Engineering Microbes for Plant Polyketide Biosynthesis

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Abstract: Polyketides are an important group of secondary metabolites, many of which have important industrial applications in the food and pharmaceutical industries. Polyketides are synthesized from one of three classes of enzymes differentiated by their biochemical features and product structure: type I, type II or type III polyketide synthases (PKSs). Plant type III PKS enzymes, which will be the main focus of this review, are relatively small homodimeric proteins that catalyze iterative decarboxylative condensations of malonyl units with a CoA-linked starter molecule. This review will describe the plant type III polyketide synthetic pathway, including the synthesis of chalcones, stilbenes and curcuminoids, as well as recent work on the synthesis of these polyketides in heterologous organisms. The limitations and bottlenecks of heterologous expression as well as attempts at creating diversity through the synthesis of novel “unnatural” polyketides using type III PKSs will also be discussed. Although synthetic production of plant polyketides is still in its infancy, their potential as useful bioactive compounds makes them an extremely interesting area of study.

Introduction

Through the process of natural selection, plants have evolved the ability to produce an array of defensive chemicals. Plant secondary metabolism, also termed specialized metabolism, represents a unique and vast pool of highly diversified molecules that qualitatively and quantitatively vary greatly within and between species [1]. While secondary metabolites are not directly involved in essential cellular functions, they play a major role in the adaptation of plants to their environment [2]. The impressive synthetic capacity of plants has long been exploited by mankind as a source of colorants, flavours, fragrances, traditional medicines and pharmaceutical drugs [3]. Presently, plant secondary metabolites represent valuable compounds for the pharmaceutical, cosmetic, agro-food, and fine chemicals industries [4].

Among plant secondary metabolites, polyketides represent a large group of structurally diverse molecules. In plants, polyketides are synthesized by type III polyketide synthases (PKSs) by the condensation of acetyl (ketide) units with a coenzyme A (CoA)-linked starter molecule [5]. The structural diversity of the plant polyketides results from the number of starter substrates that can be used by the PKSs and from subsequent modifications via regio-specific condensation, cyclization, aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfonation, and methylation reactions [6]. Compound backbones generated by these PKSs include chalcones, stilbenes, phloroglucinols, resorcinols, benzophenones, biphenyls, bibenzyls, chromones, acridones, pyrones, and curcuminoids [7]. Some of the resulting plant polyketides have been shown to possess anticancer, antimicrobial, antiviral, antioxidant, neuroprotective and oestrogenic activities [8-12]. Such potential healthprotecting effects of plant polyketides have stimulated the elucidation of their biosynthetic pathways and the development of frameworks for commercial production.

For industrial or pharmaceutical applications, the use of plant polyketides is mainly limited by their availability [13]. Significant engineering work has been carried out in recent years to increase the yield of polyketides (mainly chalcones and stilbenes) in plants [14,15]. As is the case for other plant metabolites, many polyketides tend to accumulate in small amounts and may require long growth periods to do so [16]. Purification can also be problematic, as multiple structurally similar metabolites are often present [17]. Total or semi-synthetic approaches are generally challenging and may result in racemic mixtures with relatively low yields [18,19].

Alternatively, microbes can be utilized as heterologous hosts for polyketides biosynthesis, with several advantages compared to plant and chemical synthesis. Microbes can be grown on inexpensive substrates and have very fast production cycles compared to plants. Current production methods result in microbial synthesis being significantly more environmentally friendly than chemical synthesis. Reconstruction of a plant biosynthetic pathway in microbes is still a challenging task. It first requires the stable introduction of multiple heterologous genes in the microbial host. These genes then have to be expressed and generate functional enzymes. Once functionality of the heterologous pathway has been demonstrated, the main challenge remains in reaching yields sufficient for commercialisation.

This review presents the recent development of microbial engineering for the biosynthesis of plant polyketides, yield improvement and product diversification. Current limitations and bottlenecks are also covered.
Polyketide biosynthesis in plants

Polyketides are a large group of metabolites found in bacteria, fungi and plants, which are synthesized from acyl-CoA precursors by PKS [20]. PKSs can be grouped in three distinct classes based on their biochemical features and product structure [21]. Type I refers to large modular and multifunctional enzymes, whereas type II PKSs are dissociable complexes usually composed of monofunctional enzymes that are found in bacteria [22]. Plant PKSs are part of the type III group, which comprises homomeric enzymes of relatively small size [7]. Type III PKSs are also found in bacteria [23] and fungi [24]. Type III PKSs catalyze iterative decarboxylative condensations of malonyl units with a CoA-linked starter molecule [25]. A typical type III PKS reaction involves the loading of a starter molecule, the extension of the polyketide chain and cyclization of the linear intermediate [5]. A great variety of CoA-linked starter substrates can be utilized by plant type III PKSs: acetyl-CoA, malonyl-CoA, methylmalonyl-CoA, p-coumaroyl-CoA, cinnamoyl-CoA, N-methylanthraniloyl-CoA, n-hexanoyl-CoA, isobutryl-CoA, isovaleryl-CoA and 3-hydroxybenzoyl-CoA to name a few [5,7].

Chalcone synthase (CHS) is the archetypal plant-specific type III PKSs that catalyzes sequential condensation of p-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone via a Claisen cyclization reaction (Fig. 1) [26]. Synthesis of p-coumaroyl-CoA requires the action of three enzymes. Phenylalanine ammonia lyase (PAL) catalyses the first reaction of the phenylpropanoid pathway by the deamination of phenylalanine into trans-cinnamic acid [27]. A cinnamate 4-hydroxylase (C4H) then catalyses the hydroxylation of trans-cinnamic acid to yield p-coumaric acid [28]. Alternatively, p-coumaric acid can be produced directly from tyrosine by a tyrosine ammonia lyase (TAL), bypassing the C4H intermediate [6]. While some PALs have demonstrated promiscuity towards tyrosine, distinct TALs are found in grasses and non-plant organisms [29]. A 4-coumaroyl-CoA ligase (4CL) activates p-coumaric acid through the addition of a CoA unit to generate p-coumaroyl-CoA. These three enzymes, PAL, C4H and 4CL, are required for normal growth and development and, are therefore highly conserved among plants. A significant fraction of p-coumaroyl-CoA is consumed for lignin synthesis, but it is also required for flavonoid production [30]. Phenylpropanoid pathway intermediates are also redirected into synthesis of benzoates, salicylates, coumarins, monolignols, lignans, and phenylpropenes [29]. The other CHS co-substrate, malonyl-CoA, is formed by the carboxylation of acetyl-CoA, a reaction catalyzed in the cytosol by a homomeric acetyl-CoA carboxylase (ACC) [31]. Malonyl-CoA is also required for the synthesis of fatty acids, but is produced in the plastid by a distinct ACC for this purpose [32]. After formation of naringenin chalcone (also called chalconaringenin) by CHS, a variety of flavonoids are created by the combined actions of functionalizing enzymes, namely isomerases, reductases, hydroxylases, glycosyltransferases, acyltransferases, methyltransferases and prenyltransferases [19,29,33]. Stilbene synthase (STS), another well studied representative of the plant type III PKSs, uses the same substrates as CHS, but performs an aldol cyclization reaction to produce resveratrol [34] (Fig. 1). STS is found in few diverse plants, such as grapevine, and would have evolved from CHS by a limited number of amino acids substitutions [35].

Figure 1. Naringenin chalcone and resveratrol common biosynthetic pathway. TAL, tyrosine ammonia lyase; PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; ACC, acetyl-CoA carboxylase; CHS, chalcone synthase; STS, stilbene synthase.
Figure 2. Examples of plant polyketides.

Table 1. Production of plant polyketides in microorganisms.

| Host organism | Biosynthetic components | Fed precursor | End-product | Yield (mg/L) | Ref. |
|---------------|-------------------------|---------------|-------------|--------------|------|
| *E. coli*     | PAL (Rhodotorula rubra), 4CL (Sreptomyces coelicolor), CHS (Glycyrrhiza echinata) | Tyrosine | Naringenin | 0.453 | [39] |
|               | Phenylalanine | Pinocembrin | 0.752 | |
|               | TAL (Rhodobacter sphaeroides), 4CL, CHS (Arabidopsis thaliana) | None | Naringenin | 20.8 | [40] |
|               | PAL (R. rubra), 4CL (S. coelicolor), CHS (G. echinata), CHI (Pueraria lobata), ACC (Corynebacterium glutamicum) | Tyrosine | Naringenin | 57 | [41] |
|               | Phenylalanine | Pinocembrin | 58 | |
|               | 4CL (A. thaliana), STS (Arachis hypogaea) | p-Coumaric acid | Resveratrol | 104.5 | [42] |
|               | 4CL (Nicotiana tabacum), STS (Vitis vinifera) | p-Coumaric acid | Resveratrol | 16 | [43] |
|               | 4CL, FSI (Petroselinum crispum), CHS, CHI (Petunia × hybrida), OMT (Mentha × piperita) | p-Coumaric acid | Apigenin | 0.415 | [44] |
|               | Caffeic acid | Luteolin | 0.01 | |
|               | p-Coumaric acid | Genkwanin | 0.208 | |
|               | 4CL (P. crispum), CHS (P. × hybrida), CHI (Medicago sativa), ACC (Photorhodobactus luminescens), ACS (E. coli) | Cinnamic acid | Pinocembrin | 429 | [45] |
|               | p-Coumaric acid | Naringenin | 119 | |
|               | Caffeic acid | Eriodictyol | 52 | |
|               | 4CL (Lithospermum erythrorhizon), CHS, CHI (G. echinata), STS (A. hypogaea), FNS (P. crispum), F3H, FLS (Citrus), ACC (C. glutamicum) | p-Coumaric acid | Flavonols | 33 | [46] |
|               | Fluoroacinnamic acid | Stilbenes | 171 | |
|               | 4CL | Flavanones | 102 | |
| System | Reactionants | Products | IC50 (μM) |
| --- | --- | --- | --- |
| 4CL (<i>P. crispum</i>), CHS, CHI (<i>P. × hybridus</i>), MatB, MatC (<i>Rhizobium trifolii</i>) | Cinnamic acid, <i>p</i>-Coumaric acid, Caffeic acid | Pinocembrin, Naringenin, Eriodictyol | 480, 155, 50 |
| PAL (<i>R. rubrid</i>), 4CL (<i>L. erythrochiron</i>), CUS (<i>Oryza sativa</i>) | Tyrosine, Phenylalanine, Phenylalanine/tyrosine | Bidemethoxycurcumin, Dicinnamoyl-methane, Cinamoyl-p-coumaroyl-methane | 53.4, 107, 19.2 |
| TAL (<i>Rhodotorula glutinis</i>), 4CL (<i>P. crispum</i>), CHS (<i>P. × hybridus</i>), CHI (<i>M. sativa</i>) | None | Naringenin | 84 |
| TAL (<i>Sacharothrix epatensis</i>), 4CL (<i>S. coelicolor</i>), CHS (<i>A. thaliana</i>), STS (<i>A. hypogae</i>) | None | Naringenin | 5.3 |
| 4CL (<i>P. crispum</i>), CHS (<i>P. × hybridus</i>), CHI (<i>M. sativa</i>), ACC (<i>P. luminens</i>), PGK, PDH (<i>E. coli</i>) | <i>p</i>-Coumaric acid | Resveratrol | 1.45x10³ |
| 4CL (<i>P. crispum</i>), CHS (<i>P. × hybridus</i>), CHI (<i>M. sativa</i>), F3H (<i>A. thaliana</i>), OMT (<i>Streptomyces avermitilis</i>), ACC, ACS (<i>Nocardiopsis flavirostris</i>) | <i>p</i>-Coumaric acid | 7-O-Methyl aromadendrin | 2.7 |
| TAL (<i>R. glutinis</i>), 4CL (<i>P. crispum</i>), CHS (<i>P. × hybridus</i>), CHI (<i>M. sativa</i>), AroF, PheA (<i>E. coli</i>), MatB, MatC (<i>R. trifolii</i>) | None | Pinocembrin | 40 |
| 4CL (<i>Populus trichocarpa × Populus deltoide</i>), STS (<i>V. vinifera</i>) | <i>p</i>-Coumaric acid | Resveratrol | 1.45x10³ |
| PAL (<i>Rhodotorula toruloides</i>), 4CL (<i>A. thaliana</i>), CHS (<i>Hypericum androsaemum</i>) | None | Naringenin | 7 |
| C4H (<i>A. thaliana</i>), 4CL (<i>P. crispum</i>), CHS, CHI (<i>P. × hybridus</i>) | Cinnamic acid, <i>p</i>-Coumaric acid, Caffeic acid | Pinocembrin, Naringenin, Eriodictyol | 16.3, 28.3, 6.5 |
| C4H (<i>A. thaliana</i>), 4CL, FSI (<i>P. crispum</i>), CHS, CHI (<i>P. × hybridus</i>) | Cinnamic acid, <i>p</i>-Coumaric acid, Caffeic acid | Chrysin, Apigenin, Luteolin | - |
| 4CL (<i>V. tachum</i>), STS (<i>V. vinifera</i>) | <i>p</i>-Coumaric acid | Resveratrol | 6 |
| Fusion of 4CL (<i>A. thaliana</i>) and STS (<i>V. vinifera</i>) | <i>p</i>-Coumaric acid | Resveratrol | 5.25 |
| PAL, CPR (<i>P. trichocarpa × P. deltoide</i>), C4H, 4CL (<i>Glycine max</i>), STS (<i>V. vinifera</i>) | Phenylalanine | Resveratrol | 0.31 |
| 4CL (<i>A. thaliana</i>), STS (<i>V. vinifera</i>) | <i>p</i>-Coumaric acid | Resveratrol | 391 |
| 4CL (<i>A. thaliana</i>), STS (<i>A. hypogae</i>) | <i>p</i>-Coumaric acid | Resveratrol | 3.1 |
| TAL (<i>R. sphaeroides</i>), fusion of 4CL (<i>A. thaliana</i>) and STS (<i>V. vinifera</i>) | Tyrosine | Resveratrol | 1.9 |
| OAC, TKS (<i>Cannabis sativa</i>) | Hexanoic acid | Olivetolic acid | 0.48 |
| PAL (<i>R. toruloides</i>), C4H, 4CL (<i>A. thaliana</i>), STS (<i>A. hypogae</i>), ACC (<i>S. cerevisiae</i>) | None | Resveratrol | 4.3 |
| PAL, C4H, CPR, 4CL, CHS, CHI (<i>A. thaliana</i>), TAL (<i>Rhodobacter capsulatus</i>), ARO4<sup>fl</sup> (<i>S. cerevisiae</i>) | None | Naringenin | 109 |

**Streptomyces venezuelae**

| Reactionants | Products | IC50 (μM) |
| --- | --- | --- |
| 4CL (<i>S. coelicolor</i>), CHS (<i>A. thaliana</i>), STS (<i>A. hypogae</i>) | Cinnamic acid, <i>p</i>-Coumaric acid | Pinocembrin, Naringenin, Resveratrol | 6, 4, 0.4 |
Naringenin chalcone and resveratrol are not the only plant polyketides that can result from the condensation of p-coumaroyl-CoA and malonyl-CoA. Different polyketides can be synthesised by varying the number of malonyl-CoA and p-coumaroyl-CoA molecules used in the elongation step (Fig. 2). The type of cyclization or its absence also influences the resulting product [7]. Stryglypyrone synthase (SPS) produces bisnoryangonin from the condensation of two molecules of malonyl-CoA [36]. Benzalactone synthase (BAS) catalyzes the condensation of only one molecule of malonyl-CoA without a cyclization, resulting in diketide benzalactone [37]. Similarly, curcuminooid synthase (CUS) uses one molecule of malonyl-CoA without cyclization, but produces bisdemethoxycurcumin by the condensation with p-coumaroyl-CoA [38]. Other plant PKSs catalyze the condensation of three malonyl-CoA units while employing varying starter substrates. Such PKSs include acridone synthase (ACS), phlorisovalerophenone synthase (VPS), benzophenone synthase (BPS), olivetol synthase (OLS) and 2-pyrene synthase (2PS) [7].

**Engineered microbes**

Like many plant secondary metabolites, polyketides are often difficult to extract in large amounts or to synthesize chemically. For those reasons, engineering microbes for the production of plant metabolites is emerging as an advantageous alternative. This approach typically required the construction of new metabolic pathways in the microbial host and the modification of existing ones to enhance productivity. Metabolic engineering for plant polyketide production has been conducted mainly in the two workhorse organisms *Escherichia coli* and *Saccharomyces cerevisiae*. These two microbial hosts are easy to grow and manipulate, are genetically and biochemically well characterized, and have many genetic tools available. As a eukaryote, *S. cerevisiae* presents some distinct advantages over *E. coli* for the reconstruction of plant pathways. *S. cerevisiae* has compartments similar to plant cells and can post-translationally modify proteins. The eukaryotic cellular environment is also more adequate for the expression of functional membrane proteins, such as cytochrome P450x.

*E. coli* and *S. cerevisiae* naturally produce malonyl-CoA, but lack most of the CoA-ester starter substrates needed for plant polyketide synthesis. Although precursors can be supplemented in the growth medium, the production of these substrates represents an important part of the metabolic engineering process. Most plant polyketide pathway engineering in microbes described in the literature is based on CHS and STS, with only few other examples using curcuminooid synthase and tetraketide synthase (Table 1).

**Chalcone synthases**

Hwang *et al.* [39] were the first to report the production of plant polyketides in *E. coli*. Naringenin and pinocembrin were produced by co-expressing PAL from the yeast *Rhodotorula rubra*, 4CL from the actinobacterium *Streptomyces coelicolor A3(2)*, and CHS from the plant *Glycyrrhiza echinata*. PAL could use both tyrosine and phenylalanine as substrates, yielding *p*-coumaric acid and *trans*-cinnamic acid, respectively. Similarly, 4CL catalyzed the activation of *trans*-cinnamic and *p*-coumaric acid, and CHS used both cinnamoyl-CoA and *p*-coumaroyl-CoA as starters. Very small amounts of naringenin (0.27 µg/L) and pinocembrin (0.17 µg/L) were detected in the culture medium. To increase yields, tyrosine and phenylalanine were supplied. As a result, yields of naringenin and pinocembrin were increased to 452.6 µg/L and 751.9 µg/L, respectively. The same group was then able to improve production of naringenin and pinocembrin by overexpressing an ACC from *Corynebacterium glutamicum* and by optimising culture conditions [41]. They also added a chalcone isomerase from *Pueraia lobata* to catalyze the isomerisation of naringenin chalcone to naringenin. Although the malonyl-CoA concentrations were not reported, these results suggested that an increase in the amount of malonyl-CoA, as a result of ACC overexpression, led to improved production of polyketides. Under culture conditions where *E. coli* cells were concentrated to 50 g/L, yields of naringenin and pinocembrin reached about 60 mg/L in the presence of tyrosine and phenylalanine, respectively. Watts *et al.* [40] were the first to use a true TAL enzyme from the bacteria *Rhodobacter sphaeroides*. This enzyme, while accepting both tyrosine and phenylalanine as substrates, exhibits a much higher specificity toward tyrosine [66]. By using TAL, *p*-coumaric acid was efficiently produced directly from tyrosine without producing *trans*-cinnamic acid and pinocembrin.

**Results from Miyahisa *et al.* [41] and Leonard *et al.* [56] indicated that malonyl-CoA was most probably limiting polyketide production in *E. coli* and *S. cerevisiae*. Leonard et al. [44] also evaluated flavone production in *E. coli*, using 4CL and FSI from *Petroselinum crispum*, CHS and chalcone isomerase (CHI) from *Petunia × hybrida*. Feeding assays were done with *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid, yielding respectively pinocembrin (16.3 mg/L), naringenin (28.3 mg/L), eriodictyol (6.5 mg/L), but no homoeriodictyol. However, naringenin yield was quite low (0.2 mg/L) when *trans*-cinnamic acid was the precursor, indicating that 4CH is a rate-limiting step. In a follow-up study, the same group tested two flavone synthases (FSI and FSII) in their yeast strain harbouring C4H, 4CL, and CHI [56]. The resulting strains converted fed phenylpropanoid acid precursors into the flavone molecules chrysin, apigenin, and luteolin. Yields were increased by overexpressing the yeast cytochrome P450 reductase (CPR1) and by using acetate as carbon source. CPR1 overexpression probably improved both 4CH and FSII activities. The overall increase in flavone production with acetate was hypothesized to result from increased carbon flux toward malonyl-CoA. Leonard et al. [44] also evaluated flavone production in *E. coli*, using 4CL and FSI from *Petunia × hybrida*, and 7-O-methyltransferase (OMT) from *Mentha x piperita*. *E. coli* successfully produced apigenin, luteolin and genkwanin from fed phenylpropanoid acid precursors.

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ACS catalyses the formation of acetyl-CoA from acetate and CoA. With this last approach, pinocembrin, naringenin, and eriodictyol accumulated in the culture medium to 429 mg/L, 119 mg/L, and 52 mg/L, respectively.

Leonard et al. [47] used a more elaborate metabolic engineering approach for producing flavanones from exogenous phenylpropanoid acids in E. coli. To increase availability of malonyl-CoA, a malonyl-CoA synthetase (MatB) and a putative malonate transporter (MatC) from the bacteria Rhizobium trifolii were introduced in E. coli. MatB and MatC allowed the production of malonyl-CoA from exogenous malonate, bypassing the natural metabolism from glucose. The introduction of this malonate assimilation pathway increased production of naringenin, pinocembrin and eriodictyol by 269%, 1555% and 335%, respectively. Yields were further increased by attenuating the activity of fatty acid metabolism, which competes for malonyl-CoA. This was done by adding a fatty acid synthase inhibitor to the culture medium.

Park et al. [65] reported the first plant-derived polyketide production in an organism other than E. coli and S. cerevisiae. Genes encoding 4CL from Streptomyces coelicolor and CHS from A. thaliana were expressed in Streptomyces venezuelae. Relatively small amounts of naringenin (4 mg/L) and pinocembrin (6 mg/L) were produced when fed with p-coumaric acid and trans-cinnamic acid, respectively.

In most of the strategies described previously, polyketide production required supplementation of precursors. Santos et al. [13] engineered a four-step pathway consisting of a TAL, 4CL, CHS, CHI in a tyrosine-overproducing E. coli strain, allowing naringenin production directly from glucose. Enzymes from different sources were tested, and genes were codon-optimized for E. coli expression. The performance of TAL from R. spheroides (RsTAL) and from the yeast Rhodotorula glutinis (RgTAL) was evaluated in the absence of the other downstream enzymes. Only small amounts of p-coumaric acid (1.5-5.5 mg/L) were produced with RsTAL compared to RgTAL (104-213 mg/L). Enzymatic assays with purified enzymes revealed a twelve-fold higher catalytic activity with tyrosine for RgTAL [66]. Different 4CLs, CHSs and CHIs were also evaluated. The most efficient strain was obtained with RgTAL, 4CL from Petroselinum crispum, CHS from Petunia hybrida and CHI from Medicago sativa L. Without any precursor supplementation, the strain was capable of producing 29 mg/L of naringenin and up to 84 mg/L with the addition of cerulein, a fatty acid enzyme inhibitor. Choi et al. [49] also reported the production of naringenin in E. coli without feeding tyrosine or p-coumaric acid. Their synthetic gene cluster was composed of TAL from Saccharothrix espanaensis, 4CL from S. coelicolor and CHS from A. thaliana. The yield of naringenin obtained (5.3 mg/L) was low compared to Santos et al. [13], as no further metabolic engineering was done to improve precursors level.

With the aim to improve the availability of malonyl-CoA in E. coli, Xu et al. [50] used an integrated computational and experimental approach. A customized version of the OptForce algorithm was utilized to predict a minimal set of metabolic interventions [67]. Based on that model, genes encoding a fumarase (fumC) and a succinyl-CoA synthetase (sucC) were knocked out. FumC and SucC are part of the TCA cycle and catalyze the interconversion of fumarate to malate and the reversible reaction of succinyl-CoA to succinate, respectively. Knocking out those genes partially inactivated the TCA cycle, which in turn increased naringenin production by 30% compared to the wild type strain. The model also identified target genes that if upregulated would augment the production of the precursor acetyl-CoA. By co-expressing acetyl-CoA carboxylase (ACC) with either phosphoglycerate kinase (PGK) or glyceraldehyde-3-phosphate dehydrogenase (GAPD), and pyruvate dehydrogenase (PDH), production of naringenin was increased by about 220%. Finally, overexpression of ACC, PGK and PDH in the double knockout strain resulted in the production of 474 mg/L of naringenin, the highest yield achieved so far.

Koopman et al. [64] were the first to engineer S. cerevisiae for improved tyrosine availability for the production of naringenin. Synthesis of tyrosine was increased by introducing a feedback resistant 3-deoxy-d-arabinose-heptulosonate-7-phosphate (DAHP) synthase allele (aroF66) and deleting the other allele (aroJ). Additionally, the loss of tyrosine to side products was reduced by deleting the phenylpyruvate decarboxylases (aro10, Pdc5, Pdc6). Most of the naringenin biosynthetic pathway parts (PAL, C4H, CPR, 4CL, CHS, CHI) were from A. thaliana, but a TAL from R. capsulatus was also used. The presence of PAL, C4H, CPR and TAL allowed synthesis of p-coumaric acid from both tyrosine and phenylalanine. In shake-flask cultures, approximately 50 mg/L of naringenin was produced directly from glucose and, more than 100 mg/L in controlled aerobic batch cultures.

Wu et al. [52] engineered E. coli to increase both the supply of phenylalanine and malonyl-CoA. Carbon flux toward phenylalanine was increased by overexpressing 3-deoxy-d-arabinose-heptulosonate-7-phosphate (AroF) and a feedback-inhibition-resistant chorismate mutase/prephenate dehydratase (PheA). The supply of malonyl-CoA was increased by introducing the malonate assimilation pathway from R. trifolii (MatB and MatC), as described previously by Leonard et al. [47]. The resulting strain, in which TAL (R. glutinis), 4CL (P. crispum), CHS (P × hybridra) and CHI (M. sativa) were co-expressed, allowed the production of pinocembrin (40 mg/L) without any precursor supplementation.

Stilbene synthases

Becker et al. [53] were the first to report the production of resveratrol in an engineered microorganism. The resveratrol (1.45 µg/L) was produced in S. cerevisiae from fed p-coumaric acid by co-expressing 4CL from Populus trichocarpa × Populus deltoides and STS from Vitis vinifera. Zhang et al. [57] further improved resveratrol yield in S. cerevisiae by fusing 4CL from A. thaliana and STS from V. vinifera. The 4CL:STS fusion protein increased resveratrol production by up to 15-fold compared to the co-expression of 4CL and STS. Nevertheless, the yield of resveratrol (5.25 mg/L) obtained from p-coumaric acid feeding remained relatively low. In E. coli, resveratrol was produced at a higher level. Concentrations reached 16 mg/L by co-expressing 4CL from Nicotiana tabacum and STS from V. vinifera [43], and over 100 mg/L when co-expressing 4CL from A. thaliana and STS from Arachis hypogaea [42]. A similar two-reaction approach was used in S. venezuelae with a 4CL from S. coelicolor and a STS from A. hypogaea, producing 0.4 mg/L of resveratrol [65]. Trantas et al. [58] extended the synthetic pathway in S. cerevisiae by adding a PAL and a CPR from Populus trichocarpa × P. deltoides and a C4H from Glycine max. This extended pathway synthesized resveratrol from fed phenylalanine, but with a yield of less than 1 mg/L. Sydor et al. [59] evaluated resveratrol synthesis from p-coumaric acid in different S. cerevisiae strains expressing 4CL from A. thaliana and STS from V. vinifera. Different growth media were also tested. In synthetic medium (SD), the strain CEN.PK2-1 produced 6 mg/L of resveratrol. When grown in the rich medium YEPD, yield increased dramatically to 262 mg/L. They also evaluated four industrial yeast strains for their efficiency in resveratrol production. Three strains produced resveratrol in various concentrations, but one metabolized p-coumaric acid and did not synthesis any resveratrol. The highest
yield (391 mg/L) was obtained with an S. cerevisiae strain isolated from a Brazilian sugar cane plantation. Other attempts to produce resinoviolin in E. coli and S. cerevisiae have resulted in yields not exceeding 6 mg/L [49,60,61,63].

**Curcuminoid synthase**

Curcuminoid synthase (CUS) is a type III PKS involved in the formation of diarylheptanoid compounds, such as those found in the spice turmeric [68]. An artificial curcuminoid biosynthetic pathway based on a CUS from rice (Oryza sativa) was assembled in E. coli [48]. CUS catalyzes the formation of bisdemethoxycurcumin from two molecules of p-coumaroyl-CoA and one molecule of malonyl-CoA [68]. It also accepts cinnamoyl-CoA and feruloyl-CoA as substrates to produce dicinnamylmethane and curcumin, respectively. Along with CUS, a PAL from R. rubra and a 4CL, from Lithospermum erythrorhizon were assembled in E. coli. The resulting strain produced bisdemethoxycurcumin (53.4 mg/L), dicinnamylmethane (107 mg/L) and cinnamyl-p-coumaroylmethane (19.2 mg/L) from supplemented tyrosine and/or phenylalanine. Curcumin (113 mg/L) was also synthesised when ferulic acid was fed.

**Tetraketide synthase and olivetolic acid cyclase**

Gagne et al [62] have recently reported the identification of a novel polyketide cyclase enzyme, olivetolic acid cyclase (OAC), which functions together with a type III PKS to form olivetolic acid using hexanoyl-CoA as a starter substrate. Oivetolic acid is the first polyketide intermediate in the cannabinoids biosynthetic pathway in Cannabis sativa (marijuana, hemp). Co-expression of a type III PKS from cannabis, dubbed tetraketide synthase (TKS) based on its presumed synthesis of a linear tetraketide, and OAC in yeast that was supplied with exogenous hexanoic acid allowed for the production of small amounts of olivetolic acid (0.48 mg/L). Further optimization of precursor supply, including the use of hexanoyl-CoA synthetases [69] to augment the supply of the starter substrate, may allow increased yield of this alkylresorcinolic product.

**Limitations and bottlenecks**

As with other secondary metabolites, achieving high yields of plant polyketides in microbes is a challenging task. An array of factors can impair productivity. The most obvious include the functionality of the biosynthetic pathway and the availability of precursors. The efficacy of a heterologous pathway is first affected by the concentration of its constituent enzymes. Although transcription rate can impact gene expression, there is no general correlation between mRNA and protein abundance [70-72]. Enzyme concentration is also influenced by translation-related mechanisms and protein degradation rate [73]. Codon usage is known to affect heterologous gene expression [74,75]. Although codon optimization does not necessarily improve expression, it should be part of every metabolic engineering approach. In addition to expression, enzyme efficiency (kcat/Km) can affect final productivity. Enzyme stability in the heterologous host, cofactor requirements, post-translational modification and regulation, subcellular compartmentation and feedback inhibition are other factors to consider. If possible, multiple enzymes should be tested to find the best candidates for each step of the pathway. In plants, polyketide pathways are tightly regulated so that only the required quantities of metabolites are produced [76]. In contrast, synthetic pathways assembled in heterologous hosts are not under such regulatory control. This absence of regulation can lead to growth retardation and may cause accumulation of toxic intermediates. Modulating expression of relevant genes is a commonly used strategy for balancing pathway dynamics. This can be done by tuning promoter strengths, by manipulating non-coding regions, and by altering gene or plasmid copy number [77-79]. Rate-limiting enzymes can be substituted for more efficient ones or can be improved by protein engineering [80,81].

The productivity of a synthetic pathway is greatly determined by the supply of precursor metabolites. As demonstrated by multiple research groups, tyrosine/phenylalanine and malonyl-CoA are limiting for the production of polyketides in microbes [39,41,45,47,50,54]. Although tyrosine and phenylalanine can be supplemented in the growth medium, this is not a sustainable option for cost-effective polyketide production. A more viable alternative is to increase the pool of tyrosine/phenylalanine within the polyketide-producing host, an approach successfully used by Santos et al [13]. Different groups succeeded in increasing tyrosine production in E. coli and S. cerevisiae by overproducing feedback-inhibition-resistant enzymes from the aromatic amino acid biosynthesis pathway and by removing transcriptional regulation [82-85]. Santos et al [86] subsequently improved the tyrosine titer in E. coli by using a global transcription machinery engineering approach (gTMTE) [87]. A common approach to increase the pool of malonyl-CoA is to overexpress ACC, the enzyme that catalyzed its synthesis from acetyl-CoA [41,45,63,88]. Malla et al [51] also included an acetyl-CoA synthetase to increase the carbon flux toward malonyl-CoA. Alternatively, malonyl-CoA can be synthesized from malonate by the co-expression of a malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) from Rhizobium trifolii, a strategy successfullly used in E. coli [47]. More sophisticated engineering approaches, that included the overexpression and the knockout of multiple genes, were also used to increase the pool of malonyl-CoA [50,89,90].

**Combinatorial biosynthesis of novel, unnatural plant type III polyketides**

Type III polyketides are of particular interest due to their potential as bioactive compounds with therapeutic uses. When screening for desired functionalities, the larger the library of diverse compounds one can create, the higher the chances of finding a compound that can fulfill a purpose of interest. In addition to the role of the type III PKSs, the diversity of plant polyketides also arises from the range of tailoring enzymes that perform post-polyketide modifications [91]. Because of this, the use of combinatorial biosynthesis is an ideal way to increase variety in an ever expanding library of polyketides derived from type III PKSs. Combinatorial biosynthesis entails designing artificial biosynthetic pathways containing enzyme-encoding genes from different species and/or organisms into a heterologous host. In this way, PKSs can be combined with tailoring enzymes from other organisms resulting in polyketides that would otherwise never be formed.

In 1999 McDaniel et al [92] were able to create a library of over 50 unnatural products (macrolides) by genetically modifying the erythromycin PKS genes thereby combinatorially affecting their catalytic activities in the biosynthetic pathway. Five years later, Katsuyama et al [46] used combinatorial biosynthesis and precursor-directed biosynthesis to create novel plant polyketides. This required a multi-plasmid approach, transforming E. coli with three plasmids each containing three distinct sections of the polyketide biosynthetic
pathway. The first plasmid (substrate synthesis plasmid) contained 4CL from Lithospermum erythrorhizon. The second plasmid (polyketide synthesis plasmid) contained CHS from Glycyrrhiza echinata and CHI genes from Pueraria lobata, or contained the STS gene from Arachis hypogaea. The post-polyketide modification plasmid contained either genes for flavone (FNS I gene from Petroselinum crispum) or flavonol production (flavanone 3β-hydroxylase (F3H) and flavonol synthase (FLS) genes from Citrus). The various genes from various organisms used to create this pathway define the combinatorial biosynthesis aspect of this experiment. Precursor-directed biosynthesis is a technique where cells are supplied with unnatural precursors which result in the formation of unnatural products. Natural and unnatural carboxylic acids were also exogenously introduced to the recombinant E. coli. When natural carboxylic acids were introduced, the system created corresponding natural polyketides. When unnatural carboxylic acids were introduced, the organism mainly produced the corresponding unnatural polyketides. The authors hypothesized that the reason for this was the relatively relaxed substrate specificity of their FNS I. This system resulted in the synthesis of 14 flavanones, 13 flavones, 8 flavanols, and 15 stilbenes at high yields and, the production of 20 triketide pyrones and 17 tetraketide pyrones through derailment or incorrect cyclization. Out of the 50 polyketides, 36 were novel compounds. 

Also in 2007, Chemler et al. [93] were able to create a type III polyketide pathway in Saccharomyces cerevisiae capable of utilizing acrylic acid analogues to produce novel unnatural polyketides. In this study, acrylic acid analogues were first screened in vitro for their potential as substrates for the flavonoid enzymes. In the second-stage screening, the compounds were tested for their ability to be synthesized into flavanone analogues through an in vitro assay where 4CL, CHS and CHI from petunia and a malonyl-CoA synthetase from Rhizobium trifolii were provided. The acrylic acids that passed both screenings were then provided as substrates for a S. cerevisiae strain expressing 4CL, CHS and CHI on a plasmid. This system resulted in the expression of six unnatural flavanones. In an effort to increase the amount of novel flavonoids produced, a plasmid containing flavanone 3β-hydroxylase (FHT) from Malus domestica was added to the initial strain, creating six novel dihydroflavonols from 6 acrylic acids. 

Another interesting approach to the formation of novel polyketide compounds is the manipulation or mutation of the type III PKS protein itself. In 2007, Abe et al. [94] mutated the pentaketide chromone synthase (PCS) from Aloe arborescens in order to create novel polyketides. E. coli expressing a triple mutant (F80A/Y82A/M207G) version of PCS, using malonyl-CoA as a substrate, was found to lead to the production of an unnatural novel nonaketide (naphthopyrone). In addition, control over polyketide chain length from triketide to octaketide has been demonstrated through site-directed mutagenesis of amino acid residues lining the active site of certain PKSs [94–96]. In one case, Abe et al. [96] were able to perform site-directed mutagenesis on an octaketide producing PKS, thereby creating the mutant CHS’s G207A, G207T, G207M, G207L, G207F, and G207W. The mutagenesis of this single residue led to the formation of smaller polyketides (triketides to heptaketides). Site-directed mutagenesis of a CHS has also been shown to affect its starter molecule specificity. In 2002 Jez et al. [97] were able to create a type III PKS capable of using the previously unusable substrate N-methylanthraniloyl-CoA to produce a novel alkaloid. This was accomplished by a single point mutation, F215S, in the PKS.

Concluding remarks and outlook

With the increasing availability of plant genomic sequences, the repertoire of plant biosynthetic genes is continuously expanding. These biological parts are the starting point for the reconstruction of plant secondary metabolic pathways in microorganisms [3]. The success of a defined engineering approach greatly depends on the use of adequate synthetic enzymes. After establishing the pathway in the microbial host and demonstrating the production of the desired metabolite, the next step is to increase product yield to the point of economic viability. Yield improvement currently represents the greatest engineering challenge. Metabolic limitations and bottlenecks have to be identified, and should be considered from the perspective of the whole organism. A holistic approach including rational and computational analysis is fast becoming the norm, and is advisable before carrying out significant manipulation of metabolic pathways. Not only can such an approach inform planning design, it can also be used iteratively once physiological and yield data is available from initial metabolic systems.

While informed engineering decisions are comparatively easy to make in the canonical model organisms, many promising host species currently lack sufficient characterisation to exploit fully [19,65,98]. Along with improved tools for genetic manipulation, as metabolic and regulatory data becomes available for novel hosts, more engineering will take place in these systems to take advantage of differences in their evolutionarily engineered metabolome. Such an expansion of host chassis, both highly engineered and naturally diverse, will undoubtedly complement the current molecular tuning of engineered plant polyketide pathways.

Microbial production of plant polyketides is still at an early stage and is greatly limited by the availability of characterised biosynthetic genes and the lack of knowledge about pathway structures. Recent engineering efforts have focused on CHS and STS, with most of the PKS diversity barely touched. Given all the avenues for development available to researchers, there is significant room for the field to grow.

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References

1. Pichersky E, Lewinsohn E (2011) Convergent evolution in plant specialized metabolism. Annu Rev Plant Biol 62: 549-566.
2. Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64: 3-19.
3. Facchini PJ, Bohlmann J, Covello PS, De Luca V, Mahadevan R, et al. (2012) Synthetic biosystems for the production of high-value plant metabolites. Trends Biotechnol 30: 127-131.
4. Bourgaud F, Gravor A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. Plant Science 161: 839-851.

5. Austin MB, Noel JP (2003) The chalcone synthase superfamily of type III polyketide synthases. Nat Prod Rep 20: 79-110.

6. Noel JP, Austin MB, Bomati EK (2005) Structure-function relationships in plant phenylpropanoid biosynthesis. Curr Opin Plant Biol 8: 249-253.

7. Abe I, Morita H (2010) Structure and function of the chalcone synthase superfamily of plant type III polyketide synthase. Nat Prod Rep 27: 809-838.

8. Clare N, Faure S, Martinez MC, Andriantithaina R (2011) Anticancer properties of flavonoids: roles in various stages of carcinogenesis. Cardiovasc Hematol Agents Med Chem 9: 62-77.

9. Gresele P, Cerletti C, Guglielmini G, Pignatelli P, de Gaetano G, et al. (2011) Effects of resveratrol and other wine polyphenols on vascular function: an update. J Nutr Biochem 22: 201-211.

10. Yen TL, Hsu CK, Lu WJ, Hsieh CY, Hsiao G, et al. (2012) Neuroprotective effects of xanthohumol, a flavonoid from hops (Humulus lupulus), in ischemic stroke of rats. J Agric Food Chem 60: 1937-1944.

11. Milligan SR, Kalita JC, Heyerick A, Rong H, De Cooman L, et al. (1999) Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer. J Clin Endocrinol Metab 84: 2249-2252.

12. Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55: 481-504.

13. Santos CN, Koffas M, Stephanopoulos G (2011) Optimization of a heterologous pathway for the production of flavonoids from glucose. Metab Eng 13: 392-400.

14. Wu S, Chappell J (2008) Metabolic engineering of natural products in plants; tools of the trade and challenges for the future. Curr Opin Biotechnol 19: 145-152.

15. Jeandet P, Delaunois B, Aziz A, Donnez D, Vasserot Y, et al. (2012) Metabolic engineering of yeast and plants for the production of the biologically active hydroxystilbene, resveratrol. J Biomed Biotechnol 2012: 5790089.

16. Wang Y, Chen S, Yu O (2011) Metabolic engineering of flavonoids in plants and microorganisms. Appl Microbiol Biotechnol 91: 949-956.

17. Chemler JA, Koffas MA (2008) Metabolic engineering for plant natural product biosynthesis in microbes. Curr Opin Biotechnol 19: 597-605.

18. Nicolau KC, Yang Z, Liu JJ, Ueno H, Nantemter PG, et al. (1994) Total synthesis of taxol. Nature 367: 630-634.

19. Marienhagen J, Bott M (2012) Metabolic engineering of microorganisms for the synthesis of plant natural products. J Biotechnol.

20. Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem Int Ed Engl 48: 4668-4716.

21. Staunton J, Weissman JK (2001) Polyketide biosynthesis: a millennium review. Nat Prod Rep 18: 380-416.

22. Shen B (2003) Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol 7: 285-295.

23. Funa N, Ohnishi Y, Fujii I, Shibuya M, Ebizuka Y, et al. (1999) A new pathway for polyketide synthesis in microorganisms. Nature 400: 897-899.

24. Seshime Y, Juvvadi PR, Fujii I, Kitanoto K (2005) Discovery of a novel superfamiy of type III polyketide synthases in Aspergillus oryzae. Biochem Biophys Res Commun 331: 253-260.

25. Abe I (2008) Engineering of plant polyketide biosynthesis. Chem Pharm Bull (Tokyo) 56: 1505-1514.

26. Jez JM, Ferrer JL, Bowman ME, Austin MB, Schroder J, et al. (2001) Structure and mechanism of chalcone synthase-like polyketide synthases. J Ind Microbiol Biotechnol 27: 393-398.

27. MacDonald MJ, D’Cunha GB (2007) A modern view of phenylalanine ammonia lyase. Biochem Cell Biol 85: 273-282.

28. Hotze M, Schroder G, Schroder J (1995) Cinnamate 4-hydroxylase from Catharanthus roseus, and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in Escherichia coli. FEBS Lett 374: 345-350.

29. Ferrer JL, Austin MB, Stewart C, Jr., Noel JP (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol Biochem 46: 356-370.

30. Douglas CJ (1996) Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. Trends in Plant Science 1: 171-178.

31. Konishi T, Sasaki Y (1994) Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. Proc Natl Acad Sci U S A 91: 3598-3601.

32. Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. Biosci Biotechnol Biochem 68: 1175-1184.

33. Nagel J, Culley LK, Lu Y, Liu E, Matthews PD, et al. (2008) EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyzes the biosynthesis of xanthohumol. Plant Cell 20: 186-200.

34. Austin MB, Bowman ME, Ferrer JL, Schroder J, Noel JP (2004) An aldol switch discovered in stilbene synthases mediates cyclization specificity of type III polyketide synthases. Chem Biol 11: 1179-1194.

35. Trofip S, Lant S, Rensing SA, Schroder J, Schroder G (1994) Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution. J Mol Evol 38: 610-618.

36. Herderich M, Beckert C, Veit M (1997) Establishing styrlylpyrone synthase activity in cell free extracts obtained from gametophytes of Equisetum arvense L. by high performance liquid chromatography–tandem mass spectrometry. Phytochemical Analysis 8: 194-197.

37. Abe I (2012) Benzalacetone Synthase. Frontiers in Plant Science 3.

38. Morita H, Wanibuchi K, Kato R, Sugio S, Abe I (2010) Expression, purification and crystallization of a plant type III polyketide synthase that produces diallylheptanoids. Acta Crystallographica Section F 66: 948-950.

39. Hwang EL, Kaneko M, Ohsahi Y, Horinouchi S (2003) Production of plant-specific flavonones by Escherichia coli containing an artificial gene cluster. Appl Environ Microbiol 69: 2699-2706.

40. Watts KT, Lee PC, Schmidt-Dannert C (2004) Exploring recombinant flavonoid biosynthesis in metabolically engineered Escherichia coli. ChemBiochem 5: 500-507.

41. Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, et al. (2005) Efficient production of (2S)-flavanones by Escherichia coli containing an artificial biosynthetic gene cluster. Appl Microbiol Biotechnol 68: 498-504.

42. Watts KT, Lee PC, Schmidt-Dannert C (2006) Biosynthesis of plant-specific stilbene polyketides in metabolically engineered Escherichia coli. BMC Biotechnol 6: 22.

43. Beekwilder J, Wolsinkelt R, Jonker H, Hall R, de Vos CH, et al. (2006) Production of resveratrol in recombinant microorganisms. Appl Environ Microbiol 72: 5670-5672.

44. Leonard E, Chemler J, Lim KH, Koffas MA (2006) Expression of a soluble flavone synthase allows the biosynthesis of phytoestrogen derivatives in Escherichia coli. Appl Microbiol Biotechnol 70: 85-91.
45. Leonard E, Lim KH, Saw PN, Koffas MA (2007) Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. Appl Environ Microbiol 73: 3877-3886.

46. Katsuayama Y, Funa N, Miyahisa I, Horinouchi S (2007) Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. Chem Biol 14: 613-621.

47. Leonard E, Yan Y, Fowler ZL, Li Z, Lim CG, et al. (2008) Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. Mol Pharm 5: 257-265.

48. Katsuayama Y, Matsuzawa M, Funa N, Horinouchi S (2008) Production of curcuminoids by *Escherichia coli* carrying an artificial biosynthesis pathway. Microbiology 154: 2620-2628.

49. Choi O, Wu CZ, Kang SY, Ahn JS, Uhm TB, et al. (2011) Biosynthesis of plant-specific phenylpropanoids by construction of an artificial biosynthetic pathway in *Escherichia coli*. J Ind Microbiol Biotechnol 38: 1657-1665.

50. Xu P, Ranganathan S, Fowler ZL, Maranas CD, Koffas MA (2011) Genome-scale metabolic network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA. Metab Eng 13: 578-587.

51. Malla S, Koffas MA, Kazlauskas RJ, Kim BG (2012) Production of 7-O-methyl aromadendrin, a medicinally valuable flavonoid, in *Escherichia coli*. Appl Environ Microbiol 78: 684-694.

52. Wu J, Du G, Zhou J, Chen J (2013) Metabolic engineering of *Escherichia coli* for (2S)-pinocembrin production from glucose by a modular metabolic strategy. Metabolic Engineering 16: 48-55.

53. Becker JV, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, et al. (2003) Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. FEMS Yeast Res 4: 79-85.

54. Jiang H, Wood KV, Morgan JA (2005) Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71: 2962-2969.

55. Yan Y, Kohli A, Koffas MA (2005) Biosynthesis of natural flavonoids in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71: 5610-5613.

56. Leonard E, Yan Y, Lim KH, Koffas MA (2005) Investigation of two distinct flavone synthases for plant-specific flavone biosynthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71: 8241-8248.

57. Zhang Y, Li SZ, Li J, Pan X, Cahoon RE, et al. (2006) Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and Mammalian cells. J Am Chem Soc 128: 13030-13031.

58. Trantas E, Panopoulos N, Ververidis F (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab Eng 11: 355-366.

59. Sydor T, Schaffer S, Boles E (2010) Considerable increase in resveratrol production by recombinant industrial yeast strains with use of rich medium. Appl Environ Microbiol 76: 3361-3363.

60. Shin SY, Han NS, Park YC, Kim MD, Seo JH (2011) Production of resveratrol from *p*-coumaric acid in recombinant *Saccharomyces cerevisiae* expressing 4-coumarate:CoA ligase and stilbene synthase genes. Enzyme Microb Technol 48: 48-53.

61. Wang Y, Halls C, Zhang J, Matsuno M, Zhang Y, et al. (2011) Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. Metab Eng 13: 455-463.

62. Gagne SJ, Stout JM, Liu E, Boubakir Z, Clark SM, et al. (2012) Identification of olivetolic acid cyclase from *Cannabidiol sativa* reveals a unique catalytic route to plant polyketides. Proc Natl Acad Sci U S A 109: 12811-12816.

63. Shin S-Y, Jung S-M, Kim M-D, Han NS, Seo J-H (2012) Production of resveratrol from tyrosine in metabolically engineered *Saccharomyces cerevisiae*. Enzyme and Microbial Technology 51: 211-216.

64. Koopman F, Beekwilder J, Crimi B, Houwelingen A, Hall RD, et al. (2012) De novo production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. Microb Cell Fact 11: 155.

65. Park SR, Yoon JA, Paik JH, Park JW, Jung WS, et al. (2009) Engineering of plant-specific phenylpropanoids biosynthesis in *Streptomyces venezuelae*. J Biotechnol 141: 181-188.

66. Xue Z, McCluskey M, Cantera K, Sariaslan FS, Huang L (2007) Identification, characterization and functional expression of a tyrosine ammonia-lyase and its mutants from the photosynthetic bacterium Rhodobacter sphaeroides. J Ind Microbiol Biotechnol 34: 599-604.

67. Ranganathan S, Suthers PF, Maranas CD (2010) OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. PLoS Comput Biol 6: e1000744.

68. Katsuayama Y, Matsuzawa M, Funa N, Horinouchi S (2007) In vitro synthesis of curcuminoids by type III polyketide synthase from *Oryza sativa*. J Biol Chem 282: 37702-37709.

69. Stout JM, Boubakir Z, Ambrose SJ, Purves RW, Page JE (2012) The hexanoyl-CoA precursor for cannabinoid biosynthesis is formed by an acyl-activating enzyme in *Cannabidiol sativa* trichomes. Plant J 71: 353-365.

70. Nie L, Wu G, Zhang W (2006) Correlation between mRNA and protein abundance in *Desulfovibrio vulgaris* a multiple regression to identify sources of variations. Biochemical and Biophysical Research Communications 339: 603-610.

71. Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, et al. (2004) Integrated genomic and proteomic analyses of gene expression in mammalian cells. Molecular & Cellular Proteomics 3: 960-969.

72. Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, et al. (2003) Global analysis of protein expression in yeast. Nature 425: 737-741.

73. Beyer A, Hollunder J, Nasheuer H-P, Wilhelm T (2004) Post-transcriptional expression regulation in the yeast *Saccharomyces cerevisiae* on a genomic scale. Molecular & Cellular Proteomics 3: 1083-1092.

74. Kane JF (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. Curr Opin Biotechnol 6: 494-500.

75. Angov E, Hillier CJ, Kincaid RL, Lyon JA (2008) Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. PLoS one 3: e2189.

76. Vom Endt D, Kijne JW, Memelink J (2002) Transcription factors controlling plant secondary metabolism: what regulates the regulators? Phytochemistry 61: 107-114.

77. Stephanopoulos G (1999) Metabolic fluxes and metabolic engineering. Metabolic Engineering 1: 1-11.

78. Pitera DJ, Paddon CJ, Newman JD, Keasling JD (2007) Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. Metabolic Engineering 9: 193-207.

79. Pfleger BF, Pitera DJ, Smolke CD, Keasling JD (2006) Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat Biotechnol 24: 1027-1032.

80. Bloom JD, Meyer MM, Meinhold P, Otey CR, MacMillan D, et al. (2005) Evolving strategies for enzyme engineering. Current Opinion in Structural Biology 15: 447-452.

81. Leonard E, Ajikumar PK, Thayer K, Xiao W-H, Mo JD, et al. (2010) Combining metabolic and protein engineering of a terpenoid
biosynthetic pathway for overproduction and selectivity control. Proceedings of the National Academy of Sciences 107: 13654-13659.
82. Lutke-Eversloh T, Stephanopoulos G (2007) L-tyrosine production by deregulated strains of Escherichia coli. Appl Microbiol Biotechnol 75: 103-110.
83. Lurtik MA, Vuralhan Z, Suir E, Braus GH, Pronk JT, et al. (2008) Alleviation of feedback inhibition in Saccharomyces cerevisiae aromatic amino acid biosynthesis: quantification of metabolic impact. Metab Eng 10: 141-153.
84. Chávez-Béjar MI, Lara AR, López H, Hernández-Chávez G, Martínez A, et al. (2008) Metabolic engineering of Escherichia coli for L-tyrosine production by expression of genes coding for the chorismate mutase domain of the native chorismate mutase-prephenate dehydratase and a cyclohexadienyl dehydrogenase from Zymomonas mobilis. Applied and Environmental Microbiology 74: 3284-3290.
85. Jumina D, Baidoo EKE, Redding-Johanson AM, Barth TS, Burd H, et al. (2012) Modular engineering of L-tyrosine production in Escherichia coli. Applied and Environmental Microbiology 78: 89-98.
86. Santos CN, Xiao W, Stephanopoulos G (2012) Rational, combinatorial, and genomic approaches for engineering L-tyrosine production in Escherichia coli. Proc Natl Acad Sci U S A 109: 13538-13543.
87. Alper H, Stephanopoulos G (2007) Global transcription machinery engineering: A new approach for improving cellular phenotype. Metabolic Engineering 9: 258-267.
88. Watanachaisereekul S, Lantz AE, Nielsen ML, Nielsen J (2008) Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. Metab Eng 10: 246-254.
89. Zha W, Rubina-Pitl SB, Shao Z, Zhao H (2009) Improving cellular malonyl-CoA level in Escherichia coli via metabolic engineering. Metabolic Engineering 11: 192-198.
90. Fowler ZL, Gikandi WW, Koffas MAG (2009) Increased malonyl coenzyme A biosynthesis by tuning the Escherichia coli metabolic network and its application to flavanone production. Applied and Environmental Microbiology 75: 5831-5839.
91. Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology 126: 485-493.
92. McDaniel R, Thamchaipener A, Gustafsson C, Fu H, Brlach M, et al. (1999) Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel “unnatural” natural products. Proceedings of the National Academy of Sciences 96: 1846-1851.
93. Chemler JA, Yan Y, Leonard E, Koffas MA (2007) Combinatorial mutasynthesis of flavonoid analogues from acrylic acids in microorganisms. Org Lett 9: 1855-1858.
94. Abe I, Morita H, Oguro S, Noma H, Wanibuchi K, et al. (2007) Structure-based engineering of a plant type III polyketide synthase: formation of an unnatural nonaketide naphthopyrone. Journal of the American Chemical Society 129: 5976-5980.
95. Abe I, Watanabe T, Morita H, Kohno T, Noguchi H (2006) Engineered biosynthesis of plant polyketides: manipulation of calchone synthase. Organic Letters 8: 499-502.
96. Abe I, Oguro S, Utsumi Y, Sano Y, Noguchi H (2005) Engineered biosynthesis of plant polyketides: chain length control in an octaketide-producing plant type III polyketide synthase. Journal of the American Chemical Society 127: 12709-12716.
97. Jez JM, Bowman ME, Noel JP (2002) Expanding the biosynthetic repertoire of plant type III polyketide synthases by altering starter molecule specificity. Proceedings of the National Academy of Sciences 99: 5319-5324.
98. Meyer V, Wu B, Ram AF (2011) Aspergillus as a multi-purpose cell factory: current status and perspectives. Biotechnol Lett 33: 469-476.

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