Review Article

Engineered Production of Bioactive Natural Products from Medicinal Plants

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Abstract

Plant natural products have been particularly important due to their use in food, cosmetic, and pharmaceutical industries. In particular, Traditional Chinese Medicine provides a precious potential for the discovery of bioactive natural products and development of novel modern medicines. However, the existing production methods for plant natural products such as chemical synthesis and plant extraction does not meet the current demand. Due to their environmental and economic concerns, engineered production of valuable natural products in microbial hosts has become an attractive alternative platform. This review covers the recent advances in the engineered production of plant natural products in microorganisms. A special focus was placed on the biotechnological production of plant-derived terpenoids, flavonoids, and alkaloids. Some successful examples of engineered production of plant natural products (or their precursors) such as artemisinin, paclitaxel, naringenin, quercetin, berberine, and noscapine are summarized. This clearly indicates that the engineered production method is a promising approach with various advantages over current methods.

Keywords: Flavonoids; terpenoids; alkaloids; engineered production; plant natural products

Introduction

Bioactive compounds from various natural sources have been widely used in the treatment of diseases throughout the human history. Plant natural products have been particularly important due to their use in food, cosmetic and pharmaceutical industries as fragrances, flavors, and medicines.[1] The structural complexity of natural products leads to not only their diversity but also the wide range of their biological activities such as antimicrobial, anticancer, antitumor, antifungal, antiviral, antiparasitic, antioxidant, antihypertensive, anti-inflammatory activities, and so on.[2] As a successful example, penicillin as an antibiotic has saved the lives of millions of people since its use in World War II.[3] Newman and Cragg analyzed all approved new drugs, including vaccines and biological macromolecules, over almost four decades (January 1981 – September 2019) and revealed that out of 1881 new approved drugs, there are 431 drugs obtained from natural products, botanical natural products, and natural product derivatives. Additionally, 489 drugs are synthetically produced, yet either their pharmacophore are natural products, or they mimic natural products. This analysis obviously indicated that natural products are the source of almost half of the all approved new drugs in the last four decades. In fact, natural products and their derivatives occupy a significant market share in the pharmaceutical industry, covering 63% of anticancer drugs and 56% of antibacterial agents.[4]

Natural products are isolated from various sources such as bacteria, fungi, animals, and plants. In particular, plants have been an important reservoir of natural products with many successful examples such as artemisinin, paclitaxel, camptothecin, vinblastine, podophyllotoxin, and so many others.[5-7] Historically,

Access this article online

Quick Response Code:

Website: www.wjtcm.net

DOI: 10.4103/wjtcm.wjtcm_66_21

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Received: 17-03-2020, **Accepted:** 25-04-2021, **Published:** 29-01-2022

How to cite this article: Fidan O, Ren J, Zhan J. Engineered production of bioactive natural products from medicinal plants. World J Tradit Chin Med 2022;8:59-76.

plants have been used as traditional medicine without knowing the active natural products for the treatment of many diseases for thousands of years. For instance, *Compendium of Materia Medica* as an ancient encyclopedia of Traditional Chinese Medicine (TCM), describes more than 1,000 species of plants.[8] As noted above, artemisinin is an example of modern drugs based on TCM. There is a record of the efficacy and related details of *Artemisia annua* in the treatment of malaria in China's Jin Dynasty (AD 284–384). This indicates Chinese physicians had been able to treat malaria 1700 years ago.[9] As a result of this historical route and successful studies, a Chinese scientist, Youyou Tu was awarded the 2015 Nobel Prize in Medicine due to her outstanding work on the discovery of artemisinin from *A. annua* L.[10] This clearly indicates that TCM is extremely important for natural product discovery to develop novel modern medicines.

Plants produce a wide variety of pharmaceutically important compounds as a means of evolutionary advantage in their environment. It was estimated that there are more than 200,000 plant secondary metabolites with the functions of signaling and defense mechanism against other organisms.[11-13] Once a promising plant natural product is discovered, the main bottleneck for industrial or medical applications is the ability to produce enough compound due to the fact that plant natural products accumulate at varying quantities over long growth periods and are affected by environmental and regional factors.[14] For example, paclitaxel (Taxol®) is an effective anticancer agent used in the treatment of various cancer cells. Two to four fully–grown trees of *Taxus brevifolia* have to be sacrificed to extract the necessary amount for the treatment of a single patient.[15-18] On the other hand, usage of organic solvents is required for the extraction and purification processes due to the presence of structurally similar compounds.[14,19] For these reasons, extraction of promising natural products from plants is time-consuming and environmentally unfriendly and leads to pricy and unaffordable drugs to many patients.[20]

Chemical synthesis has also been used to produce medicinally important plant natural products. Nevertheless, the structural complexity of many plant natural products makes the chemical synthesis impractical due to the difficulties in creating chiral molecules.[21,22] Even though there are chemical methodologies to synthesize the complex plant natural products, it involves numerous steps, leading to very low yields, as occurred in the example of total synthesis of taxol and the increase in accumulated waste and consumed resources in each step.[23,24] Moreover, chemical synthesis often necessitates the use of heavy metal catalysts, strong acids and bases, and organic solvents. This not only makes the chemical synthesis unsustainable but also results in the unrealistic prices of some natural products such as nicotianamine ($350,000 per gram).[16,25] Thus, chemical production of structurally complex plant natural products is extremely challenging, time-consuming, expensive, and environmentally unfriendly.

Cultivation of plant cells, tissues or organs, and fermentation of engineered microorganisms are an promising way to produce a plant natural product using biotechnological approaches.[26] However, engineering of plant cells is more technically difficult and time-consuming than microbes. In addition, unlike microbial cells, plant cells are larger, relatively inflexible due to the rigid cellulose cell wall, and prone to aggregation. They also suffer from unpredictable productivity, slow growth, and high sensitivity to shear stress in bioreactors. Therefore, the cultivation of plant cells for the production of promising natural products still remains challenging.[27]

In recent years, biotechnological production of medicinally important natural products in engineered microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Aspergillus,* and *Streptomyces* species has been more attractive and popular in the era of genomics, which exhibits several advantages over chemical synthesis, cell and organ cultures, and plant extraction. First, microorganisms grow rapidly and thus need a shorter overall process time than chemical synthesis, plant cell cultures, and regular plant cultivation to obtain the same amount of plant natural product.[28-30] Second, microbial production of plant natural products generates a less complex mixture of plant secondary metabolites, leading to relatively easier downstream processing and quality control compared to plant extraction. Also, unlike chemical synthesis, it does not require harsh reaction conditions and the usage of toxic solvents, heavy metals, strong acids or bases. Therefore, biotechnological production of plant natural products in engineered microorganisms is more environmentally friendly and sustainable.[14,19,31] It is also more economically viable and cost-effective compared to plant extraction and plant cell cultures due to the fact that engineered microorganisms can utilize renewable and cheap carbon sources such as cellulose, hemicellulose, and even CO_2.[32,33] Another important advantage of microbial production is that fermentation techniques for microorganisms are readily available to scale up from flasks to large-scale fermenters, while large-scale production is still challenging in traditional chemical synthesis, plant extraction, and plant cell cultures.[14,31] Furthermore, heterologous expression of biosynthetic enzymes in microorganisms helps elucidate the biosynthetic pathways, which can facilitate the improvement of the yield and productivity of natural products through metabolic and strain engineering strategies.[34-36] Furthermore, the direct genetic manipulation of biosynthetic genes in plant species is often challenging due to a lack of genetic tools. By contrast, the development of available and accessible genetic tools for microbial production paves the way for the characterization and modification of biosynthetic pathways.[28,37,38] Most importantly, with the advances in synthetic biology, the reconstitution of plant natural product biosynthetic pathways in engineered microbes using combinatorial biosynthesis opens the door for the generation of novel "unnatural" natural products for drug discovery.[7,21,39]

The current synthetic biology techniques and the general workflow for the engineered production of plant natural products in the microorganisms are illustrated in Figure 1.

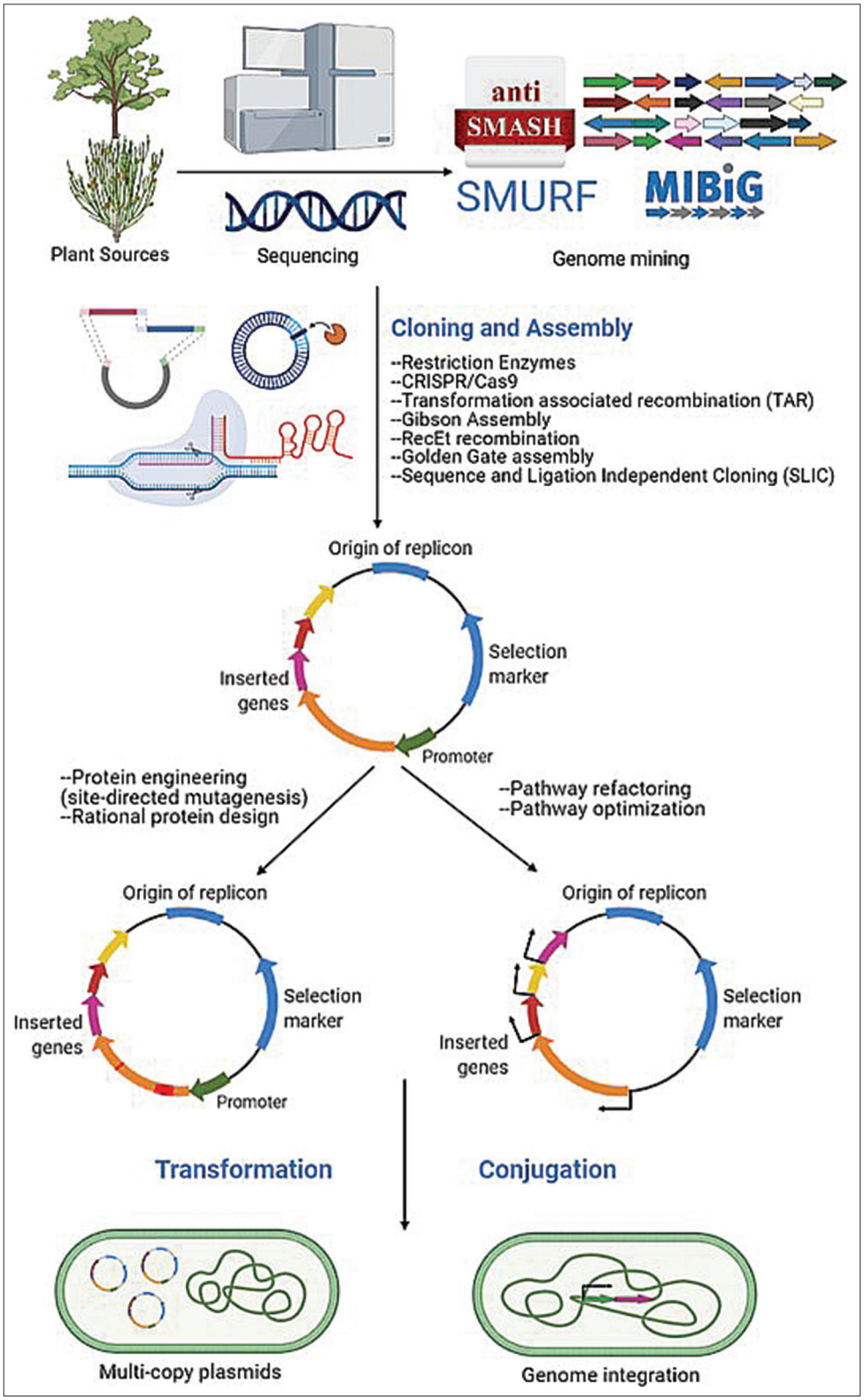

Figure 1: General workflow for the engineered production of plant natural products in microorganisms and some of the current synthetic biology strategies used for the construction of microbial platforms

The detailed review articles on these techniques are available in the literature.[40-43] Thus, this review only covers the recent progresses in the engineered production of plant natural products in the microorganisms. It touches upon the biotechnological production of plant-derived natural products such as terpenoids, flavonoids, and alkaloids in the host microorganisms. For each medicinally important class of plant natural products, the successful engineered production

examples such as artemisinin, paclitaxel, naringenin, quercetin, berberine, and noscapine will be summarized.

Flavonoids

Flavonoids are polyphenolic natural products produced by all terrestrial plants. There are more than 9000 flavonoids extracted from plants, representing an important class of plant natural products.[44,45] Herbs used in TCM are also an important source for flavonoids, and many bioactive flavonoids have been extracted from those medicinal plants.[46-48] Flavonoids possess various biological functions in the plants such as the protection against plant pathogens and UV-radiation, signaling during the activation of nodulation, auxin transport, and the coloration of flowers. In addition to such benefits to plants, flavonoids exhibit beneficial properties for human health with diverse biological activities such as antioxidant, antiviral, antimicrobial, anticancer, anti-obesity, anti-diabetic, and anti-Alzheimer's.[49,50] Because of their health-benefiting properties, flavonoids have been utilized in pharmaceuticals, nutraceuticals, functional foods, and cosmetics, with an estimated total market value of USD 1.05 billion in 2021.[51]

Structurally, flavonoids have a C-15 carbon skeleton with a characteristic C_6-C_3-C_6 carbon framework. Based on the alterations on heterocyclic C-ring and the linkage between B-ring and C-ring, flavonoids are categorized into several subclasses: flavones, flavonols, flavonones, flavononols, anthocyanidins, flavan-3-ols, chalcones, and isoflavones.[51,52] All of them are synthesized through a common phenylpropanoid pathway which begins with tyrosine or phenylalanine as the primary precursor. Tyrosine ammonia-lyase (TAL) and phenylalanine ammonia-lyase (PAL) converts tyrosine and phenylalanine to flavonoid intermediates, respectively. While tyrosine is directly converted to *p*-coumaric acid by TAL, phenylalanine is firstly deaminated to cinnamic acid by PAL; then cinnamic acid can be oxidized by cinnamate 4-hydroxylase (C4H) to generate *p*-coumaric acid, which is subsequently converted to *p*-coumaroyl-CoA by 4-coumarate: CoA ligase (4CL). Chalcone synthase adds 3 units of malonyl-CoA to *p*-coumaroyl-CoA through decarboxylative condensation for the biosynthesis of the chalcones, which are converted to different flavonoids by tailoring enzymes such as isomerases, oxido-reductases, hydroxylases, and postmodification enzymes such as glycosyltransferases, acyltransferases and methyltransferases [Figure 2].[53-56]

Naringenin

Naringenin is widely distributed in plants, particularly edible fruits such as tomatoes, grapes, figs, and *Citrus* species. It is a very common plant flavonoid, which also serves as one of the key biosynthetic intermediates for many other flavonoids. Naringenin exhibits several biological activities such as antioxidant, anticancer, antibacterial, antiviral, anti-obesity, antiadipogenic, anti-inflammatory, and cardioprotective effects. Due to its benefits to human health, naringenin has been tested in preclinical trials.[57,58] In a recent preprint, with

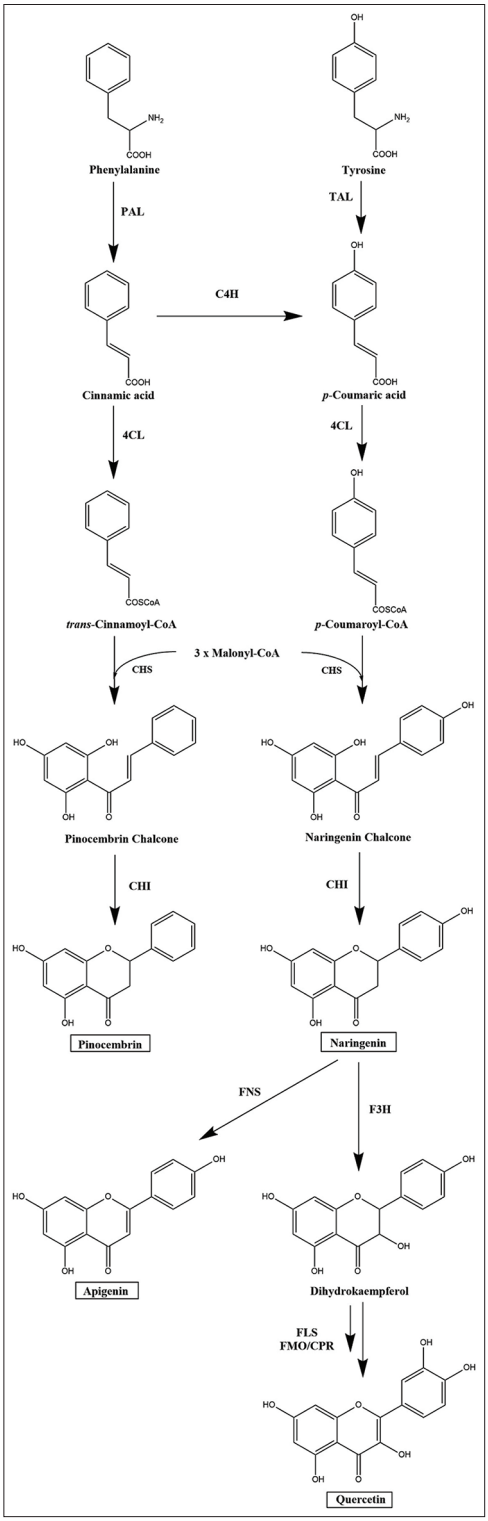

Figure 2: Biosynthetic pathway of selected flavonoids. PAL: Phenylalanine ammonia-lyase, TAL: Tyrosine ammonia-lyase, C4H: Cinnamate 4-hydroxylase, 4CL: 4-Coumarate:CoA ligase, CHS: Chalcone synthetase, CHI: Chalcone isomerase, FNS: Flavone synthetase, F3H: Flavonoid 3-hydroxylase, FLS: Flavonol synthetase, FMO: Flavonoid 3'-monooxygenase, CPR: Cytochrome P450 reductase

an *in silico* approach, phytochemicals from Indonesian herbal medicine (Jamu) were docked against severe acute respiratory

syndrome coronavirus-2 (SARS-CoV-2) 3-chymotrypsin-like protease (3CLpro). As one of the phytochemicals in this study, naringenin yielded the highest binding energy, showing its potential as an anti-SARS-CoV-2 agent.[59] Other studies also indicated that naringenin could be further investigated on its potential anti-SARS-CoV activity through the inhibition of main protease, 3CLpro, and the reduction of angiotensin-converting enzyme receptors activity.[60,61] Therefore, naringenin holds great potential to be used for the treatment of various diseases.

Naringenin, as illustrated in Figure 2, is biosynthesized through the phenylpropanoid pathway in which CHS condenses 3 units malonyl-CoA to coumaroyl-CoA to generate naringenin chalcone, which is subsequently converted to naringenin by CHI.[62] Interestingly, it has also been reported that the soil bacterium, *Streptomyces clavuligerus* can use a distinct enzyme, naringenin cytochrome P450 for the isomerization of naringenin chalcone to yield naringenin.[63] Since the biosynthetic pathway and responsible enzymes have been elucidated for naringenin biosynthesis in plants, the engineered production of naringenin has been tested in different microbial hosts such as *E. coli, S. cerevisiae,* and *Yarrowia lipolytica*.[64-66] Some of the successful examples of engineered production of naringenin, particularly the ones with a significant yield improvement, are summarized below.

E. coli was the firstly-chosen microbial host for the engineered production of naringenin. In one of the early studies, Hwang *et al*. isolated the necessary genes from different sources, including yeast, soil bacteria, and plant. They cloned PAL from the yeast *Rhodotorula rubra*, 4CL from the actinomycete *Streptomyces coelicolor* A3(2), and CHS from the licorice plant *Glycyrrhiza echinata*. Without any codon optimization, the naringenin titer achieved in this study was 0.45 mg/L.[64] In another study, 4CL and CHS were cloned from *Arabidopsis thaliana*, while TAL was cloned from *Rhodobacter sphaeroides*. The expression of these genes without any codon optimization in *E. coli* led to the titer of up to 20.8 mg/L, which was the highest yield achieved in terrific broth medium.[67] Moreover, increasing precursor supply was an effective approach to enhance the production of naringenin. For this purpose, Fowler *et al*. overexpressed 4CL, CHI, and CHS, as well as acetyl-CoA carboxylase (ACC) and biotin ligase (BPL). The latter ones were expressed to increase the malonyl-CoA supply in *E. coli*, which resulted in a titer of 69 ± 8 mg/L in a batch fermentation. To further improve the precursor supply, they knocked out *sdhA, adhE*, *brnQ,* and *citE* genes from the genome of *E. coli*. This increased the titer of naringenin to 215 ± 18 mg/L in the batch fermentation.[68] In another study, malonyl-CoA supply was enhanced by the genome-scale metabolic network modeling, for which the carbon flux is directed toward malonyl-CoA by the knockout of *fumC* and *sumC* genes and the overexpression of *acc*, *pgk*, and *pdh* genes in *E. coli* BL21Star. This led to a significant increase in the production of naringenin, 474.2 mg/L in a batch fermentation.[69] With a similar approach, Wu *et al*. downregulated *adhE*, *fabF*, *fabB,* and *fumC* genes in *E. coli* BL21(DE3) using clustered regularly interspaced short palindromic repeats interference (CRISPRi) system. The overexpression TAL, 4CL, CHS, and CHI in the engineered *E. coli* strain improved the yield of naringenin up to 421.6 mg/L.[70]

Yeast has also been used as the host for engineered production of naringenin. Jiang *et al*. introduced PAL from *Rhodosporidium toruloides*, 4CL from *A. thaliana,* and CHS from *Hypericum androsaemum* into *S. cerevisiae* AH22. The titer of naringenin was only 7 mg/L in a shake-flask fermentation.[71] Koopman *et al*. also expressed PAL1, C4H, CPR1, 4CL3, CHS3 and CHI1 from *A. thaliana* in *S. cerevisiae* with additional genetic modifications, which overall led to the production of naringenin at 108.9 mg/L. Without genetic modifications, the expression of only the naringenin biosynthetic yielded naringenin at a level less than 1.36 mg/L.[65] This indicates that enhancing precursor supply is crucially important in the engineered production of naringenin in yeast. In a recent study, one of the well-studied nonconventional yeast, *Y. lipolytica*, was also used for the engineered production of naringenin from a mixture of xylose and glucose. The titer of naringenin was initially 239 mg/L with a constitutive expression. Inducible expression, in which xylose became both inducer and substrate, enhanced the yield further. In addition, the overexpression of xylose reductase, xylose transporter, and xylitol dehydrogenase resulted in the improved production of naringenin with a titer of 715.3 ± 12.8 mg/L in shake-flask fermentation.[66] This study clearly shows that xylose-based production in *Y. lipolytica* is a promising candidate for the engineered production of naringenin.

Apigenin

Apigenin is another attractive flavonoid due to its lower toxicity, health-promoting effects, and various biological activities.[72] It exhibits anticancer, anti-inflammatory, antioxidant, and antidepressant activities. Its beneficial role in Alzheimer's disease, insomnia, amnesia, and diabetes has also been reported. In addition, the supplementation of apigenin in the treatment of various diseases such as Alzheimer's disease, insomnia, anxiety disorder and depression, and knee osteoarthritis was investigated in clinical trials, and promising results were reported for each of these diseases.[73] Furthermore, antibacterial, antifungal, antiviral, and antiparasitic activities have been extensively studied and showed promising antimicrobial activity against a wide variety of pathogenic microorganisms.[72]

Apigenin is biosynthesized from naringenin by flavone synthetase in plants [Figure 2]. Nevertheless, similar to most plant natural products, the abundance of apigenin in plants is often quite low, and its production in plants is restricted due to seasonal or regional variations; the extraction process of pure apigenin from complex mixtures is also an obstacle to the production of apigenin from plants. Thus, engineered production in microbial hosts offers an efficient alternative approach for large-scale and environmentally friendly

production.[74] Miyahisa *et al*. conducted a combinatorial biosynthesis strategy for the production of a few flavonoids including apigenin. In this study, *CHS*, *CHI,* and *FNS I* genes were from plant sources, whereas gene encoding for cinnamate/coumarate: CoA ligase (*ScCCL*), which shows the same function as 4CL, and PAL gene are from *S. coelicolor* A3(2) and yeast *R. rubra*, respectively. The overexpression of these genes in *E. coli* cells led to the production of apigenin at 13 mg/L on tyrosine supplementation in a shake flask fermentation. In the same study, with the expression of additional plant genes, other flavonoids such as chrysin, galangin, and kaempfrol were also produced upon the supplementation of phenylalanine with the titers of 9.4, 1.1, and 15.1 mg/L, respectively.[75] In another study, apigenin production in *E. coli* BL21(DE3) from tyrosine was performed through the overexpression of TAL from *Saccharothrix espanaensis*, CHS from *Populus euramericana*, 4CL from *Oryza sativa*, and CHI from *Medicago truncatula*. The titer of apigenin in this engineered strain was 13 mg/L. However, the optimization of different constructs of the genes led to the improvement of the titer to 30 mg/L. The production of apigenin was further enhanced to 55 mg/L through the optimization of culture conditions such as temperature, time, and cell density.[76] *E. coli* BL21(DE3) as an engineered production platform for apigenin was also utilized in an *E. coli-Streptomyces* co-culture system for the generation of O-methylated phenylpropanoids. *E. coli* overexpressing *4CL*, *CHS*, *CHI,* and *FNS* genes from various plant sources led to the production of apigenin at 52 mg/L upon 1.2 mM 4-coumaric acid supplementation in a shake-flask fermentation.[77] All these successful examples with better yields promise hope for the engineered production of apigenin in microbial hosts.

Quercetin

Quercetin, as a major polyphenolic compound, is found in various fruits and vegetables such as berries, capers, cilantro, dill, apples, and onions. It is one of the important plant natural product with various biological activities.[78] Quercetin exhibits strong antioxidant activity, which prevents the oxidation of low-density lipoproteins through scavenging free radicals and chelating transition metal ions. It is, therefore, a promising molecule in the prevention and treatment of certain diseases, such as cancer, atherosclerosis, and chronic inflammation.[79] Particularly, its anticancer properties have been extensively studied against various cancer cell lines.[80,81] In addition, quercetin displays antimicrobial, antiviral, anti-inflammatory, and anti-allergic activities. The cardiovascular disease prevention and beneficial effects in neurological disorders such as Alzheimer's disease have also been reported for quercetin.[82] Recently, quercetin has attracted special attention due to its potential anti-SARS-CoV-2 activity. *In silico* and *in vivo* studies revealed that quercetin may interfere at the different steps of coronavirus entry and replication cycle such as 3CLpro, papain-like protease (PLpro), and RNA nucleoside triphosphatases (NTPase)/helicases.[83,84] It is already in the process of clinical trials for its effect on prophylaxis and treatment of COVID-19 (ClinicalTrials.gov Identifier: NCT04377789).

The biosynthetic pathway of quercetin is relatively longer compared to various flavonoids such as naringenin. After narigenin is formed, it is hydroxylated at C3 by F3H. Then, further reduction and hydroxylation reactions by FLS and FMO/CPR lead to the formation of quercetin [Figure 2]. Engineered production of quercetin was first conducted in *E. coli* as a heterologous host. In this study, a plant P450 flavonoid 3', 5'-hydroxylase (F3'5'H) fused with a P450 reductase cloned from *Catharanthus roseus* were overexpressed in *E. coli* BL21(DE3) along with the functional expression of 4CL, CHS, CHI, FLS, and F3H isolated from different plant species such as *P. crispum*, *A. thaliana*, *Malus domestica* and *Petunia* x *hybrida*. The metabolic engineering of *E. coli* led to the production of kaempferol and quercetin. The highest titer of quercetin (0.05 mg/L) was obtained in terrific broth with *p*-coumaric acid precursor supplementation.[85] Yeast was also used as a host microorganism for the production of quercetin. Trantas *et al*. performed the first study of quercetin production in yeast. In this study, the aforementioned genes required for the biosynthesis of quercetin were cloned from *Vitis vinifera, Glycine max,* and *Solanum tuberosum*. The overexpression of these genes in *S. cerevisiae* resulted in a titer of 0.38 mg/L in defined media under optimum growth conditions.[86] In another study, the sources of the genes were as following: 4CL from *P. crispum*, CHS from *P. hybrida*, CHR from *Astragalus mongholicus*, CHI from *Medicago sativa*, F3H from *A. mongholicus*, FLS from *A. thaliana*, CPR from *C. roseus*, and FMOs from *Fragaria ananassa* and *Petunia hybrid*. These genes were synthesized after codon optimization. Unlike other studies, flavonoids were produced directly from glucose in this study. For this purpose, high *p*-coumaric acid-producing yeast strain was further engineered with the overexpression of required genes for quercetin biosynthesis. The highest titer of quercetin (20.38 ± 2.57 mg/L) was achieved in a shake-flask fermentation.[87] This study clearly reveals that engineering of the biosynthetic pathways, development of efficient precursor-producing strains, and optimization of the expression of the corresponding proteins help to improve the production of quercetin.

Pinocembrin

Pinocembrin is one of the major flavonoids in propolis. It is also found in various plants, such as ginger roots, wild marjoram, *Peperomia* and *Piper* genera as well as *Asteraceae* families. Pinocembrin has various biological activities including anti-inflammatory, antioxidant, antibacterial, and neuroprotective activities. It is also a promising agent for the treatment of some diseases such as endotoxin shock, cancer, and cardiovascular diseases.[88-90] Due to its ability to pass through the blood-brain barrier, pinocembrin has also been investigated in the treatment of neurodegenerative diseases. In addition, it is a promising drug candidate for treating ischemic stroke.[88] For this purpose, pinocembrin is already in Phase II clinical trials (ClinicalTrials.gov Identifier: NCT02059785). The bioinsecticidal activity of pinocembrin against *Spodoptera frugiperda* was also reported with promising results, which

indicated that it might be used as a stimulo-deterrent diversionary strategy in crop protection.[91] Similar to naringenin and quercetin, pinocembrin has recently been studied for its potential anti-SARS-CoV-2 activity. Particularly, the ethanolic extract of propolis, which includes pinocembrin as well as other flavonoids, was investigated using a molecular docking approach. It was found that pinocembrin displayed one of the highest binding energy to angiotensin-converting enzyme 2 (ACE2) and showed its potential antiviral activity against SARS-CoV-2.[92]

Similar to naringenin, pinocembrin biosynthesis is governed by CHS and CHI with a difference in the precursor. CHS condenses 3 units malonyl-CoA to *trans*-cinnamoyl-CoA to generate pinocembrin chalcone, which is subsequently converted to pinocembrin by CHI.[93] Engineered microbial production of pinocembrin has been tried in different hosts. *E. coli* and *S. cerevisiae* are the commonly chosen hosts for pinocembrin production.[51] Several examples of pinocembrin production in microbes with higher titers are summarized here.

Hwang *et al*. constructed an artificial gene cluster including PAL from *R. rubra*, 4CL from *S. coelicolor* A3(2) and CHS from *G. echinata*, which were overexpressed in *E. coli* to produce a plant-specific flavanone, pinocembrin. The highest pinocembrin titer in this study was 0.75 mg/L upon glucose and phenylalanine supplementation. This titer was achieved when they added the T7 promoter in front of each gene in the artificial gene cluster.[64] Another artificial biosynthetic gene cluster was constructed and included the same enzymes as well as CHI from *Pueraria* plant. Two subunit genes of ACC from *Corynebacterium glutamicum* were also overexpressed in *E. coli* under the T7 promoter. Due to the increased malonyl-CoA supply, the titer of pinocembrin reached approximately 60 mg/L with the 3 mM tyrosine or phenylalanine supplementation.[94] Another study focused on engineering central pathways to increase the precursor supply to the flavanone biosynthetic pathway. Four ACC subunits from *Photorhabdus luminescens* (PlACC) under a constitutive promoter led to a 576% increase in the flavanone titer. Overexpression of PIACC with biotin ligase from the same species enhanced the yield up to 1166%. They also conducted the amplification of the acetate assimilation pathway along with overexpression of ACC. Overall, pinocembrin titer was improved by 1379%, which corresponded to 429 mg/L pinocembrin in engineered *E. coli*.[95] Wu *et al*. also attempted to engineer the central metabolic pathways, yet they used the CRISPRi system for efficiently channeling carbon flux toward malonyl-CoA. Furthermore, they investigated the effect of cultivation pH and introduced a two-stage pH control strategy because it is found that high pH favors the upstream pathway, whereas low pH is in favor of the downstream pathway. With this new cultivation approach and engineered central pathway with CRISPRi, recombinant *E. coli* cells produced pinocembrin at 525.8 mg/L. This might open a door for the large-scale production of pinocembrin.[96] *S. cerevisiae* was also utilized as host for pinocembrin production. However, the titers were low compared to *E. coli*. For instance, in one study, the titer was 16.3 mg/L upon cinnamic acid supplementation.[93] Another study reported the production of pinocembrin at only 11.6 mg/L in engineered yeast.[97] It is obvious that engineering the central metabolic pathways to increase the precursor supply is essential for enhanced production of pinocembrin, which can bring the engineered production in microbes from lab-scale to large-scale.

Terpenoids

Terpenoids (isoprenoids) are the most diverse family of natural products which consists of more than 80,000 different entities,[98] and they commonly exist in higher plants, such as vegetative tissues, flowers, and roots.[99] Based on the numbers of five-carbon isoprene units, terpenoids are usually classified into eight groups: hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and polyterpenoids ($C_{>40}$).[100] As important secondary metabolites in plants, these natural products feature various bioactivities and extensive applications in the food, cosmetics, and pharmaceutical industries.[101,102] There are some typical examples of commercially useful terpenoids: monoterpenoid menthol is used in foods and cosmetic industry due to its flavor and fragrance;[103,104] sesquiterpenoid artemisinin is used worldwide as an antimalarial drug;[105] ginsenoside compound K has been in the clinical trials for its anti-diabetic, neuroprotection and liver protection properties;[106] and diterpenoid paclitaxel has been developed to be an important anticancer drug.[107]

All terpenoids are synthesized from the universal 5-carbon precursors isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). There are generally four steps to form terpenoids [Figure 3]: (1) Both mevalonate (MVP) pathway (active in the cytosol) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (active in the plastid) play an important role in IPP, and DMAPP formation; (2) The sequential head-to-tail condensation of one, two, and three units of IPP to DMAPP yields the precursors for monoterpenes (geranyl pyrophosphate: GPP, C_{10}), sesquiterpenes (farnesyl pyrophosphate: FPP, C_{15}), and diterpenes (geranylgeranyl pyrophosphate: GGPP, C_{20}); meanwhile, condensation of two molecules of FPP generates the backbone of triterpenoids; (3) The primary terpene skeletons (terpenoid core structures) are assembled by terpenoid synthases (TPSs); (4) Terpenoid core structures are further modified to a variety of terpenoids by different tailoring enzymes, such as cytochrome P450 hydroxylases, dehydrogenases, reductases, oxidases, isomerases and methyl-, acyl-, and glycosyltransferases.[101,108,109]

Artemisinin

A. annua L. is an annual herb used in traditional Chinese medicine for treating fever and malaria.[110] Moreover, *A. annua* extracts exhibit a variety of biomedical and pharmaceutical

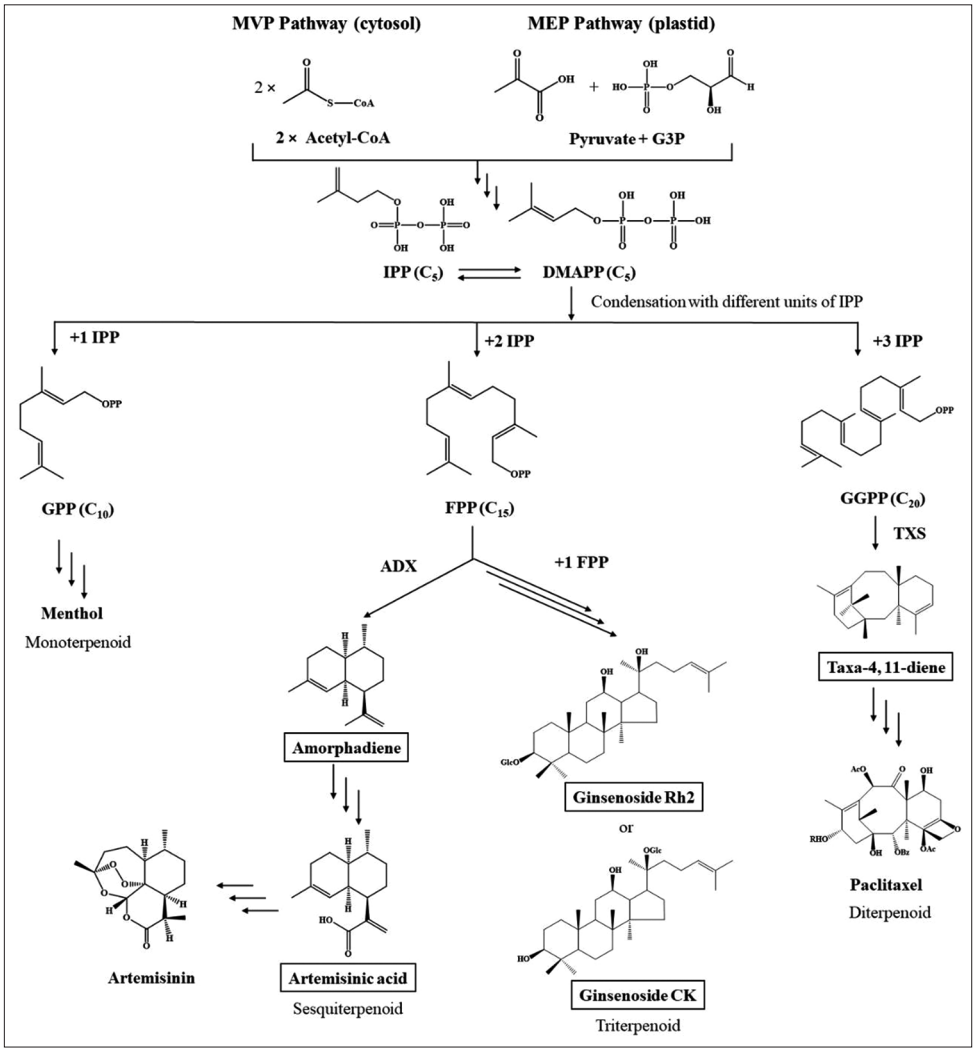

Figure 3: Biosynthetic pathway of selected terpenoids. MVP: Mevalonate pathway, MEP: 2-C-methyl-D-erythritol-4-phosphate pathway, G3P: Glyceraldehyde 3-phosphate, IPP: Isopentenyl diphosphate, DMAPP: Dimethylallyl diphosphate, GPP: Geranyl pyrophosphate, FPP: Farnesyl pyrophosphate, GGPP: Geranylgeranyl pyrophosphate, ADX: Amorphadiene synthase, TXS: Taxadiene synthase

applications.[111-113] Artemisinin is an effective compound for the treatment of malaria and against multidrug-resistant malaria parasite *Plasmodium falciparum*,[114,115] which is a sesquiterpene lactone endoperoxide extracted from *A. annua*.[116] Besides antimalarial activity,[117] artemisinin and its derivatives have broad biological applications due to their anticancer,[118] antiviral,[119] and anti-inflammatory activities.[120] The extraction of artemisinin from plants is not environmental-friendly and limited by the plant sources. Total synthesis of artemisinin is hard to achieve,[121] but the semi-synthetic microbial process for the production of artemisinin and its immediate precursor, artemisinic acid is a more effective and achievable approach.[122,123]

In order to satisfy the increasing demand of this antimalarial natural compound, Ro *et al*. engineered an artemisinic acid-producing strain of *S. cerevisiae* with a production titer of 100 mg/L through an engineered mevalonate acid (MVA) pathway. For the purpose of increasing the FPP production in *S. cerevisiae*, the expression of genes *tHMGR* and *upc2-1* were upregulated; however, *ERG9* was downregulated since it converts FPP into sterols as a competing pathway. Then, they introduced the amorphadiene synthase gene (*ADS*) from *A. annua* into the high FPP producing strain EPY224 to convert FPP to amorphadiene with a titer of 153 mg/L, which is 500-fold higher than previous work. Finally, a novel cytochrome P450 monooxygenase (*CYP71AV1*) gene was cloned from *A. annua* that performs the oxidation of amorphadiene to artemisinic acid. The expression of CYP71AV1 in the amorphadiene-producing strain EPY224 led to an artemisinic acid titer of 32 ± 13 mg/L in a shake-flask culture.[124]

To further enhance the yield, Paddon *et al*. continuously developed strains of *S. cerevisiae* for more efficient biotechnological production of artemisinic acid. They constructed strain Y692 by expressing a cytochrome b_5 from *A. annua* (*CYB5*). Specifically, they expressed a chromosomally

integrated copy of *CYB5* with a strong promotor (*GAL7*) in strain Y657, which has low CPR1 (*CYP71AV1* cognate reductase) expression level. Subsequently, they expressed the plant artemisinic aldehyde dehydrogenase (*ALDH1*) from *A. annua* in strain Y692, resulting in strain Y973 with the production of 25 g/L artemisinic acid in the fermentation flask culture. Finally, after examining the putative alcohol dehydrogenase (*ADH1*), they proposed the three-steps oxidation from amorphadiene to artemisinic acid: (1) *CYP71AV1*, *CPR1* and *CYB5* oxidize amorphadiene to artemisinic alcohol; (2) *ADH1* oxidizes artemisinic alcohol to artemisinic aldehyde; (3) *ALDH1* oxidizes artemisinic aldehyde to artemisinic acid.[125] This study shows that the discovery of the plant dehydrogenase and cytochrome enzyme *CYB5* further increased the artemisinic acid production titer apparently.

Y. lipolytica was also used for engineered production of artemisinin precursors. Marsafari and Xu used lipogenic acetyl-CoA pathways and the endogenous mevalonate pathway of *Y. lipolytica* for amorphadiene production. They identified the optimal hydroxy methylglutaryl coenzyme A reductase and expressed codon-optimized *ADS* from *A. annua* in *Y. lipolytica*, yielding 23.8 mg/L amorphadiene. Furthermore, by increasing the *ADS* copy numbers (×2), amorphadiene titer reached 71.39 mg/L with a 1.65-fold improvement. Based on this improvement, further overexpression of *ERG12* for removing the rate-limiting step in the mevalonate pathway, amorphadiene production was increased by 1.83-fold compared with the control strain. Finally, by adding 1 mg/L cerulenin, which blocks lipids synthesis and inhibits fatty acid synthesis, the titer reached 171.5 mg/L in a shake flask.[126] Therefore, blocking the competing pathways for target compounds is an effective method to improve the production titer.

Ginsenosides

Ginsenosides are glycosylated triterpenes and almost exclusively produced in *Panax* species with antidiabetic, antioxidant, anticancer, antitumor, cytotoxic, antiviral, and antibiotic properties.[127-131] Moreover, ginsenosides play an essential part in enhancing physical performance by increasing resistance to stress and aging and improving the protection of blood vessels.[132,133] Meanwhile, these natural products have also been used in the food and cosmetic industries.[134,135] *Panax* Linn. is called the ginseng genus, which belongs to the Order *Umbelliferales*, Family *Araliaceae*.[129] Among them, *Panax notoginseng* (Chinese ginseng, also called Sanchi ginseng), *Panax ginseng* (Asian or Korean ginseng), and *Panax quinquefolius* (American ginseng) have been used for more than 4000 years in Asia as a traditional herbal medicine.[136,137]

Ginsenoside compound K (GCK) is a tetracyclic triterpenoid compound isolated from plant *P. ginseng* with anti-tumor, anti-inflammatory, antioxidant, and anti-aging activities.[138,139] Furthermore, it has functions in treating heart disease and depression, improving immunity, and enhancing learning ability.[140] As a heterologous host, *Y. lipolytica* was used for engineered biosynthesis of terpenoids due to its native MVA pathway, which supplies the prerequisite precursors IPP and DMAPP.[141,142] Also, convenient genetic engineering tools, robust acetyl-CoA biosynthesis, and energy supply systems are other reasons for *Y. lipolytica* to be chosen as a host microorganism.[143-145] Li *et al*. constructed a recombinant GCK pathway in *Y. lipolytica*, including dammarenediol II synthase (DS), cytochrome P450 monooxygenase enzyme (PPDS) and UDP-glycosyltransferase (UGT1) from *P. ginseng,* and NADPH-cytochrome P450 reductase (ATR1) from *Arabidopsis thailiana*, which produced 5.1 mg/L GCK. By overexpression of the MVA pathway key genes (*tHMG1*, *ERG9* and *ERG20*) and the fusion of *PPDS* and ATR1, the production of GCK was increased up to 161.8 mg/L in a 5-L fed-batch fermentation.[146]

Ginsenoside Rh2 is a rare protopanaxadiol (PPD)-type triterpene saponin isolated from *P. ginseng* with known anti-tumor, anti-oxidant, anti-diabetes, anti-proliferative, anti-metastatic, and immune-system-enhancing activities.[147] When ginsenoside Rh2 and anti-tumor drugs are applied together, the tumor suppression efficacy can be enhanced, and the side effects of chemotherapy are reduced.[148] Zhuang *et al*. integrated the *M7-1* gene (the mutant of glycosyltransferase UGT51) into the engineered *S. cerevisiae* ZD-PPD-016(URA3-) for PPD production to produce Rh2 from glucose or glycerol. As a result, this engineered strain (ZY-M7) produced Rh2 at 6.08 mg/L. After deleting the *EGH1* gene (hydrolyzes Rh2 into PPD) and integrating additional copies of the mutant *M7-1* gene into *S. cerevisiae* chromosome, this new constructed strain (ZY-M7(4)-EΔ) improved glucosyltransferase activity and Rh2 production up to 22.2 mg/L. Moreover, by overexpressing both *PGM1* and *UGP1* genes involved in UDP-glucose biosynthesis, the titer of Rh2 was further enhanced to 36.7 mg/L. Through the integration of the gene cassette *PPDS-75tATR2*, encoding self-sufficient P450 fusion protein, the Rh2 production titer in new engineered strain reached 45 mg/L. Eventually, the highest titer of 292 mg/L was achieved at 120 h with fed-batch fermentation in a 5-L bioreactor.[147]

Paclitaxel

Paclitaxel was first isolated from the bark of the Pacific yew tree (*T. brevifolia* or *Taxus baccata* Nutt.) with potent anticancer activity.[149,150] It also exhibits significant cytotoxic and antileukemic activities.[151] Paclitaxel, approved by FDA for the treatment of breast cancer, represses cell-cycle replication and facilitates microtubule assembly in the late M or G2 phases.[152] Taxadiene is the first committed intermediate for the biosynthesis of paclitaxel.[153] The extraction of paclitaxel from plant is problematic, and many *Taxus* species are in danger because of high demand.[24] Therefore, microbial production of paclitaxel or its precursors represents a potential solution.

To achieve this goal, Abdallah *et al*. made the efforts in engineering *Bacillus subtilis* as the host cell for paclitaxel production. They expressed the plant-derived taxadiene

synthase (*TXS*) enzyme in *B. subtilis*, which is responsible for the conversion of the precursor GGPP to taxadiene. They created a synthetic operon with *B. subtilis* genes encoding the MEP pathway (*dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, *ispG*) and *ispA* for providing FPP and overexpressed these eight enzymes in the biosynthetic pathway to increase GGPP production. Furthermore, the *crtE* gene, encoding geranylgeranyl pyrophosphate synthase (GGPPS), was introduced to increase the precursor supply. Finally, 17.8 mg/L of taxadiene was achieved by overexpression of the MEP pathway enzymes and *IspA* together with GGPPS, which is an 83-fold increase compared to the strain only expressing *TXS* and using *B. subtilis* inherent pathway.[154] This study shows that precursor supply is an important factor to increase the taxadiene production titer.

Compared with *B. subtilis*, *E. coli* is a more commonly used host cell. Ajikumar *et al*. reported a multivariate-modular approach for improving taxadiene titer. They divided the taxadiene metabolic pathway into two parts: a native upstream MEP pathway for the biosynthesis of precursors and a heterologous downstream pathway for terpenoid biosynthesis; they aimed at enzymatic bottleneck genes (*dxs*, *idi*, *ispD*, and *ispF*) for overexpression by an operon (*dxs-idi-ispDF*) to increase IPP production via upstream MEP pathway. Meanwhile, a synthetic operon of downstream genes GGPPS and a TXS were constructed to convert IPP and DMAPP into paclitaxel. Inducible promoters were used to control their relative gene expression.[155] Finally, approximately 1 g/L of taxadiene was achieved in the engineered *E. coli*, demonstrating that *E. coli* is a useful candidate for producing paclitaxel precursors.

Alkaloids

Alkaloids are low-molecular-weight, nitrogen-containing compounds. They have been discovered in about 20% of plant species with various chemical structures, and most of them originate from amino acids. Around 12,000 alkaloids found in plants have the function of protecting the hosts from herbivores and pathogens. Due to their potent biological activity, some alkaloids are used as pharmaceuticals, stimulants, narcotics, and poisons.[156] It is noteworthy that different types of alkaloids have their exclusive biosynthetic pathways, which is different from most other secondary metabolites.[157] In this review, we mainly use benzylisoquinoline alkaloids (BIAs) and terpene indole alkaloids (TIAs) as the examples [Figure 4].

BIAs are a group of pharmaceutically important alkaloids with around 2,500 defined structures.[158] For example, morphine is opioid analgesic isolated from *Papaver somniferurn*;[159] berberine is antibacterial agents obtained from *Hydrastis canadensis*;[160] and noscapine is a cough suppressant also extracted from *P. somniferum*.[161] BIAs are biosynthesized through (*S*)-reticuline from L-tyrosine. (*S*)-Reticuline is a nonnarcotic alkaloid that is used for antimalarial and anticancer drug development. What's more, (*S*)-reticuline is a central pathway intermediate for the biosynthesis of many other BIAs.[162] BIA biosynthesis features the condensation of two tyrosine derivatives, namely tyramine and dopamine, which are respectively decarboxylated from tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA) separately by tyrosine decarboxylase. The condensation, also called Pictet-Spengler-type reaction, is catalyzed by norcoclaurine synthase (NCS). The important intermediate compound (*S*)-reticuline is converted from (*S*)-norcoclaurine by 6-*O*-methyltransferase (6OMT), *N*-methyltransferase (CNMT), P450 hydroxylase (NMCH), and 4'-*O*-methyltransferase (4'OMT). Finally, (*S*)-reticuline was further decorated by modifying enzymes to yield various BIAs.[156,157,163]

TIAs are complex nitrogen-containing plant-derived metabolites, which include about 3,000 compounds.[164,165] This type of natural products is found in various plant species from the *Apocynaceae*, *Loganiaceae*, *Rubiaceae*, *Icacinaceae*, *Nyssaceae*, and *Alangiaceae* plant families.[166] Many TIAs have medicinal functions, such as the anticancer compound vinblastine and antimalarial drug quinine.[167,168] Strictosidine is considered as the essential scaffold and undergoes various redox reactions and rearrangements, which leads to thousands of TIA derivatives.[164,165,169] In the TIA biosynthetic pathway, tryptophan is firstly converted to tryptamine via tryptophan decarboxylase to generate the indole moiety in TIAs. Secologanin is formed through nonmevalonate terpene biosynthesis and constitutes the terpenoid component in TIAs. Finally, tryptamine and secologanin are condensed by strictosidine synthase (STR) to form the intermediate strictosidine, which is the common precursor to form all TIAs.[157]

Reticuline

Reticuline is a BIA isolated from Ocotea duckei Vattimo. Ocotea genus contains many species which are used for treating various diseases with symptoms such as pain, neuralgia, dyspepsia, and anorexia in the north and north-west of Brazil. Pharmaceutical studies on O. duckei Vattimo plant show that it has major central effects, and reticuline is the major alkaloid in the extract of this plant.[170] Reticuline has antispasmodic, antifungal, and anti-inflammatory effects and activity on accelerating hair growth.[171-174] Moreover, reticuline possesses potent central nervous system depressant action, which produces alteration of behavior pattern, prolongation of pentobarbital-induced sleep, reduction in motor coordination and D-amphetamine-induced hypermotility, and suppression of the conditioned avoidance response.[175] Hence, besides being an important intermediate compound for all BIAs synthesis, reticuline itself also has many biological activities.

To synthesize this valuable natural product in an environmentally-friendly way, Nakagawa *et al*. engineered an (*S*)-reticuline-producing *E. coli* from glucose or glycerol with a titer of 33.9 mg/L. The constructed strain includes three pathways: (1) Pathway for L-tyrosine over-production: L-tyrosine over-producing strain was constructed with a disrupted *tyrR* gene in *E. coli* BL21(DE3), encoding a repressor of several genes connected with the shikimic acid pathway. In

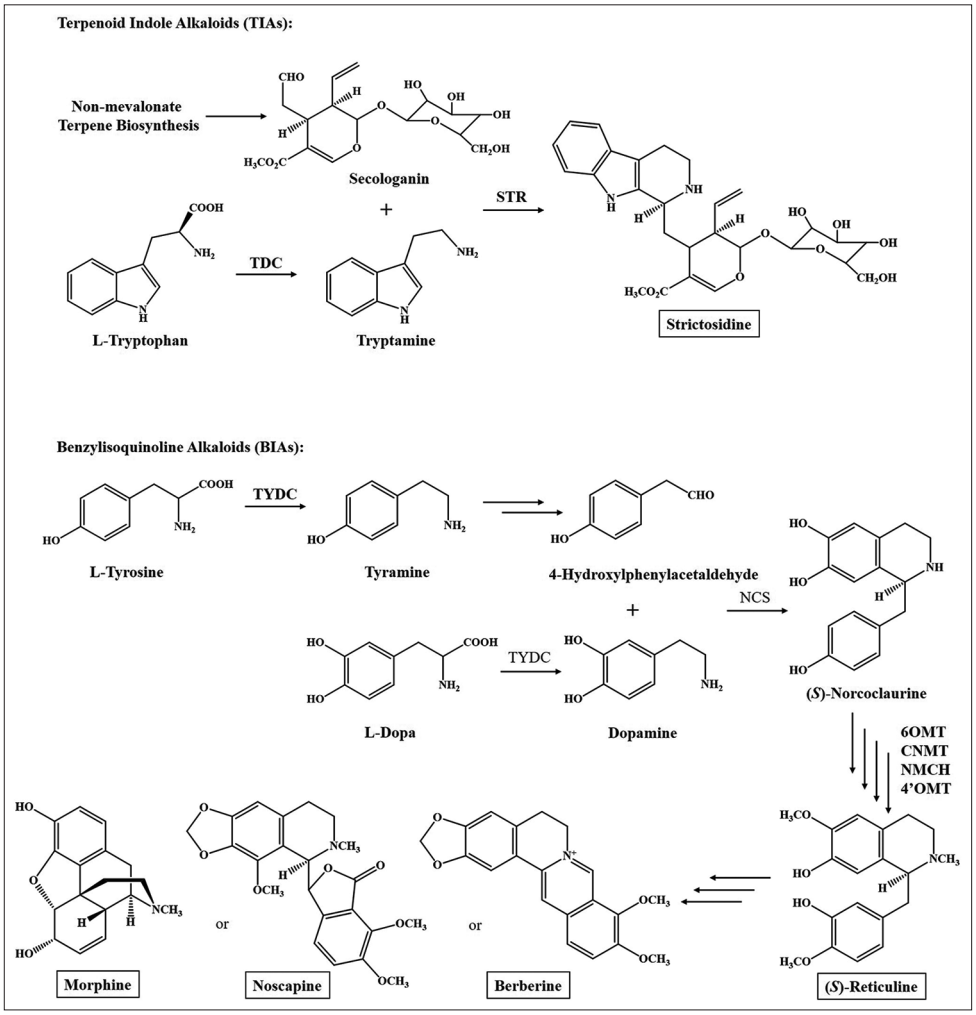

Figure 4: Biosynthetic pathway of selected alkaloids. TDC: Tryptophan decarboxylase, STR: Strictosidine synthase, TYDC: Tyrosine decarboxylase, NCS: Norcoclaurine synthase, 6OMT: 6-O-methyltransferase, CNMT: N-methyltransferase, NMCH: P450 hydroxylase, 4'OMT: 4'-O-methyltransferase

addition, the overexpression of four enzymes was introduced for the further enhancement of tyrosine production: feedback resistant (fbr) 3-deoxyd-arabino-heptulosonate-7-phosphate synthase (*aroG*^{fbr}), fbr-chorismate mutase/prephenate dehydrogenase (*tyrA*^{fbr}), phosphoenolpyruvate synthetase (*ppsA*), and transketolase (*tktA*); (2) Pathway for dopamine production from L-tyrosine: the plasmid containing tyrosinase (*TYR*) and L-DOPA specific decarboxylase (*DDC*) was transformed into the L-tyrosine over-producing strain; (3) Pathway for (*S*)-reticuline production from dopamine: Five genes for (*S*)-reticuline biosynthesis were expressed in the dopamine-producing strain: monoamine oxidase (*MAO*), norcoclaurine synthetase (*NCS*), 6-*O*-methyltransferase (*6OMT*), coclaurine *N*-methyltransferase (*CNMT*), and 4'-*O*-methyltransferase (*4'OMT*).[158,162] With the decent production titer, this method might be a promising tool for large-scale production of (*S*)-reticuline.

S. cerevisiae is another useful host for (*S*)-reticuline production. DeLoache *et al*. conducted the engineered production of (*S*)-reticuline in *S. cerevisiae* using glucose as substrate. To achieve this goal, they discovered an enzyme-coupled biosensor DOPA dioxygenase for the upstream intermediate L-DOPA. Meanwhile, they found out an active tyrosine hydroxylase (*CYP76AD1*) using this sensor and increased its L-DOPA and dopamine titers by 2.8-and 7.4-fold, respectively through PCR mutagenesis. Co-expression of L-DOPA decarboxylase led to dopamine production of 10.8 mg/L, with a 7.4-fold improvement in titer with their best mutant enzyme (*F309L*) compared with wild-type enzyme (*CYP76AD1*). Finally, this pathway was used to reconstitute the seven-enzyme pathway for (*S*)-reticuline production from L-tyrosine. Although only 80.6 μg/L of (*S*)-reticuline was produced, which is much lower than *E. coli*, this work provides an alternative method for producing valuable BIAs.

Noscapine

Noscapine is extracted from *Papaver somniferum*. This natural product belongs to the phthalideisoquinoline type alkaloids. Noscapine is a nonnarcotic antitussive and has been used as an

oral drug worldwide for more than 50 years.[176,177] Moreover, it is a microtubule-modulating anticancer agent, which exhibits fewer side effects than traditional chemotherapy drugs. This makes the compound a promising anticancer drug, and it has come into usage in some countries.[178-180] In addition, many noscapine derivatives have higher therapeutic potential for specific cancer cell lines because of enhanced *in vitro* and *in vivo* activities or water solubility, and all noscapinoids are currently derived from noscapine.[180,181] Recent studies show that noscapine might be a promising drug candidate for SARS-CoV-2, which attenuates cytokine release and repurposes against SARS-CoV2 protease.[182,183] Representative examples of engineered production of noscapine in *S. cerevisiae* are summarized below.

Even though the chemical structure of noscapine was elucidated more than 100 years ago, its biosynthetic pathway remained unclear for a long period. Winzer *et al*. discovered a biosynthetic gene cluster for noscapine biosynthesis from *P. somniferum* in 2012. It includes three *O*-methyltransferases (*PSMT1*, *PSMT2*, *PSMT3*), four cytochrome P450s (CYPs) (*CYP82X 1*, *CYP82X 2*, *CYP82Y1*, and *CYP719A21*), an acetyltransferase (*PSAT1*), a carboxylesterase (*PSCXE1*), and a short-chain dehydrogenase/reductase (*PSSDR*).[184] Based on this important finding, many researchers studied the detailed biochemical characterization of these enzymes.[185-187] These fundamental contributions paved the way for further constructing the noscapine biosynthetic pathway in yeast. In 2016, Li *et al*. assembled the gene cluster of noscapine pathway in *S. cerevisiae*. After heterologous expression of 16 plant enzymes in yeast through a 14-step biosynthetic pathway, 0.678 mg/L of noscapine was produced from the simple alkaloid norlaudanosoline.[188] This study demonstrates that engineered microbial production is a powerful tool to biosynthesize valuable natural product, which cannot be easily achieved with chemical synthesis.

In order to increase the noscapine titer, Li *et al*. reconstructed *de novo* biosynthetic pathway for noscapine production in *S. cerevisiae* with more than 30 enzymes from plants, bacteria, mammals, and yeast itself, including 7 plant endomembrane-localized plant enzymes. They began with two platform (*S*)-reticuline-producing strains.[189,190] The reticuline-producing strains biosynthesize reticuline through the overexpression of 17 enzymes over five genetic modules. They introduced 12 additional plant enzymes and integrated three additional genetic modules into the reticuline-producing platform strains CSY1060 and CSY1061. Furthermore, two base noscapine-producing strains CSY1149 and CSY1150 were constructed and produced noscapine with titers of 120.0 and 227.1 ng/L, respectively. After optimizing the expression of pathway enzymes, host endogenous metabolic pathways, and fermentation conditions, around 2.2 mg/L of noscapine was produced with a 18,000-fold improvement.[188,191] Although further improvement of pathway efficiency is still needed to increase the titer of noscapine, this provides a unique opportunity to biosynthesize valuable alkaloids such as noscapine in yeast.

Others

Morphine

Opiates have been used to treat human disease for more than 3500 years, and they are isolated from *P. somniferum* (opium poppy). Among them, morphine is a widely prescribed analgesic for application in postoperative and cancer pain with high efficacy as well as its antimalarial activity.[192,193] However, to satisfy the increasing demand for morphine, tens to hundreds of thousand tons of opium poppy biomass are extracted by industrial process method annually.[194] Scientists intended to use chemical synthesis to tackle this problem, but it turned out to be unviable due to the complex regio-and stereochemistry.[195] Although the complex, branched, multi-step architecture of BIAs leads to many challenges in engineering these biosynthetic pathways, engineering microbial strains is still an attemptable strategy for opiates production.

The morphine biosynthetic pathway was reconstructed in *S. cerevisiae* by Thodey *et al*. through expressing codon-optimized dioxygenases thebaine 6-*O*-demethylase (*T6ODM*), codeinone reductase 1.3 (*COR1.3*), and codeine *O*-demethylase (*CODM*), which are flanked by unique yeast promoters and terminators individually. They were assembled into a single yeast artificial chromosome (YAC) vector (pYES1L). Morphine was detected with the low production of 0.2 mg/L in the culture medium upon the supplementation of thebaine. To enhance the flux through the pathway, they supplied 100 mM co-substrate 2-oxoglutarate into the culture medium and observed a more than 10-fold increase in morphine titer (2.5 mg/L) in standard medium. Finally, they optimized relative enzyme expression levels and found the most favorable gene copy number ratio of *T6ODM: COR1.3*:*CODM* as 2:1:3. This led to a morphine titer of 5.2 mg/L.[194] From this study, we can conclude that gene copy number optimization, co-substrate supply enhancement are effective strategies to improve morphine biosynthesis in yeast.

Strictosidine

C. roseus (known as Madagascar periwinkle or Sadabahar) is a flowering plant species and attracts much pharmaceutical interest because it produces various important secondary metabolites used in medicine. Two typical examples are vincristine and vinblastine, which are used in the pharmaceutical industry for their anticancer properties.[196] Strictosidine is another important compound found in *C. roseus* with antimicrobial activity.[197] Although there are not many reports about its bioactivities, its role as an intermediate for all 3,000 known BIAs renders it an attractive target compound.[198] Hence, analysis of the enzymes and corresponding genes for strictosidine biosynthesis by *C. roseus* made it possible to produce this complex molecule in microbial hosts.

Brown *et al*. developed a strictosidine-producing yeast strain by introducing 21 new genes and deleting three genes from *S. cerevisiae* genome. The engineered strain 4 has 15 plant-derived genes, five additional copies of yeast genes, one

animal-derived gene, and three gene deletions. After solving the major problem of geraniol hydroxylation resulting from poor activity of the geraniol 8-hydroxylase (G8H) enzyme, strictosidine was generated with trace amounts. Subsequently, *de novo* strictosidine production titer was increased to 0.5 mg/L by introducing codon-optimized G8H into strain 4.[198] This study illustrates that reconstruction of plant natural product biosynthetic pathways in microbial host for the strictosidine production is a promising approach for generating more high-value BIAs.

Berberine

Berberine is an alkaloid existing in *H. canadensis*, the Chinese herb Huanglian, and many other plants, such as the Berberidaceae and Ranunculaceae families. It has been used as traditional Chinese and Japanese medicine for a long period[199] with antibiotic,[160] immunostimulant,[200] antitumor,[201] and antimotility properties.[202] Due to the broad spectrum of antimicrobial activity, the extract of berberine-containing plants or berberine was used as a folklore remedy to get rid of dysentery and infectious diarrhea in China for centuries.[203] It was also reported that berberine-containing natural medicine can be used for the treatment of food allergy.[204] Microbial biosynthesis of berberine can decrease doubling times so that it has better productivity than plant cell cultures.[205] Therefore, some scientists are exploring novel methods to biosynthesize this valuable natural product through microorganisms.

Galanie and Smolke firstly reported the heterologous production of berberine in *S. cerevisiae*, which was engineered to express seven heterologous enzymes. An initial scale of 0.2-L bioreactor led to the production of berberine at 39 μg/L. Afterward, they found (*S*)-tetrahydroprotoberberine oxidase (*STOX*) that generates berberine from canadine. Strains CSY1025 and CSY1026 were constructed respectively by assembling an expression cassette with yeast codon-optimized *Berberis wilsonae* STOX (*yBwSTOX*) into pCS3203 with the strong glycolytic promoter *PTPI1* and the late-stage dextrose-repressed promoter *PHXT*. These strains can produce <3 μg/L of berberine from *rac*-norlaudanosoline after 96 h of growth in culture tubes.[206] Even though only a low level of berberine was detected, this study open the doors for further improvement in the biosynthesis of berberine in microbial hosts to produce it with better yield.

Conclusion

The current production techniques for bioactive plant natural products such as chemical synthesis and plant extraction does not satisfy the demand. They also have led to environmental and economic concerns. Therefore, engineered production of valuable plant natural products in microbial hosts has become an attractive alternative. Some of the successful examples of engineered production of plant natural products are summarized in this paper, which clearly proves that engineered production method is a promising approach with various advantages. With the advances in synthetic biology and metabolic engineering, microbial production of plant natural products will lead the field. Particularly, recent developments in genome editing, expression and cloning such as CRISPR/Cas9, Gibson Assembly, DNA synthesis and sequencing will not only further facilitate the engineered production, but also take it to the industrial scale with improved yields using bioprocess and strain engineering techniques. For the later one, the rate-limiting and competing pathways, and unavailability of enough precursor are the major bottlenecks. Metabolic pathway rewiring and fine-tuning of the expression have helped to overcome these bottlenecks and enhance the overall yield of pharmaceutically important natural products. Thus, the engineered production of high-quality plant natural products will be economically viable in near future.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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