasling and S. W. Kim, *Biotechnol. Bioeng.*, 2006, **94**, 1025.
47 H. S. Choi, S. Y. Lee, T. Y. Kim and H. M. Woo, *Appl. Environ. Microbiol.*, 2010, **76**, 3097.
48 S. Yamano, T. Ishii, M. Nakagawa, H. Ikenaga and N. Misawa, *Biosci., Biotechnol., Biochem.*, 1994, **58**, 1112.
49 Y. Miura, K. Kondo, T. Saito, H. Shimada, P. D. Fraser and N. Misawa, *Appl. Environ. Microbiol.*, 1997, **64**, 1226.
50 H. Shimda, K. Kondo, P. D. Fraser, Y. Miura, T. Saito and N. Misawa, *Appl. Environ. Microbiol.*, 1998, **64**, 2676.
51 L. H. Yao, Y. M. Jiang, J. Shi, F. A. Tomás-Barberán, N. Datta, R. Singanusong and S. S. Chen, *Plant Foods Hum. Nutr.*, 2004, **59**, 113.
52 S. Kumar and A. K. Pandey, *Sci. World J.*, 2013, **2013**, 162750.
53 B. Weisshaar and G. I. Jenkins, *Curr. Opin. Plant Biol.*, 1998, **1**, 251.
54 S. Tropf, B. Kärcher, G. Schröder and J. Schröder, *J. Biol. Chem.*, 1995, **270**, 7922.
55 S. Horinouchi, *Curr. Opin. Chem. Biol.*, 2009, **13**, 197.
56 C. L. Steele, M. Gijzen, D. Qutob and R. A. Dixon, *Arch. Biochem. Biophys.*, 1999, **367**, 146; W. Jung, O. Yu, S. C. Lau, D. P. O'Keefe, J. Odell, G. Fader and B. McGonigle, *Nat. Biotechnol.*, 2000, **18**, 208.
57 T. Akashi, T. Aoki and S. Ayabe, *Plant Physiol.*, 2005, **137**, 882.
58 E. I. Hwang, M. Kaneko, Y. Ohnishi and S. Horinouchi, *Appl. Environ. Microbiol.*, 2003, **69**, 2699.
59 J. A. Kyndt, T. E. Meyerb, M. A. Cusanovichb and J. J. Van Beeumen, *FEBS Lett.*, 2002, **512**, 240.
60 M. Hotze, G. Schröder and J. Schröder, *FEBS Lett.*, 1995, **374**, 345; D. Pompon, B. Louerat, A. Bronine and P. Urban, *Methods Enzymol.*, 1996, **272**, 51.
61 K. T. Watts, P. C. Lee and C. Schmidt-Dannert, *ChemBioChem*, 2004, **5**, 500.
62 E. Leonard, K. H. Lim, P. N. Saw and M. A. Koffas, *Appl. Environ. Microbiol.*, 2007, **73**, 3877.
63 E. Leonard, Y. Yan, Z. L. Fowler, Z. Li, C.-G. Lim, K.-H. Lim and M. A. Koffas, *Mol. Pharm.*, 2008, **5**, 257.
64 P. Xu, S. S. Ranganathan, Z. L. Fowler, C. D. Maranas and M. A. Koffas, *Metab. Eng.*, 2011, **13**, 578.
65 S. Ranganathan, P. F. Suthers and C. D. Maranas, *PLoS Comput. Biol.*, 2010, **6**, e1000744.
66 J. Wu, G. Du, J. Zhou and J. Chen, *Metab. Eng.*, 2013, **16**, 48.
67 H. Jiang, K. V. Wood and J. A. Morgan, *Appl. Environ. Microbiol.*, 2005, **71**, 2962.
68 Y. Yan, A. Kohli and M. A. Koffas, *Appl. Environ. Microbiol.*, 2005, **71**, 5610.
69 F. Koopman, J. Beekwilder, B. Crimi, A. van Houwelingen, R. D. Hall, D. Bosch, A. J. A. van Maris, J. T. Pronk and J.-M. Daran, *Microb. Cell Fact.*, 2012, **11**, 155.
70 J. V. W. Becker, G. O. Armstrong, M. J. van der Merwe, M. G. Lambrechts, M. A. Vivier and I. S. Pretorius, *FEMS Yeast Res.*, 2003, **4**, 79.
71 Y. Zhang, S. Z. Li, J. Li, X. Pan, R. E. Cahoon, J. G. Jaworski, X. Wang, J. M. Jez, F. Chen and O. Yu, *J. Am. Chem. Soc.*, 2006, **128**, 13030.
72 T. Sydor, S. Schaffer and E. Boles, *Appl. Environ. Microbiol.*, 2010, **76**, 3361.
73 E. Trantas, N. Panopoulos and F. Ververidis, *Metab. Eng.*, 2009, **11**, 355.
74 E. Leonard, Y. Yan and M. A. Koffas, *Appl. Environ. Microbiol.*, 2005, **71**, 8241.
75 J. A. Chemler, Y. Yan, E. Leonard and M. A. Koffas, *Org. Lett.*, 2007, **9**, 1855.
76 M. Naesby, S. V. S. Nielsen, C. A. F. Nielsen, T. Green, T. Ø. Tange, E. Simón, P. Knechtle, A. Hansson, M. S. Schwab, O. Titiz, C. Folly, R. E. Archila, M. Maver, S. van Sint Fiet, T. Boussemghoune, M. Janes, A. S. S. Kumar, S. P. Sonkar, P. P. Mitra, V. A. Kumar Benjamin, N. Korrapati, I. Suman, E. H. Hansen, T. Thybo, N. Goldsmith and A. S. Sorensen, *Microb. Cell Fact.*, 2009, **8**, 45.
77 W. Jung, O. Yu, S. M. Lau, D. P. O'Keefe, J. Odell, G. Fader and B. McGonigle, *Nat. Biotechnol.*, 2000, **18**, 208.
78 L. Ralston, S. Subramanian, M. Matsuno and O. Yu, *Plant Physiol.*, 2005, **137**, 1375.
79 E. Leonard and M. A. Koffas, *Appl. Environ. Microbiol.*, 2007, **73**, 7246.
80 Y. Katsuyama, I. Miyahisa, N. Funa and S. Horinouchi, *Appl. Microbiol. Biotechnol.*, 2007, **73**, 1143.
81 S. R. Park, J. A. Yoon, J. H. Paik, J. W. Park, W. S. Jung, Y. H. Ban, E. J. Kim, Y. J. Yoo, A. R. Han and Y. J. Yoon, *J. Biotechnol.*, 2009, **141**, 181.
82 S. R. Park, J. H. Paik, M. S. Ahn, J. W. Park and Y. J. Yoon, *J. Microbiol. Biotechnol.*, 2010, **20**, 1295.
83 S. R. Park, M. S. Ahn, A. R. Han, J. W. Park and Y. J. Yoon, *J. Microbiol. Biotechnol.*, 2011, **21**, 1143.
84 B. G. Kim, E. J. Joe and J. H. Ahn, *Biotechnol. Lett.*, 2010, **32**, 579.
85 B. G. Kim, Y. Lee, H. G. Hur, Y. Lim and J. H. Ahn, *Phytochemistry*, 2006, **67**, 387.

86 S. H. Sung, B. G. Kim and J. H. Ahn, *J. Microbiol. Biotechnol.*, 2011, **21**, 854.
87 S. Malla, M. A. Koffas, R. J. Kazlauskas and B. G. Kim, *Appl. Environ. Microbiol.*, 2011, **78**, 684.
88 E. Leonard, J. Chemler, K. H. Lim and M. A. Koffas, *Appl. Microbiol. Biotechnol.*, 2006, **70**, 85.
89 Y. Katsuyama, N. Funa and S. Horinouchi, *Biotechnol. J.*, 2007, **2**, 1286.
90 Y. Yan, J. Chemler, L. Huang, S. Martens and M. A. Kaffas, *Appl. Environ. Microbiol.*, 2005, **71**, 3617.
91 S. Malla, R. P. Pandey, B. G. Kim and J. K. Sohng, *Biotechnol. Bioeng.*, 2013, **110**, 2525.
92 B. G. Kim, H. J. Kim and J. H. Ahn, *J. Agric. Food Chem.*, 2012, **60**, 11143.
93 H. J. Kim, B. G. Kim and J. H. Ahn, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 5275.
94 M. Brazier-Hicks and R. Edwards, *Metab. Eng.*, 2013, **16**, 11.
95 M. K. Julsing, A. K. Koulman, H. J. Woerdenbag, W. J. Quax and O. Kayser, *Biomol. Eng.*, 2006, **23**, 265.
96 J. A. Chemler and M. A. Koffas, *Curr. Opin. Biotechnol.*, 2008, **19**, 597.
97 Q. Zhang, J. O. Rich, I. C. Cotterill, D. P. Pantaleone and P. C. Michels, *J. Am. Chem. Soc.*, 2005, **127**, 7286.
98 H. Minami, J. S. Kim, N. Ikezawa, T. Takemura, T. Katayama, H. Kumagai and F. Sato, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 7393.
99 J. H. Roh, J. Wouters, E. Depiereux, H. Yukawa, M. Inui, H. Minami, H. Suzuki and H. Kumagai, *Biochem. Biophys. Res. Commun.*, 2000, **268**, 293.
100 H. Minami, E. Dubouzet, K. Iwasa and F. Sato, *J. Biol. Chem.*, 2007, **282**, 6274.
101 A. Nakagawa, H. Minami, J. S. Kim, T. Koyanagi, T. Katayama, F. Sato and I. Kumagai, *Nat. Commun.*, 2011, **2**, 236.
102 D. Na, S. M. Yoo, H. Chung, H. Park, J. H. Park and S. Y. Lee, *Nat. Biotechnol.*, 2013, **31**, 170.
103 J. W. Lee, D. Na, J. M. Park, J. Lee, S. Choi and S. Y. Lee, *Nat. Chem. Biol.*, 2012, **8**, 536.