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Metabolic Engineering and Synthetic Biology of Plant Natural Products – a Minireview

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Review article

Metabolic engineering and synthetic biology of plant natural products – A minireview

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ABSTRACT

Plant natural products include a diverse array of compounds that play important roles in plant metabolism and physiology. After elucidation of biosynthetic pathways and regulatory factors, it has become possible to metabolically engineer new capabilities *in planta* as well as successfully engineer whole pathways into microbial systems. Microbial expression systems for producing valuable plant compounds have evolved to incorporate polyculture and co-culture consortiums for carrying out robust biosynthesis strategies. This review focuses on four classes of plant secondary metabolites and the recent advances in generating useful compounds in microbial expression platforms and in plant metabolic engineering. They are the flavonoids, alkaloids, betalains, and glucosinolates.

1. Introduction

Plant secondary metabolites comprises a diverse set of compounds that have been shown to serve many roles in plants. Secondary metabolites are indirectly involved in many different stages of plant development and impart an expansive set of traits that increase survivability. These traits include increasing the likelihood of plant-pollinator interactions, facilitating root nodule formation from nitrogen-fixating bacteria, imparting distinctive taste and color, and protecting from UV light [1–6]. Additionally, plant metabolites impart antimicrobial properties and can prevent bacterial infections [7]. An extensive library of natural products are consumed as part of the human diet that convey such benefits as acting as medicines for treating cancer, natural dyes, dietary supplements, and more [8–11]. Plant secondary metabolism and its many complexities have captivated the interests of researchers for decades. There is significant commercial interest in many of these metabolites and there exists a strong incentive to capitalize on their production [12–15]. This can be done either through metabolic engineering of plants for value-added or enhanced production of desirable compounds or reduction of undesirable compounds. Additionally, use of microbes as production "factories" using synthetic biology approaches has application in the pharmaceutical and nutraceutical industries.

Engineering plant secondary metabolism begins with elucidating the biosynthetic pathways that lead to a desired product as well as specific regulation of individual enzymes. For metabolic engineering of plants, elucidating regulatory factors and impact on changes on the metabolic and physiological networks are important to consider.

Expressing metabolites in microbial systems requires a robust balance of enzymes, cofactors, ATP and other metabolites. This is, perhaps, more amenable for those pathways with fewer enzymes, however more complex pathways have also been successfully incorporated into microbial systems [16]. Unraveling the biosynthetic pathways for secondary plant metabolites, combined with advances in engineering through metabolic reconstitution in microbial systems, have paved the way for significant advances in the relatively new field of synthetic biology. Numerous publications have demonstrated efficient or proof of concept production of several classes of plant natural products in microbial systems [17–25]. Dramatic advances in metabolic pathway engineering using microbial production systems has already proven to be a useful tool for producing plant secondary metabolites [26–31]. This minireview provides information on advances in flavonoid, alkaloid, betalain, and glucosinolate bioengineering.

2. Technology

Engineering reliable biosynthesis of plant secondary metabolites currently benefits from many new and developing tools designed to inform pathway construction and optimize pathway development. These include a host of ever-expanding -omics databases, computational pathway simulations paired with custom enzyme designs, and directed evolution approaches that seek to maximize output of the

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desired product [32]. Advances in microbial engineering have allowed for a diverse range of plant compounds to be synthesized. This list includes pharmaceutical precursors, dyes, dietary supplements, cosmetics, fuels, and more [33–37]

One of the most important questions to consider when developing a microbial platform for biosynthesizing plant compounds is the choice of microbial host organism. There exist a number of specimens from the fungal and bacterial kingdoms useful as secondary metabolite expressions hosts, notably *Escherichia coli*, *Saccharomyces cerevisiae*, and more recently, *Corynebacterium glutamicum and Yarrowia lipolytica* [22,27,38–44]. These microbes are relatively easy to grow and there exists a substantial body of research detailing diverse genetic manipulation and metabolic modifications of these organisms [45, and references there in]. *E. coli* has been described as having inexhaustible engineering potential while *S. cerevisiae* is a go-to yeast for its ability to incorporate complex biosynthesis pathways [46,47].

Various approaches have arisen for tackling difficulties in reconstituting multiple pathways into one microbial expression system. The ability to culture multiple strains in one bioreactor to carry out a complex biosynthesis strategy is a powerful tool recently employed to produce a range of complex plant compounds [46,47,48,49 and references there in]. These approaches allow greater diversification of product biosynthesis while simultaneously reducing the metabolic load on a cell line. Recent advances have used different strains of *E. coli* in a balanced ratio so that batch fermentation of all strains produces the desired product. Using different strains of the same organisms in "polycultures" allows streamlining growth factors, antibiotics, and downstream processing [16].

Other techniques have used microbial consortia that combine different microbes in the same culture [20]. Elucidating growth conditions that support multiple microbes can occur in different ways but is usually determined experimentally. This may include assessing inoculation ratios from seed cultures, monitoring growth using biosensors, or supplementing the less prolific organism with additional nutrition to balance growth [50]. The most successful growth strategies employ all of the above [20,48,50,51].

Microbial consortia can be constructed artificially, semi-synthetically, or synthetically [48]. The former of the three involves using microbes that already live symbiotically in nature with no genetic manipulations, whereas the latter involves genetically modifying all microbes used in the consortia. Semi-synthetic construction combines a naturally occurring microbe with a genetically modified one. The type of relationship between the microbial hosts being cultured may be synergistic, commensal, or mutualistic [48]. A synergistic relationship divides the metabolic load between the consortia for a combined output that is more efficient than using one microbe alone. Commensal interactions benefit one microbe without harming or benefiting the other, whereas a mutualistic one benefits both microbes. For a full description of these constructions and interactions see Bhatia et al. and Roell et al. [48,50].

The interaction types described above are by no means comprehensive as novel consortia platforms are currently being explored. For example, a 4th mode of interaction has been identified, designated here as "synergistic-sacrificial," wherein a division of labor between two strains enhances production of the desired product but requires programmed cell death of one strain [51]. This involved the use of a genetic circuit in one *E. coli* strain that combined expression of a glucosidase with lytic genes from a T4 phage. Upon reaching maximal expression of the glucosidase, the lytic genes were triggered causing the first strain to lyse, thus releasing the glucosidase enzyme [51]. Glucosidase accumulation in the culture medium catalyzed the conversion of cellobiose, a common intermediary found in the industrial processing of biomass, into glucose. This was then used as a carbon source by a second *E. coli* strain to drive the production of isopropanol (IPA). This consortium platform exhibited cellobiose conversion rates 4 fold faster (6 h vs. 24 h) and IPA production amounts approximately 3-fold higher (5.8 ± 0.5 mM vs. $16-19$ mM) than a single microbe engineered to conduct both cellobiose conversion and IPA production [51]. This example highlights the potential for microbial consortium platforms to convert biomass waste products into energy while simultaneously driving production of useful compounds. These platforms perform better than single microbe systems and have the potential to be used in engineering plant natural products.

In a recent example, a microbial consortium was developed to produce oxygenated taxanes, a class of chemotherapeutic diterpenes that cannot be feasibly produced in one microbe [20,52]. This was accomplished by dividing taxadiene synthesis and oxygenation between engineered strains of *E. coli* and *S. cerevisiae*. The *E. coli* strain was engineered to produce taxadiene and metabolize xylose (supplied as food) to acetate [20]. *S. cerevisiae* was engineered to use acetate as a fuel source and oxygenate taxadiene [20]. Conditions for growth of recombinant *S. cerevisiae* and *E. coli* were determined individually before attempting co-culture. Their ability to carry out their respective functions was also determined beforehand experimentally. The recombinant *S. cerevisiae* was treated with stock taxadiene to evaluate its oxygenation potential and experiments were carried out to determine the yeast's ability to grow using acetate as the sole food source. Similarly, the engineered *E. coli* strain was verified to produce taxadiene and acetate at the necessary levels [20].

Successful co-culture was achieved by inoculating seed cultures of *S. cerevisiae* and *E. coli* into a defined medium (13.3 g/L KH2PO4, 4 g/L (NH4)2HPO4, 1.7 g/L citric acid, 0.0084 g/L EDTA, 0.0025 g/L CoCl2, 0.015 g/L MnCl2, 0.0015 g/L CuCl2, 0.003 g/L H3BO3, 0.0025 g/L Na2MoO4, 0.008 g/L Zn(CH3COO)2), 0.06 g/L Fe(III) citrate, 0.0045 g/L thiamine, 1.3 g/L MgSO4, pH 7.0) supplemented with yeast extract (40 g/L) and glucose or xylose (20 g/L). The yeast to *E. coli* seed culture inoculation ratio was determined experimentally. Fermentation was carried out in a bioreactor wherein dissolved oxygen levels, pH, and temperature were all optimally maintained at 30–40 %, 7.0, and $20-30$ °C respectively. A recent review on co-culturing different microbes expounded on the biotechnological potential of this method and provided examples of its utility for chemical biosynthesis and other fields [48].

Modifying natural product biosynthetic pathways can also occur *in planta*. Generating a knock-out line or introducing a new gene into a plant not normally carrying out a particular reaction has proven to be quite useful in confirming the function of a gene or protein [2,22,95,107,108]. New techniques have also emerged that make use of "nanomaterial-based delivery systems" that can target specific organelles without having to fully integrate new DNA into the host plant genome [53].

Examples of transgenic plants abound and their production has evolved to serve different functions [54,55]. Recently, one approach involved creating a transgenic morning glory that co-expresses AmAS1 and Am40CGT, which drives accumulation of aureusidin glucosides in order to produce flower variants with unique color [56]. These compounds belong to the aurone subclass of flavonoids and are partially responsible for yellow coloration in flower petals [57]. Additional studies have sought to genetically engineer accumulation of plant secondary metabolites known for their health benefits in staple crops, such as corn or wheat. These approaches have shown great promise and are the subject of several recent reviews [55,58,59]. While some successes have been made using transgenic plants as a source of pharmaceutical or nutraceutical compounds, accumulating specific secondary metabolites in microbial systems has proven to be a more desirable approach in some cases [60,61].

3. Flavonoids

Flavonoids belong to the phenolic class of plant secondary metabolites and serve many functions in both plants and animals. There exist thousands of identified flavonoid compounds that are divided into 9

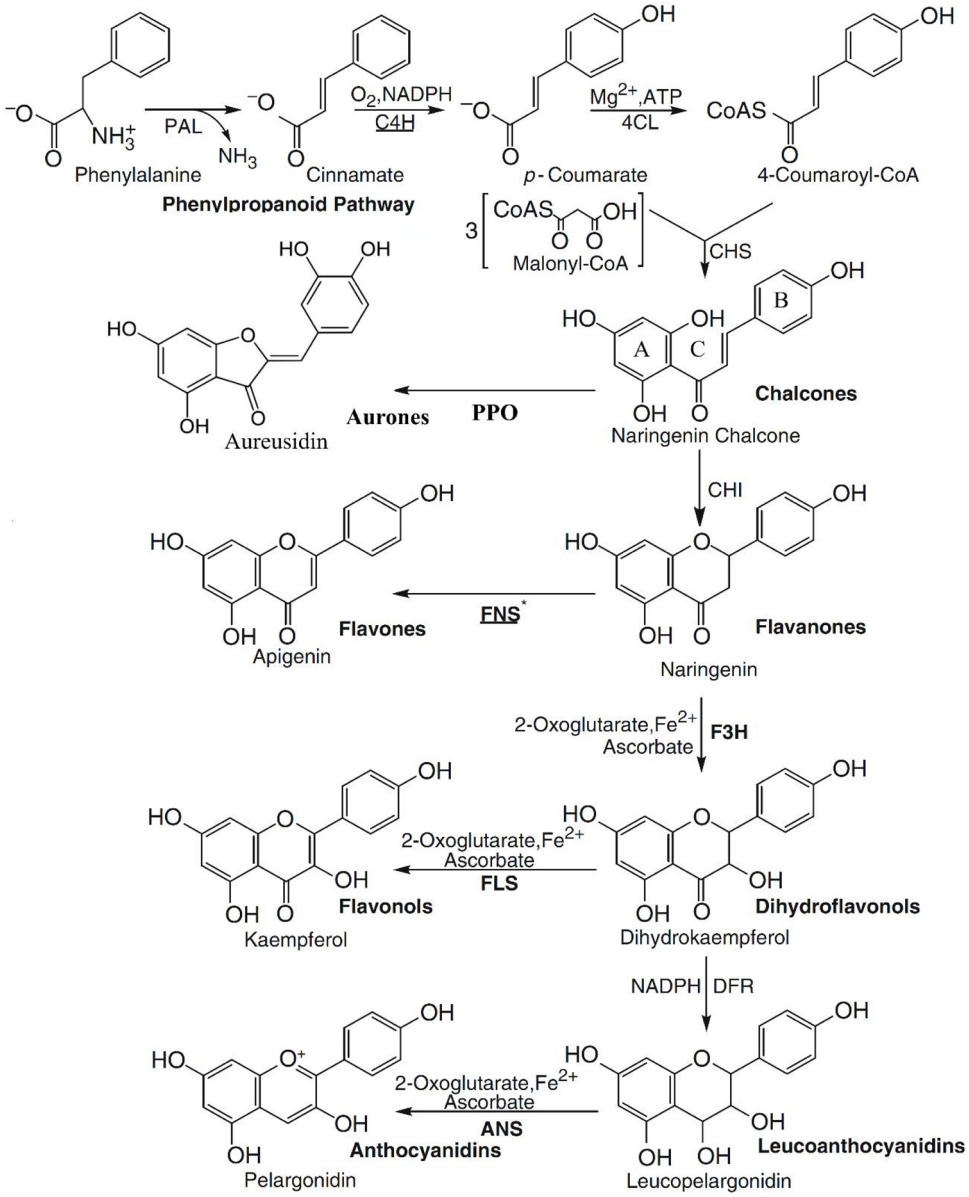

Fig. 1. Biosynthesis of flavonoid classes through the phenylpropanoid pathway (adapted from [66]). Enzymes shown are phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), plant polyphenol oxidase (PPO), chalcone isomerase (CHI), flavone synthase (FNS), flavanone 3β-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS).

subclasses based on the oxidation level and conformation of the middle ring (Fig. 1). They serve many roles in plants including modulating various defensive traits, such as UV light protection in leaves or protection from herbivores [62,63]. Flavonoids have been shown to act as signaling compounds by assisting in the formation of symbiotic colonization of *Rhizobia* species, a critical relationship in plants that rely on *Rhizobia* for nitrogen fixation [2,64]. Additionally, flavonoids have been shown to modulate plant-insect interactions and to play a role in feeding, as well as stimulating or discouraging oviposition behavior [65].

The ubiquity of flavonoids in edible plants ensures that for herbivorous/omnivorous mammals, they are a routine part of dietary metabolism. Studies suggest they impart many physiological benefits such as reducing inflammation and increasing radical scavenging capabilities. Indeed, numerous flavonoid extracts are marketed as dietary supplements [67].

Many flavonoids are associated with health benefits in humans and some show the potential to inhibit angiogenesis and upregulate apoptosis in cancerous cells [68–72]. A recent investigation into the anti-cancerous potential of flavonoids extracted from blueberries highlights the importance of these compounds. The study showed that the principal flavonoids present in *Vaccinium myrtillus* (quercetin, kaempferol, and gentisic acid) had significant apoptotic effects on colorectal cancer cells [73].

The extensive use and application of plant flavonoids has driven refinement of recombinant means to produce these compounds. The core pathways for flavonoid biosynthesis have been elucidated and their chemical/biological potential has been thoroughly reviewed [66,74–76]. While all terrestrial plants synthesize flavonoids, the class of flavonoid, specific final products, and amount produced can vary significantly [26]. Furthermore, derivatives of flavonoids that do not occur in nature have been synthesized and are being investigated for their pharmaceutical potential [77].

Flavonoid biosynthesis begins with the production of naringenin chalcone from 4-coumaroyl CoA and 3 malonyl CoA, which is catalyzed by chalcone synthase (CHS) [78]. The chalcone backbone subsequently

gives rise to a series of other reactions resulting in the synthesis of other flavonoid subclasses (Fig. 1). Chalcones can be converted to aurones by plant polyphenol oxidase (PPO) or to flavanones by chalcone isomerase (CHI). Flavanones can be converted to flavones by flavone synthase (FNS) or to dihydroflavonols by flavanone 3−OH transferase. In turn, dihydroflavonols can be converted to flavonols by flavonol synthase (FLS) or to leucocyanins by dihydroflavonol reductase(DFR). Anthocyanins are synthesized from leucocyanidins by a two step process catalyzed by anthocyanin synthase (ANS) followed by glycosylation. Reconstituting these pathways requires encoding all genetic information relevant to the synthesis of the desired flavonoids into the microbial host genome. Furthermore, expression of plant genes necessary for biosynthesis must be fine-tuned to avoid undesirable product pathways and upregulate genes that lead to adequate precursor synthesis [44 and references there in]. Modifications to the final product may also be needed. These include glucosylation, prenylation, methylation, sulfation, and more. Flavonoids have been shown to be more biologically active when glycosylated making this a desirable modification for many synthesis platforms [79,80]. These steps have been successfully carried out in both *E. coli* and *S. cerevisiae* [81,82].

A recent endeavor to produce flavonols and anthocyanidin-3-O-glucosides made use of *E. coli* polycultures, a mix of *E. coli* strains each designed to carry out a specific biosynthesis step [16]. This system began by generating *E. coli* cultures with plasmids that overexpressed phenylpropanoic acids, specifically p-coumaric acid and caffeic acid. The first strain used xylose, glucose, and glycerol to produce a precursor pool of phenylpropanoic acids used in chalcone synthesis. A second strain carried out production of a flavanone, which was designed to be converted to a dihydroflavonol by a third strain. This allowed a fourth strain to achieved production of the anthocyanin callistephin [16]. A 5th strain was proposed that could be used for further downstream modifications to synthesize non-natural flavonoid compounds. Both the flavonoid and stilbene classes of polyphenols have been altered in microbial organisms to produce non-natural variants, with some possibly having medical or pharmaceutical importance [83,84].

When attempting to fine tune a microbial synthesis platform for flavonoid biosynthesis, notable obstacles have been overcome. One example highlights the rapidly improving potential for optimizing commercial biosynthesis of these compounds. This example involves the precursor malonyl CoA. Three malonyl CoA are combined with 4-Coumaroyl CoA to produce chalcone, the flavonoid backbone from which different classes of flavonoids are derived. Malonyl CoA availability has been shown to be a limiting factor when trying to express flavonoids in microbial systems [31,85]. Recently, *C. glutamicum* has been used to express naringenin and resveratrol at production titers markedly improved due to engineering strategies that increased malonyl CoA availability [44]. A similar endeavor to modulate malonyl CoA metabolism used *E. coli* and CRISPR editing to produce naringenin at greater amounts due to a larger malonyl CoA pool [86].

Another approach used the oleaginous yeast *Y. lipolytica* to engineer hydroxylated flavonoid production [27]. This microbe provides a lipophilic environment that is beneficial for the expression of plant enzymes needed to produce flavonoids, such as chalcone synthase and cytochrome P450 reductase [78]. Furthermore, *Y. liplytica* has a naturally high flux of malonyl-CoA [27]. This microbe benefitted from engineered expression of genes responsible for chalcone synthase and cytochrome p450 reductase, as well as genes that upregulate malonyl-CoA production. The engineered strain was able to produce titers of naringenin at concentrations of 250 mg/L and 134 mg/L respectively [27].

4. Alkaloids

Different structural classes of alkaloids exist that include benzylisoquinoline alkaloids, monoterpene indole alkaloids, bisbenzylisoquinoline alkaloids, and the tropane and nicotine alkaloids (Fig. 2) [87,88]. Numerous pathways exist for synthesizing these compounds but all originate from precursor amino acids, such as tryptophan or aspartic acid. For example, the monoterpene indole pathways begin when L-tryptophan is converted to tryptamine, which is then used as a substrate to form strictosidine, the precursor for all monoterpene indole derivatives (Fig. 2) [89].

Alkaloids are used in plants for self-defense and can exhibit direct toxicity to herbivorous organisms [90]. Alkaloids may also inhibit or alter important metabolic pathways within the consuming animal. Plants use alkaloids as nitrogen sinks for suppling pools of chemicals important to the synthesis of wound-healing metabolites [91,92]. Like flavonoids, alkaloids have also been shown to influence plant-insect interactions and can deter insect feeding [90]. Elucidated pathways of monoterpene indole alkaloids (MIAs) have shown that when under attack, *Catharanthus roseus* deglucosylates the alkaloid strictosidine-4, converting it into a dialdehyde compound that can cross link proteins [93,94]. This mechanism of defense has also been found for glucosinolates found in mustard oil discussed further below [95].

Plant alkaloids have long been used by humans, with their usage recorded prominently throughout ancient history and into today [88, 96 and references there in]. The thousands of plant alkaloids currently known to exist represent a vast pharmacopeia of useful compounds that include medicines, stimulants, and many psychoactive substances [97,98]. Consequently, there is a strong incentive to produce a wide range of alkaloid compounds in microbial systems, with many recent reviews highlighting its potential to transform drug manufacture and other fields of industry [33,99,100].

Chemotherapeutic alkaloids such as taxol are derived from plant sources, as are other alkaloids such as opiates for treating pain [101,102]. These compounds are fundamental to modern medicine yet extracting high value medicinal compounds from plant sources can be complicated by agricultural or environmental factors [103]. Biosynthesizing plant alkaloids in microbial systems is not subject to these constraints. Consequently, optimizing microbial engineering towards the synthesis of useful alkaloids has garnered an abundance of research interest. Recent advances have been made regarding complete biosynthesis of alkaloids in microbial systems, semi-synthesis of alkaloids, and engineering alkaloids *in planta* [104].

Complete biosynthesis of alkaloids in microbial systems offers the greatest potential for generating a safe and affordable basis for pharmaceutical manufacture. A proof of concept manufacturing process was recently described using *S. cerevisiae* to engineer benzylisoquinoline alkaloids used in opiate production, specifically hydrocodone [105]. This process achieved titers of less than 1 μg/L, which fell short of the 5 g/L projection needed to be a suitable alternative to poppy cultivation, yet an improved process was reported shortly thereafter that used engineered *E. coli* to synthesize the alkaloid thebaine, a precursor used in the semi-synthetic production of hydrocodone and codeine [106]. At titers of approximately 2 mg/L, this method showed a 300-fold increase in production capacity over previous methods. Furthermore, these precursors were used to biosynthesize hydrocodone in *S. cerevisiae*, demonstrating that complete biosynthesis of valuable alkaloid compounds in microbes is possible [106]. Rapidly advancing techniques in microbial engineering could reduce the need for plant-based extraction of alkaloids as well as lowering the cost of chemically based synthesis strategies. However, not all alkaloids can be completely synthesized in microbes. A recent review discussed in great detail the various chemical and biochemical routes needed for complete biosynthesis of many alkaloids [107].

Employing complex chemical syntheses of alkaloid based drugs often cannot meet the demand for large-scale production, resulting in limited supplies of many lifesaving therapeutics. Semisynthesis of alkaloids involves using microbe biosynthesis platforms to generate high levels of precursor alkaloids used in drug manufacture. The desired precursor is biosynthesized in the microbe of choice, extracted, and subjected to further synthesizing steps. This technique has emerged as

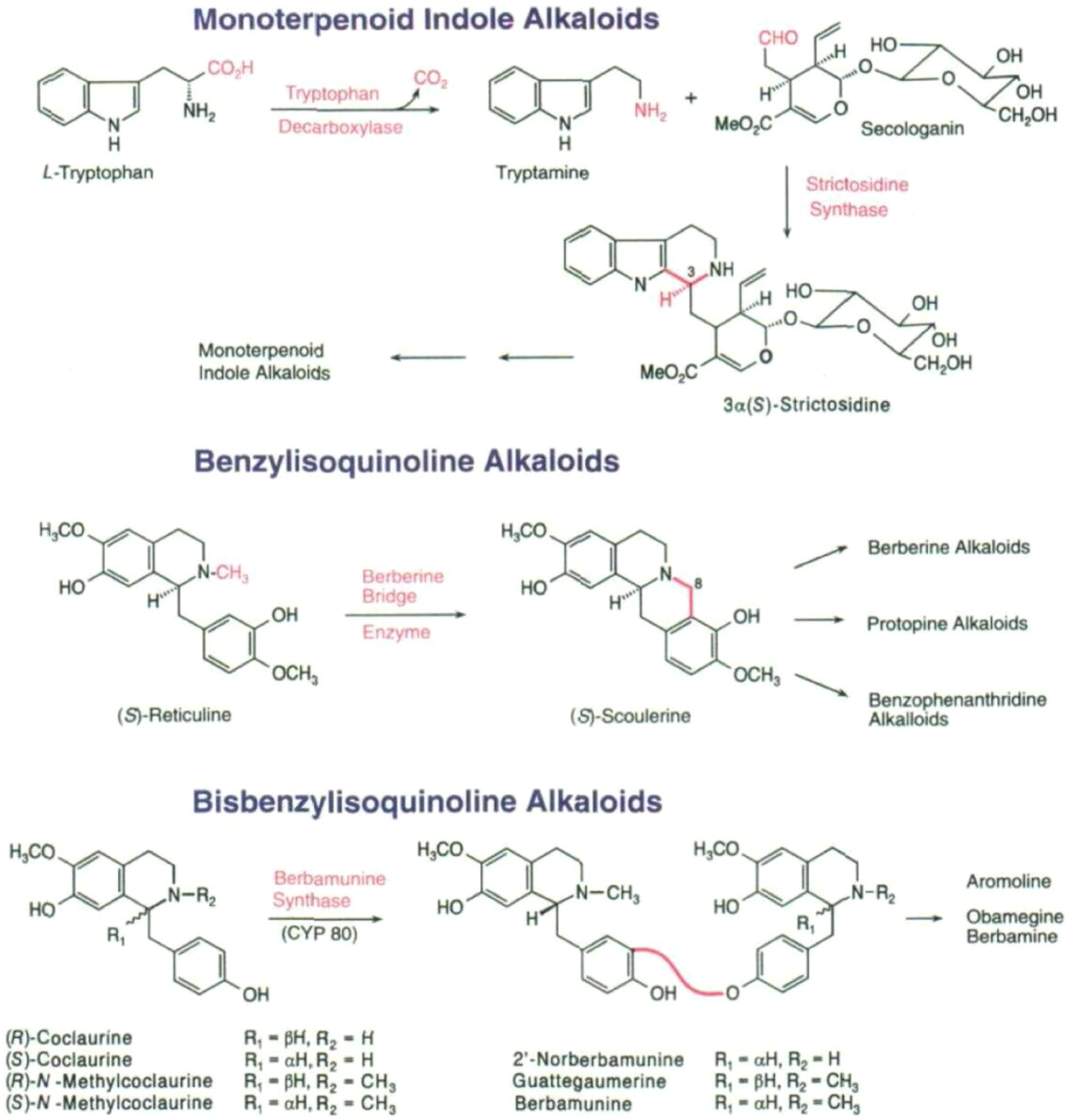

Fig. 2. Pathways for 3 subclasses of alkaloids (adapted from [87]).

an attractive way to simplify multistep synthesis strategies and drive down the cost of drug manufacture. A recent approach engineered *E. coli* and *S. cerevisiae* to produce guaia-6,10(14)-diene via the mevalonate pathway [104]. This starting material is used in the synthesis of englerin A, a chemotherapeutic agonist of the transient receptor potential (TRP) channels implicated in some cancers [108–110]. For alkaloids that cannot be completely expressed in microbial systems, this semisynthetic approach shows great promise in expanding drug availability and reducing cost of manufacture, as highlighted in recent reviews [33,107,111].

Despite exciting developments in full and semi biosynthesis of alkaloids, plant extraction remains a vital component of producing important alkaloid drugs [112]. Significant research has been focused towards engineering optimized and enhanced expression of alkaloids in plants [113–116]. The opium poppy, *Papavar somniferum*, is perhaps the most recognizable plant source due to its supply of alkaloids necessary for synthesizing pain relieving medication. Not surprisingly, gene editing techniques have been employed to upregulate expression of metabolites that regulate alkaloid biosynthesis. Recently, a CRISPER/Cas9 system was designed to knock out the 4′OMT2 gene in *P. somniferum*, which has been shown to control biosynthesis of thebaine, noscapine, and morphine [99,113]. This technique generated a transgenic poppy plant that had markedly reduced biosynthesis of benzylisoquinoline alkaloids, indicating alkaloid biosynthesis can be regulated in transgenic plants using CRISPER/Cas9 gene drives [113]. Other plants, such as *C. roseus*, have been targeted for genetically engineering their production of the anti-cancerous vinca alkaloids vinblastine and vincristine [103,116,117]. A recent review highlighted a wide scope of engineering practices for upregulating vinca alkaloids in this plant [114]. Hairy root cultures of the Iranian flower *Hyoscyamus senecionis* and *H. muticus*, were also recently targeted for engineering of the anticholinergic and deliriant tropane alkaloids [115].

5. Betalains

Betalains are nitrogen containing compounds produced largely in plants in the Caryophyllales such as cacti and red beets. They are synthesized from tyrosine and fall into two main subclasses, the betacyanins that are red/purple and the betaxanthins that are yellow (Fig. 3).

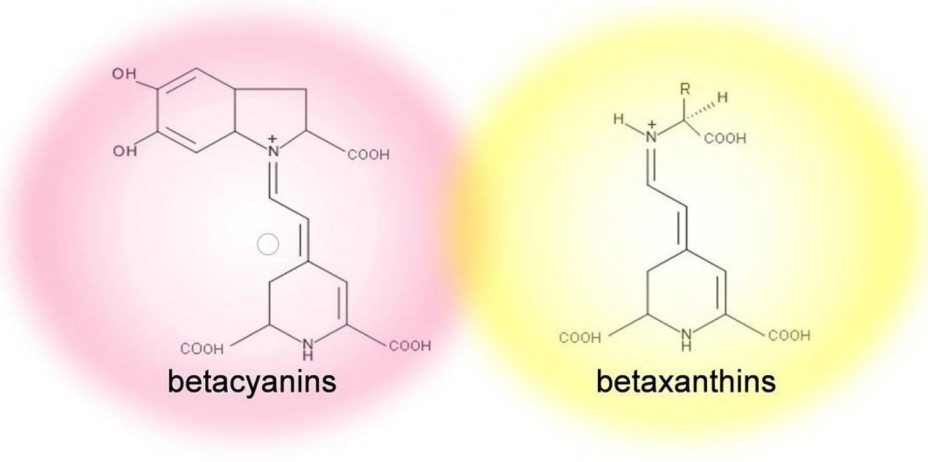

Fig. 3. The two main subclasses of betalains, betacyanins and betaxanthins [21].

Because of the similarity of colors of betacyanins and the anthocyanin flavonoid pigments, significant research has been conducted with respect to biosynthesis and addressing the interesting phenomenon that anthocyanins are not found in plants producing betalains even as other flavonoid compounds are found in these plants [118,119]. Different scenarios have been suggested for this ranging from loss of function to gene deletion. Richardson [37] and Polturak et al. [38] recently published results from a study of transcriptome and metabolite composition in *Mirabilis jalapa* that showed that anthocyanin synthase was present and highly expressed however the gene contained a deletion in the active site [37,38] rendering the enzyme inactive. These data support the idea of loss of function as one possible explanation of this exclusivity between betalains and anthocyanins and how anthocyanin production could have been lost over time in this group of plants. It will be interesting to see if this pattern of the ANS gene being present and expressed but having a loss of function through mutation will observed in other betalain producing plants, or if there are other variations.

Betalains are known for the vibrant colors that they impart to plants where they are found, serving as pollinator cues as well as for their potent antioxidant properties [35,120, and references there in]. Synthesis of these compounds in plants is increased in response to stress [120]. Several contributions to improvement of human health have been identified that have contributed to the increased interest in these compounds. These include positive effects on hypertension, cancer, and more and has recently been reviewed [121,122]. These properties, including the ability to use these compounds as natural food colorants and dyes, show significant potential for commercial applications [123].

Elucidation of the biosynthetic pathway has been sought for decades and was resolved through recognition that some steps involve spontaneous (i.e., not enzyme catalyzed) reactions[35,124] and references there in]. As stated previously, betalains are synthesized from tyrosine leading to production of a key intermediate, betalamic acid. This is done by a cytochrome P450 converting tyrosine to L-DOPA followed by conversion of L-DOPA to betalamic acid through the action of DOPA 4,5-dioxygenase. Synthesis of betaxanthins occurs by spontaneous condensation of betalamic acid with amino acids such as glycine or proline or from condensation with amines such as tyramine or dopamine [35 and references therein]. Synthesis of betacyanins occurs through spontaneous condensation of betalamic acid with cyclo-DOPA followed by derivatization or “decorating” reactions such as addition of sugars and/or acyl groups [40]. As a result, over 70 naturally occurring betalains have been identified from plants [35,124].

This group of compounds has found renewed interest as a target of metabolic engineering at least in part due to the properties mentioned above as well as elucidation of betalain biosynthesis. Recent reviews have been published by Polturak and Aharoni [35,125]. These reviews included consideration of biosynthesis, metabolism, applications, and current state of metabolic engineering. Because of the interest in this field, significant publications have appeared since those reviews. Additional factors to consider for engineering in plants includes tyrosine and other amino acid partitioning [126,127]. Other key contributions to the metabolic engineering toolbox have included identification of key genes and transcription factors [120,125,126], and references therein].

Tian et al. published results of their work to engineer betanin into rice endosperm as a health promoting food additive to increase health benefits of rice as well as provide a potential source of raw material for commercial supplement production [128]. Because of the relative simplicity of the pathway, they introduced only 3 genes to be overexpressed in rice, meloS, BvDODAIS, and BcCYP76ADIS. This work included determination of antioxidant capacity, grain yield, and quality of starch granules. The latter two parameters were comparable to wild type, while the transgenic rice had 4 − 6x higher antioxidant activity. This as well as the successful engineering of betalain synthesis into microbes, serve as concrete examples of the success of metabolic engineering of this pathway and likely will lead to further successful applications [22,122].

6. Glucosinolates

Glucosinolates are plant secondary natural products derived from both aliphatic and aromatic amino acids that are notably found in the Brassicales and related plants. Their structure is composed of a sulfonated oxime, a thioglucose moiety, and a side chain originating from the aliphatic and aromatic amino acids (Fig. 4) [129–131]. Further structural diversity occurs through modification reactions. A rigorous vetting of structures and compound identification has recently been published by Blazevic et al. [131]. To date, there are over 150 naturally occurring glucosinolates identified from plants.

Like the cyanogenic glycosides, glucosinolates are compartmentalized from degrading enzymes in plant tissues. Following mechanical disruption of tissue, they are hydrolyzed by endogenous myrosinases, a specific group of glucosidases that act on glucosinolates [130,131]. The products of hydrolysis include isothiocyanates and thiocyanates that have diverse biological activity (Fig. 5). In plants, these compounds provide defense against insects and microbes, as the hydrolysis products can lead to production of phytoalexins [132,133]. They have been shown to serve as antifeedant defense compounds for both insects and animal foragers, as well as feeding attractants and oviposition signals in insects preferring crucifers [129, 130, 132 and references therein].

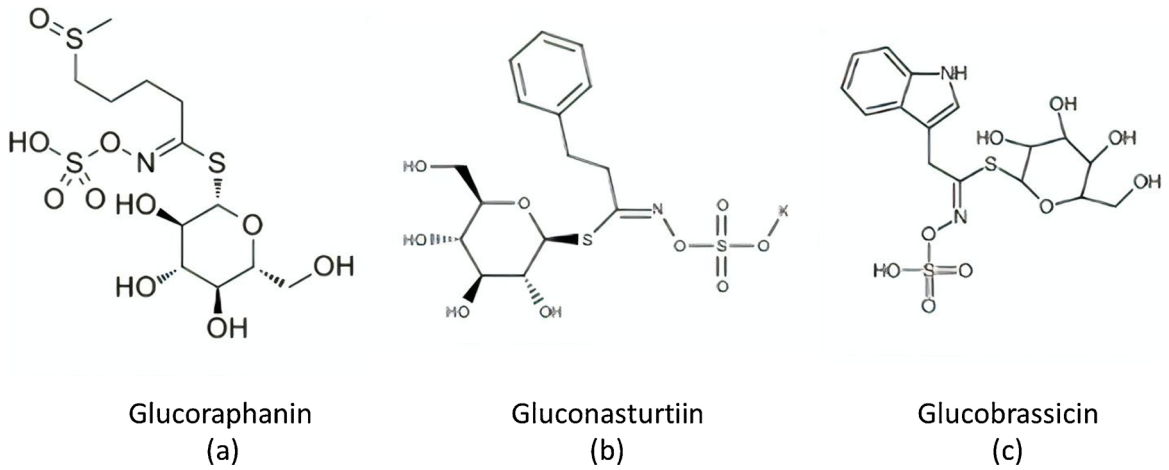

Fig. 4. Examples of glucosinolates synthesized from a) methionine, b) phenylalanine, and c) tryptophan.

In addition to the mustard and other taste properties of the Brassica, glucosinolates also impact animal health and nutrition. Examples of health benefit include cancer prevention and inhibition of proliferation, stimulation of the immune system, and reduction of heart disease [135,136 and references therein]. Nutritional and health considerations include bioavailability of glucosinolates and/or their breakdown products isothiocyanates [137 and references therein].

Because of the benefits to plants and animals, there is significant interest in the metabolic engineering of glucosinolates into plants, enhancement of production in plants, and commercial production of these compounds for use as nutritional supplements. As stated previously, for successful engineering it is imperative that the biosynthetic pathway, metabolism, regulation, and physiological impact of altering metabolism in the target organism is understood.

Blazevic et al. recently published a comprehensive review of glucosinolate biosynthesis [131 and references therein]. It includes consideration of synthesis from aliphatic and aromatic amino acids as well as key studies to identify genes and elucidate their functions (see also [138]); seven biosynthetic steps have been fully elucidated. Arabidopsis synthesizes glucosinolates thereby providing an amenable model system and powerful tool for identification of key genes and potential regulatory factors [139,140]. Transformation of genes into non-glucosinolate producing plants or systems was an important approach to further confirm function of the cytochrome P450 enzymes as well as the hydrolytic enzymes [139–141]. Knowledge of key genes in Arabidopsis has been used to identify potential genes in other species through comparative genetics [25].

Glucosinolate metabolism and regulation in plants has been studied in several systems including consideration of levels in different tissues and different stages [24,25,138,141]. One important aspect is the use of tryptophan for synthesis of both indole glucosinolates and indole acetic acid (IAA) and the metabolic balance of tryptophan use between these pathways [138,142]. MYB transcription factors have been identified that control synthesis [e.g [132,143, and ref therein]. Circadian regulation has also been established [132].

Recent reviews on development of tools and strategies for metabolic engineering and on use of *E. coli* as a host have shown the significance of these contributions and the advancements in technological approaches [32,144]. Glucosinolate metabolic engineering has encompassed transformation of genes into plants or systems that do not normally synthesize glucosinolates, as well as efforts to improve production in crop plants to improve nutritional value [24,25,139,141]. Building upon transformation of *E. coli* to produce glucoraphanin, Petersen et al. recently were able to engineer *E. coli* to produce benzyl glucosinolate [29,145]. They used a series of strategies evaluating host strain and culture conditions as well as monitoring protein expression levels to optimize levels of production and to further increase synthesis five-fold [146]. In a perhaps somewhat related effort, Liou et al. have

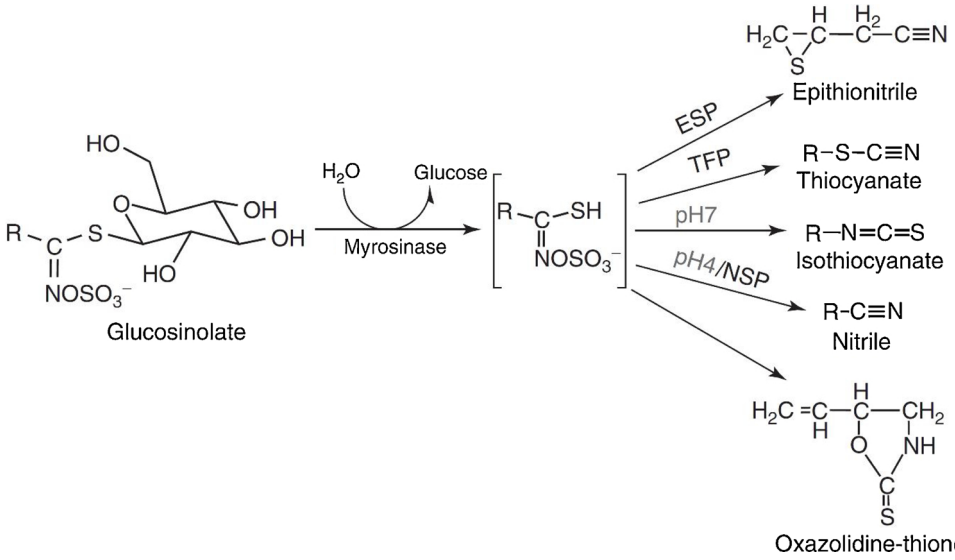

Fig. 5. Possible products of glucosinolate hydrolysis [134]. ESP = epithiospecifier protein; TFP = thiocyanate forming protein; NSP = nitrile specifier protein.

recently identified the operon used by a human gut symbiont responsible for transformation of glucosinolates into health promoting isothiocyanates by engineering this operon into a non-metabolizing strain and showing gain of function [147].

7. Summary

This mini-review summarized recent advances in synthetic biology and metabolic engineering for four classes of plant natural products; flavonoid, alkaloids, betalains, and glucosinolates. The importance of plant natural products to both plants and animals coupled with advances in technological approaches has resulted in being able to engineer plants for value-added crops, increased resistance/defense, enhanced coloration, and more. As we learn more about *in planta* regulation and control of metabolism and metabolic networks, we will continue to see increased success and efficiency in this area.

An overview of recent technological advances in synthetic biology was also given. Emphasis was placed on microbial platforms as a highly useful tool for engineering these natural products with additional consideration given to microbial consortia. The ability to culture robust assortments of microbes with varying functionality has dramatically expanded to allow a wider scope of products to be engineered in increasingly efficient ways. These technological breakthroughs are continuing to develop at a rapid pace and coincide with new discoveries regarding the use of plant natural products. This is especially so for those compounds with benefits to human health. Improved approaches for synthetic biology and scalable production for pharmaceutically effective compounds as well as nutraceuticals have been significant. Further optimization and technological advances as well as continued enlightenment of the intricacies of the metabolic networks involving plant natural products will continue to advance the field.

Authorship contribution statement

Both authors contributed equally to the preparation and writing of this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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