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Production and engineering of terpenoids in plant cell culture

Susan C Roberts

Terpenoids are a diverse class of natural products that have many functions in the plant kingdom and in human health and nutrition. Their chemical diversity has led to the discovery of over 40,000 different structures, with several classes serving as important pharmaceutical agents, including the anticancer agents paclitaxel (Taxol) and terpenoid-derived indole alkaloids. Many terpenoid compounds are found in low yield from natural sources, so plant cell cultures have been investigated as an alternate production strategy. Metabolic engineering of whole plants and plant cell cultures is an effective tool to both increase terpenoid yield and alter terpenoid distribution for desired properties such as enhanced flavor, fragrance or color. Recent advances in defining terpenoid metabolic pathways, particularly in secondary metabolism, enhanced knowledge concerning regulation of terpenoid accumulation, and application of emerging plant systems biology approaches, have enabled metabolic engineering of terpenoid production. This paper reviews the current state of knowledge of terpenoid metabolism, with a special focus on production of important pharmaceutically active secondary metabolic terpenoids in plant cell cultures. Strategies for defining pathways and uncovering rate-influencing steps in global metabolism, and applying this information for successful terpenoid metabolic engineering, are emphasized.

Terpenoids, also known as isoprenoids, are perhaps the most diverse family of natural products synthesized from plants, serving a range of important physiological and societal functions. Over 40,000 different terpenoids have been isolated from plant, animal and microbial species[1,2]. Plant terpenoids are classified as primary metabolites necessary for cellular function and maintenance, or secondary metabolites that are not involved in growth and development. Primary metabolic terpenoids include gibberellins, carotenoids and sterols, which serve basic functions such as cell growth modulation and plant elongation, light harvestation and photoprotection, and membrane permeability and fluidity control. Secondary metabolic terpenoids are often commercially attractive because of their uses as flavor and color enhancers, agricultural chemicals, and medicinals. A wide range of terpenoids (**Fig. 1**) have demonstrated pharmaceutical activity against human ailments such as cancer[3–5] (for example, taxanes, including paclitaxel from *Taxus* spp., and terpenoid indole alkaloids (TIAs), including vincristine and vinblastine from *Catharanthus roseus*), malaria (for example, artemisinin from *Artemisia annua*), and HIV[3] (for example, coumarins, including calanolide A from *Calophyllum lanigerum*). Because of the commercial significance of terpenoids, the application of metabolic engineering strategies to both increase supply (for example, production of medicinally valued terpenoids either from the natural plant or via plant cell culture; see below) and improve product properties (for example, disease and pest resistance in crop plants, enhanced fragrance in flowering plants, enhanced flavor in fruits and vegetables) is of great societal importance. This review will highlight both the exciting progress and the challenges still to be met in the production and engineering of terpenoids in plants and plant cell cultures, with a focus on the pharmaceutically active terpenoids.

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Terpenoid biosynthesis

Terpenoids are a class of compounds derived from the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyldiphosphate (DMAPP), also called isoprene units (**Scheme 1**). Terpenoid building blocks are then formed through condensation of additional IPP moieties via prenyltransferases. Monoterpenoids are derived from geranyl pyrophosphate (GPP, C_{10}), sesquiterpenoids are derived from farnesyl pyrophosphate (FPP, C_{15}), and diterpenoids are derived from geranylgeranyl pyrophosphate (GGPP, C_{20}). Even higher order terpenoids are possible through condensation of these intermediates to larger precursor moieties. For example, sterols are derived from the triterpenoid squalene (C_{30}), which contains six isoprene units through condensation of two molecules of FPP, and carotenoids (C_{40}) are largely formed through condensation of two molecules of GGPP to yield eight-isoprene-unit compounds. After the formation of the acyclic terpenoid structural building blocks (for example, GPP, FPP, GGPP), terpene synthases act to generate the main terpene carbon skeleton. Additional transformations often involving oxidation, reduction, isomerization, and conjugation enzymes decorate or alter the main skeleton with varied functional groups to yield the tremendously diverse terpenoid family of compounds.

Two distinct pathways generate the universal C_5 precursors IPP and DMAPP (**Scheme 2**). The classic mevalonate (MVA) pathway was discovered in the 1950s and was assumed to be the sole source of the terpenoid precursors IPP and DMAPP. The MVA pathway is active in bacteria, plants, animals and fungi and functions in the cytosol to generally supply the precursors for production of sesquiterpenes and triterpenes. Recently, labeling experiments in bacteria and plants revealed the presence of an alternate pathway to IPP and DMAPP supply (for

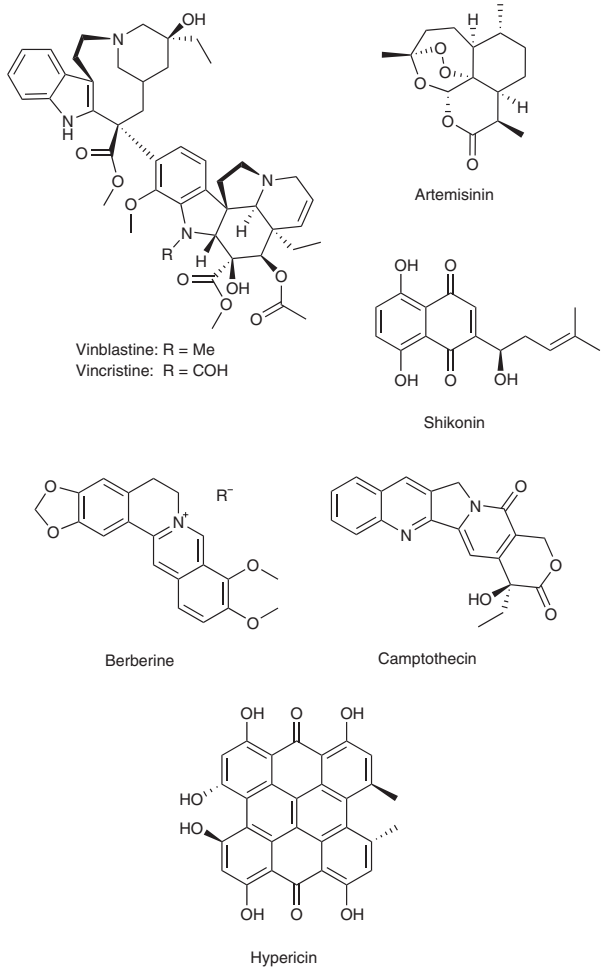


Figure 1 Structures of industrially relevant terpenoids and terpenoid-derived compounds.

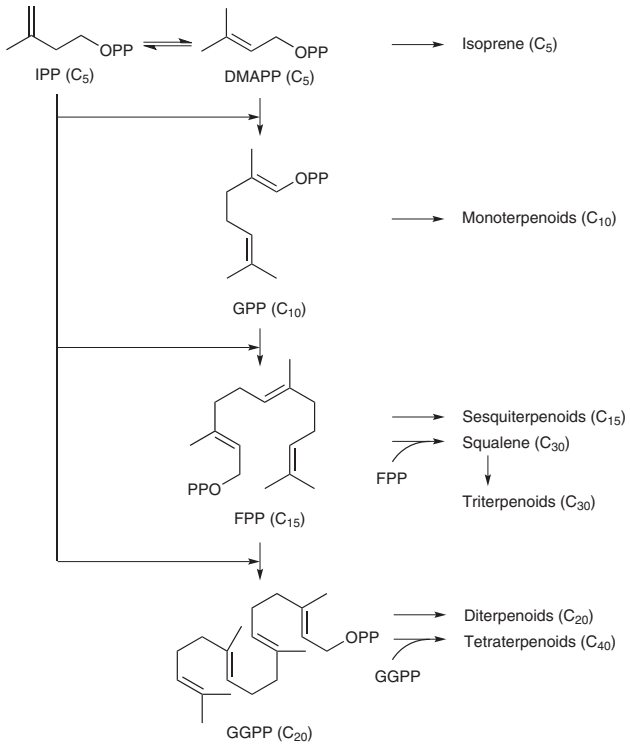
reviews in plants refer to refs. 6–8). This pathway, which is named after the first committed precursor, 2-*C*-methyl-D-erythritol-4-phosphate (MEP; the pathway is also sometimes referred to as the DXP pathway), is plastidial in nature and is generally used to supply precursors for the production of monoterpenoids, diterpenoids and tetraterpenoids. Although interaction between these pathways is still largely undiscovered, recent evidence has revealed an exchange of intermediates between the cytosol and the plastid[9–11]. Conversely, isotope labeling studies in tobacco detect little metabolite exchange between compartments in sesquiterpene biosynthesis[12]. Metabolic engineering of these pathways would benefit significantly from a more complete understanding of this compartmentalization and transport.

Terpenoid supply and study via plant cell culture

Although some terpenoids are produced in relatively large quantities from natural sources (for example, essential oils, resins and waxes), often high-value terpenoid products are found in low abundance in nature, with secondary terpenoids typically less than 2–3% of total dry weight. For example, approximately 10,000 kg of dry bark is required to supply 1 kg of paclitaxel[13], which translates to harvestation of the bark of 2–4 mature trees to supply enough paclitaxel to treat a single patient[14]. Extraction from natural sources is also highly variable depending on the source plant, location and season of harvest, and can be adversely affected by unpredictable environmental circumstances. Because of the high value of these terpenoid-derived products and the unreliability upon harvest from natural sources in meeting both patient treatment and clinical evaluation needs, significant effort has been directed toward development of renewable and environmentally friendly production processes. Alternate routes to terpenoid supply include total chemical synthesis, semisynthesis from isolated precursors, genetic engineering of plant pathways in microbial hosts, and plant cell culture (for example see ref. 15). Each method offers distinct advantages and disadvantages depending on the specific system of interest. Total synthesis is not reliant on field-grown material, but the often complex chemistry involves use of harsh solvents and typically results in low product yields, as is the case for paclitaxel (reviewed in ref. 16) and artemisinin[17] supply.

Transfer of terpenoid biosynthetic pathways into microbial hosts offers advantages such as environmentally friendly chemistry, use of inexpensive carbon sources, and capability of genetic manipulation to increase yields[18] (see **Table 1** for a comparison of bacterial and plant cells). Additionally, microbial cells are more amendable to use in large-scale processes. Smaller cells are relatively insensitive to shear stress induced via impeller mixing in bioreactors. Doubling times are significantly shorter and therefore culture times can be minimized, increasing throughput and decreasing the chance for contamination. The production phase is typically uncoupled from the growth phase for microbial products, so two-phase systems with optimal media formulations for growth and production can be used. Plant metabolites are sometimes concurrent with the exponential growth phase of culture, as is the case with paclitaxel[19], and therefore two-phase culture is not always possible. Despite the obvious advantages to engineering microbial cells for terpenoid supply, some plant terpenoid metabolic pathways are either too complex for efficient transfer or are partially undefined, and therefore complete pathways cannot be reconstituted in microbial systems. Additionally, cytochrome P450 (CYP450) hydroxylases are often heavily involved in decorating the carbon skeleton of complex terpenoid metabolites (for example, paclitaxel and related taxanes), and expression of redox partners for P450 enzymes presents challenges for advanced terpenoid synthesis in microbial systems[20]. Thus, production in the native species is sometimes required, and in these circumstances plant cell culture is a viable option.

Plant cell culture provides an environmentally friendly, renewable alternative for secondary metabolite supply. As opposed to natural harvest, culture conditions can be strictly controlled to meet stringent US Food and Drug Administration requirements. Manipulation of process and environmental conditions and/or metabolic engineering of key pathways can significantly enhance both selectivity and yield. Additionally, new compounds can be synthesized through either controlled regulation of inherent biosynthetic pathways or metabolically engineered pathways. Plant cell culture has been explored in the production of numerous terpenoid and terpenoid-derived compounds, most notably high-value pharmaceutical products such as the TIAs from *Catharanthus roseus*[21], taxanes from *Taxus* sp.[15,22,23], and artemisinin from *Artemisia annua*[24]. Cell culture has also been investigated for production of other pharmaceutically active agents such as shikonin from *Lithospermum erythrorhizon*[25], berberine from *Coptis japonica*[26,27], camptothecin from both *Camptotheca acuminata*[27,28] and *Nothapodytes foetida*[29], and hypericins from *Hypericum perforatum*[30]. Many processes have been developed using plant suspension cultures as a result of the ease in scale-up and application of traditional optimization strategies developed for microbial and mammalian cell systems. However, secondary metabolite production is often higher in differentiated tissues; thus hairy root cultures that have been transformed genetically with *Agrobacterium* spp. have also been widely used[31,32]. *Taxus* spp. cell suspension culture represents



Scheme 1 Overview of terpenoid biosynthesis and the generation of terpenoid starting blocks. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) condense to form higher order terpenoid precursors, including the monoterpenoid precursor geranyl diphosphate (GPP), the sesquiterpenoid precursor farnesyl diphosphate (FPP) and the diterpenoid precursor geranylgeranyl diphosphate (GGPP). Two molecules of FPP condense to form the triterpenoid precursor squalene, and two molecules of GGPP condense to form higher order tetraterpenoids.

a success of plant cell culture technology in the United States with its proven feasibility by Bristol-Myers Squibb and Phyton Biotech, Inc. (a DFP Pharmaceuticals company) in supplying paclitaxel for Bristol-Myers Squibb's Taxol formulation. In addition, Samyang Genex has commercialized a process for the production of paclitaxel via plant cell culture[33]. Plant cell culture is not only useful in the supply of valuable terpenoids, but can also be extremely valuable in isolating previously unidentified terpenoid metabolic genes. The best example is elucidation of the paclitaxel biosynthetic pathway (see below for more details).

A key challenge to the regular commercial use of plant cell culture technology is variability in product accumulation. Unlike microbial systems, in which productivity is predictable, plant culture systems demonstrate significant variability in product formation. In fact, variability has been cited as the most important issue facing the use of *Taxus* spp. cell cultures for the commercial supply of paclitaxel[34]. The highest levels of paclitaxel accumulation have been observed to differ by ten-fold between cell lines and within cell lines sampled over time[35]. Because of the nature of plant cellular division and the resilience of the pectin-rich middle lamella that cements cells together, aggregates containing anywhere from ~2–50 cells are typical in plant cell cultures. Owing to the size of these plant cell aggregates, which may reach 2 mm in diameter, heterogeneous microenvironments related to oxygen and nutrient availability are created within an aggregate. Cells toward the center of an aggregate are metabolically and morphologically distinct from cells near the periphery of an aggregate[36]. The presence of such microenvironments (in which *Taxus* spp. cellular metabolism is altered) within the same culture may contribute to the observed variability in paclitaxel yield. The majority of current research is directed toward understanding variability by analyzing total culture averages (that is, sampling an entire batch of cells and averaging over replicate batches). Our laboratory has recently developed flow cytometric population analysis methods for characterizing heterogeneity in *Taxus* spp. cell cultures[36–40]. As more information is uncovered at the single-cell level for plant cell cultures, populations can be better engineered for enhanced terpenoid productivity and stability.

Bioengineering of terpenoids in plant cell culture

The production of high-value terpenoid products via plant cell culture is an attractive proposition, but low yields and high levels of variability in metabolite accumulation complicate the regular use of plant cell culture technology for terpenoid supply at the large scale. Although there have been key recent advances in defining terpenoid biosynthetic pathways and in understanding their regulation (see below), there still exist numerous challenges associated with engineering plant cells for enhanced accumulation of terpenoid compounds: undefined terpenoid secondary metabolite biosynthetic pathways, need for coordinated regulation of multiple rate-influencing steps, lack of system-wide analysis on plants and plant cell cultures due to the fact that so few plant genomes have been sequenced, inability to transfer information derived from whole-plant studies to the cell culture environment, variability in terpenoid product accumulation over time and among cultures sampled at the same time, lack of plant inducible promoters, difficulty in applying traditional genetic transformation methods to key plant cell culture systems, and compartmentation of terpenoid metabolism. Despite these challenges, significant advances have been made in recent years toward realizing the goal of engineering terpenoid metabolism in plant cell cultures. The following sections will highlight these important advances and suggest potential strategies for engineering terpenoid metabolism, with a specific focus on the medicinal secondary metabolites for which most of the significant advances in plant cell culture technology have been made.

To engineer terpenoid pathways, rate-influencing steps must be identified. Most often the rate limitation occurs within the biosynthetic pathway. Because many terpenoid biosynthetic pathways remain partially undefined, a variety of methods have been used to identify terpenoid biosynthetic pathway genes and elucidate pathway regulation: mutant selection, gene silencing, gene overexpression via genetic engineering, precursor feeding, application of metabolic inhibitors, and enzyme induction with elicitation (the use of an exogenous compound to induce gene expression). One of the most commonly applied elicitors of terpenoid metabolism is jasmonic acid, or its methyl ester methyl jasmonate. Since the first publication of the use of jasmonic acid as an inducer of secondary metabolite biosynthesis in plant cell cultures in 1992[41], there has been a steady increase in the number of publications that contain the terms 'plant' and either 'jasmonic acid' or 'methyl jasmonate' (1,024 in 2002–2006 versus 526 in 1997–2000 versus 199 in 1992–1996; courtesy of Web of Science, Thomson Scientific). Enzyme elicitation is a powerful tool for identifying regulatory steps in terpenoid biosynthesis.

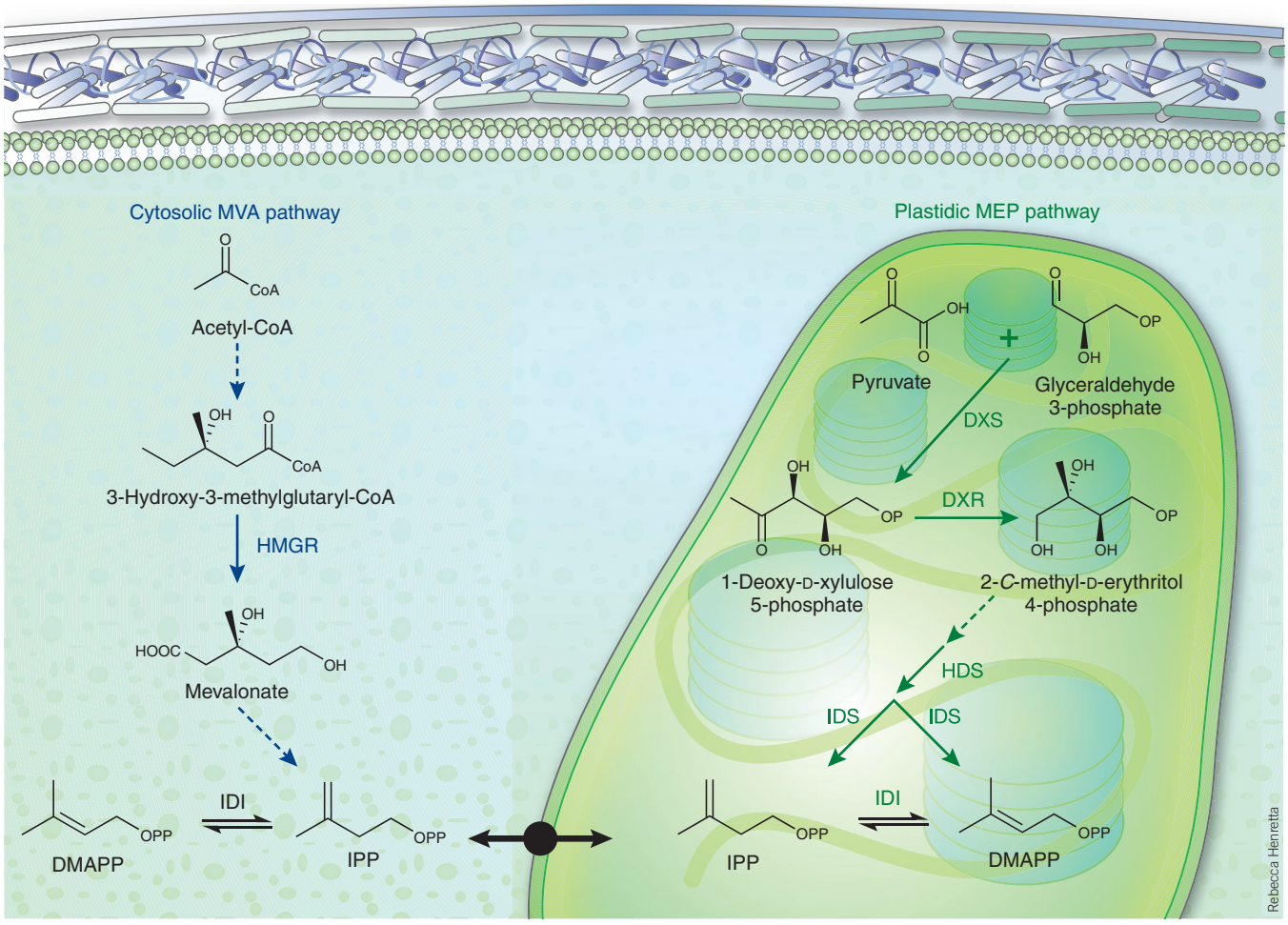
Terpenoid biosynthesis for precursor supply

A common strategy for engineering terpenoid synthesis is to focus on enhancing flux to increase the precursor pools of IPP and DMAPP, by cloning the necessary genes and understanding regulation of both the MVA and MEP supply pathways. All of the pathway genes have been identified (reviewed in refs. 7,42), but the relative contribution of particular enzymes to global flux control through the pathway and between compartments to supply terpenoids is largely unknown. A great deal of evidence has emerged on the influence of isolated enzymes on terpenoid

accumulation (presented below), but a complete picture of flux control needed for optimal metabolic engineering requires more system-wide studies, which so far are lacking.

MVA pathway for supply of IPP and DMAPP. Although many important terpenoids and terpenoid-derived compounds synthesized via plant cell culture originate from IPP and DMAPP produced through the MEP pathway, recent studies demonstrating cross-talk between the MVA and MEP pathways suggest that engineering of the MVA pathway for IPP formation could be useful in controlling plastidial terpenoid biosynthesis. The classic MVA pathway is well defined, and numerous studies have demonstrated the importance of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). HMGR is strictly regulated in plant systems, and elicitor-induced HMGR genes have been cloned and characterized in numerous plant systems including tobacco, tomato, potato, rice and most recently pepper[43], with induction often correlated with increased terpenoid biosynthesis. For example, overexpression of HMGR led to increased sterol accumulation in tobacco[44] and tomato[45]. Cloning and expression analysis of HMGR genes have recently commenced in important commercial plant cell culture systems. An HMGR gene from *Taxus media* belonging to a small gene family was cloned that showed high homology to other plant HMGRs[46]. An analysis of HMGR expression in hairy root cultures of *A. annua* L. demonstrated temporal control, but did not correlate with accumulation of artemisinin[47]. A hamster HMGR gene was overexpressed in *C. roseus* hairy roots, resulting in differential patterns of alkaloid accumulation, which suggests a potential role of HMGR in regulating alkaloid biosynthesis[48].

MEP pathway for supply of IPP and DMAPP. Extensive research has been undertaken to elucidate MEP pathway regulation (for pathway summary and relation to terpenoid synthesis see refs. 6,49,50 and **Scheme 2**). Early pathway enzymes have proven to be rate-influencing in terpenoid accumulation in a variety of systems. For example, overexpression of 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS) is correlated with increases in essential oil accumulation in transgenic lavender[51], ginkgolide accumulation in *Ginkgo biloba*[52], carotenoid accumulation in *Arabidopsis thaliana*[53] and beta-carotene accumulation in the oil palm *Elaeis guineensis* Jacq[54]. Overexpression of DXP reductoisomerase (DXR) in peppermint increased monoterpene essential oil production by 40–60%[55]. Transgenic *A. thaliana* plants with altered DXS and DXR enzyme levels were collected through screening for changed resistance to metabolic inhibitors of the MEP pathway. Downregulation of DXR resulted in phenotypes representing decreased terpenoid production, whereas upregulation of DXR resulted in positive increases in



Scheme 2 Compartmentalized biosynthesis of IPP and DMAPP. (Left) Via the cytosolic mevalonate (MVA) pathway. HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IDI, isopentenyl diphosphate isomerase. (Right) Via the plastidic MEP pathway. DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HDS, hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; IDS, isopentenyl diphosphate: dimethylallyl diphosphate synthase; IDI, isopentenyl diphosphate isomerase. Dashed arrows indicate more than one step.

Table 1 Comparison of bacterial and plant cell characteristics for use in bioprocesses

Characteristic	Bacterial cells	Plant cells
Size	1 μm	20–50 μm
Shear stress	Insensitive	Sensitive
Doubling time	<1 hour	Days
Batch fermentation time	Days	Weeks
Product accumulation	Typically extracellular	Often cell-associated
Production phase	Uncoupled from growth	Often growth-associated
Variability in accumulation	Low	High
Product yields	High	Low
Post-translational processing	Simple	Advanced
Compartmentalization	None	Highly compartmentalized

plastid-derived terpenoids such as chlorophylls and carotenoids[56].

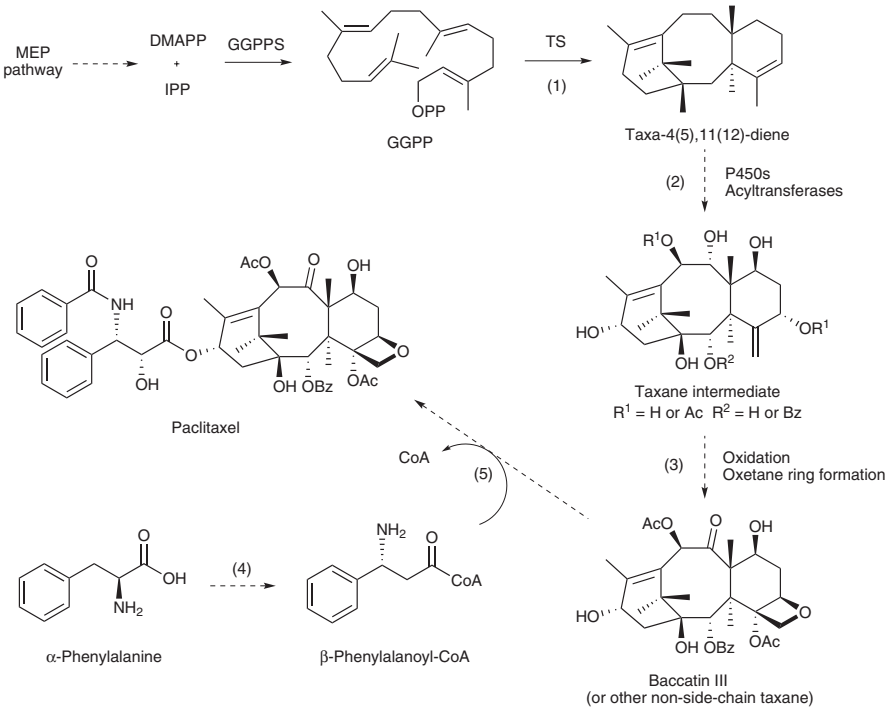
The influence of the final steps in the MEP pathway, which are catalyzed by the enzymes 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (HDS) and isopentenyl diphosphate:dimethylallyl diphosphate synthase (IDS), as well as the interconversion reaction between IPP and DMAPP, which is catalyzed by isopentenyl diphosphate isomerase (IDI), were recently investigated by post-transcriptional silencing using the tobacco rattle virus (TRV)[57]. The silencing of HDS and IDS resulted in plants with leaves that contained <4% of the chlorophyll and carotenoids of control plants. Additionally, silencing the isomerization step resulted in plants with an 80% reduction in pigments compared with controls. These results demonstrate the usefulness of gene silencing in understanding terpenoid pathway regulation and suggest that IDI is required for a fully functional MEP pathway.

Cloning and expression analysis of MEP pathway genes has recently commenced in commercially important plant cell culture systems. The fifth enzyme in the MEP pathway, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), was recently cloned and characterized from *Taxus media*[58]. The MEP pathway was recently shown to be functional in *A. annua* root cultures[59]. Clones for DXS and DXR were isolated from complementary DNA libraries, and functionality was confirmed through complementation analysis in *Escherichia coli*. Both culture age and light level influenced transcript levels, with continuous light being most beneficial. Because many plant cell culture processes for the supply of secondary metabolic terpenoids operate in the dark to maximize productivity, plastids other than chloroplasts are functional. Further studies concerning operation and regulation of the MEP pathway in nongreen plastids such as amyloplasts, chromoplasts or proteinoplasts will provide new insight for engineering these plant cell culture systems.

Inhibitor studies with fosmidomycin (inhibits MEP pathway by inhibiting DXR), mevinolin (inhibits MVA pathway by inhibiting HMGR), mevastatin (inhibits MVA pathway by inhibiting HMGR), D,L-glyceraldehyde (DLG; inhibitor of IPP transport into the plastids) and sodium pyrophosphate (NaPP; substrate analog of IPP that interferes with plastid uptake of IPP) were conducted on *Taxus* spp. suspension cultures to determine the relative contribution of the MVA and MEP pathways in taxane accumulation. In studies with *Taxus baccata*, taxane production was significantly more negatively influenced by addition of fosmidomycin as compared with mevinolin, which suggests a larger contribution of IPP supply from the MEP pathway[60,61]. Decreased taxane production with mevastatin addition suggests that the MVA pathway may contribute to IPP supply[62]. Inhibition of IPP transport into the plastids resulted in decreased taxane production, particularly in the late-stage culture period[62,63] (that is, day 14), again suggesting some supply of IPP from the MVA pathway. Inhibitor studies need to be analyzed with caution owing to potential issues with specificity, solubility, uptake, and transport to correct compartment. The percentage contribution of each pathway for IPP supply for taxane production can only be definitively determined from labeling studies, which have not yet been conducted.

Terpenoid secondary metabolite biosynthesis and regulation

Many plant secondary metabolite pathways are partially undefined, and therefore elucidation of these pathways and their regulation is necessary to better direct biosynthesis in plant cell cultures. This section will focus on advancements in *Taxus* spp. for production of the anticancer agent



Scheme 3 Biosynthesis of paclitaxel and related taxanes. DMAPP and IPP are generated from the plastidic MEP pathway and condensed by GGPP synthase (GGPPS) to form GGPP. (1) The first committed precursor of taxane biosynthesis, taxa-4(5),11(12)-diene is generated by taxadiene synthase (TS). (2) A series of P450 hydroxylations and acylations generate a putative taxane intermediate. (3) Non-side-chain taxanes (including baccatin III) are produced through both an oxidation reaction and oxetane ring formation. (4) The side chain moiety is derived from α-phenylalanine. (5) The side chain is added to a putative non-side-chain taxane precursor (likely baccatin III), and paclitaxel and other side chain taxanes are formed. Ac, acetyl; Bz, benzoyl. Dashed arrows indicate more than one step.

paclitaxel, although the strategies presented here are generally applicable to other important plant secondary metabolic terpenoid systems.

Paclitaxel is a cyclic and polyoxygenated diterpenoid containing several functional group substitutions, an oxetane ring, and a phenylisoserine-derived side chain. Paclitaxel biosynthesis begins with formation of GGPP and putatively involves 19 additional steps[64]. The taxane biosynthetic pathway as it is currently known can organizationally be divided into the following steps (**Scheme 3**): (1) formation of the first committed taxane precursor, taxadiene; (2) addition of seven oxygen functional groups and decoration of the carbon core with acylation reactions; (3) oxidation of ketone functionality and formation of the oxetane ring; (4) assembly of the side chain; (5) conjugation of the side chain to a taxane intermediate (likely baccatin III) and final modification reactions to generate the side chain–containing taxanes such as paclitaxel. Early work in discerning pathway steps was performed on whole plant tissues, but logistical challenges of *Taxus* spp. plant collection and the slow-growing nature of saplings prompted researchers to use *Taxus* spp. cell cultures as a renewable source of material[65]. Using direct molecular approaches, the Croteau laboratory developed a highly successful strategy for uncovering taxane biosynthetic pathway steps, which involved using cell-free systems to demonstrate enzyme activity through the addition of putative substrates and the application of precursor feeding studies with either plants or cell cultures, followed by isolation of the metabolite product[65]. To acquire the cDNAs encoding each pathway enzyme, homology-based searches were used in conjunction with a differential display library generated through comparison of unelicited cultures with those elicited with methyl jasmonate[65].

In addition to gaps in our understanding of the paclitaxel biosynthetic pathway, other aspects of paclitaxel metabolism remain mysterious. Within the pathway, regulation can be investigated by comparing gene expression time courses to taxane accumulation in cell culture. Recently, expression of ten pathway genes was compared over time in both unelicited and methyl jasmonate–elicited cell cultures[66]. Downstream steps in the pathway were identified as excellent candidates for rate limitations, as transcripts were not present in unelicited cultures as determined through both northern blotting and RT-PCR. These data additionally determined a preference for one branch in the taxane biosynthetic pathway[66], thereby providing further clues into how best to control metabolic flux.

However, major questions remain. For example, several of the genes responsible for oxygenation in the core taxane ring structure have not been identified, and CYP450 reductases responsible for electron shuttling to the CYP450 hydroxylases or monooxygenases have not been characterized. Additionally, no regulatory elements related to methyl jasmonate signaling or paclitaxel biosynthesis, such as transcriptional activators, have been cloned from *Taxus* spp. Outside of the direct biosynthetic pathway, regulatory steps in secondary metabolism may exist in transport of intermediates and products either to intracellular compartments or to the extracellular space in plant cell cultures, or in product degradation either in the intracellular or extracellular space (**Fig. 2**). Transport and degradation of paclitaxel *in planta* and in plant cell cultures also remain unknown. A plasma membrane ATP-binding cassette–type transporter was found to be involved in secretion of defense terpenoids from *Nicotiana plumbaginifolia* cell cultures[67], and analogous specific transport proteins may also be involved in taxane secretion.

Nondirect approaches have been used to determine putative biosynthetic genes and regulatory elements in other areas of paclitaxel metabolism. For example, a random sequencing strategy using a paclitaxel-accumulating cDNA library has identified ten new CYP450s in paclitaxel-accumulating *Taxus cuspidata* cells[64]. These CYP450 clones have a high similarity (>75%) to known paclitaxel-related CYP450

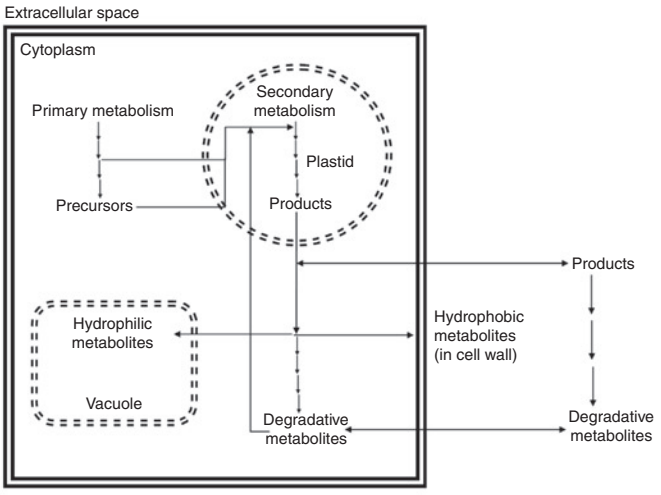


Figure 2 Overview of plant cellular metabolism for plastid-derived secondary metabolites such as paclitaxel in plant cell culture. Primary metabolic intermediates are largely produced in the cytosol and transported to the plastid for conversion via secondary metabolic pathways. Secondary metabolites are transported from the plastid for either storage or secretion to the extracellular medium. Hydrophilic secondary metabolites are stored in aqueous compartments such as the vacuole, whereas hydrophobic secondary metabolites are stored in the cell wall. Products can be degraded either intracellularly or extracellularly, and degradative metabolites can be transported in and out of the cell and reused in both primary and secondary metabolism.

oxygenases and low similarity to other plant cytochrome hydroxylases, which strongly implies a relationship to paclitaxel biosynthesis. To identify nonbiosynthesis-related regulatory genes, a more global approach is necessary. Analysis of differentially expressed genes between two physiological states (for example, paclitaxel nonaccumulating versus paclitaxel accumulating) can provide a wealth of information concerning genes involved with regulating a particular state. For example, a differential display method was applied to identify genes related to paclitaxel biosynthesis[68]. mRNA populations were compared from *Taxus chinensis* cells before and after commencing paclitaxel synthesis, and one clone was determined to be specifically expressed in the paclitaxel-production phase of culture. Although the putative gene was not further characterized, this study demonstrates the potential for this large-scale approach to identify new genes. Differential display methods have also been used to elucidate alkaloid gene expression in leaf and root samples from *C. roseus*[69], to probe the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti*[70] and to identify new genes involved in xylogenesis of *Eucalyptus*[71].

Progress in metabolic engineering of terpenoids in plants

Some of the most promising results have been observed with engineering precursor availability through targeting the MVA and MEP pathways (reviewed in refs. 72,73). Terpenoid metabolic engineering of downstream secondary metabolite pathway steps has been more limited, in part owing to the lack of both pathway definition and understanding of regulation, but some successes have been reported for taxanes, alkaloids and artemisinin. In one of the first published examples, synthesis of scopolamine was induced in *Atropa belladonna* upon introducing the gene encoding hyoscyamine-6β-hydroxylase[74]. Farnesyl diphosphate synthase (FPPS) produces the sesquiterpene precursor FPP, and overexpression of a cotton FPPS gene in transgenic *A. annua* resulted in three- to four-fold higher yield of artemisinin in hairy roots[75]. Recently, an exciting paper

demonstrated that targeting coexpressed amorpha-4,11-diene synthase (ADS) and FPPS to the plastids in tobacco dramatically increases accumulation of the artemisinin precursor amorpha-4,11-diene[12]. One of the most well-studied and successful systems in regards to metabolic engineering is TIA biosynthesis in *C. roseus*, in which improved alkaloid yields and profiles have been achieved with a variety of transformation conditions (for a recent review see ref. 21).

Genetic engineering of *Taxus* spp. cell suspensions with putative rate-influencing genes has not been possible owing to lack of a reliable method for gene transfer. However, a new *Agrobacterium* spp.–mediated transformation protocol has recently been established for *Taxus* spp. suspension cultures with stable transformation up to 20 months[76], which now enables metabolic engineering of *Taxus* spp. cells with key regulatory genes. Aside from this recent report, taxane biosynthetic pathway genes have been functionally expressed in both *E. coli*[77] and *Saccharomyces cerevisiae*[78], and five sequential genes were transformed into a single yeast host[78]. Although the pathway intermediate taxadiene was observed, products following the first CYP450 hydroxylation were not detected[78]. This problem may be alleviated with enhancement of the required redox activity. Coexpression in yeast of a CYP450 reductase along with a taxane biosynthetic CYP450 hydroxylase (10β-hydroxylase) resulted in a significant enhancement in hydroxylase activity compared with yeast lacking the cotransformed reductase[79]. Recently, the first steps to engineering paclitaxel synthesis in an alternate plant host have been accomplished[80,81]. A taxadiene synthase isolated from *T. baccata* was constitutively expressed in *A. thaliana* to produce taxadiene[80]. Growth retardation and a decrease in total photosynthetic pigments suggested flux diversion from other terpenoid pathways. The taxadiene synthase was additionally constitutively expressed in transgenic tomato fruit, reaching yields greater than 600 times higher than maximal yields reported in *A. thaliana*[81]. Expression of key taxane biosynthetic pathway enzymes in alternate host systems will enable not only supply of pathway intermediates, but also systems for characterization of newly discovered pathway genes. Expression of the complete taxane biosynthetic pathway in alternate hosts is highly unlikely given the pathway's complexity, so engineering directly in *Taxus* spp. is the most promising approach to increase supply of paclitaxel.

Future directions in metabolic engineering of terpenoids

Despite the recent advancements and promising results in terpenoid metabolic engineering, there are still numerous areas in which significant progress can be made. Metabolic engineering efforts would be enhanced through system-wide analysis of plants and cell cultures. Plant reaction networks are significantly more complex than microbial systems. Pathway redundancy and involvement of multiple intracellular compartments complicate flux analysis efforts. Manipulation of single enzymatic steps has resulted in unpredictable success. In engineering of carotenoid synthesis, the first committed enzyme, phytoene synthase (PSY), has been targeted for metabolic engineering. However, overexpression of PSY led to growth defects in tomato due to flux diversion from gibberellin synthesis[82]. This example demonstrates the importance of understanding the overall impact of single-gene manipulation on additional downstream products. Because secondary metabolic pathways are often undefined, a promising approach to effective metabolic engineering is the use of specific transcription factors[83,84]. Because many secondary metabolites are defense-related, their regulation is tightly coordinated both temporally and spatially in higher plants and presumably cell cultures, and transcription factors can be used to control either a whole pathway or specific branches to produce a desired terpenoid yield and distribution.

Currently, only two plant genomes have been completely sequenced—rice and *A. thaliana*—and therefore traditional "omics" analysis on plants and cell cultures has been largely limited to these systems. Some challenges include annotation of plant genes based solely on sequence homology to genes of known or predicted function in other systems[85]. For example, only 15% of putative plant CYP450s have been functionally characterized biochemically[85]. Therefore, annotation based on absolute knowledge of function is highly limited. Because terpenoids are so diverse and numerous it is challenging to identify all terpenoid and terpenoid-derived compounds through metabolomics analysis within a plant or cell culture system. For example, over 350 taxanes have been isolated[86,87], with more than 30 different taxanes accumulating simultaneously in cell culture[88]. Therefore, most metabolite analyses with *Taxus* spp. focus on identification and quantification of key taxane metabolites such as paclitaxel and baccatin III. However, identification and quantification of the complete taxane metabolite pool as well as other terpenoid-derived compounds present under particular conditions within a cell culture would further enable the design of strategies to enhance yield of desired taxanes, suggesting which pathways should be inhibited and which pathways should be induced. The integration of plant genomics, transcriptomics, proteomics and metabolomics datasets is in its infancy, but initial results are promising[85] and progress is being made in the mathematical modeling of plant metabolic pathways[89]. Genome-wide functional genomics analysis has primarily been limited to model plant systems, but recently a comprehensive profiling analysis of both transcripts (417) and metabolites (178) was performed in *C. roseus* cell cultures[90]. Gene-to-gene and gene-to-metabolite networks were generated, and they helped uncover regulatory differences in key alkaloid metabolic pathways. These data can be applied to better understand secondary metabolism and regulation in this important medicinal plant system.

Overall, exciting and important advances have been made in both characterization and redirection of metabolic pathways involved in terpenoid synthesis. Manipulation of plant metabolism through metabolic engineering is significantly more complicated than manipulation of the more well-studied microbial systems, but it is becoming increasingly possible owing to advances in systems analysis of plants and cell cultures. Plant cell culture technology is an excellent platform both for supply of terpenoid compounds and for identification and isolation of new terpenoid biosynthetic and regulatory genes. Currently, paclitaxel supply depends on plant cell culture technology and is a major success story for this emerging technology in the United States.

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COMPETING INTERESTS STATEMENT

The author declares no competing financial interests.

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