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Metabolic engineering for plant natural product biosynthesis in microbes

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Plant natural products (NPs) not only serve many functions in an organism's survivability but also demonstrate important pharmacological activities. Isolation of NPs from native sources is frequently limited by low abundance and environmental, seasonal, and regional variation while total chemical synthesis of what are often complex structures is typically commercially infeasible. Reconstruction of biosynthetic pathways in heterologous microorganisms offers significant promise for a scalable means to provide sufficient quantities of a desired NP while using inexpensive renewable resources. To this end, metabolic engineering provides the technological platform for enhancing NP production in these engineered heterologous hosts. Recent advancements in the production of isoprenoids, phenylpropanoids, and alkaloids were made possible by utilizing a variety of techniques including combinatorial biosynthesis, codon optimization, expression of regulatory elements, and protein engineering of P450s.

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Introduction

Plant secondary metabolites are chemicals not necessary for a cell's immediate survival but serve in a plethora of roles to give an organism an evolutionary advantage in its ultimate survival and reproduction. These compounds are thought to be the result of evolutionary design described as either a 'target-based' model [1] because of sophisticated product/receptor interactions or 'diversity-based' model [2,3] since secondary pathways produce a large number of related molecules with only few having known biological activity. Either way, the relatively high percentage of secondary metabolites that are biologically active compared to large synthetic libraries underscores the argument that natural products (NPs) are underexploited in drug discovery [4•]. For instance, biopharmaceuticals entering Phase I testing have a 25% success rate of final regulatory approval while the success rate for a conventional drug is only 6% [5]. During the past 20 years, pharmaceutical companies have shied away from NPs because their complex structures typically make total synthesis impractical. Indeed, combinatorial synthetic molecules have on average less than one chiral center per molecule while approved pharmaceuticals have multiple chiral centers [6]. Instead, companies have embraced automated high-throughput screening (HTS) and combinatorial chemistry to supply the vast chemical libraries required to discover rare drug hits. In the wake of HTS, NP discovery programs were de-emphasized or eliminated altogether because of the limited size and supply of NP libraries to provide the sheer number of compounds HTS demanded [7]. Still, during the past 25 years a little over half of pharmaceuticals brought to market are either NPs or structurally derived from or related to a NP [4•].

Once a potent NP is identified, the main limiting factor for drug discovery is the ability to produce enough material for clinical applications [8]. The biological activity and potency of NPs are derived largely from their complex structures involving regio-specific functionalization and chirality. Total synthesis poses many challenges for chemists, and although often successful, has very little practical value for the development of complex NPs for research and therapeutics. Indeed, elegant methodologies exist for complex NPs but the numerous steps involved can result in low overall yields as illustrated by the total synthesis of Taxol® which has an overall yield of just 0.02% [9]. Usually, a synthetic route for a complex molecule becomes impractical if the total process requires over 10 separate steps, since with each step the overall yield decreases and the waste and resources increase.

Extraction from the native plant sources is an alternative that is often problematic: plant NPs tend to accumulate at low quantities over very long growth periods, purification of the desired compound requires separation from a multitude of other compounds of similar structure and yields are subject to regional and environmental factors. Plant cell cultures can standardize yield fluctuation issues but challenges include slow doubling times and complex separation. Significant work has been made on improving primary and secondary NP yields in native

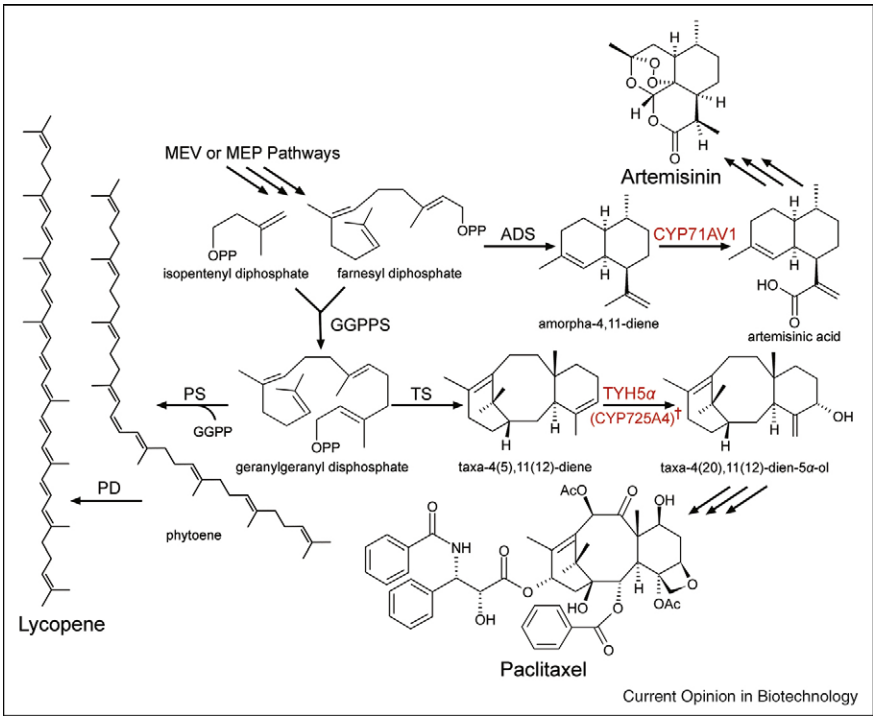
plant hosts and plant cell cultures have been reviewed extensively [10].

Biosynthesis of chemicals and biomaterials from recombinant microorganisms has arisen as a competitive alternative to traditional chemistry-based routes. For instance, cultures of tractable strains are readily scalable from the bench-top to industrial-sized fermentors unlike synthetic chemistry. Large-scale microbial fermentations address many of the issues mentioned regarding extracting a NP from its native plant source. In addition, recombinant microbes are typically void of competing pathways to transgenic metabolism, thus producing specific enantiomeric distinct products and intermediates, which minimize downstream purification often to one step. Microbial fermentations also have the advantages of having rapid doubling times, allowing much shorter production times and their growth which is based on inexpensive renewable feed stocks (carbon and nitrogen sources), can be strictly controlled. Unfortunately, however, only a handful of NPs are currently produced from recombinant microorganisms; this is because perhaps the largest challenge that has yet to be overcome is the fact that often times the metabolic pathway leading to a plant NP is mostly or completely unknown. This review will summarize the successes in the production and yield improvements of plant NPs, including isoprenoids, phenylpropanoids, and alkaloids, by expressing their heterologous biosynthetic pathways in microorganisms. It is important to note that an equally diverse number of secondary metabolites exist in microbial sources [11].

Challenges of producing plant NPs in microbes

Several techniques such as metagenomics, transcriptomics, proteomics, metabolomics, and phenomics [12] are necessary first steps for the identification of involved genes, enzymes, and metabolites of a plant NP biosynthetic pathway. Once a heterologous pathway is introduced to a microbial host, the next challenge is to boost production so it becomes a commercially viable platform. Metabolic engineering is the platform technology applied for addressing the second limitation in NP heterologous production, namely the production optimization of a desired compound usually in engineered genetically tractable microbial strain such as *Escherichia coli* and *Saccharomyces cerevisiae*. Yields of small molecules produced in their native hosts have been improved through classic strain improvement strategies consisting of repetitive cycles of random mutagenesis coupled with a HTS method [13]. Metabolic engineering provides the means to achieve yield improvements of recombinant small

Figure 1



A representative version of the isoprenoid biosynthetic pathway discussed in this article. MEV, mevalonate; MEP, methyl erythritol phosphate; ADS, amorphadiene synthase; CYP71AV1, amorphadiene oxidase; GGPPS, geranylgeranyl diphosphate synthase; TS, taxadiene synthase; TYH5α, taxadiene 5α-hydroxylase; PS, phytoene synthase; PD, phytoene desaturase; GGPP, geranylgeranyl diphosphate; †CYP725A4 was recently suggested to be a taxadiene cyclase not a TYH5α [16]. Multiple arrows indicate multiple enzyme reactions. Cytochrome P450 enzymes are indicated in red.

molecules, such as plant NPs, in shorter time frames and in a focused and directed manner.

Engineering of isoprenoid biosynthesis

Isoprenoids (also known as terpenoids) represent the largest class of known plant metabolites including carotenoids and sterols with the most notable examples being Taxol®, lycopene, and artemisinin (Figure 1). All isoprenoids originate from the common five-carbon building blocks, isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMPP), which are derived from either the mevalonate (MEV) pathway or the methyl erythritol phosphate (MEP) pathway, depending on the organism.

Taxol

Taxol® (paclitaxel), produced in *Taxus brevifolia* (Pacific yew), is a classical example of the plant kingdoms' ability to produce chemotherapeutics. Taxol® is a complex and highly functionalized terpenoid that has been approved for the treatment of refractory ovarian and metastatic breast cancer. Its production is based on a semisynthetic route that chemically converts a paclitaxel intermediate isolated from the needles or cell cultures of various *Taxus* species [14]. However, the desire for a robust biosynthetic route using microorganisms still exists in order to meet growing demands [15].

The biosynthesis of paclitaxel involves approximately 20 biochemical steps [16] that do not necessarily operate in a linear fashion. As some steps of the metabolic pathway are missing, identification and reclassification of the involved steps continues [17–19]. Recent progress has been made in the heterologous expression of the preliminary paclitaxel biosynthesis genes and optimization of paclitaxel intermediates in microbial hosts. Reconstitution of the first five committed steps to synthesize taxdien-5α-acetoxyl-10β-ol was attempted in *S. cerevisiae* [20]. The recombinant strain produced taxadiene, the molecular core of paclitaxel, at about 1 mg/L but only trace amounts of the expected taxadien-5α-ol was detected. It was concluded that the main bottleneck resulted from poor P450 activity but a recent reclassification of the P450, CYP725A4, has shown that a novel taxene 5(12)-oxa-3(11)-cyclotaxane was the sole product [17], suggesting that CYP725A4 is not involved in paclitaxel synthesis but is rather a diversification branch point toward cyclotaxenes. More recent work successfully boosted the production of taxadiene in yeast 40-fold through metabolic engineering by using codon optimization, combinatorial biosynthesis, and introducing regulatory elements to inhibit competitive pathways [21•].

Artemisinin

Artemisinin is an antimalarial drug isolated from *Artemisia annua* L (family Asteraceae; commonly known as sweet wormwood) and is highly effective against multidrug resistant strains of *Plasmodium falciparum*. Supply from natural sources is limiting and too expensive for most malaria sufferers while the cost of its total synthesis is prohibitive [22]. A semisynthesis route [23] could potentially be cost effective by starting with artemisinic acid that can be produced from a genetically engineered microbe. The first committed step of artemisinin biosynthesis, namely the conversion of farnesyl diphosphate (FPP) into amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS) was performed in *S. cerevisiae* but low yields were reported [24]. Optimization of the FPP precursor in yeast proved pivotal to boosting amorphadiene production and the identification and expression of amorphadiene oxidase (AMO), a cytochrome P450, and a CPR from *A. annua* allowed the production of artemisinic acid in significant quantities [25••].

Unlike yeast, *E. coli* only harbors the MEP pathway to generate FPP and its derivatives and optimization of the MEP pathway is a viable means to increase FPP availability [26]. An alternative approach was used to increase isoprenoid products by successful reconstitution and optimization of the MEV pathway from yeast in *E. coli* along with ADS [27–29]. However, to produce artemisinic acid in *E. coli*, one must address the expression of the P450 enzyme, AMO. Indeed, a high titer production of artemisinic acid was achieved using an *E. coli* platform expressing an engineered AMO that was codon optimized and had its membrane anchor altered [30]. The next logical step would be to couple engineered P450s with a balanced MEV or MEP pathway to achieve even higher terpenoid yields in *E. coli*.

Lycopene

Carotenoids are a subfamily of isoprenoids that are the most widely distributed yellow, orange, and red natural pigments synthesized in bacteria, algae, and fungi. Commercially available carotenoids such as lycopene, β-carotene, and astaxanthin are used as food colorants, animal feed supplements, and for nutritional and cosmetic purposes. More recently, carotenoids have received attention for their significant antioxidant activities and for playing important roles in inhibiting the onset of chronic diseases [31].

Overexpression of three exogenous genes including geranylgeranyl diphosphate synthase (GGPPS), phytoene synthase (PS), and phytoene desaturase (PD) was sufficient to produce the red-color lycopene in *E. coli* [32]. To identify additional genes to enhance production, a shotgun genomic library was screened in *E. coli* for enhanced coloration. Three regulatory elements and one directly involved in the MEP pathway were found to increase lycopene accumulation [33]. An alternative strategy was employed to increase lycopene concentrations by performing gene knockouts. Using both systematic (model-based) and combinatorial (transposon-based) approaches,

a number of targets were identified leading to the construction of two triple knockout mutants [34••]. Further characterization of these two mutants in a high cell density fermentor led to high production levels [26]. Most recently, enhanced lycopene accumulation in *E. coli* was reported using a hybrid approach to identify the best combination of gene expressions and knockouts [35].

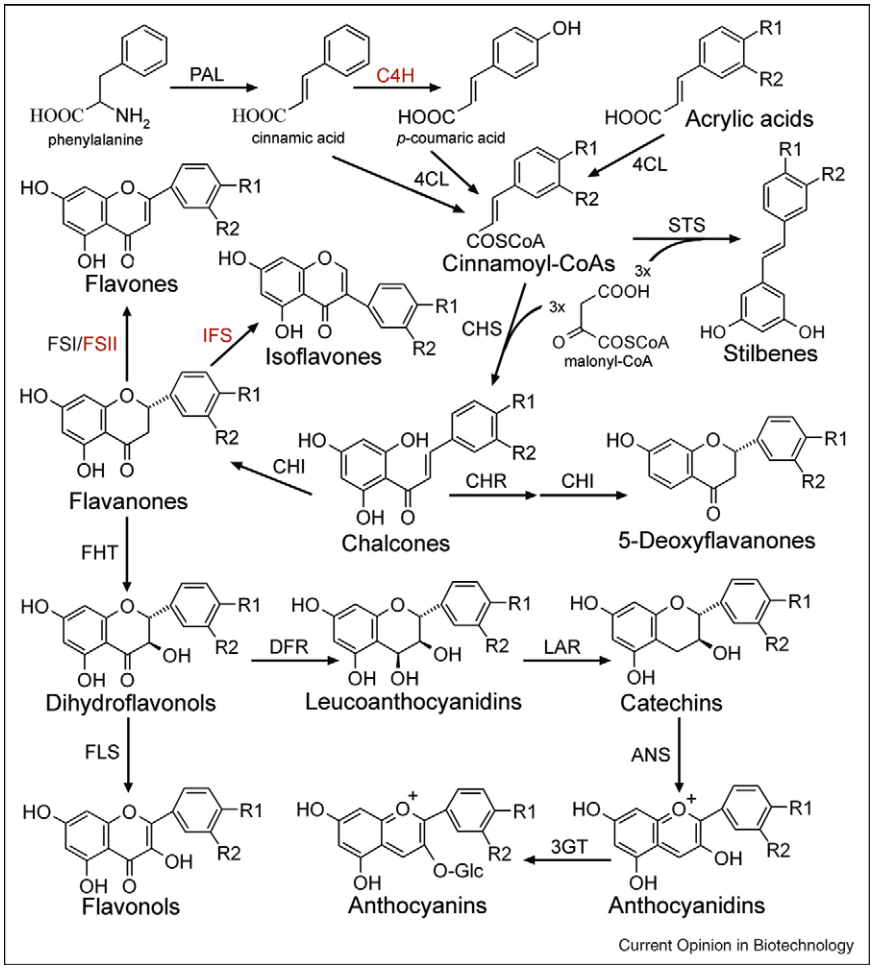
Engineering of phenylpropanoid biosynthesis

Phenylpropanoids are a diverse group of plant secondary metabolites derived from the condensation of several malonyl-CoA molecules and starter units derived from phenylalanine or tyrosine (Figure 2). Phenylpropanoids, including flavonoids and stilbenes, possess extraordinary antioxidant activity and estrogenic, antiviral, antibacterial, and anticancer activities [36]. The health-protecting effects of flavonoids have stimulated significant research toward the elucidation of their biosynthetic networks, as well as the development of production platforms using genetically tractable hosts.

Flavonoids

Both *E. coli* and *S. cerevisiae* have been used for the metabolic engineering of recombinant flavonoid pathways. The initial steps in the phenylpropanoid pathway required expression of a phenylalanine ammonia lyase (PAL) and a cinnamate 4-hydroxylase (C4H) in yeast [37] to first deaminate phenylalanine into cinnamic acid and then undergo hydroxylation to yield *p*-coumaric acid. Alternatively, tyrosine ammonia lyase (TAL) was expressed in *E. coli* and yeast [38] to directly deaminate tyrosine into *p*-coumaric acid. By also expressing a 4-coumaroyl-CoA ligase (4CL) and a chalcone synthase (CHS), a type II polyketide synthase, a racemic mixture

Figure 2



The main flavonoid biosynthetic pathway. R1, R2 = H and/or OH for natural flavonoids; R1, R2 = H, OH, F and/or NH_3 for unnatural flavonoid analogs. PAL, phenylalanine lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; CHI, chalcone isomerase; CHR, chalcone reductase; FSI, soluble flavone synthase; FSII, cytochrome flavone synthase; IFS, isoflavone synthase; FHT, flavanone 3β-hydroxylase; DFR, dihydroflavonol reductase; LAR, leucoanthocyanidin reductase; FLS, flavonol synthase; ANS, anthocyanidin synthase; 3GT, 3-*O*-glycosyltransferase. Cytochrome P450 enzymes are indicated in red.

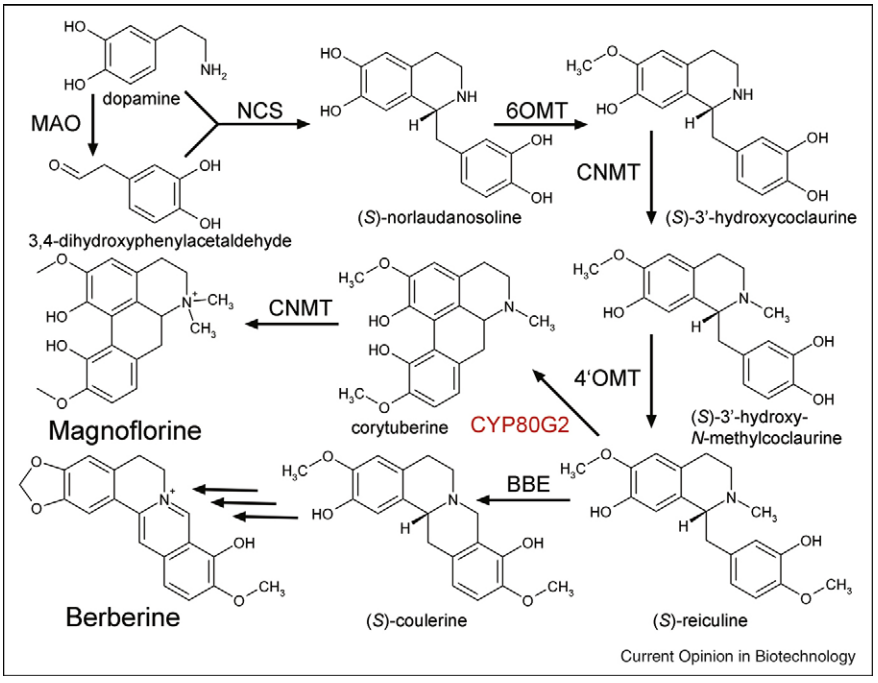
of flavanones was produced in yeast [39]. The addition of a chalcone isomerase (CHI) presumably enriched the enantiomeric pool of the natural (2*S*)-flavanones while improving yields when yeast cultures were fed with cinnamic acids [40]. Expression of a chalcone reductase (CHR) along with the flavanone pathway led to the production of both 5-deoxyflavanones and 5-hydroxyflavanones [41].

Additional expression of a flavone synthase (FNS) with the flavanone pathway produced several flavones in both yeast [42] and *E. coli* [43,44] while expression of a flavonol 3β-hydroxylase (FHT) along with either a flavonol synthase (FLS) [43,45] or a dihydroflavonol reductase (DFR) with a leucocyanidin reductase (LAR) [46] yielded flavonols or catechins, respectively. It is important to also note that in just the past two years, metabolic engineering has been employed to greatly improve *E. coli*'s efficiency to produce various flavonoids by focusing on increasing intracellular pools of biosynthetic pathway cofactors. For instance, anthocyanin synthesis relies on UDP-glucose as the sugar donor molecule to the aglycone. Overexpression of UDP-glucose biosynthesis genes with an anthocyanidin synthase — 3-*O*-glucosyltransferase (ANS-3GT) fusion in *E. coli* yielded high anthocyanin concentrations [47]. Further yield improvements were obtained by deleting competitive pathways for UDP-glucose [48]. Similarly, flavanone production was increased dramatically by optimizing intracellular levels of the cofactor malonyl-CoA by overexpressing the multicomponent acetyl-CoA carboxylase (ACC) and other acetate assimilating enzymes [49••]. Using an alternate strategy, malonate was supplemented into the culture medium while expressing a malonate carrier protein (MatC) and malonyl-CoA synthetase (MatB) to give similar high levels of flavanones [48]. By also inhibiting fatty acid biosynthesis using sublethal levels of the antibiotic cerulenin, flavanone production levels were reportedly further improved [48]. The biosynthetic pathways in both *E. coli* and *S. cerevisiae* can also be used for mutasynthesis by converting functionalized cinnamic acid analogs into their corresponding novel flavonoid analogs including novel stilbenes, flavanones, flavones, flavonols, and dihydroflavonols [45,48,50].

Stilbenes

Stilbenes are another class of phenylpropanoids that have gained much attention, particularly resveratrol, which is touted as an antioxidant [51] and for its ability to increase the lifespan of yeast, fruit fly, round worm [52], and fish [53] by activating siruin deacetylases [54]. *S. cerevisiae* reportedly produced resveratrol from endogenous phenylalanine using a combination of PAL, 4CL, and stilbene synthase (STS) [55]. Furthermore, *E. coli* expressing 4CL and STS converted supplemented cinnamic

Figure 3



A representative version of the alkaloid biosynthetic pathway discussed in this article. MAO, monoamine oxidase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; 4′OMT, 3′-hydroxy *N*-methylcoclaurine 4′-*O*-methyltransferase; CYP80G2, P450 hydroxylase; BBE, berberine bridge-forming enzyme; multiple arrows indicate multiple enzyme reactions. Cytochrome P450 enzymes are indicated in red.

acids into their respective stilbenes at much higher levels [45,56].

Engineering of alkaloid biosynthesis

Alkaloids are low-molecular-weight nitrogenous compounds with significant medicinal applications as analgesics, stimulants, and chemotherapeutics. Owing to their numerous applications, production platforms for their synthesis have been engineered, mainly based on the use of plant cell and tissue cultures. In the past, the successful synthesis of *Nicotiana*, *Hyoscyamus*, *Datura*, and *Atropa tropane* alkaloids, the isoquinoline alkaloids of *Coptis* and *Eschscholtzia californica*, and the terpenoid indole alkaloids of *Catharanthus roseus* has been demonstrated (reviewed in [57,58]). In addition, the biosynthesis of alkaloid analogs in *Catharanthus roseus* has also been reported [59,60]. Some of the recent successes in reconstituting alkaloid biosynthesis in microorganisms as well as the use of combinatorial biosynthesis for the construction of novel alkaloid structures will further be reviewed.

Benzylisoquinoline alkaloids are among the most potent and yet complex alkaloids in nature (Figure 3). Their biosynthesis begins with the conversion of tyrosine to dopamine and 4-hydroxyphenylacetaldehyde (4HPAA). Dopamine and 4HPAA are then condensed to yield (*S*)-norcoclaurine which is the central precursor to all isoquinoline alkaloids, a reaction catalyzed by norcoclaurine synthase (NCS). (*S*)-Norcoclaurine is then sequentially converted to coclaurine by *S*-adenosyl methionine (SAM)-dependent norcoclaurine 6-*O*-methyltransferase (6OMT), to *N*-methylcoclaurine by coclaurine *N*-methyltransferase (CNMT), to 3′-hydroxy-*N*-methyl coclaurine by the CYP80G2 P450 hydroxylase, and then to (*S*)-reticuline by 3′-hydroxy *N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT).

Because the first steps in benzylisoquinoline alkaloid biosynthesis are difficult to reconstruct in a prokaryote because of the presence of plant P450 monooxygenases, Minami *et al.* used plant NCS, 6OMT, CNMT and 4′OMT, and a microbial monoamine oxidase (MAO) to synthesize reticuline from dopamine in *E. coli* [61•]. In order to synthesize aporphine-type alkaloids, corytuberine synthase and CNMT were cloned in *S. cerevisiae* to synthesize magnoflorine from reticuline, while the expression of berberine bridge enzyme led to the synthesis of scoulerine also from reticuline. Although overall production levels were low, this is the first report of the microbial production of alkaloids and can provide the means to create new alkaloid compounds through combinatorial biosynthesis.

In another work, the biosynthesis of an expanded array of benzylisoquinoline alkaloids in the sanguinarine/berberine and morphinan branches in *S. cerevisiae* was recently demonstrated [62•]. Relatively high-yield reticuline production from norlaudanosoline was first accomplished, followed by the biosynthesis of (*S*)-scoulerine and salutaridine from (*S*)-reticuline and (*R*)-reticuline, respectively. This work highlights the diversity of alkaloid products that can be synthesized in microbial hosts by combining activities from diverse organisms.

Engineering of P450s

A common feature of plant biosynthetic pathways is the use of one or more cytochrome P450 enzymes to functionalize a molecule's core structure. Heterologous expression in eukaryotes like yeast can naturally support the membrane-bound enzymes and efforts have been made to further optimize such heterologous expression [63]. However, expression of P450s in prokaryotes is hampered by a number of factors including improper protein folding or membrane translation, general toxicity, and lack of post-translational modifications. Although a number of strategies have been developed for mammalian P450s [64], very few examples exist for engineering functional plant P450s in *E. coli in vivo*. To address the membrane translation issue, most examples focus on altering a P450s membrane anchor to be similar to that of the N-terminus of the successfully expressed recombinant bovine P450 17A1 [65]. In doing so, small amounts of hydroxylated flavonoids were synthesized [66] and using a similar approach relatively high amounts of terpenoids [30] and isoflavones [67] were obtained. Along with engineering the membrane anchor, efforts have been made to mimic protein complexes found in native plants by creating P450 chimeras. In one instance, the P450 was fused to the enzyme responsible for yielding the substrate for the P450 [68] while other examples linked the P450 with a supporting CPR using a flexible mini-linker [66,67]. A future approach could involve the engineering of the native, soluble *Bacillus megaterium* P450 to hydroxylate substrates involved in plant secondary metabolism, as it has been demonstrated in other cases [69].

Conclusions

NP biosynthesis in microbial hosts still remains an engineering challenge despite significant progress in the last 10 years. During that time, significant strides have been made that allow the introduction of large metabolic networks and their regulatory elements in heterologous hosts, the functional expression of microsomal hydroxylases in prokaryotes through protein engineering and the redirection of carbon flux in the cell for enhancing production yields. Still, however, significant challenges remain that present golden opportunities for the development of an array of techniques that can benefit several other biochemical engineering efforts: the rapid elucidation of metabolic pathways through the combination of 'omics' technologies such as genomics, functional genomics, and metagenomics; the improved expression of microsomal P450s through protein engineering of prokaryotic enzymes; the fine tuning of the transcription of

native and heterologous pathways utilizing tools from synthetic biology; and finally the development of more general methodologies that will allow the HTS of large libraries of phenotypes for efficient production yields.

It is at the same time important to underline that the rapid advancement in the area will be difficult without addressing the issue of the multitude of regulations that currently restrict its large-scale application. It is therefore important for a parallel effort to be placed on educating the general public about the great opportunities metabolic engineering has to offer for meeting ever-increasing needs in human health and overall well-being. This will be perhaps the main approach to sustain research efforts in the field and will help it flourish even further.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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