Biosynthesis of Plant Isoprenoids: Perspectives for Microbial Engineering

James Kirby[1] and Jay D. Keasling[1,2,3,4]

[1]California Institute of Quantitative Biomedical Research, University of California, Berkeley, California 94720; email: jimkirby@berkeley.edu

[2]Departments of Chemical Engineering and Bioengineering, University of California, Berkeley, California 94720; email: keasling@berkeley.edu

[3]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

[4]Joint BioEnergy Institute, Emeryville, California 94608

Annu. Rev. Plant Biol. 2009. 60:335–55

First published online as a Review in Advance on January 9, 2009

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

This article's doi: 10.1146/annurev.arplant.043008.091955

1543-5008/09/0602-0335$20.00

Key Words

terpenes, P450, yeast, *Escherichia coli*, gene discovery

Abstract

Isoprenoids are a large and highly diverse group of natural products with many functions in plant primary and secondary metabolism. Isoprenoids are synthesized from common prenyl diphosphate precursors through the action of terpene synthases and terpene-modifying enzymes such as cytochrome P450 monooxygenases. Many isoprenoids have important applications in areas such as human health and nutrition, and much effort has been directed toward their production in microbial hosts. However, many hurdles must be overcome in the elucidation and functional microbial expression of the genes responsible for biosynthesis of an isoprenoid of interest. Here, we review investigations into isoprenoid function and gene discovery in plants as well as the latest advances in isoprenoid pathway engineering in both plant and microbial hosts.

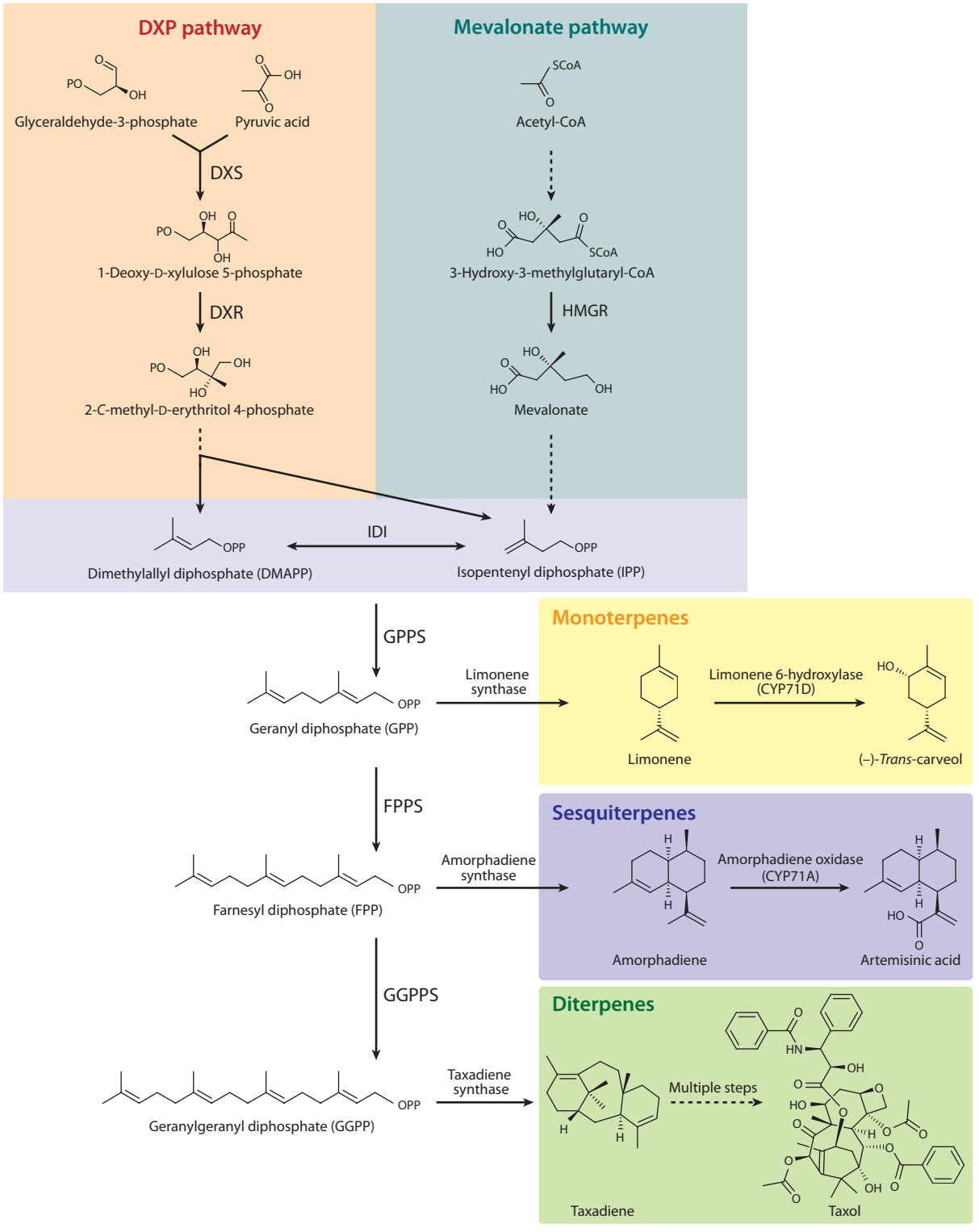
Contents

INTRODUCTION

Isoprenoids (also referred to as terpenes) constitute one of the most diverse groups of natural products in nature. In plants, isoprenoids range from essential and relatively universal primary metabolites, such as sterols, carotenoids, quinones, and hormones, to more unique and sometimes species-specific secondary metabolites that may serve roles such as plant defense and communication (52). Isoprenoids are classified into groups according to the number of carbons they contain; the major groups of interest here are monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), and triterpenes (C30). All isoprenoids are synthesized via the two universal C5 building blocks: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These universal precursors can be produced by either of two routes: the mevalonate pathway or the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway (**Figure 1**). These pathways are distributed throughout nature, and as a rule of thumb the mevalonate pathway is prevalent in eukaryotes and archaea, whereas the DXP pathway is widespread in eubacteria. However, there are many exceptions to this pattern; for example, several eubacteria are known to utilize the mevalonate pathway instead of the DXP pathway, with some species carrying genes from both pathways, whereas the protozoan parasite *Plasmodium falciparum* relies on the DXP pathway (26). Both pathways are expressed in plants but differ in their localization. The mevalonate pathway enzymes are located in the cytosol, whereas the DXP enzymes are found in the plastid. The exact origin of the DXP pathway genes in plants is not clear from phylogenetic studies because most of the plant DXP genes do not branch with their cyanobacterial counterparts (plastids are thought to have evolved

Figure 1

Overview of the isoprenoid biosynthetic pathways. The 1-deoxy-D-xylulose-5-phosphate (DXP) and mevalonate pathways are responsible for production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) from central metabolites. Key enzymes of each pathway are shown: 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductase (DXR), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and IPP-DMAPP isomerase (IDI). IPP and DMAPP are then converted to terpene synthase precursors through the action of the prenyltransferases geranyl diphosphate (GPP) synthase (GPPS), farnesyl diphosphate (FPP) synthase (FPPS), and geranylgeranyl diphosphate (GGPP) synthase (GGPPS). One example of a terpene synthase reaction and downstream processing reaction(s) (such as P450 oxidation) is given for each category of isoprenoid shown. Multiple steps are indicated by dashed lines.



IPP: isopentenyl diphosphate

DMAPP: dimethylallyl diphosphate

DXP: 1-deoxy-D-xylulose-5-phosphate

GPP: geranyl diphosphate

FPP: farnesyl diphosphate

GGPP: geranylgeranyl diphosphate

from a cyanobacterium-like progenitor). One theory proposes that the genes currently found in plants were acquired subsequent to the origin of plastids, and lateral transfer of genes from eubacteria played a role (52).

The mevalonate pathway consists of six steps that transform acetyl-CoA to IPP, followed by an IPP isomerase that maintains a balance between IPP and DMAPP. The DXP pathway consists of seven enzymes that transform glyceraldehyde-3-phosphate and pyruvic acid to IPP and DMAPP in a ratio of 5:1. An IPP isomerase gene (*idi*) is present in only a minority of bacterial species that utilize the DXP pathway (24), and this gene is nonessential in *Escherichia coli* (35). IDI may play a more important role in plants than in bacteria, however, because gene silencing of *IDI* in *Nicotiana benthamiana* resulted in an 80% reduction in pigments (carotenoid and chlorophyll) compared with controls (71). The mevalonate pathway enzymes have received much attention over the past 30 years, and several regulatory mechanisms have been uncovered (96). In contrast, the DXP pathway was fully elucidated only in 2002 and is less well understood in terms of both requirements and regulation (1).

Downstream of both pathways, prenyltransferases convert IPP and DMAPP to longer chain isoprenoid precursors: geranyl diphosphate (GPP, the C_{10} precursor to monoterpenes), farnesyl diphosphate (FPP, the C_{15} precursor to sesquiterpenes and triterpenes), and geranylgeranyl diphosphate (GGPP, the C_{20} precursor to diterpenes). In plants, synthesis of terpenes is compartmentalized such that monoterpenes and diterpenes (as well as carotenoids and chlorophylls) are produced via the DXP pathway in the plastid, whereas sesquiterpenes and triterpenes are made in the cytosol via the mevalonate pathway (**Figure 2**). Several studies provide evidence for an exchange of prenyl diphosphate precursors between the cytosol and the plastid. However, the extent of this cross talk is limited and cannot compensate for loss of flux through one pathway, as demonstrated by studies using *Arabidopsis thaliana* DXP mutants (14).

Many isoprenoids have found applications in medicine and agriculture and as nutraceuticals, flavors, and fragrances (31). Some of these natural products are considered to be attractive targets for bioengineering owing to their market value as commercial products and/or their impact in areas such as human health. For example, carotenoids have many applications in the food (e.g., β-carotene), animal feed (e.g., astaxanthin), cosmetics (e.g., tocopherols), and health supplement industries. In the midst of a growing awareness of the health benefits of carotenoids as antioxidants and the use of lutein in particular for prevention of age-related macular degeneration disease and related disorders, the total carotenoid market value has been projected to surpass $1 billion by the end of this decade (25).

Isolation of the heavily decorated diterpene paclitaxel from *Taxus brevifola* (Pacific yew) led to the development of the promising anticancer drug Taxol®, which has since proven to be effective in the treatment of several forms of cancer and has already exceeded a market value of $1 billion (93). However, ensuring initial supplies of Taxol to meet the growing number of clinical applications met with controversy surrounding the environmental impact on old-growth forests. The bark of 2000–3000 *T. brevifola* trees is estimated to be required for commercial production of 1 kg of Taxol; this is equivalent to harvesting a 100-year-old tree to extract one dose of the drug (39, 44). A semisynthetic route was later devised, involving purification of a Taxol precursor from yew tree needles, and this process has now been replaced by production via plant cell culture (**http://www.epa.gov/greenchemistry/pubs/pgcc/winners/gspa04.html**) (44).

Artemisinin, a sesquiterpene produced in the leaves of *Artemisia annua* (sweet wormwood) is widely considered to be the best treatment for uncomplicated malaria when used as part of a combination therapy (66). The cost of the commercially available drug, however, is prohibitive to many people in developing countries who are suffering from malaria. A microbial source of artemisinin has been proposed to

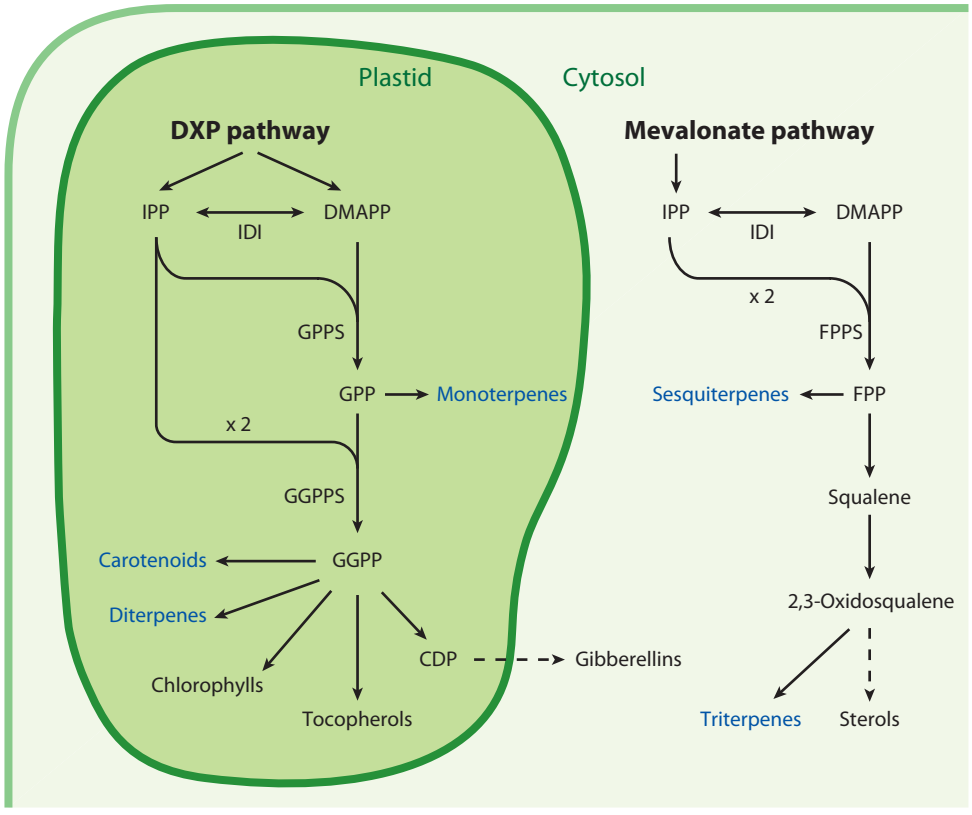


Figure 2

Generalized schematic of isoprenoid biosynthesis in plants. Note that although the reactions shown here form the commonly accepted prenyl phosphate pathways, some evidence exists for the expression of a GPPS in the cytosol (15) and for the presence of significant levels of FPP in the plastid (99). The presence of GGPP in the cytosol has also been postulated for the geranylgeranylation of proteins (14). CDP, copalyl-diphosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; FPP, farnesyl diphosphate; FPPS FPP synthase; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; GPPS, GPP synthase; GGPPS, GGPP synthase; IDI, IPP isomerase; IPP, isopentenyl diphosphate. Dashed lines indicate multiple steps.

ensure a supply of the drug at a significantly lower cost than the current production method of extraction from the plant (36). In many cases such as those of artemisinin and Taxol, several different approaches have been explored to achieve the goal of an economical and reliable source for high-value products with minimal environmental impact. Typically the different options explored for sourcing plant isoprenoids include extraction from its natural source (16); total or partial chemical synthesis (39); improvement of existing plant sources through breeding (23), genetic engineering (101), or cell culture (83); and production in a microbial host (3). In the case of commercial production of Taxol, all the common routes have been considered and the method used for production has shifted over time.

The major challenges associated with the development of a microbial host for plant isoprenoid biosynthesis include engineering of isoprenoid precursor pathways and the discovery and successful expression of the necessary biosynthetic genes. One advantage of the microbial system is its adaptability for synthesis of analogs through the introduction of new

genes or mutation of existing pathway genes. For instance, more than 350 taxoid products have been detected in *Taxus* spp., but progress toward clinical evaluation of many of these is limited by the quantities available in the plant (44). Here we review our current understanding of isoprenoid biosynthesis and function in plants and how they inform gene discovery and engineering endeavors, as well as more recent advances in microbial platforms for heterologous production.

DISCOVERY OF APPLICATIONS FOR ISOPRENOIDS

The Role of Indigenous Knowledge

Considering the many flavors and fragrances that are available in the world of plant essential oils, it is perhaps not surprising that human knowledge and practical use of these and other sources of isoprenoids date back several millennia. Some of the earliest records of traditional medicine, originating in Mesopotamia more than 4500 years ago, report the use of several plant oils, many of which are still in use today (63). Dating from ancient Roman times, one of the first pharmacological manuscripts describes medicinal uses for the latex from the succulent *Euphorbia resinifera*; the likely active component was identified 2000 years later as the diterpene derivative resiniferatoxin (8). Today, the World Health Organization (WHO) estimates that 80% of the world's population relies on traditional remedies for their primary healthcare, whereas 50% of the most commonly prescribed U.S. drugs either contain, or were developed from, natural products (63).

The role of indigenous knowledge in the discovery of isoprenoid-based drugs or agricultural agents should not be underestimated, and according to the 1992 Convention on Biological Diversity, this role should be acknowledged in the form of conservation and benefits sharing (22). Artemisinin was isolated and demonstrated to be an effective treatment for malaria as a result of a large-scale effort in China, where modern scientists worked alongside practitioners and historians of traditional Chinese medicine. The discovery was made following much research into China's earliest materia medica and in particular a 1700-year-old report from the famous physician Ge Hong entitled "Emergency Prescriptions Kept Up One's Sleeve" (41). Ethnobotanical research into current traditional medicine practices has also led to important discoveries of isoprenoid function. The diterpene 12-deoxyphorbol 13-acetate, or prostratin, was discovered by the U.S. National Cancer Institute (NCI) to display promising activities against HIV following research into current practices by traditional Samoan healers (34). Prostratin is currently in the early stages of drug development following a profit-sharing agreement put in place between the NCI and Samoan healers (21).

The Role of Plant Investigations

Whereas ethnobotanical studies may provide valuable leads for isoprenoids of value to human medicine, studies in plant biology can do the same for agriculturally important compounds. Plant isoprenoids, whether produced constitutively or in response to a stimulus, often act as deterrents against assault by pathogens and herbivores. The optimal defense theory predicts that constitutive accumulation of isoprenoids will be focused on tissues with the highest fitness value, such as younger growth and reproductive organs (69). This theory appears to hold true in many cases; for instance, feeding on cotton (*Gossypium herbaceum*) by beet armyworm larvae (*Spodopterta exigua*) induces terpene production in younger but not older leaves (13). The prediction of a functional role for a particular plant isoprenoid typically involves a wide range of disciplines, from ecology and physiology to analytical chemistry, but may begin with traditional or common knowledge of a plant's natural resistance or toxicity. For example, the neem tree (*Azadirachta indica*) has been known in India for thousands of

years to possess insecticidal properties—it was used to protect stored grain from pests, among other applications. The many constituents of neem oil are still under evaluation, with a wide range of proposed uses, but a highly modified triterpene constituent, azadirachtin, has been clearly demonstrated to have insect antifeedant and growth regulating activity (16).

Correlations between isoprenoid levels in a plant and resistance to attack have been discovered in many ways. Another study of the response of cotton to feeding by beet armyworm larvae revealed that the number of leaf glands (which are typically rich in isoprenoids) increased in density after feeding. High-performance liquid chromatography (HPLC) analysis showed a significant increase in specific sesquiterpene heliocides, which was correlated with deterrence of further feeding (60). To demonstrate a protective role for isoprenoids in Norway spruce (*Picea abies*), trees were treated with methyl jasmonate (MeJA, a common signal molecule in plant defense systems), and an increase in both terpene levels and resistance to the spruce bark beetle were observed (28).

If a gene responsible for biosynthesis of an isoprenoid is already known, its overexpression or deletion in the plant can provide useful functional information. Expression of linalool synthase (FaNES1) in *A. thaliana* led to a large increase in levels of the monoterpene and its derivatives and also a significant increase in resistance to the aphid *Myzus persicae* (2). Conversely, oat (*Avena strigosa*) mutants deficient in triterpene saponins displayed increased sensitivity to a range of fungal pathogens (73).

DISCOVERY OF ISOPRENOID BIOSYNTHETIC GENES

Elucidation of genes required for biosynthesis of an isoprenoid not only facilitates functional studies in the plant, but also paves the way for engineering higher production levels in plant or microbial hosts. Following biosynthesis of the universal isoprenoid prenyl diphosphate precursors, the next step in an isoprenoid pathway entails conversion of the prenyl diphosphate into a cyclic product by a terpene synthase (TPS). In the majority of cases a carbocation is generated following cleavage of the diphosphate group and the synthase then acts as a chaperone to guide a cascade of molecular events that ultimately results in a terpene olefin product. However, in the case of a few (class II) TPSs a carbocation is generated through protonation of a double bond, and the cyclic product retains the diphosphate group. A common example of this second mechanism is the biosynthesis of copalyl-diphosphate, precursor to gibberellins and phytoalexins (**Figure 2**). The mechanisms, versatility, and diversity of terpene synthases were explored in recent reviews (14, 20).

Although a TPS cyclization reaction may occasionally constitute the terminal step in a plant secondary metabolism pathway, it is much more common for further modifications of the terpene olefin to take place. These modifications may at later points in the pathway involve the addition of acyl-, aryl-, or sugar moieties, but usually begin with oxidation of the terpene olefin through the action of cytochrome P450 monooxgenases (P450s). P450s are capable of catalyzing a wide variety of chemical reactions, from hydroxylation and epoxidation to ring formation, aryl migration, and carbon-carbon bond cleavage (61). Plant P450s, belonging to Class II of the P450 group, are found in the endoplasmic reticulum, where they act in concert with an NADPH-dependent cytochrome P450 reductase (CPR). P450s are categorized into families and subfamilies depending on the degree of protein sequence identity; for example, enzymes from family CYP1 (cytochrome P450 family 1) share more than 40% identity, whereas those from subfamily CYP1A share more than 55% identity (87). The fact that plants typically encode several hundred P450s (272 are found in *Arabidopsis*, 458 in rice) can make gene discovery projects particularly challenging (87).

MeJA: methyl jasmonate

TPS: terpene synthase

P450: cytochrome P450 monooxygenase

Tools for Gene Discovery

Some useful tools for the discovery of new TPS and P450 genes have arisen from insights gained from plant biology studies. Although the availability of many P450 and TPS gene sequences has in many cases facilitated the successful design of PCR primers for gene isolation, this approach when used alone can be limiting in its scope. For instance, there are higher numbers of terpene-modifying P450s within certain CYP families, but any correlation postulated between a terpene structure and a P450 family is by no means reliable. Even if the assumption of the target family proves to be correct, there may be many representatives from that family in the plant in question, and other connections between the terpene and the target gene must be made to narrow the field. The isolation of the gene *CYP71AV1* from *Artemisia annua*, encoding the P450 responsible for conversion of the sesquiterpene amorphadiene into artemisinic acid, provides a good example (82). For this work, CYP71-specific PCR primers were designed on the basis that in other plants of the Asteraceae family sesquiterpenes similar to amorphadiene are oxidized by CYP71 enzymes. Isolation of the correct gene, however, required the use of cDNA made from a glandular trichome-enriched cell preparation as a template for the PCR. Thus, knowledge of the site of biosynthesis of the terpene in question proved to be invaluable. Glandular trichomes, specialized structures found on the leaf surfaces of many plants, are frequently the production and storage sites for volatile mono- and sesquiterpenes (86). The use of glandular trichome preparations played a central role in the pioneering work of Rodney Croteau and coworkers (55), including the isolation of two regiospecific limonene hydroxylase genes from mint. Latex is also known to be a site for terpene biosynthesis in many plants; in the case of *Hevea brasiliensis* the use of cDNA isolated from latex has been instrumental in elucidating genes involved in rubber biosynthesis (19, 67, 79), and several other members of the Euphorbiaceae are known to accumulate a variety of isoprenoid secondary metabolites in their latex (43), although none of the P450s involved in the synthesis of these compounds have yet been isolated.

In many cases an elicitor known to induce isoprenoid biosynthesis is used to uncover new genes. Infection of soybean (*Glycine max*) with the fungus *Phytophythora sojae* was found to induce expression of several P450s involved in isoflavonoid biosynthesis (90, 92). One gene that is also induced under the same conditions, although it belongs to the same family as the isoflavonoid P450s (CYP93), was later found to encode a hydroxylase that acts on the related triterpenes β-amyrin and sophoradiol (90). Triterpenes derived from β-amyrin and sophoradiol, known as soyasaponins, are thought to protect plants from phytopathogenic fungi by interacting with sterols in the fungal cell membrane to cause a loss in membrane integrity (98).

The tomato linalool synthase gene (*LeMTS1*) was isolated from plants infested with spider mites and subsequent studies showed that gene expression was induced in stem trichomes by jasmonic acid (95). In fact, plant hormones, and particularly jasmonates, have been commonly used as elicitors of isoprenoid biosynthesis. Nine full-length TPS genes were recovered from Norway spruce (*Picea abies* L. Karst) using needles collected from trees treated with MeJA (57). Perhaps the most impressive demonstration of the use of elicitors for gene discovery is the case of a MeJA-induced cDNA library from a *Taxus cuspidata* cell culture (45). Following up on previous studies that demonstrated that MeJA significantly increases Taxol production in *Taxus* cell cultures (49), a cDNA library was made from pretreated cultures and approximately 8,500 clones were randomly selected and sequenced. Within this relatively small sample size, expressed sequence tags (ESTs) representing all members of the DXP pathway were identified along with a GGPP synthase, whereas the Taxol pathway TPS gene (taxadiene synthase) was present in the library at an abundance of almost 5%. Further analysis of

the sequences revealed 10 new putative P450 taxoid hydroxylases and six new acyl/aroyl transferases that are most likely also involved in the Taxol pathway (45). This work also serves to illustrate the likelihood of a higher incidence of sequence homology between proteins of the same metabolic pathway: The new putative P450s share >75% sequence similarity with previously identified P450s from the pathway, compared with <35% similarity with most other plant P450s, whereas the acyl/aroyl transferases share >65% similarity as a group. Although several examples exist of likely convergent evolution in plant secondary metabolic genes, the genes of a particular pathway, sharing temporal and spatial expression patterns, more likely have arisen through gene duplication and divergence (77).

More high-throughput technologies may play an increasing role in gene discovery as the need arises; one example is the use of robotics for tandem gene expression coupled with arrays of potential substrates. The prediction of P450 function based on molecular modeling may also prove fruitful for plant genes, following in the footsteps of work done with bacterial and mammalian P450s (87).

GENE EXPRESSION AND PATHWAY ENGINEERING IN PLANTS

Plants may be engineered for increased isoprenoid production with an aim to purify the isoprenoid component, as a means of increasing the nutritional value of food crops, or to enhance the fitness of the plant itself by increasing resistance to herbivores, pests, or pathogens. Of course, gene expression and pathway engineering studies can also advance our understanding of both the regulation and function of isoprenoid biosynthesis in plants. Although microbial engineering offers many advantages over plant systems for product synthesis and purification, many plant isoprenoid biosynthetic pathways are only partially elucidated and are therefore not currently amenable to microbial transformation.

Carotenoids

Perhaps the most high-profile early work on engineering higher isoprenoid levels in plants came about as a means of tackling malnutrition due to vitamin A deficiency in developing countries. The carotenoids α- and β-carotene serve as precursors to vitamin A in humans, but are scarce in most staple crops of the developing world and absent in polished (white) rice. Successful engineering of phytoene synthase, phytoene desaturase, and lycopene β-cyclase into rice resulted in formation of β-carotene and β-xanthophylls, and carotenoid concentrations reached 1.6 $\mu g\ g^{-1}$ in the rice endosperm (101). Similar results were obtained in rice transformed with only the phytoene synthase and desaturase genes (101), and it was later demonstrated that lycopene β-cyclase and downstream genes are constitutively expressed in rice endosperm (85). Improvements were made over the original strain through a systematic evaluation of phytoene synthase genes from carotenoid-rich plant sources, culminating in a 23-fold increase in carotenoid levels (37 $\mu g\ g^{-1}$) in a line harboring the phytoene synthase gene from maize (72). However, because lutein and zeaxanthin levels were actually much lower in many of the improved lines compared with the original golden rice, further engineering to boost levels of these important carotenoids would be beneficial.

The discovery of a cauliflower gene involved in the differentiation of noncolored plastids into chromoplasts provides a complementary approach to engineering higher carotenoid levels in plants. Chromoplasts, which sequester excess carotenoids produced in the chloroplast, were found to be more numerous in cauliflower plants that express the *Orange* (*Or*) gene (53). Expression of *Or* in potato, under control of the granule-bound starch synthase (GBSS) promoter, resulted in a sixfold increase in carotenoid levels in tubers (54). In addition, increases in certain carotenoids upon *Or* overexpression may provide insights into rate-limiting steps in the potato carotenoid pathway; for example, the increase in phytoene from zero

to approximately 10 μg g^{-1} suggests a limitation in the phytoene desaturation step (54). Increasing the number or size of chromoplasts, thus providing a sink for carotenoid production, may become a very powerful tool when combined with metabolic engineering strategies.

Menthol

Menthol is a widely used monoterpene with a market value of approximately $300 million that constitutes the primary component of peppermint essential oil. Much work has gone into elucidating the menthol biosynthetic pathway (**Figure 3**) as well as engineering plants with enhanced menthol yield and purity (23). One of the most successful engineering steps was not in the dedicated menthol biosynthetic pathway, but in the precursor DXP pathway: Overexpression of deoxyxylulose phosphate reductase (*dxr*), driven by the CaMV 35S promoter, resulted in a 40% increase in oil production (56). The quality of peppermint oil was improved through a reduction in yield of a pathway side

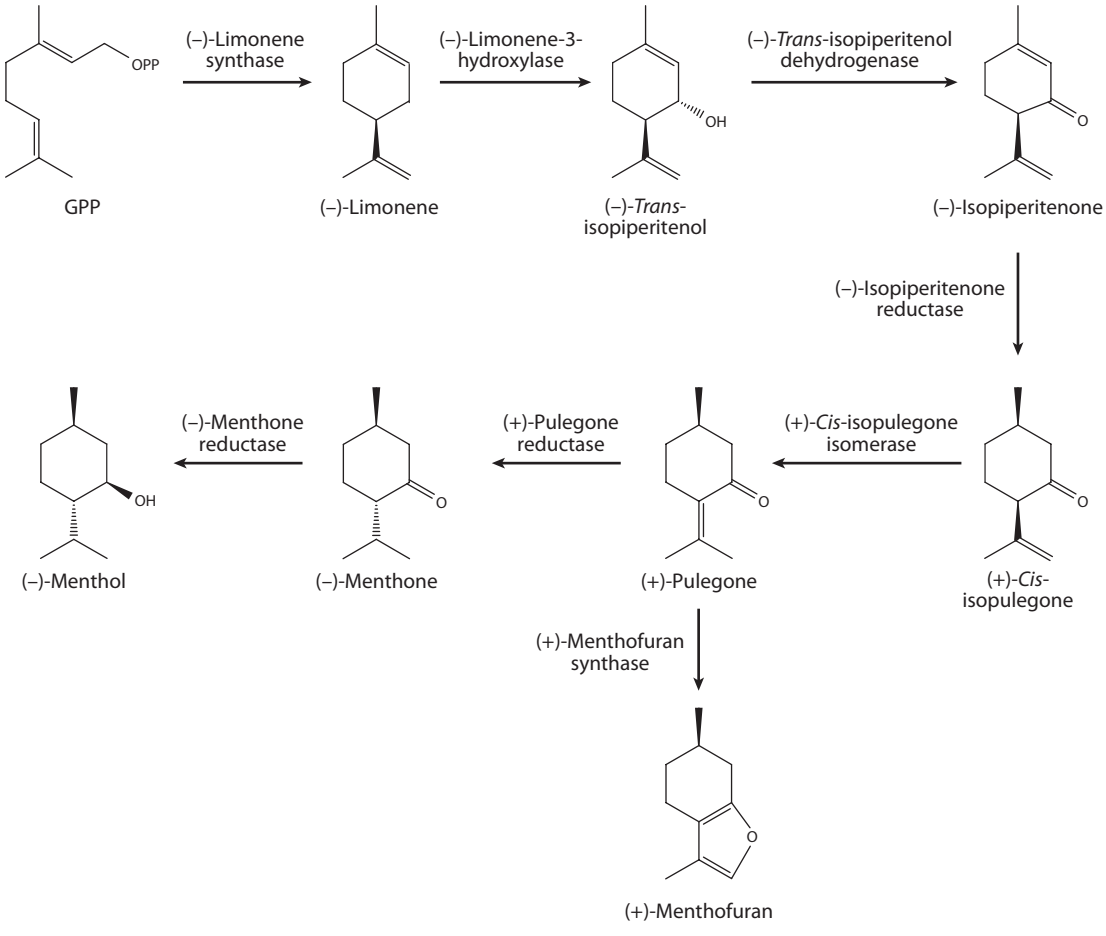


Figure 3

The menthol biosynthetic pathway of peppermint (*Mentha* × *piperita*). Geranyl diphosphate (GPP) is derived from the plastidic 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. Engineering of the pathway improved menthol yield and purity and also revealed an inhibitory mechanism of (+)-pugelone reductase by the pathway side product (+)-menthofuran. For an in-depth review of the pathway see Reference 23.

product, (+)-menthofuran, by expressing a menthofuran synthase antisense RNA (56). To evaluate further steps for improvement of the menthol pathway a kinetic mathematical model was combined with experimental data to determine the biochemical processes underlying changes observed in the pathway under low-light conditions (81). The model predicted that (+)-pugelone reductase [which converts (+)-pugelone to the menthol precursor (−)-menthone] is inhibited by the (+)-pugelone-derived side-product (+)-menthofuran with a K_i of 300 μM. This prediction was confirmed by measurement of (+)-menthofuran concentrations in glandular trichome secretory cells, which turned out to be 20 mM under low-light conditions but less than 400 μM under normal light.

Crossing Plant Compartments

In general, engineering of plants for improved isoprenoid biosynthesis has been dominated by studies on the isoprenoid precursor pathways. Several studies have shown that overexpression of deoxyxylulose phosphate synthase (*dxs*), which catalyzes the first step of the DXP pathway, leads to increases in plant isoprenoid levels (29, 33, 50, 62). Overexpression of a key regulatory enzyme of the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), has met with less success, but does significantly increase triterpene sterol production in tobacco (18, 84). Perhaps the most innovative isoprenoid metabolic engineering work performed in plants to date takes advantage of the compartmentalization of the two isoprenoid pathways. Because monoterpenes are normally produced in the plastid and sesquiterpenes are normally produced in the cytosol, Wu and coworkers (99) reasoned that flux may be redirected to specific terpene products more readily by targeting the cell compartment not normally used for production of that class of isoprenoid. For example, expression of amorphadiene synthase (ADS) in the cytosol of tobacco results in amorphadiene levels in the leaf of approximately 1 ng g^{-1} fresh weight at best. However, expression of both ADS and FPP synthase genes in the chloroplast (the same nuclear promoter was used for ADS, but the protein was targeted to the chloroplast through the addition of a signal sequence) results in amorphadiene levels of more than 25 μg g^{-1} fresh weight, a 25,000-fold improvement. A large part of this effect is most likely due to the early redirection of flux from the DXP pathway to amorphadiene through expression of FPP synthase. Although FPP is a central regulatory point in the cytosolic mevalonate pathway, it is not expected to play a major role in regulation of the plastidic DXP pathway because GGPP is synthesized in a single step from GPP, bypassing FPP (**Figure 2**). In contrast, expression of patchoulol synthase in the plastid without an accompanying FPP synthase gene resulted in the accumulation of significant quantities of the sesquiterpene patchoulol, reopening the question of how much FPP is produced in, or imported into, the plastid (99). The same study involved a second strategy converse to the amorphadiene approach: production of the monoterpene limonene in the cytosol instead of its normal plastidic location. However, in this case limonene levels were higher in the plastid, a result probably attributable to higher flux through the plant DXP pathway than the mevalonate pathway.

ADS: amorphadiene synthase

GENE EXPRESSION AND PATHWAY ENGINEERING IN MICROBES

In contrast to plants, microbial systems are more amenable to large-scale engineering projects where many different modifications may be compared and combined. However, microbes do suffer some technical drawbacks compared with plants, particularly when it comes to expressing heterologous genes such as P450s.

Engineering the Mevalonate and DXP Pathways

A broad review of mevalonate and DXP pathway engineering covers many of the earlier

developments in this field (59), whereas a more recent review provides a comprehensive table of the titers of isoprenoids achieved in various metabolic engineering studies (3). Early work on engineering the DXP pathway in *E. coli* was initially focused on overexpression of DXP pathway enzymes, usually resulting in moderate increases in isoprenoid production levels. As is the case in plants, most studies have focused on the first two pathway enzymes, DXS and DXR (59) (**Figure 1**). A significant advance in *E. coli* isoprenoid engineering came about through the successful heterologous expression of the mevalonate pathway, which, when coexpressed with ADS, resulted in amorphadiene titers of more than 110 mg L^{-1} (58). Improvements in the mevalonate pathway in *Saccharomyces cerevisiae* have usually centered on the main pathway regulatory enzyme HMGR, and particularly on a truncated form of the enzyme that lacks the membrane-bound domain through which HMGR degradation is regulated (3, 30). However, more recent work describes a successful three-pronged approach that involves expression of a truncated HMGR, downregulation of squalene synthase to reduce flux from FPP to sterols, and expression of a mutant transcription factor (*upc2-1*) to upregulate several mevalonate pathway genes at once, resulting in amorphadiene levels of 150 mg L^{-1} when ADS is coexpressed (74, 82).

Optimization of culture conditions as well as scale-up of cultures to lab-scale bioreactors can lead to further improvements in productivity. Production of amorphadiene in *E. coli* engineered with the mevalonate pathway reached 400 mg L^{-1} in a two-phase bioreactor in which dodecane was used to sequester amorphadiene released from the cells (64). Lycopene production in engineered *E. coli* was also improved (reaching 220 mg L^{-1}) by high-density fermentation in which oxygen and pH levels were optimized (5). Further improvements to microbial hosts for enhanced isoprenoid production will likely require more global approaches to discover limiting factors and balance pathway flux, some of which are described below.

Global Approaches to Improving Pathway Flux

Combinatorial approaches can be effective in finding the most favorable permutation of genetic modifications for pathway optimization, particularly when used together with isoprenoid products that can be detected easily, such as carotenoids. A strain of *E. coli* engineered for production of β-carotene was used to screen a library of mutants in which native promoters were replaced by the bacteriophage T5 promoter (104). The highest producing strain resulting from this study harbored a combination of four T5 promoter replacements in isoprenoid pathway genes. This combination of modifications would probably not have come about through rational design, especially because one of the overproduced proteins (octaprenyl-diphosphate synthase, IspB) normally diverts flow away from FPP to quinone biosynthesis. The role of IspB in increasing carotenoid production in *E. coli* has yet to be investigated, but it demonstrates the utility of nonbiased combinatorial studies for discovery of (unexpected) genes that influence isoprenoid pathway flux.

A lycopene-producing strain of *E. coli* was used to evaluate mutations that were previously identified as potential influences on carotenoid production (46). Many of these genes had been identified as targets for lycopene overproduction in two earlier studies: a shotgun library of overexpression mutants (47) and a library combining both systematic and random (transposon-mediated) knockouts (4). The combinatorial evaluation of these gene knockouts and overexpressions was performed in a two-dimensional array that reached a global maximum production in a strain containing a triple knockout and double overexpression, in combination with two genes already overexpressed in the base strain (46).

Optimization of zeaxanthin biosynthesis in *E. coli* was attempted by constructing five operons, each containing five zeaxanthin pathway genes in a different order so that their transcription levels would vary according to

distance from the promoter (65). Zeaxanthin levels varied by approximately fourfold between the five operons, reaching 800 μg g^{-1} dry weight in the best-producing strain. The method for reordering the genes within the operon, known as ordered gene assembly in *Bacillus subtilis* (OGAB), proved to be a useful tool for this combinatorial approach, although more recent ligation-free assembly methods may be more convenient (97).

A novel tool termed global transcription machinery engineering (gTME), involving mutation of the *E. coli rpoD* gene (which encodes the main sigma factor, σ^{70}), has been applied to lycopene-producing strains of *E. coli* (6). A single round of gTME can produce *rpoD* mutants that outperform triple knockout strains previously developed using extensive screening programs. Mutants that led to an increase in lycopene production in one genetic background were not found to deliver the same result in a different strain, indicating that the transcription reprogramming is genotype-specific.

In the absence of a suitable colorimetric method, biosensors can provide alternative screens for use with global or combinatorial engineering approaches. A mevalonate biosensor was developed using an *E. coli* strain that is auxotrophic for mevalonate and expresses the green fluorescent protein (GFP) (75). This screen was applied to a library of mutants developed from an *E. coli* strain expressing the yeast mevalonate pathway; intergenic sequences of the mevalonate pathway genes were varied to alter mRNA stability and processing, and the biosensor screen yielded a mutant that produced sevenfold more mevalonate than the original strain (76).

Finally, -omics approaches can provide a wealth of information for metabolic pathway engineering but have not been widely used for isoprenoid applications so far. However, transcriptional analysis by microarray has been used in combination with targeted metabolite profiling to successfully improve flux through the heterologous mevalonate pathway in *E. coli* (51). In this study, an accumulation of HMG-CoA inhibited fatty acid biosynthesis in *E. coli* and led to membrane stress, but simultaneous feeding of the cultures with certain fatty acids counteracted the HMG-CoA–induced growth inhibition.

Expression of P450s in Microbial Hosts

One of the major hurdles to overcome in engineering isoprenoid pathways in microbial hosts is achieving functional expression of enzymes responsible for downstream processing of terpene olefins, particularly P450s. Plant P450s are usually membrane-bound enzymes and require the presence of a colocalized CPR partner. *E. coli* and most other bacteria do not have any native P450s, whereas the eukaryote *S. cerevisiae* does contain native P450s and is generally the preferred host for P450 expression (37). Expression of a plant TPS gene in a microbial host can benefit from codon optimization of the TPS coding sequence (27, 58), which has also proven to be the case for expression of P450 genes, particularly in *E. coli*, in which codon bias is stronger than in yeast (10, 17). Although one case demonstrates that the native *E. coli* flavodoxin reductase is at least partially successful in providing a reductase partner for plant P450 expression, in most cases a plant CPR is required for activity in *E. coli* (9). A single CPR serves as a reductase partner for many P450s in the native plant and is therefore not expected to be particularly attuned to any one P450 (*A. thaliana* has two CPR genes compared with 272 P450 genes). However, expression of a CPR originating from the same plant as the P450 can improve productivity in a microbial host (17). The expression of translational P450-CPR fusion proteins has proven to be a successful strategy in some cases of functional P450 expression in *E. coli* (40, 42). When using *S. cerevisiae* as a host for P450 expression, the native yeast CPR may also serve as a reductase partner but overexpression of the gene is necessary to achieve moderate levels of P450 activity (94). Whereas most P450 expression studies have involved *E. coli* or *S. cerevisiae*, a third less commonly used vehicle for P450 expression is

via baculovirus promoters in Sf9 or Tn5 insect cells (87).

No reliable guidelines or rules have been developed for microbial P450 expression. One of the factors that influences functionality of P450s expressed in *E. coli* is modification of the N terminus of the protein through expression of truncated or mutated versions of the original plant gene. Early work with mammalian P450s demonstrated that N-terminal truncation of the protein yielded the best results for expression in *E. coli* (32). Since then, improvements in functional expression of plant P450s in *E. coli* have also been made using this approach, although no consensus protein sequence or length has emerged so far (17, 40). Choice of *E. coli* host strain also significantly impacts P450 expression or activity. For example, DS410 was the only successful strain out of several candidates for expression of cinnamate 4-hydroxylase (40), whereas a choice of DH1 over DH10B yielded a 1000-fold increase in the oxidation of amorphadiene (17).

MUTATION OF ISOPRENOID BIOSYNTHETIC ENZYMES

Mutation of Terpene Synthases

Prior to mutagenesis studies using TPS genes, prenyltransferases were found to be highly similar in protein sequence and amenable to the acquisition of altered specificities through mutation. For example, mutation of an FPP synthase into a GGPP synthase through a single amino acid substitution demonstrated the similarity of prenyltransferase catalytic sites (68). Since then, many studies have demonstrated the plasticity of TPSs through the construction of mutants with altered product profiles or completely new activities (88). Many TPS mutations have resulted in enzymes with broad product ranges that may be useful in mechanistic studies or for functional product bioassays. However, enzymes with higher specificity are more likely to find applications in an industrial setting. Site-specific saturation mutagenesis of a (+)-δ-cadinene synthase resulted in an enzyme that maintained its specific activity and produced a new sesquiterpene product with a high degree of fidelity (germacrene D-4-ol at 93%) (103). This work led to the development of a mathematical model for the prediction of mutations likely to generate novel TPS activities (102). A single amino acid substitution found to convert iso-kaurene or *ent*-kaurene synthase into a specific pimaradiene synthase is particularly interesting because kaurene is the primary metabolite precursor to gibberellin, whereas pimaradiene is a secondary metabolite (100). This mutation, which may be applicable to all plant kaurene synthases, is significant not only from the perspective of TPS evolution from primary to secondary metabolism, but also opens up intriguing possibilities for engineering.

Mutation of P450s

In view of the difficulties associated with microbial expression of plant P450s, many groups have considered the use of bacterial P450s as a substitute. $P450_{cam}$ from *Pseudomonas putida* and P450 BM-3 from *Bacillus megaterium* are the two best known bacterial P450s and the two first P450 enzymes for which crystal structures were solved (78, 80). $P450_{cam}$ requires a bacterial ferredoxin and ferredoxin reductase for completion of the electron transfer reaction, but BM-3 is a self-contained protein that contains both P450 and reductase domains. Applications for these two enzymes are not limited to the reactions that they naturally catalyze, because many studies have demonstrated the generation of new activities through mutagenesis. Analysis of the active site of $P450_{cam}$, which normally oxidizes camphor to 5-*exo*-hydroxycamphor, and consideration of the differences between the structures of camphor and α-pinene led to rationally designed $P450_{cam}$ mutants capable of oxidizing (+)-α-pinene to (+)-*cis*-verbenol (11). A slightly different $P450_{cam}$ mutant produced (+)-*cis*-verbenone (32%) in addition to (+)-*cis*-verbenol (55%), suggesting the possibility for catalysis of multiple oxidative steps by a single mutant. BM-3, which naturally hydroxylates fatty acids of chain length C12 to C18, is the fastest known P450 enzyme and expresses well in *E. coli* (12). Rational

mutagenesis of BM-3 for hydroxylation of shorter-chain fatty acids resulted in a triple mutant that was unexpectedly capable of hydroxylation of various alkanes and cycloalkanes, indicating some promise for engineering the enzyme for isoprenoid substrates (7). Whereas these two bacterial P450s use relatively small substrates and may be best suited for mutation for activity on mono- or sesquiterpenes, the steroid 15β-hydroxylase (CYP106A2) from *B. megaterium* P450 hydroxylates a variety of steroids and therefore may be more suited for larger isoprenoid substrates such as triterpenes (38).

The construction of chimeras between bacterial and plant P450s may provide a means of generating soluble enzymes with improved activity in microbial hosts such as *E. coli*. A fusion of the human liver P450 2C9 and $P450_{cam}$ resulted in a soluble P450 that retained catalytic activity in *E. coli* while using reducing equivalents from the bacterial putidaredoxin and putidaredoxin reductase (91). A computation algorithm (SCHEMA) designed to identify optimal crossover points for generating protein fusions is an effective tool in designing P450 chimeras (70).

APPROACHING A COMPLETE MICROBIAL SYSTEM

Production of artemisinic acid, precursor to the antimalarial drug artemisinin, in both *E. coli* and *S. cerevisiae* illustrates the utility of using microbial hosts for isoprenoid production. A 30–60% reduction in the cost of artemisinin combination therapies is projected following the switch from plant-extracted artemisinin to a microbial source (36). In addition to benefits for low-income patients, the new drug source will alleviate pressure on agricultural land and circumvent environmental hazards associated with current plant extraction methods. The technology to deliver a microbial source of artemisinic acid came about through a combination of plant gene discovery, microbial isoprenoid pathway engineering, codon optimization, P450 expression studies, gene expression balancing, growth optimization, two-phase fermentation, and a global analysis of changes in cell metabolism in response to the new pathway (17, 36, 48, 51, 58, 64, 74, 82, 89). Titers of amorphadiene have increased 10 million–fold in *E. coli* to more than 480 mg L^{-1} through a decade of metabolic engineering work and have reached 150 mg L^{-1} in *S. cerevisiae* (36, 82, 89). Conversion of amorphadiene to artemisinic acid in *E. coli* through functional expression of the P450 *CYP71AV1* from *A. annua* required a good deal of troubleshooting, but artemisinic acid yields of more than 300 mg L^{-1} were ultimately achieved (17). Expression of *CYP71AV1* in yeast did not prove to be as troublesome, and the conversion efficiency of amorphadiene was found to be high, with artemisinic acid levels exceeding 100 mg L^{-1} (82). Improvement of both the *E. coli* and yeast systems are ongoing and either system could ultimately prove to be the best choice for industrial production.

Whereas artemisinin has proven to be a good case for microbial isoprenoid production, some isoprenoids such as Taxol are considerably more complex and will require a great deal of work to achieve total biosynthesis in a microbial host. In the meantime, it is encouraging to note that advances in plant cell culture techniques, coupled with an understanding of Taxol pathway elicitors, have allowed the replacement of destructive plant extraction methods with cleaner biotechnological solutions (83).

SUMMARY POINTS

1. Isoprenoids have many diverse functions in their native plant species that range from universal primary metabolites such as gibberellin to family- or genus-specific secondary metabolites such as artemisinin. Many of the secondary metabolites serve roles in plant defense or communication.

2. Many applications have been found for plant isoprenoids through indigenous knowledge and plant biology studies. Elucidation of isoprenoid pathway genes usually depends on some knowledge of the site of biosynthesis and/or elicitors that induce expression of pathway genes.
3. Major advances have been made within the past decade in engineering both microbial and plant hosts for enhanced isoprenoid production. Many of the more successful approaches have involved expression of exogenous or mutant genes rather than overexpression of endogenous genes.
4. Enzymes that modify the initial terpene synthase product, typically belonging to the cytochrome P450 monooxgenase (P450) family, pose the greatest challenges in terms of both gene discovery and microbial expression.

FUTURE ISSUES

1. Advancement of current engineering efforts may be best facilitated by either combinatorial approaches coupled with practical screening methods or global approaches to balancing pathway expression and cell metabolism.
2. The development of methods for the functional expression of P450s in microbial hosts, particularly *E. coli*, remains a key issue in the development of microbial platforms for isoprenoid biosynthesis.
3. The use of P450 crystal structure data coupled with molecular modeling technology and rational mutagenesis may facilitate the generation of soluble bacterial P450s adapted to specific isoprenoid substrates.

DISCLOSURE STATEMENT

J.D.K. has a consulting relationship with and a financial interest in Amyris and a financial interest in LS9, both biofuel companies.

ACKNOWLEDGMENTS

Isoprenoid research in the Keasling laboratory has been funded by the National Science Foundation, the Office of Naval Research, the University of California Discovery Grant Program, Maxygen, Diversa, the Bill & Melinda Gates Foundation, and the U.S. Department of Energy through the Joint BioEnergy Institute.

1. IspH, the final enzyme in the DXP pathway, is characterized.

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Plant Biology
Volume 60, 2009

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