REVIEWS IN ADVANCE

Engineering of Secondary Metabolism

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Abstract

Secondary (specialized) metabolites, produced by bacteria, fungi, plants, and other organisms, exhibit enormous structural variation, and consequently display a wide range of biological activities. Secondary metabolism improves and modulates the phenotype of the host producer. Furthermore, these biological activities have resulted in the use of secondary metabolites in a variety of industrial and pharmaceutical applications. Metabolic engineering presents a powerful strategy to improve access to these valuable molecules. A critical overview of engineering approaches in secondary metabolism is presented, both in heterologous and native hosts. The recognition of the increasing role of compartmentalization in metabolic engineering is highlighted. Engineering approaches to modify the structure of key secondary metabolite classes are also critically evaluated.

INTRODUCTION

Secondary or specialized metabolites are small molecules that are not essential for life, although these compounds likely confer evolutionary advantage to the producer organism (62, 96). Although primary metabolic processes (e.g., glycolysis, amino acid biosynthesis) are found in many or all species of life, the pathways that lead to secondary metabolites are specialized, with particular pathways found in a taxonomically restricted group of organisms. Secondary metabolites exhibit enormous structural variation and consequently display a wide range of biological activities.

Classes of Secondary Metabolites

Secondary metabolites are classified according to biosynthetic origin (**Figure 1**). Terpenes are derived from precursors [dimethylallylpyrophosphate (DMAPP) and isopentylpyrophosphate (IPP)] containing five carbons (73) and are further classified by size. Polyketides are synthesized from acetate units and are also subdivided into several classes (95). In plants, polyketide biosynthesis intersects with aromatic amino acid biosynthesis to generate phenylpropanoid structures, such as flavonoids, flavonones, stilbenes, and anthocyanins (143). Some carbohydrates can also be classified as secondary metabolites (93), and saccharides that are unique to secondary metabolism often decorate the core structure of many secondary metabolites. Peptide metabolites are built from amino acids using either the ribosome (118) or dedicated enzymes (nonribosomal peptide synthetases) (146). Alkaloids encompass a broad range of metabolites with the sole commonality of a basic nitrogen atom (5, 34, 152, 163).

To date, secondary metabolites have been isolated from all domains of life. Those from bacteria were among the first to be discovered and were subject to the first engineering efforts. From the eukaryotic kingdom, fungi and plants are known to be prolific sources of secondary metabolites. Notably, animals do not produce many such compounds, most likely because these organisms have other ways of interacting with the environment (e.g., movement). Secondary metabolites have also been isolated from archaea, although the number of examples reported is relatively low; this may be in part due to challenges associated with culturing many species of this domain.

Metabolic Pathway Elucidation

The biosynthetic genes and/or regulatory elements of a metabolite must be characterized prior to metabolic engineering. The study of bacterial-derived secondary metabolites was hugely accelerated by Hopwood and coworkers' discovery that the biosynthetic genes for the polyketide actinorhodin are clustered on the genome of *Streptomyces* (125). This phenomenon of clustering is now known to be generally true for all bacterial secondary metabolic pathways. Bacterial genomes can be sequenced and subjected to genome mining to rapidly identify new metabolic pathway clusters for functional characterization (13). Over the past decade, it has become clear that the vast majority of fungal secondary metabolite pathways are also clustered on the genome (15, 20, 25).

Although an ever-increasing number of metabolic pathway clusters are being identified in plant genomes (113), not all plant pathways are clustered; the extent to which clustering prevails will be clarified as more plant genomes become available. Because genes of a plant metabolic pathway are frequently coordinately regulated, a known biosynthetic gene can be used as bait to search for undiscovered genes that share similar expression profiles using transcriptomic (RNA-seq) data (132). Additionally, transcriptome-metabolome comparisons can point to potential biosynthetic genes by correlating gene expression with the presence of the secondary metabolite of interest (156).

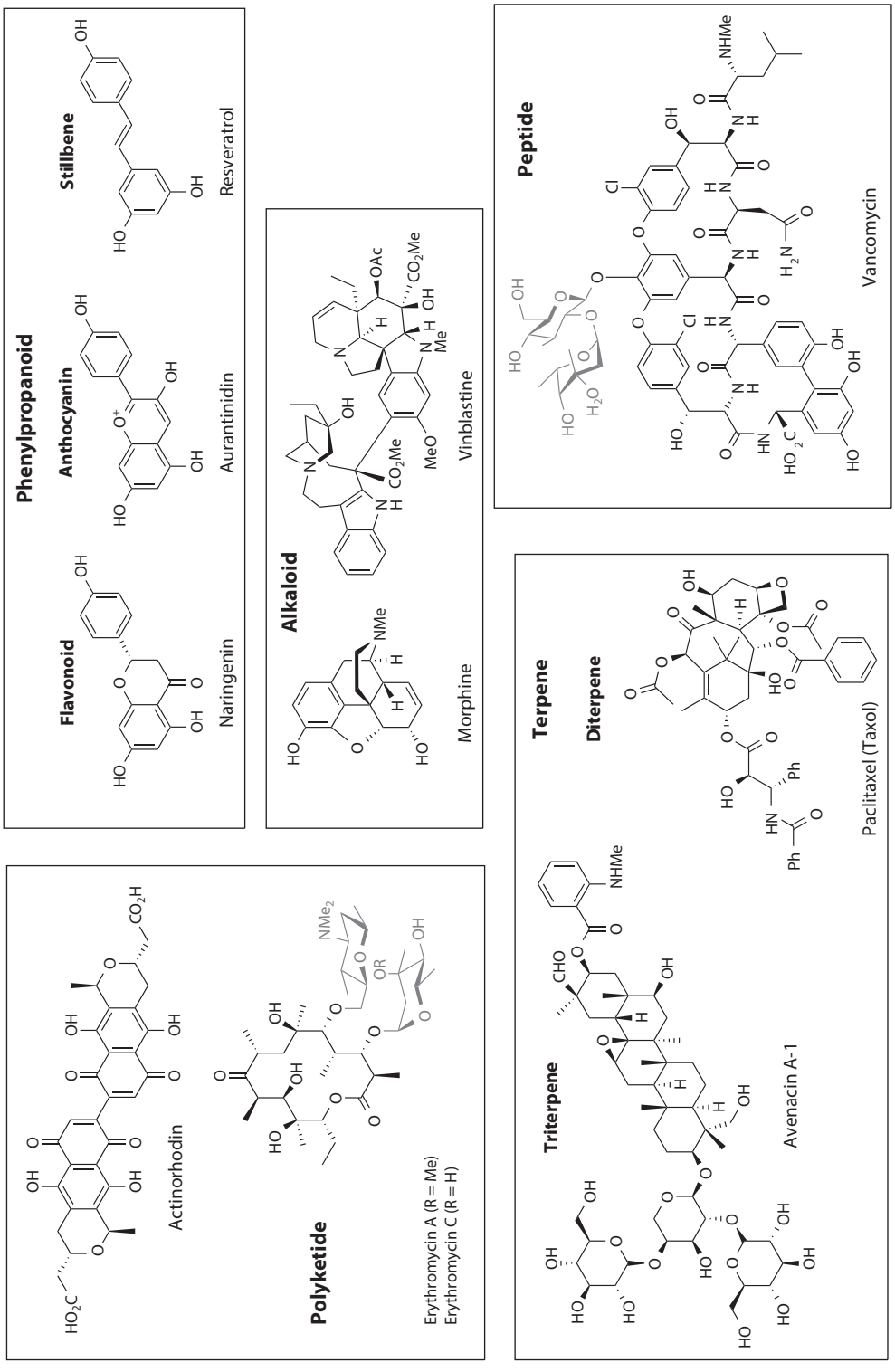

Figure 1

Representative members of the major classes of secondary metabolites. Carbohydrates unique to secondary metabolism decorate the structures of many of these compounds (*gray*).

Principles of Metabolic Engineering

Secondary metabolites have been used for pharmaceutical, cosmetic, and agrochemical purposes for thousands of years. Even today, secondary metabolites account for more than one-third of all therapeutic compounds (110). Although the pharmaceutical industry has largely turned to relatively inexpensive synthetic compounds as a source of new drug leads, in the past decade there has been renewed interest in secondary metabolites (83). This is likely due to the increasing recognition that secondary metabolites are evolutionarily preselected for biological activity; secondary metabolites are termed privileged scaffolds. Unfortunately, many secondary metabolites are produced in small quantities by the producer organism, and these organisms can be slow growing or impossible to culture under laboratory conditions. Metabolic engineering of biological systems has the potential to be a scalable, selective, and cost-effective way to access these high-value molecules with good yields and levels of purity. Furthermore, modifications to the structures of secondary metabolites can often improve or change the biological activity of the compound (26). Pathway engineering can be used to modify the structure of the secondary metabolite, or to make completely novel molecules, with new or improved biological properties. Finally, engineering of secondary metabolism can improve the function or fitness of the host organism.

The choice of host is an essential first consideration in metabolic engineering. Manipulation of the native producer to enhance yields has a long history in industry. Nonengineering strategies, such as random mutation followed by screening or selective breeding, have led to enhancements in secondary metabolite levels, indicating that the yields in the native host can be substantially improved. If the native producer can be genetically manipulated, then engineering the native producer is a viable option. Additionally, if the metabolite is being used to confer a beneficial property, such as enhanced defense or nutritional benefit, to the host, then engineering must take place in the native producer. However, metabolic pathways are tightly regulated, and overexpressing a single biosynthetic enzyme usually has a limited impact on yield. Recent advances have demonstrated that the expression of transcription factors can be used to achieve more global upregulation of secondary metabolism (11). Additionally, altering the site of biosynthesis also has the potential to override the endogenous regulatory controls (151).

Transfer of entire metabolic pathways into a heterologous host is an increasingly recognized alternative to using the native producer. Although heterologous hosts for secondary metabolite production were reported as early as 1985, the development of modern molecular biology tools has made heterologous expression much more accessible. Genes can now be synthesized relatively inexpensively, allowing the codon usage of foreign genes to be optimized for the host organism; this typically results in improved protein expression. Techniques to incorporate genes into dedicated locations in the chromosome ensure stable and controllable gene expression. Isolation and characterization of a wide range of promoters (constitutive and inducible) allow tuning of protein expression levels; it is important to note that high expression levels do not always lead to the highest product yields, and substantial tuning of gene expression plays a crucial role in many successful metabolic engineering efforts. Finally, the ability to assemble large blocks of DNA has made heterologous reconstitution of lengthy and complex pathways possible.

Four heterologous hosts are assessed in this review: *Streptomyces*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana*. *Streptomyces*, a soil bacterium that is a prodigious producer of secondary metabolites, was the first genus used for modern metabolic engineering efforts (47). Because *Streptomyces* produces many secondary metabolites, minimal upstream engineering is required to ensure that the appropriate building blocks for the foreign metabolite are available. *Streptomyces* is typically used as a host for pathways from other related bacteria, and there are usually minimal issues with gene expression.

Although *Streptomyces* is still used widely as a host for many bacterial pathways, compared with *E. coli Streptomyces* is relatively slow growing, and far fewer genetic tools are available (23, 61). However, *E. coli* is not a major producer of secondary metabolites, so reconstitution in this host typically requires substantial engineering of the native cellular metabolism to ensure that a sufficient amount of precursors are available to build the desired secondary metabolite. Early examples demonstrated that *E. coli* could be used to reconstitute bacterial products, although more recent work has demonstrated that plant metabolites can also be reconstituted successfully in this host. However, many plant enzymes, such as cytochrome P450s, are membrane bound, making expression in a bacterial host problematic (21). Additionally, many plant and some fungal pathways use compartmentalization of biosynthetic enzymes to control biosynthesis (53), and compartmentalization cannot be easily replicated in a bacterial host.

S. cerevisiae (baker's yeast), although slower growing than *E. coli*, can be transformed readily; efficient homologous recombination enables stable integration of genes into the chromosome, and a host of genetic tools are available. Numerous metabolic engineering approaches have been developed for *S. cerevisiae* (76, 135). Yeast as a heterologous host has been particularly important for plant-derived pathways such as complex terpenes (136); because plant cytochrome P450s can be challenging to express in *E. coli*, yeast remains the more common choice of host for plant-derived pathways. Additionally, proteins can be targeted to different cellular compartments in a yeast host, which can be an important aspect of a metabolic engineering strategy.

N. benthamiana, a relative of tobacco, is emerging as a practical expression host for certain secondary metabolite pathways. This species is relatively fast growing and can be transformed efficiently, and coexpression of large numbers of genes (gene-stacking), which is required for reconstitution of lengthy metabolic pathways, has been successfully demonstrated. Both stable and transient expression of pathway enzymes are possible, with exceptionally high levels of protein expression achieved in some transient expression systems (130). Despite this, microbial expression hosts are still more convenient for many laboratories, and *N. benthamiana* has so far been used primarily for expression of secondary metabolites derived from plants.

Assessment of yield must be taken on a case-by-case basis. An acceptable yield depends on how much of the metabolite is required, how much is produced by the native producer, and the value of the biological activity of the compound. Generally, in microbial hosts, titers over 1 g/L are considered outstanding.

A critical survey of engineering approaches in secondary metabolism is presented here. Specific examples have been provided to illustrate key developments in the field; unfortunately, many excellent examples have not been included because of space constraints. Additionally, several rigorous reviews in engineering secondary metabolism have been published recently (45, 121, 148, 154).

PATHWAY RECONSTITUTION IN HETEROLOGOUS HOSTS

An important strategy in secondary metabolic engineering is reconstitution of an entire metabolic pathway into a new host. Although many natural producer organisms are difficult to culture or transform, heterologous expression allows the use of a convenient host that can be genetically manipulated and is safe and cheap to culture. Below, the major heterologous hosts that are used in metabolic engineering are assessed, along with examples of important secondary metabolites that have been produced using these hosts. Other pathway hosts are being developed, including the gram-positive bacterium *Bacillus subtilis* (141), the filamentous fungus *Aspergillus nidulans* (88, 129, 155), the microalga *Chlamydomonas reinhardtii* (44), the moss *Physcomitrella patens* (7), and the liverwort *Marchantia polymorpha* (59).

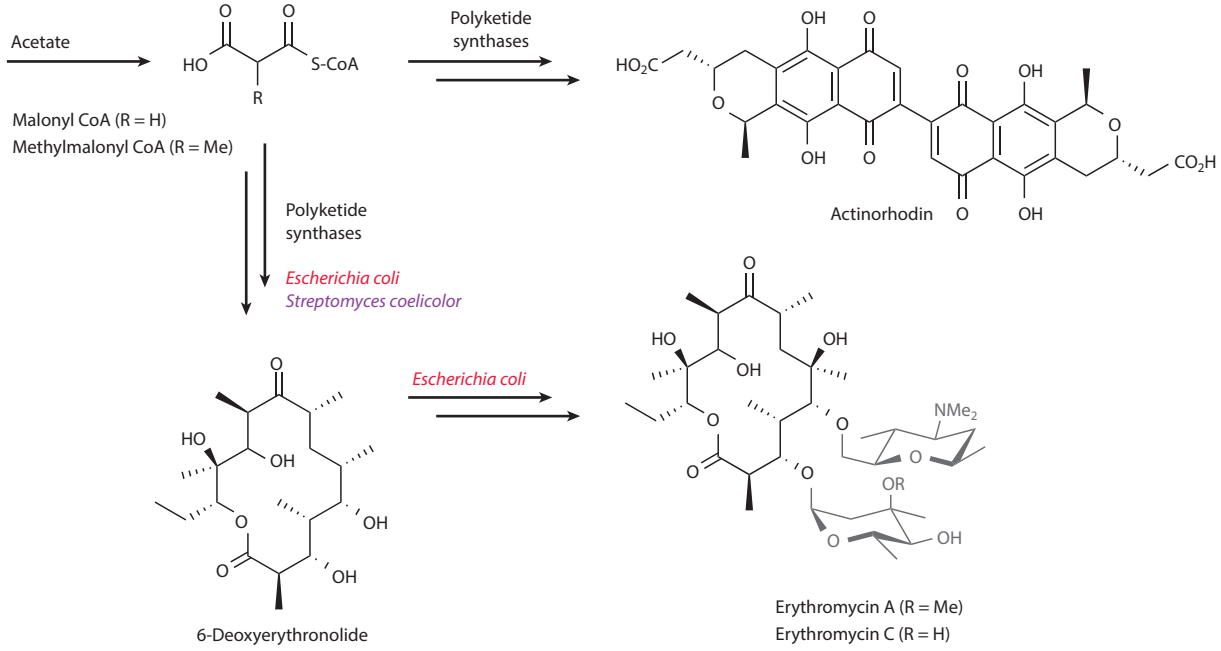

Figure 2

Polyketides have been the workhorse of engineering efforts in secondary metabolism in both *Streptomyces* and *Escherichia coli*. Actinorhodin and erythromycin biosynthetic pathways are shown.

Streptomyces

Streptomyces is a genus of soil bacteria that is a rich producer of secondary metabolites, particularly polyketide and peptide-type antibiotics (57). Secondary metabolites derived from *Streptomyces* were among the earliest targets of metabolic engineering; nearly 30 years ago, Hopwood and coworkers reported the reconstitution of actinorhodin (**Figure 2**), an aromatic polyketide secondary metabolite from *Streptomyces coelicolor*, into the heterologous host *Streptomyces parvus* (91). Since then, a range of genetic tools has been developed for *Streptomyces*, most notably *S. coelicolor*, including promoters for gene expression and vectors to integrate large inserts into the chromosome (48). Because *S. coelicolor* produces polyketide- and peptide-derived secondary metabolites, this host requires minimum upstream engineering to heterologously produce these classes of secondary metabolites.

The power of *S. coelicolor* as a heterologous host was further demonstrated in 1994 when Khosla and coworkers reported the first reconstitution of 6-deoxyerythronolide B, a precursor to the polyketide antibiotic erythromycin (**Figure 2**), in titers of 40 mg/L (68). Many other secondary metabolites have subsequently been expressed in this host (48, 89). An optimized *S. coelicolor* strain, in which the gene clusters of the four major metabolites have been deleted (47), has recently been developed, which greatly simplifies isolation of heterologous metabolites when used as a host.

Escherichia coli

E. coli is a well-characterized prokaryotic host with a wealth of genetic tools and a fast doubling period. As such, this organism has served as the host for numerous metabolic engineering efforts.

Erythromycin: a bacteria-derived polyketide. The reconstitution of a complex polyketide secondary metabolite into *E. coli* was first reported in 2001 (120). The metabolite 6-deoxyerythronolide B, the precursor for the polyketide erythromycin, was produced in *E. coli* at titers comparable to those of an industrially relevant native producer strain. In addition to expressing the biosynthetic enzymes (polyketide synthases) on plasmids, a biosynthetic pathway for the precursor methylmalonyl-CoA was introduced, along with the enzyme required for post-translational modification of the polyketide synthase. Erythromycin has been used as an exemplar for metabolic engineering, and numerous improvements of this initial system have been reported (64, 65, 106, 145). Most notably, reconstitution of the more highly derivatized end product of 6-deoxyerythronolide B, erythromycin C, was reported in 2005 at titers of <1 mg/L (119), and erythromycin A was reported in 2010 in titers of approximately 10 mg/L (159). The production of erythromycins from 6-deoxyerythronolide B requires the addition of 17 additional genes responsible for deoxysugar biosynthesis and macrolide tailoring. Many other bacterially derived natural products have been produced in *E. coli*, indicating that this is a robust choice of host for secondary metabolites from bacteria secondary metabolism (23).

Paclitaxel (Taxol): a plant-derived diterpene. Paclitaxel is a diterpene produced by yew (*Taxus brevifolia*, *Taxus baccata*) that has anticancer activity. The terpene precursors, DMAPP and IPP, can be produced from either the methylerythritol-phosphate (MEP) or mevalonic acid (MVA) primary metabolic pathways (**Figure 3*a***). *E. coli* uses the MEP pathway exclusively. A precursor to paclitaxel, taxadiene, was produced at titers of 1 g/L in *E. coli* (2) by overexpressing all of the downstream taxadiene biosynthetic enzymes, along with four rate-controlling MEP genes to ensure adequate amounts of terpene precursors (**Figure 3*b***). Importantly, the MEP genes and the downstream genes were constructed as two separate modules and the levels of the modules were optimized together, emphasizing the importance of balancing the two parts of the pathway together, as opposed to considering pathway enzymes in isolation (154). The next step of the pathway, catalyzed by a cytochrome P450 that hydroxylates taxadiene, was then introduced. It is challenging to express plant P450s into *E. coli* because these enzymes are membrane bound and often require engineering of the N terminus for functional expression in *E. coli* (2). Although yields dropped after introduction of this P450, nevertheless approximately 100 mg/L of hydroxylated taxadiene intermediate was observed. Whether plant-derived pathways containing numerous P450s can be efficiently expressed in *E. coli* remains to be seen (66). The precursors for diterpene biosynthesis have also been improved by combinatorial mutagenesis of some of the upstream biosynthetic enzymes in *E. coli* (82).

Artemisinin: a plant-derived sesquiterpene. Artemisinin is a sesquiterpene isolated from sweet wormwood (*Artemisia annua*) that has potent antimalarial activity (**Figure 3*c***). Production of amorpha-4,11-diene (amorphadiene), a biosynthetic intermediate of artemisinin, has been engineered in *E. coli* (92). In this case, the MVA precursor pathway, which is not naturally present in *E. coli*, was engineered into the host and used as the source of the IPP/DMAPP precursor supply (92). A completely heterologous pathway may not be subject to the regulatory controls of the native host and could therefore be more useful for overproduction efforts. This system was subsequently improved by identifying two rate-controlling steps of amorphadiene biosynthesis and then tuning the expression levels of these enzymes, resulting in a titer of 300 mg/L (4). After substantial optimization of the cytochrome P450 that acts on amorphadiene, titers of artemisinic acid of 100 mg/L could be achieved (21). The merits of using the MEP or the MVA pathway for metabolic engineering of terpene biosynthesis are currently being debated (102).

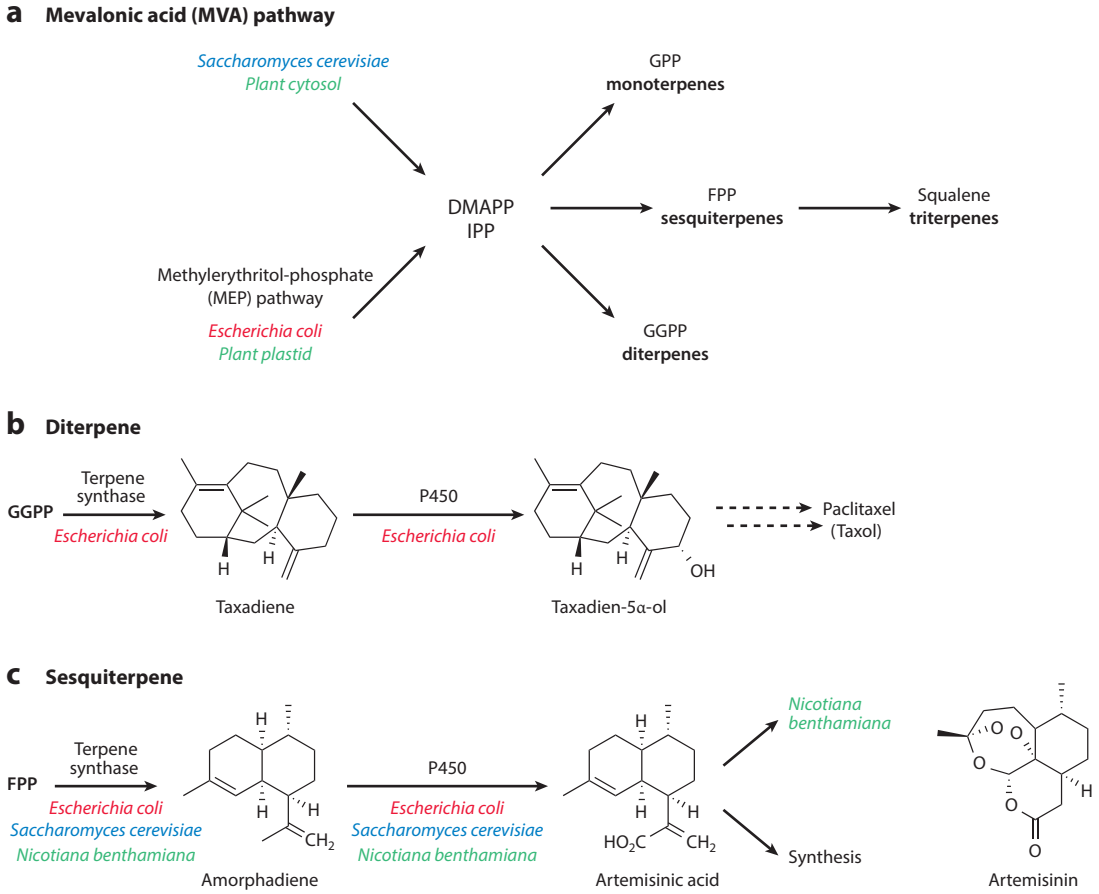

Figure 3

Terpene secondary metabolism. (*a*) All terpenes are derived from either the mevalonic acid (MVA) or methyl-erythritol phosphate (MEP) pathways, depending on the organism or location of biosynthesis. (*b*) Intermediates for Paclitaxel (diterpene) can be reconstituted in *Escherichia coli*. (*c*) The biosynthesis of artemisinin (sesquiterpene) and corresponding biosynthetic intermediates is described for *E. coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana*.

Reticuline: a plant-derived benzylisoquinoline alkaloid. Reticuline is the precursor for all benzylisoquinoline alkaloids, which are produced in a variety of plants, such as the opium poppy (*Papaver somniferum*), and include the opiates morphine, codeine, and noscapine. Engineering a five-gene pathway for reticuline in *E. coli* from an exogenously supplied dopamine precursor was first reported in yields of >10 mg/L (100). *E. coli* does not have the intrinsic metabolic capacity to produce alkaloids, and this system was subsequently improved in 2011 when *E. coli* was engineered to produce dopamine de novo using pathway enzymes from a variety of organisms (bacterial and mammalian). In this system, reticuline could be produced from glycerol at yields of 6 mg/L (108).

Plant-derived phenylpropanoids. Phenylpropanoids from plants, which are composed of stilbenes, flavonoids, flavonols, flavonones, and anthocyanins, have beneficial health properties (**Figure 1**) (122, 140). Production of flavonones in *E. coli* by transformation of a plasmid harboring three heterologous genes was first reported in 2003 in yields up to 11 μg/L, when the precursor

aromatic amino acid (phenylalanine or tyrosine) was added exogenously (56). These yields have been substantially improved over the past decade; by testing enzymes from numerous plant sources and tuning expression levels, titers of 84 mg/L of the common flavonoid naringenin have been achieved from glucose (131). Resveratrol, a stilbene found in red wine, has been produced in excellent titers (2.3 g/L) when the intracellular malonyl-coenzyme A pool was enhanced (84).

Saccharomyces cerevisiae

S. cerevisiae (baker's yeast) is a common eukaryotic host for metabolic engineering. Efficient homologous recombination enables stable integration of genes into the chromosome, and many genetic tools are available for this organism. Yeast also contains different cellular compartments, which can be exploited in metabolic engineering strategies.

Artemisinin: a plant-derived sesquiterpene. A prominent case study of metabolic engineering in yeast is production of artemisinic acid (123), an advanced biosynthetic precursor of artemisinin that can be chemically modified to generate artemisinin (**Figure 3*c***) (116). In this case, the MVA pathway—the pathway used naturally by yeast for terpene precursor production—was optimized by overexpressing rate-controlling genes and by deleting genes responsible for precursor diversion into sterol biosynthesis. Additionally, a novel cytochrome P450 involved in artemisinic acid formation was identified. Overall titers in this strain were 100 mg/L. This process was improved by the discovery of two additional enzymes that assisted with the formation of a carboxylic acid present on artemisinic acid, allowing titers of at least 25 g/L (117, 147), which are sufficiently high to enable commercialization. In a related approach, an engineered cytochrome p450 was introduced into the system to oxidize amorphadiene into the alternative semisynthetic substrate dihydroartemisinic acid (32).

Strictosidine: a plant-derived monoterpene indole alkaloid. Strictosidine is a key intermediate in the biosynthesis of the monoterpene indole alkaloids, which includes the anticancer agents vinblastine and vincristine from *Catharanthus roseus* (**Figure 4*a***). Strictosidine is derived from a monoterpene, a class of terpene that yeast does not naturally produce. However, yeast can be engineered, by expressing key enzymes of the upstream MVA pathway and dedicated monoterpene synthases, to direct flux into monoterpene synthesis (58). For strictosidine biosynthesis, a total of 21 genes were introduced into yeast to increase levels of monoterpene precursors and to convert the precursor to the final product. This is one of the longest plant pathways to be reconstituted into yeast, and titers of strictosidine at 0.5 mg/L were reported (16).

Plant-derived isoquinoline alkaloids. A number of benzylisoquinoline alkaloid pathways have been partially reconstituted into *S. cerevisiae* (**Figure 4*b***). Although the upstream pathway leading from tyrosine to the biosynthetic intermediate reticuline can be expressed in *E. coli*, these upstream enzymes have not been expressed in an active form in yeast until recently (31). Production of reticuline from glucose in yeast was achieved by developing an enzyme-coupled biosensor to identify a tyrosine hydroxylase that was active when expressed in yeast. Prior to this recent development, all isoquinoline alkaloids expressed in yeast have been produced from an exogenously supplied substrate, the commercially available compound norlaudanosoline. Hawkins et al. (52) reported production of the key intermediate reticuline from norlaudanosoline, and the downstream alkaloids scoulerine, tetrahydrocolumbamine, salutaridine, and tetrahydroberberine, which are derived from reticuline. Enzyme expression levels could be tuned and correlated with product titers (52). A subsequently reported system could produce the highly derivatized alkaloids codeine,

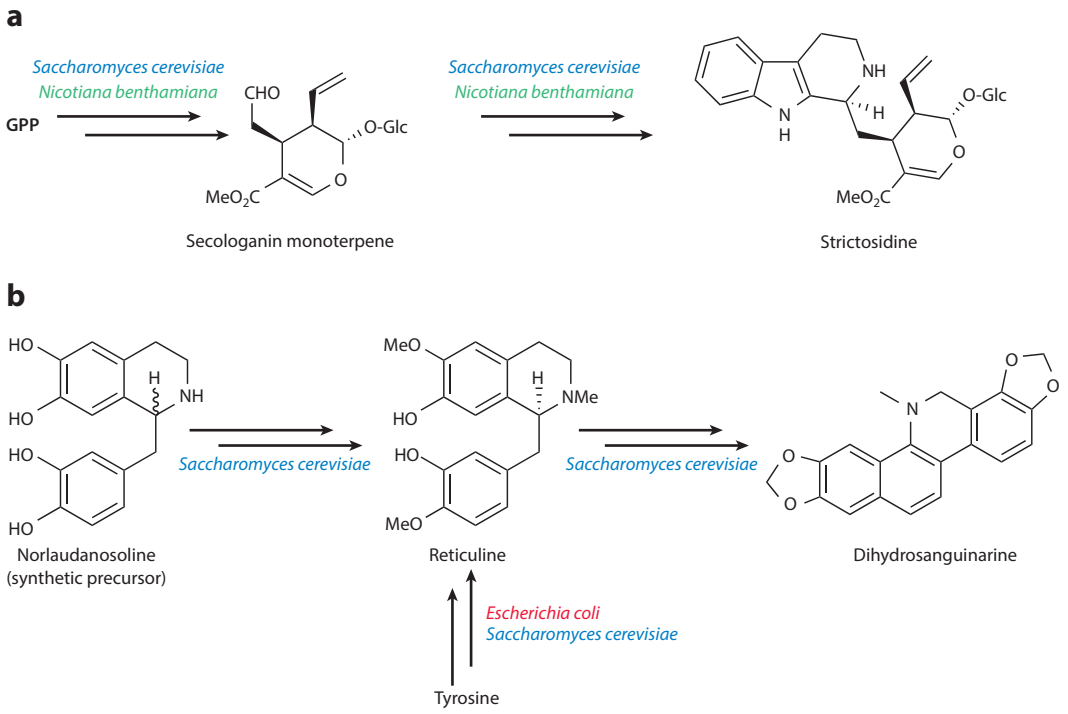

Figure 4

Alkaloid metabolism. (*a*) The biosynthesis of the alkaloid strictosidine has been reconstituted in both *Saccharomyces cerevisiae* and *Nicotiana benthamiana*. (*b*) Alkaloids such as dihydrosanguinarine can be produced in *S. cerevisiae* from the synthetic intermediate norlaudansoline. The native biosynthetic intermediate reticuline can be produced in *Escherichia coli*.

morphine, hydromorphone, hydrocodone, and oxycodone from the advanced intermediate thebaine (137). Dihydrosanguinarine has also been produced in yeast from norlaudanosoline by introduction of a 10-gene pathway into *S. cerevisiae*. Yields were 1.5% from the norlaudanosoline precursor (40).

Plant-derived phenylpropanoids. The stilbene resveratrol was first reconstituted in yeast from p-coumaric acid at titers of 1 μg/L by expressing two genes required for resveratrol biosynthesis on plasmids (10), and subsequently improved to approximately 300 μg/L (139). Naringenin, the central precursor of all flavonoids, was reconstituted by introducing three genes from the phenylpropanoid pathway into *S. cerevisiae* under an inducible promoter to yield titers of approximately 7 mg/L of naringenin (63). More complex derivatives of naringenin have been reconstituted from phenylalanine in metabolically engineered yeast strains harboring plasmids with heterologous biosynthetic genes in yields up to 7 mg/L (139). After extensive optimization of expression levels, de novo production of the flavonoid naringenin from glucose has been achieved (75). Notably, the yields for phenylpropanoids are consistently higher in an *E. coli* host, which is surprising because yeast is phylogenetically closer to the native plant producer.

Fungal-derived polyketides. Fungi are increasingly recognized as a rich source of secondary metabolites. A number of fungal-derived polyketide pathways have been successfully

reconstituted in *S. cerevisiae*. In a breakthrough study, a large, multifunctional polyketide synthase from *Aspergillus terreus* responsible for lovastatin biosynthesis was expressed in *S. cerevisiae* (90). Additional pathways have since been reconstituted in product titers of approximately 1–15 mg/L (e.g., 126, 161). Filamentous fungi produce a host of other secondary metabolite classes, some of which have also been successfully expressed in yeast at good titers (60).

Nicotiana benthamiana

N. benthamiana, a relative of tobacco, has been used as a practical expression host. This plant is relatively fast growing and can be transformed efficiently, and multiple genes can be expressed (stacked) using both stable and transient expression.

Plant-derived glucosinolates. The amino acid–derived glucosinolate metabolites benzylglucosinolate and glucoraphanin, which have health-giving properties for humans and insect deterrence properties for the host plant, were reconstituted in *N. benthamiana* using a transient expression system (43, 99). These examples were one of the first proof-of-concept studies demonstrating that a large number of genes (14) could be stacked in a plant host to successfully produce a desired metabolite at reasonable yields (e.g., 0.4 μmol glucoraphanin/g dry weight of plant tissue). Expression cassettes containing multiple genes could be expressed under the control of a single promoter by using a viral 2A autoprocessing sequence. This pathway was also the basis for a study involving engineering of transport.

Strictosidine: a plant-derived monoterpene indole alkaloid. The first reconstitution of strictosidine, the common precursor for all monoterpene indole alkaloids, was performed in the *N. benthamiana* host (98). Ten genes encoding the strictosidine pathway, along with two genes to boost terpene precursor formation, were transiently expressed on individual plasmids. From these experiments, an advanced precursor of strictosidine was isolated. Co-infiltration of early intermediates of the pathway along with the plasmids improved yields of strictosidine. Upstream biosynthetic intermediates appeared to be derivatized by endogenous *N. benthamiana* enzymes (54), thereby reducing the flux into end-product synthesis. Unfortunately, although the rich metabolism of *N. benthamiana* ensures an ample supply of precursors for secondary metabolite biosynthesis, these native enzymes can derivatize some foreign biosynthetic intermediates, diverting them into dead-end products. Successful genome editing has recently been demonstrated in *N. benthamiana* (109), and it may be possible to use this technology to create *N. benthamiana* hosts that are more optimized for metabolite production (e.g., by eliminating certain endogenous secondary metabolite enzymes).

Artemisinin: a plant-derived sesquiterpene. Biosynthesis of artemisinin—not an advanced intermediate—was achieved in a stable transgenic tobacco line in 2011 (37). Transgenic plants expressed five plant- and yeast-derived genes from the mevalonate and artemisinin pathways. These five genes were cloned into a single vector, targeted to mitochondria, which appeared to enhance yields. Overexpression of one of the rate-limiting MVA genes was also important in achieving good titers. Artemisinin was produced at 7 μg/g dry weight plant tissue, which is lower than the native plant producer *Artemisia annua*. However, the transgenic plant has not yet been subjected to optimization efforts. Transient expression of these same enzymes in *N. benthamiana* does not lead to artemisinin, but yields of artemisinic acid reached 4 μg/kg of fresh weight plant tissue (138).

Plant-derived triterpenes. A three-gene module from a triterpene gene cluster from oat was transiently expressed in *N. benthamiana* as three individual genes to produce a glycosylated, acetylated triterpene product in yields of 40 nmol/mg fresh weight of plant tissue (104). Substantial improvements in triterpene production levels in tobacco have also been achieved by changing the location of biosynthesis.

PATHWAY ENGINEERING IN NATIVE HOSTS

Titers of secondary metabolites have been improved by random mutation or selective breeding of the native host, but genetic manipulations of the native producer—if the host is amenable to genetic manipulation—often result in modest changes to the product profile. Secondary metabolism is tightly and redundantly regulated, requiring innovative strategies to alter titer levels in the native host.

Turning on Cryptic Clusters in Native Microbial Producers

The genomes of many bacteria and fungi encode gene clusters for secondary metabolites that are not expressed under laboratory conditions. Although heterologous expression of these clusters is one way to access the product encoded by these molecules, engineering the native host to activate these clusters is an attractive alternative (8). Provided that the native host can be genetically manipulated, the awakening of silent clusters can allow rapid functional characterization of the corresponding product.

In the bacteria *Streptomyces*, several approaches have been used to awaken secondary metabolite production. Constitutive expression or deletion of a regulatory element, usually present within the gene cluster, often stimulates metabolite production (18, 49, 81). Insertion of a heterologous promoter in front of selected cluster genes has also proven successful (114). Certain point mutations to the ribosome have been linked to overproduction of silent clusters, although this method is not widely used for discovery of new secondary metabolite pathways (8). Finally, random mutagenesis has been coupled to a promoter-reporter system that selects for mutants in which transcription from the targeted gene cluster is activated (51). *Aspergillus*, a genus of fungi that produces a diverse range of secondary metabolites, is also highly amenable to gene cluster activation (24, 85). One early study fused a heterologous promoter to a transcription factor associated with the cluster, leading to expression of the product (11). Given that many fungal gene clusters contain a dedicated transcription factor, this is a widely applicable approach. Chromatin modifications have also been linked to regulation of secondary metabolites, and silencing of biosynthetic gene clusters has been reversed in *A. nidulans* by removal of genes involved in histone methylation, suggesting that expression of secondary metabolite gene clusters is mediated by chromatin configuration (14).

The complexity of plant metabolism and plant genome biology makes identification of cryptic gene clusters in plants more challenging. However, as more plant genomes become available, analogous strategies to identify and mine cryptic or unexpressed pathway in plants may become an important tool in plant secondary metabolite discovery.

Enhancing and Introducing Pathways in Native Plant Producers

Introduction of desirable traits into plants can be achieved by manipulation of secondary metabolism. Engineering the metabolism of native, nonmodel plant producers poses substantial challenges, but an enormous range of tools for gene overexpression, knockdown, and regulation has been developed in the past several years (157) since the earliest demonstrations of secondary

metabolite engineering in plants (e.g., 97). Both silencing and overexpression of transcription factors and key pathway enzymes have been used to modify secondary metabolite profiles in plants. The push strategy refers to using early pathway enzyme genes to push the flux downstream, whereas the pull strategy refers to expression of downstream genes to draw the substrates toward the final product (157). Although in a few cases these engineering efforts are used for the overproduction and isolation of a specific product (e.g., 160), in many cases the goal is to create plants with improved taste, scent, color, or defensive properties. However, the legislation concerning genetically modified plants may limit widespread application of these new plants; for discussion of one high profile case study on Golden Rice, see Reference 33.

Enzyme overexpression. Tomatoes are a principal dietary source of flavonoids and anthocyanins, secondary metabolites that are highly beneficial for human health. Flavonol biosynthesis was upregulated in tomato by transforming tomato with a flavonoid biosynthetic gene from petunia, leading to an increase of up to 78-fold in flavonols in the peel (105). The taste of tomatoes was improved by overexpressing a monoterpene synthase from basil under the control of a ripening-specific promoter. Notably, the increased monoterpene resulted in a decrease in lycopene, the red pigment of tomato, also derived from the terpene pathway (28).

Overexpression of certain secondary metabolite enzymes can enhance the plant's defense response. Maize was transformed with a terpene synthase gene from oregano that yields a sesquiterpene that attracts beneficial nematodes. The resulting maize plants constitutively emitted this sesquiterpene, resulting in substantially less root damage in field trials (30). A terpene synthase that makes a sesquiterpene that confers herbivore resistance is found in wild species of tomatoes but has been lost during the cultivation process. This terpene synthase was expressed in the trichomes of cultivated tomato, and the herbivore resistance of the wild species was restored (12). Recently, wheat was engineered to release the sesquiterpene (E)-β-farnesene, the alarm pheromone for pest aphids (17). However, these plants showed no reduction of aphids in the field. Natural defense from aphids involves a pulsed emission of (E)-β-farnesene, suggesting that aphids may become habituated to the continuous levels of the compound produced by the transgenic lines (17, 79). These experiments demonstrate how both the identity and expression profiles of secondary metabolism can be used to enhance the fitness of crop plants.

Enzyme downregulation. Improvements in the profile of peppermint oil were made by silencing a synthase that generates an undesirable monoterpene side product. Combined with overexpression of an MEP pathway gene, a 60% increase in oil with low levels of the undesirable side-product was achieved in field trials (80). To modify flower color in carnation, antisense suppression was used to block the expression of a key gene in the anthocyanin pathway. The transgenic plants exhibited a range of flower colors caused by slight decreases in anthocyanin production to flowers that lacked any color at all. Unexpectedly, transgenic plants with severe color modification were more fragrant than control plants because of higher levels of a biosynthetically unrelated compound, methyl benzoate (164). These unexpected effects often occur when engineering native metabolism. Genome editing methods, which are becoming routine for nonmodel plants (78), will allow faster and more selective knockdown of genes to impact secondary metabolism.

Transcription factors. Typically, transcription factors control production of a group of metabolites, as these regulators usually control expression of more than one gene (157). Expression of heterologous transcription factors has resulted in dramatic impacts on product profiles. In early experiments, when anthocyanin pathway–specific transcriptional activators from maize were transformed into *A. thaliana*, anthocyanins were produced in root, petal, and stamen tissues of the

transgenic plants (86). More recently, after a transcription factor from *A. thaliana* involved in anthocyanin biosynthesis was introduced into rose, both phenylpropanoids and terpenes that contribute to scent and color were upregulated in the resulting transgenic rose plants (165). When a regulatory gene that represses several signaling pathways controlled by light was silenced in tomato using RNA interference, flavonoid and carotenoid (terpene) content increased significantly (29). Anthocyanin production has been enhanced in tomato fruits by overexpressing two transcription factors involved in upregulating anthocyanin biosynthesis in snapdragon (19). Tomatoes that were colored purple throughout the fruit tissue resulted, and they contained the highest levels of anthocyanins ever reported in tomato. The search for transcription factors that control levels of high-value natural products is a burgeoning area in plant metabolism (142).

ENGINEERING LOCATION OF SECONDARY METABOLISM

Many eukaryotic biosynthetic pathways, particularly in plants, are highly compartmentalized at the inter- and intracellular levels (50, 53). In addition to the linear design of metabolic pathways, successful engineering must consider this third dimension (45). This is a relatively new area of engineering within secondary metabolism, but early successes indicate that this approach should be further explored.

An emerging approach is to synthesize secondary metabolites in compartments where the metabolic process does not normally occur (150). For example, localizing terpene biosynthetic pathways to the plastids of plants has been shown to result in high levels of product (53). In plants, terpene biosynthesis generally occurs in either the cytosol via the mevalonic acid (MVA) pathway to make sesquiterpenes and triterpenes, or in the plastid via the methylerythritol phosphate (MEP) pathway to make monoterpenes and diterpenes. In a pioneering study, a sesquiterpene synthase, along with an upstream enzyme that makes the terpene precursor, was expressed in tobacco plastids, resulting in up to 1,000-fold higher accumulation of sesquiterpene levels when compared to levels achieved when the enzymes were expressed in the cytosol (151). Similar experiments were performed for triterpenes, which are also naturally produced in the cytosol. When two triterpene biosynthetic enzymes were expressed in tobacco plastids, up to 90-fold higher triterpene levels were observed (150). For reasons that are not clear, terpene production in the cytosol appears to be much more tightly regulated than it is in the plastid (1). In a subsequent study, the entire six-gene MVA pathway (normally expressed in the cytosol) was transferred to the plastid of tobacco, and this appeared to stimulate higher levels of terpene production in the plant compared to negative controls (77). Again, whether the MEP or MVA pathways would generally yield higher titers remains unclear. Notably, multiple genes for expression in the plastid can be stacked in an operon and coregulated by transcription from the same promoter as a polycistronic pre-mRNA. Use of such plasmids has resulted in up to tenfold improvement in titer of the plastid-produced product compared to controls (87).

Targeting biosynthetic enzymes to the mitochondria has also resulted in improved yields of terpenes and other compounds—up to a 20-fold improvement in levels of a sesquiterpene was found when the pathway was expressed in yeast mitochondria (38). New profiles of terpene products were also observed after changing localization of certain pathway enzymes to the mitochondria in the model plant *Arabidopsis thaliana* (69). It is speculated that these increases in titer after compartmentalizing a specific pathway may be due to higher local enzyme concentrations, increased availability of intermediates, the prevention of intermediates being diverted into other pathways, or interference of these intermediates with other cellular pathways (6).

The spatial separation of pathway enzymes implies that dedicated transporters exist to shuttle the intermediates to the correct location (112). A number of transporters involved in secondary

metabolism have been identified (112), but more study is required to evaluate whether approaches involving transporters will be a generally useful approach in metabolic engineering. One highly successful example of transport engineering involves the glucosinolates, which are synthesized in roots and then shuttled to seeds. Two transporters involved in moving glucosinolates to the seeds were identified in *A. thaliana*, and when knocked down, glucosinolates could no longer be detected in seeds (111). Because glucosinolate-free seeds from certain crops are highly desirable for feedstocks, this is an important demonstration of how transport engineering can introduce a desirable trait into plants.

ENGINEERING APPROACHES TO PRODUCE UNNATURAL PRODUCTS

Modifying the chemical structures of secondary metabolites can often improve or change the biological activity of the parent compound (26). Genetic manipulation of biosynthetic pathways is a powerful way to make unnatural analogs, or completely novel molecules, with new or improved biological properties (101).

Mutasynthesis

Unnatural products are produced when the biosynthesis of the natural starting material is genetically blocked, and the producing organism is forced to utilize exogenously supplied precursors exclusively for product biosynthesis. This strategy, termed mutasynthesis, was first applied several decades ago to yield novel antibiotics in *Streptomyces* (134). This strategy has been applied to countless microbial systems with great success, with too many examples to list individually (71). Precursor feeding is not practical for plants, but an example of mutasynthesis with plant cultures has been reported (127).

Swapping Pathway Enzymes

Enzymes can be swapped among pathways to yield new metabolic processes that produce novel products, provided that downstream enzymes can recognize unnatural intermediates. One of the earliest examples showed that gene transfer between strains producing actinorhodin and several other related aromatic polyketides could be used to make actinorhodin analogs (55). These experiments have been expanded over the decades, largely facilitated by powerful heterologous expression hosts. In one recent example, fungal synthases that produce aromatic polyketides were expressed as random pairs in a yeast heterologous host to create a diverse library of molecules, including one member with an unprecedented skeleton and unique biological activity (153).

The saccharides that decorate the core scaffold of secondary metabolites can also be readily modified by enzyme swapping. For example, the saccharides that decorate erythromycin can be changed by substituting in saccharide pathways from other related polyketides, albeit at reduced titer levels (67). Again, these experiments are greatly facilitated by the heterologous expression systems that allow different biosynthetic genes to be switched in and out rapidly. Furthermore, glycosyltransferases from secondary metabolite pathways can act in reverse, allowing the sugar and aglycone moieties to be readily exchanged, allowing a wide diversity of scaffold-saccharide combinations to be synthesized (158). Many examples of this glyco-randomization approach have been successfully reported (42).

Plant pathways that use stand-alone enzymes are particularly amenable to pathway swapping. For example, libraries of flavonoid compounds using heterologous production systems

in *S. cerevisiae* (see Plant-Derived Phenylpropanoids) have been developed. Combinations of flavonoid biosynthetic genes along with unnatural substrates lead to new analogs, with some having new biological activities (22). The assembly process has been further expanded by using seven flavonoid pathways randomly combined on yeast artificial chromosomes, with a combination of natural and unnatural precursors (107). Plant-derived triterpenes, which can also be reconstituted in *S. cerevisiae* (27, 72), can be combined in different combinations with tailoring enzymes (P450s, glycosyltransferases) to produce both natural and unnatural triterpenes (41, 103).

Specialized enzymes decorate secondary metabolites with unusual functional groups that impart unique biological activity, with halogenation being one important example. To introduce this important functional group more widely into secondary metabolism, a foreign chlorinase gene was transformed into *Streptomyces coeruleorubidus* to yield a chlorinated analog of the native antibiotic product (124). A similar strategy was reported in which a halogenase from a bacterial pathway was transformed into medicinal plant cultures to yield unnatural chlorinated and brominated alkaloids (128). Although fluorinated compounds are known in nature, the only known natural fluorinated product is fluoroacetate. To add the fluoro functionality to polyketide secondary metabolites, a pathway to synthesize the required substrate, fluoromalonyl-CoA, was developed and successfully interfaced with a polyketide pathway to generate a fluorinated analog of a 6-deoxyerythronolide B biosynthetic intermediate (144). These examples highlight how metabolic engineering can take a relatively rare chemical modification (e.g., halogenation) and allow it to be incorporated into a broader array of metabolic products.

Instead of secondary metabolite analogs, completely novel compounds can be generated by randomly combining biosynthetic enzymes from a wide range of sources. This approach was implemented in a yeast host to create a library of more than 70 compounds, most of which have not been described previously (74).

Enzyme Engineering

Enzyme engineering is a critical element to metabolic engineering. It is beyond the scope of this review to cover the applications of enzyme engineering to secondary metabolism in detail (9), but a few key examples are discussed below.

A major focus has been on polyketide biosynthetic pathways that generate macrolide-type polyketides such as erythromycin (**Figure 2**) (149). The enzymes that generate these polyketides are large, multidomain enzymes, with one module dedicated to each substrate that is added to the product. Polyketide analogs can be generated by cutting out an acyltransferase (AT) domain and replacing it with another having different substrate specificity (94, 120, 159). Although the downstream modules appear to be highly tolerant of the resulting unnatural biosynthetic intermediates, crucial protein-protein interactions are disrupted by this cut-and-paste approach. Better protein designs have been enabled by understanding the structural interactions that occur within and between modules (35, 46). These interactions are also responsible for polyketide size: Additional changes in polyketide structure can be achieved by mutation at the protein-protein interface so that two successive rounds of chain elongation are catalyzed, resulting in a bigger polyketide product (70).

Nonribosomal peptide synthetases, which construct peptides, function much like polyketide synthases. However, instead of an AT domain selecting an acyl substrate, an adenylation (A) domain selects an amino acid substrate. Nonribosomal peptide synthetases have been used to demonstrate how directed evolution can be used to restore the activity of the enzyme after foreign A domains are cut and pasted into the protein (39). The problems created by the cut-and-paste

approach can be avoided altogether by using point mutations to modify the substrate specificity of the native A domain. By mutating active site residues, coupled with a rapid mass spectrometry–based screening technique, a large number of A domain variants with alternate substrate specificity profiles could be generated (36). Ribosomal-generated peptides such as lantibiotics have proven to be highly amenable to structural engineering. These pathways use a ribosomal encoded peptide that is subjected to a variety of posttranslational modifications to yield the final secondary product. A leader peptide sequence, which is cleaved after biosynthesis, provides the recognition elements required for peptide modification following translation. The structure of the peptide product can therefore readily be altered by standard site-directed mutagenesis, provided it is attached to the leader peptide (133). Strategies to eliminate the requirement for a leader peptide have recently been reported (115).

CONCLUSIONS

Engineering of secondary metabolism, which aims to enhance yield, increase chemical diversity, and/or generate species with enhanced phenotypes, poses both substantial challenges and opportunities. If the goal is to isolate the secondary metabolite, then the choice of host is framed in terms of the best titers in the highest purity. Alternatively, metabolic engineering may be employed to alter the properties of the host: for example, engineering the secondary metabolism of a plant might confer increased disease resistance. In these cases, the host for which the application is being designed must be used. The host may not be sequenced, or have appropriate genetic tools available, and embedded regulatory networks can tightly and redundantly control the levels of product production, usually to low levels. Nevertheless, a number of spectacularly successful outcomes for engineering native host metabolism are highlighted. Innovative solutions such as disrupting regulatory elements or moving the location of the metabolic pathway can be sufficient to override tight regulatory controls.

The use of heterologous hosts for production of secondary metabolites has often been framed in the terminology of synthetic biology, in which a neutral chassis is available for the installation of metabolic parts that can be rapidly combined to make the desired molecule. However, expressing pathways in heterologous hosts to commercially viable titers has a high failure rate and typically requires substantial optimization (and innovation) before success is achieved. Nevertheless, the advancements that have been made over the past two decades suggest that this approach has enormous potential to harness metabolism that would otherwise be out of reach. The choice of a heterologous host is still largely empirical, with the general assumption that the closer the host is to the native producer, the higher the titers, although there are exceptions. As more examples are reported, more reliable guidelines for which host is best suited for which metabolite may become clearer. Culturing combinations of hosts, with each one encoding the portion of the metabolic pathway for which it is best suited, may also be an option (162).

Artemisinin is an exemplar of a successful heterologous production platform; semisynthetic artemisinin derived from an *S. cerevisiae* host has been produced at titers high enough to enable commercialization. However, it is important to note that the native artemisinin producer, the plant *Artemisia annua*, remains an important source of this compound, highlighting that natural production sources can also be efficient production platforms. The artemisinin example has also brought to light broader social issues that can arise in the wake of a successful metabolic engineering project; it is not clear how the economic situation of the farmers who grow *A. annua* in the developing world will be impacted by an alternative production source. Despite all of these challenges, engineering of secondary metabolism is enabling much wider applications of these high-value, complex molecules.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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