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MINI-REVIEW

# Perspectives and limits of engineering the isoprenoid metabolism in heterologous hosts

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**Abstract** Terpenoids belong to the largest class of natural compounds and are produced in all living organisms. The isoprenoid skeleton is based on assembling of C5 building blocks, but the biosynthesis of a great variety of terpenoids ranging from monoterpenoids to polyterpenoids is not fully understood today. Terpenoids play a fundamental role in human nutrition, cosmetics, and medicine. In the past 10 years, many metabolic engineering efforts have been undertaken in plants but also in microorganisms to improve the production of various terpenoids like artemisinin and paclitaxel. Recently, inverse metabolic engineering and combinatorial biosynthesis as main strategies in synthetic biology have been applied to produce high-cost natural products like artemisinin and paclitaxel in heterologous microorganisms. This review describes the recent progresses made in metabolic engineering of the terpenoid pathway with particular focus on fundamental aspects of host selection, vector design, and system biotechnology.

**Keywords** Metabolic engineering · Synthetic biology · Terpenoids · Artemisinin · Paclitaxel · Biosynthesis

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## Introduction: terpenoids and their role in nutrition and medicine

Terpenoids belong to the largest class of natural products with important medicinal and industrial properties, and today approximately 25,000 terpenoid structures have been elucidated and isolated from organisms like plants, microorganisms, insects, and various marine organisms (Gershenzon and Dudareva 2007). Despite the enormous structural complexity, terpenoids are constructed from two basic isoprene building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be coupled in nearly any way as defined by the enzymatic setup in a species.

Terpenoids serve a number of different functions: for instance, steroids for membrane fluidity and hormone signaling, carotenoids for photosynthesis in plants and as antioxidant agents (Howitt and Pogson 2006), quinones for electron transport (Berry 2002), and dolichol for protein glycosylation (Lehrman 2007). Especially in plants and in marine invertebrates, many terpenoids have been found with significant pharmaceutical importance. Indeed, the terpenoids encompass a vast number of bioactive compounds like artemisinin, paclitaxel, and menthol; most of these molecules are used in pharmaceutical, cosmetic, or food industries. Compounds like limonene, menthol, and carotenoids are available in discrete quantities in nature, and they can be produced and purified rather simply. But terpenoids like artemisinin and paclitaxel are rare and have low abundance, causing supply problems.

Furthermore, enzymes from both mevalonate (MVA) and 2*C*-methyl-D-erythrotol-4-phosphate (MEP) pathways can be targeted for medicinal purposes. Two examples, statins for MVA pathway and fosmidomycin for the MEP one, will be discussed shortly.

Inhibitors of the MVA pathway are widely used for treatment of hypercholesterolemia and osteoporosis. Statin

drugs inhibit the enzyme hydroxy methylglutaryl CoA (HMG-CoA) reductase (HMGR)-depleting cells of downstream isoprenoids including farnesyl pyrophosphate (FPP) and geranyl-geranyl pyrophosphate (GGPP). FPP is a key intermediate in synthesis of cholesterol, and its depletion results in decreased *de novo* cholesterol synthesis leading to increased low-density lipoprotein (LDL) receptor expression and decreased LDL cholesterol in vivo (Goldstein and Brown 1990).

One of the first natural inhibitors of HMGR was mevastatin (compactin, ML-236B), which was isolated from *Penicillium citrinium* in 1976 (Endo et al. 1976). When mevastatin was initially administered to rats, it inhibited cholesterol biosynthesis, but it also caused unacceptable hepatocellular toxicity. Subsequently, mevinolin or lovastatin, a more active metabolite, was isolated from *Aspergillus terreus* in 1979 (Alberts 1988, 1990). Compared to mevastatin, lovastatin was a more potent inhibitor of HMGR but did not cause hepatocellular toxicity when given to rats. Lovastatin, therefore, became the first of this class of cholesterol-lowering agents to be approved for clinical use in humans. Since then, several new statins, both natural and chemically modified, have become commercially available, including pravastatin, simvastatin, fluvastatin, atorvastatin, cerivastatin, and, most recently, pitavastatin and rosuvastatin (Illingworth and Tobert 2001).

While statins inhibit the HMGR from the MVA pathway, the second enzyme of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, converting 1-deoxy-D-xylulose-5-phosphate (DXP) into the branched compound MEP (Takahashi et al. 1998a), is the molecular target for fosmidomycin. The MEP pathway enzymes are highly conserved but show no homology to mammalian proteins (Lange et al. 2000). This implies that the use of specific inhibitors should result in novel antimicrobial drugs with broad-spectrum activity and little toxicity to humans. Fosmidomycin, a phosphonic acid derivative originally isolated from *Streptomyces lavendulae*, has been shown to have antibacterial activity against most gram-negative bacteria (Mine et al. 1980). It represents the first example of a new class of antimalarial drugs that block the MEP pathway localized in the apicoplast of *Plasmodium falciparum*. During the trials, fosmidomycin has been shown to be well tolerated and to act rapidly (Borrmann et al. 2004). Furthermore, its tolerance and efficacy towards acute urinary tract infection and uncomplicated malaria in humans were good (Lell et al. 2003).

Fosmidomycin is also extensively used as a tool to inhibit the biosynthesis of natural compounds coming from the MEP pathway, for example carotenoids (Laule et al. 2003), paclitaxel (Palazon et al. 2003), or various isoprenoids (Rodríguez-Concepción et al. 2004). Moreover, it has been proven to be a potent herbicide inhibiting the MEP pathway even at very low concentrations.

Metabolic engineering of rare and complex compounds, like artemisinin or paclitaxel, is a new concept in synthetic biology and considered as a new avenue for the production of rare and high-cost natural products. In both pathways, the majority of biosynthetic enzymes is known (Table 1), making the introduction of the complete primary and secondary pathway in heterologous hosts possible. The main idea is the complete transfer of a plant terpenoid pathway to microorganisms to optimize the biosynthetic production rate by fermentation of the genetically engineered microorganisms. But pathways in both bacteria and fungi meant to be used as target hosts are regulated through complex networks. Furthermore, the regulation of the terpenoid pathway is far from being simple, and control mechanisms are rather unknown. In this review, the challenges and promising recent developments in terpenoid pathway engineering will be discussed.

## General aspects of terpenoid biosynthesis in plants and microorganisms

Nature has developed two biosynthetic pathways for the production of the terpenoid backbones, namely MVA and MEP (Fig. 1). The MVA pathway has been known since 1958, when it was described by Bloch (1992). They received the Nobel prize in 1964 for the description of the cholesterol pathway. For almost 40 years, it was thought that all isoprenes were descended from the MVA pathway, although studies indicated that not all of the produced terpenoid structures can be explained solely by the MVA pathway. For bacteria, there was also no direct support for biosynthetic production of isoprenes through the MVA pathway (Rohmer et al. 1993; Lichtenthaler 2000).

In 1993, an alternative pathway for IPP biosynthesis was reported for bacteria (Rohmer et al. 1993) through intensive analysis of $^{13}C$ labeling in hopanoid production by the microorganisms studied. Soon after the publications describing the alternative pathway, others reported the same pathway in algae, diatoms, and protozoa (Lichtenthaler 2000; Kuzuyama 2002). The nomenclature for this alternative pathway was confusing as various names have been used in literature, like DOXP-, DXP-, MEP-, Rohmer-, or non-mevalonate pathway. Only recently, the pathway was officially named the "MEP pathway", according to the first official committed precursor in the biosynthetic route in *Escherichia coli* (Phillips et al. 2008).

Both pathways produce the same final product IPP; however, delivery of precursors depends on different metabolic routes. The MVA pathway is fed by the precursor acetyl-CoA obtained through the citrate cycle. The MEP pathway is mainly fed by D-glyceraldehyde 3-phosphate, produced as a product of the pentose phosphate pathway and pyruvate created from precursors descendent of a number of metabolic routes, including the citrate cycle and glycolysis

**Table 1** Engineering studies of the mevalonate pathway (alternative gene and protein names are given in parenthesis)

| Enzyme activity in pathway | | *S. cerevisiae* | | *A. thaliana* | | Engineering studies |
|---|---|---|---|---|---|---|
| Substrate and cofactors | Accepted by enzyme | Gene | Reference | Gene | Reference | |
| Acetyl-CoA | Acetoacetyl-CoA thiolase (**1**) | *ERG10* (YPL028W, LPB3, TSM0115) | (Hiser et al. 1994) | *AT5G48230.1* | (Carrie et al. 2007) | (Rodriguez-Vargas et al. 2002) |
| Acetoacetyl-CoA<br>Acetyl-CoA | HMG-CoA synthase (HMGS) (**2**) | *ERG13* (YML126C, HMGS) | (Rudney and Ferguson 1959) | *MVA1* | (Montamat et al. 1995) | |
| 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) | HMG-CoA reductase (HMGR) (**3**) | *HMG1* (YML075C) | (Basson et al. 1986) | *HMG1* | (Caelles et al. 1989; Learned and Fink 1989; Enjuto et al. 1994; Ohyama et al. 2007) | (Shimada et al. 1998)<br>(Verwaal et al. 2007)<br>(Suzuki et al. 2004) |
| NADPH | | *HMG2* (YLR450W) | | *HMG2* | | (Muñoz-Bertomeu et al. 2007) |
| Mevalonate<br>ATP | Mevalonate kinase (**4**) | *ERG12* (YMR208W, RAR1) | (Oulmouden and Karst 1990) | *AT5G27450.1* | (Riou et al. 1994) | |
| Mevalonate-5-phosphate<br>ATP | Phosphomevalonate kinase (**5**) | *ERG8* (YMR220W) | (Tsay and Robinson 1991) | | | |
| Mevalonate-5-diphosphate<br>ATP | Mevalonate pyrophosphate decarboxylase (**6**) | *MVD1* (YNR043W, ERG19) | (Toth and Huwyler 1996; Hélène et al. 1999) | *AT2G38700.1* | | |
| $\Delta^3$-Isopentenylpyrophosphate (IPP) | Isopentenyl diphosphate: dimethylallyl diphosphate isomerase (**7**) | *IDI1* (YPL117C, BOT2, LPH10) | (Anderson et al. 1989) | *IPP1*<br>*IPP2* | (Campbell et al. 1998) | |

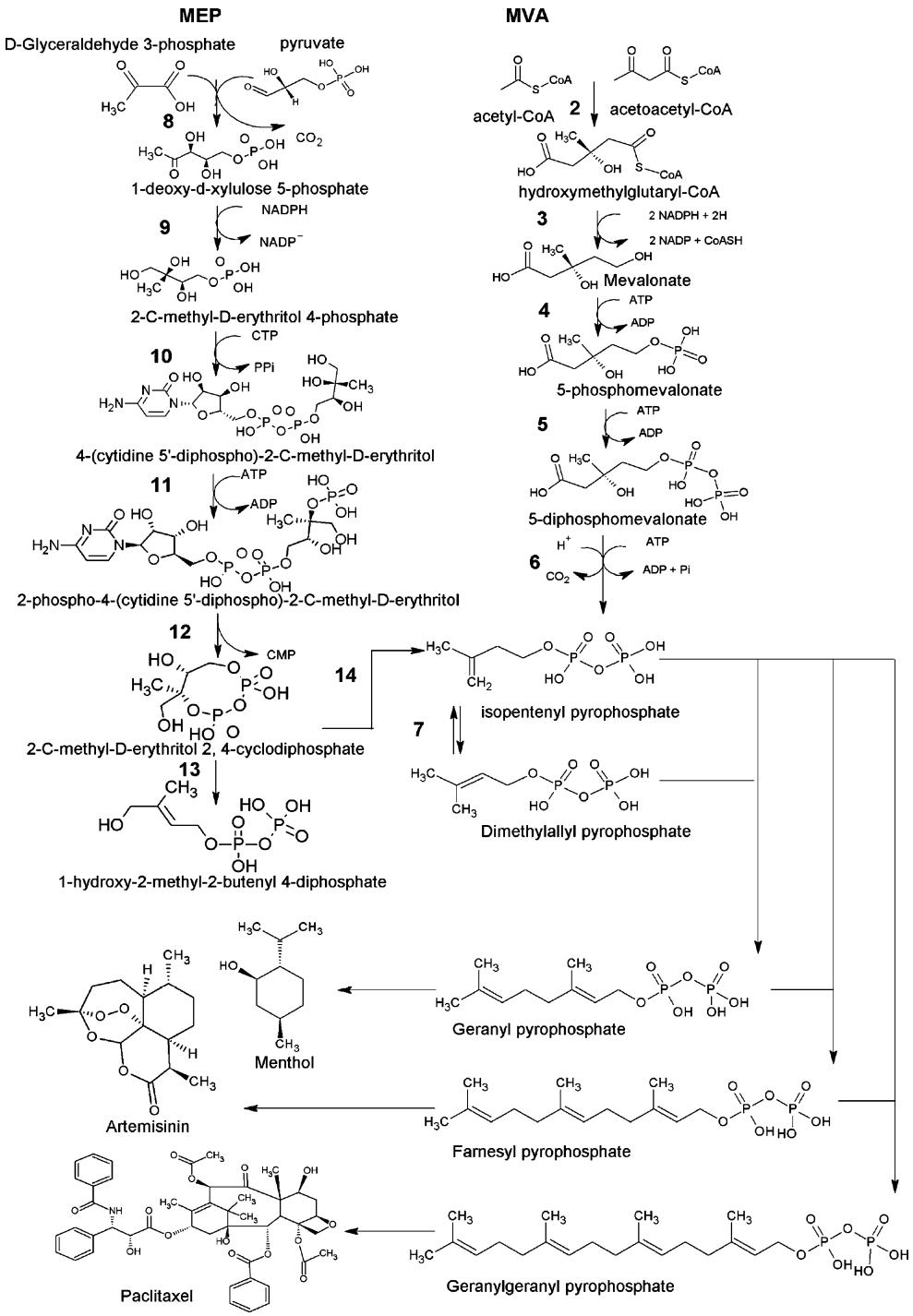


**Fig. 1** General overview of isoprenoid pathway from both the MVA as well as the MEP pathways, according to KEGG database (Kanehisa et al. 2008). Enzymes (*numbered*) are found in Tables 1 and 2. The overviews only show the enzymes that are unique for either the prokaryotic or eukaryotic organisms. We did not include the production step of acetoacetyl-CoA as this can be produced by prokaryotic cells. This prokaryotic pool of acetoacetyl-CoA has been successfully used as precursor for the production of isoprenoids by recombinant MVA pathway in *E. coli* (Harada et al. 2009)

(Kanehisa and Goto 2000; Kanehisa et al. 2006, 2008). However, in photosynthetic organisms and in anaerobic conditions, the MEP pathway is mainly fed through production of the same precursors by the Calvin cycle (Seemann et al. 2006; Lichtenthaler 2007).

Higher plants exhibit both pathways, while prokaryotes, with some exceptions (Boucher and Doolittle 2000; Martin et al. 2003), have the MEP pathway, and other eukaryotes may possess either the MEP or MVA pathway. In addition, higher plants have separated both pathways in different compartments. The MVA pathway is localized in the cytosol, while the MEP pathway is separated in plastids. The coexistence of both pathways in higher plants and also intracellular trafficking of precursors makes higher plants unique compared to prokaryotes and some eukaryotes having only one of them. This interchange of metabolites, called "cross talking," occurs in varying degrees between species and depends on physiological conditions (Bick and

Lange 2003; Hemmerlin et al. 2003; Laule et al. 2003; Dudareva et al. 2005; Hampel et al. 2005; Skorupinska-Tudek et al. 2008). The regulatory system controlling this cross talk is not yet fully understood (Kobayashi et al. 2007) and remains a major challenge in biochemistry and metabolic engineering.

Increasing the flux by single-enzyme introduction or exogenous precursor supply may improve productivity. The introduction of the *Saccharomyces cerevisiae* MVA pathway in *E. coli* allowed increased production of terpenoid precursors, but accumulation was documented to be toxic. The toxicity problem was solved by introducing a truncated HMGR (Pitera et al. 2007), which is not affected by downstream sterol-induced phosphorylation. The phosphorylation guides degradation (Goldstein and Brown 1990) of the mammalian (Jingami et al. 1987) and plant HMGR (Harker et al. 2003). For recombinant production of the carotenoid lycopene, it has been documented that part introduction of the MVA pathway in *E. coli* increased the lycopene production ten times compared to the original recombinant control (*E. coli* K12 MG1655). The highest yield was obtained when investigators switched the genetic background from *E. coli* K12 MG1655 to *E. coli* BL21 (DE3). This last strain can be of advantage because of its genetic adaption to recombinant protein expression, by a lack in certain proteases and the presence of genes for low-occurring tRNAs.

Various terpene synthases are known to convert the terpenoid backbone into pathway-specific structures; however, some of them are less specific and can produce more than one structure (Christianson 2008). This promiscuity can also be simulated by the introduction of specific point mutations within plasticity residues. This powerful technique is known as "directed evolution" based on site-directed mutagenesis. In this review, we do not focus on recent advances of directed evolution, but we recommend reviews from Platis and Labrou (2008) and Jäckel et al. (2008). As an example, Wilderman and Peters (2007) demonstrated that, after site-directed mutagenesis, a single mutation could change the product biocatalysis from abietadiene to pimaradien (Xu et al. 2007). Within these enzymes, converting the common terpenoid backbone, like geranyl pyrophosphate (GPP), FPP, or GGPP, in the first step, a divalent metal, generally magnesium, is used to stabilize the pyrophosphate group. After this first committed enzymatic step towards secondary relevant terpenoids, the pyrophosphate is lost and more species-specific enzymes (e.g., cytochromes) are in place to create the known broad diversity of structures (Croteau et al. 2005; Covello et al. 2007).

Sesquiterpenoids and diterpenoids are mostly characterized by macrocyclic rings as exemplified in artemisinin, paclitaxel, and ginkgolide. The ring closing step is performed by the enzyme class of terpenoid cyclases. A comparison between terpenoid cyclases showed that, despite the lack of sequence identity, structural similarity is high (Liang et al. 2002; Picaud et al. 2005). The major difference between terpenoid synthases derived from plant and microorganisms is the presence of an N-terminal sequence in plant terpene cyclases. This N-terminal sequence is similar to glucosyl hydrolase (Picaud et al. 2005) and is crucial for the enzyme activity (Back and Chappell 1996), though the specific function remains unknown. The diversity of the secondary terpenoids is caused by promiscuity, mutations, and various enzymes acting downstream on the terpene skeleton and may explain chemotypes within a family or species as we know for *Mentha x piperita* or *Thymus vulgaris*. This variation can be used for selection of highly producing individuals for commercial medicinal plant cultivation, commercial microbiological fermentation, and more sophisticated strategies in metabolic engineering.

Metabolic fluxes can differ among families, species, and even within a multicellular organism. An example is the production of astaxanthin by various hosts. Several studies were performed on the production capacities of yeasts, plants, and microalgae. They showed that wild-type production levels of astaxanthin differ dramatically between hosts. *Chlorella zofingiensis* (a microalga) produces about 0.1%, *Nicotiana tabacum* around 0.24% (Hasunuma et al. 2008), and *Xanthophyllomyces dendrorhous* as an industrial-used high producer between 0.4% and 2.5% of dry weight (An et al. 1989), and the highest production in a wild-type organism is the microalga *Haematococcus pluvialis* with production levels around 3% of dry weight (Lorenz and Cysewski 2000; Bhosale and Bernstein 2005). Next to accumulation levels, the produced enantiomer is also different in various species. For instance, in *X. dendrorhous* the all-*trans* isomer is mainly produced (Visser et al. 2005), where *H. pluvialis* produces mainly the all-*cis* isomer (Jackson et al. 2008).

## Metabolic engineering of the terpenoid pathway

### The concept of metabolic engineering

Metabolic engineering is considered as one of the major concepts in biotechnology. At the moment, genetic engineering allows the transfer of a biosynthetic pathway to any selected host. The isoprenoid pathway is an example how biosynthetic-relevant genes can be reassembled from different biological sources (e.g., plants, bacteria, and fungi) in a heterologous microorganism. Engineering of plant terpenoids into microbial hosts has been focused mainly on isoprenoid-derived compounds such as carote-

noids (Schmidt-Dannert et al. 2000), artemisinin, and paclitaxel (Newman et al. 2006; Withers and Keasling 2007). The production of carotenoids and artemisinin demonstrates that complex natural products can be produced by microbial fermentation with yields approaching commercial relevance. Development of a microbial production platform for the biosynthesis of complex terpenoids offers large-scale and cost-effective industrial production via fermentation, which is independent from climate (too dry, too wet) and cultivation risks (pest controls, weeds, soil conditions). Well-characterized and genetically fairly easy to manipulate heterologous hosts like *E. coli* and *S. cerevisiae* allow very specific engineering of biosynthetic pathways for increased yields and generating novel compounds. Although extensive engineering is frequently required to successfully reconstitute biosynthetic pathways in these hosts, the potential to manipulate the biosynthesis is a significant advantage when compared to engineering in poorly characterized host strains or nonmicrobial systems.

## Selecting microorganisms for engineering of terpenoid pathways

Commonly, the host *E. coli* and *S. cerevisiae* are favored in metabolic engineering, but other hosts can be used as well. Both laboratory domestic organisms share the advantages of having been studied over decades, and the elucidation of their genome, transcriptome, and metabolome is nearly complete; however, in silico models are yet to exist (Feist et al. 2009). In theory, the possibility exists to adapt every host for the production of natural compounds, but, as various organisms exhibit different genetic properties, they also react distinctively to genetic manipulations and to the introduced recombinant genes. By selecting a host other than *E. coli* or *S. cerevisiae*, it is wise to choose directly microorganisms with a genuine existing high precursor flux. For instance, the yeast *X. dendrorhous* is industrially known for carotenoid accumulation (Verdoes et al. 2003; Liu and Wu 2007; Niklitschek et al. 2008). Since this yeast is specialized to produce long-chain terpenoids, it is easier to use its ability for the production of carotenoid structures. An additional advantage is that they are already adapted to possible toxicity of high-level production.

Although nearly all organisms are able to produce terpenoid structures (Goldstein and Brown 1990; Hunter 2007), their strategy and diversity vary dramatically between species. Synthetic biology provides now the tools to overcome these problems and to explore the rich variety of enzymes by which nature is capable of creating imaginable structures. This can be exploited by metabolic engineers: by selecting the right host for recombinant expression, it is possible to increase production levels by minimal adaption of the genetic system.

Prerequisite for metabolic engineering of either a plant or microorganism for different classes of terpenoids is a profound understanding of the isoprenoid biosynthesis at a molecular level. The primary MVA and MEP pathways leading to the biosynthesis of GPP, FPP, and GGPP are well characterized. The knowledge about related secondary natural product biosynthesis is still insufficient. Artemisinin is an excellent example that early steps have been elucidated, but enzymes involved in the late-stage biosynthesis and largely responsible for construction of the trioxan ring skeleton remain yet unknown.

## Engineering the primary metabolism

The first enzyme committed to the MEP pathway (Fig. 1 and Table 2) is 1-deoxy-D-xylulose-5-phosphate synthase (DXS). This enzyme is documented to be a rate-limiting step in the MEP pathway (Estévez et al. 2001) and, as a consequence, it is often subject to overexpression with the goal to increase production of GPP and, in extension, monoterpenes.

A recent example of DXS overexpression in *Lavandula latifolia* resulted in a 4.6-fold increase of essential oils in leaves and a 1.74-fold increase in flowers compared with the control (Muñoz-Bertomeu et al. 2006). However, Botella-Pavia and Besumbes proved that hydroxymethylbutenyl diphosphate reductase (HDR), a late enzyme in the MEP pathway that simultaneously synthesizes IPP and DMAPP, functions as a rate-determining enzyme, rather than DXS in transgenic *Arabidopsis thaliana* grown under light conditions. In their experimental setup with recombinant *A. thaliana* expressing taxadiene synthase (TXDS), DXS was overexpressed, and taxadiene production increased by sixfold compared to the plant line solely expressing the taxadiene synthase. At the same time, constitutive coexpression of TXDS and HDR resulted in 13 times higher production of taxadiene (TXD; Botella-Pavía et al. 2004). This study clearly indicates that pathway regulation is essential and that more knowledge in this field is important for a better understanding of requirements in metabolic engineering and in flux improvement (Carrari et al. 2003).

A well-known regulation mechanism in the MVA biosynthetic pathway is represented by the enzyme HMGR (Fig. 1 and Table 1). This enzyme converts the substrate HMG-CoA to mevalonate and is a rate-determining step in the MVA pathway (Chappell et al. 1995). Several groups have successfully taken the approach to overexpress this gene to increase the production of downstream isoprenoids. This enzyme is inhibited by phosphorylation as a control mechanism and is stimulated by accumulation of downstream sterols. Accumulation of phytosterols in seeds was observed in transgenic tobacco plants expressing a mutated form of HMGR from *Arabidopsis thaliana*. The serine recognized by the HMGR-phosphorylase was changed to

**Table 2** Engineering studies of the non-mevalonate pathway (alternative gene and protein names are given in parenthesis)

| Enzyme activity in pathway | | *E. coli* | | *A. thaliana* | | Engineering studies |
|---|---|---|---|---|---|---|
| Substrate and cofactors | Accepted by enzyme | Gene | Reference | Gene | Reference | |
| Glyceraldehyde-3-phosphate<br>Pyruvate | 1-deoxy-D-xylulose-5-phosphate synthase **(8)** | *DXS* | (Lois et al. 1998) | *CLA1* | (Estévez et al. 2000) | (Muñoz-Bertomeu et al. 2006) |
| 1-deoxy-D-xylulose-5-phosphate<br>NADPH | 2-*C*-methyl-D-erythritol-4-phosphate synthase/1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) **(9)** | *IspC* (*DXR*, *yaeM*) | (Takahashi et al. 1998b) | *DXR* | (Schwender et al. 1999) | (Carretero-Paulet et al. 2006) |
| 2-*C*-methyl-D-erythritol-4-phosphate<br>CTP | 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-4-phosphate synthase **(10)** | *IspD* (*YgbP*) | (Rohdich et al. 1999) | *IspD* | (Rohdich et al. 2000) | |
| 4-diphosphocytidyl-2-*C*-methyl-D-erythritol<br>ATP | 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase **(11)** | *IspE* (*YchB*) | (Lüttgen et al. 2000) | *ATCDPMEK* (*IspE*) | | |
| 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate | 2-*C*-methylerythritol-2,4-cyclodiphosphate synthase **(12)** | *IspF* | (Herz et al. 2000) | *IspF* | (Hsieh and Goodman 2006) | |
| 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate | 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate reductase/hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase **(13)** | *IspG* (*GcpE*) | (Hecht et al. 2001) | *GcpE* | (Querol et al. 2002) | |
| 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate | 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (HDR) **(14)** | *IspH* (*LytB*) | (Rohdich et al. 2002) | *AT4G34350.1* (*IspH*) | (Hsieh and Goodman 2005) | (Botella-Pavía et al. 2004) |
| $\Delta^3$-Isopentenylpyrophosphate (IPP) | Isopentenyl diphosphate: dimethylallyl diphosphate isomerase **(7)** | *Idi* | (Hahn et al. 1999) | *IPP1*<br>*IPP2* | (Campbell et al. 1998) | (Lee et al. 2004) |

an alanine, causing insensitivity to the phosphorylation and thus reduced degradation. Though accumulation of phytosterols was observed only in seeds and not in leaves, the authors did not describe the presence of other isoprene derivates, whose concentration could be augmented by the expected increased presence of mevalonic acid (Hey et al. 2006). Another example of HMGR modification is the truncation of the N-terminal membrane anchor domain and expression of solely the catalytic C-terminal domain of the protein. The truncated form of HMGR (tHMGR) increased the flux through the MVA pathway as it is unresponsive to negative feedback by FPP (Gardner and Hampton 1999; Muñoz-Bertomeu et al. 2007).

Strategies to maximize IPP production are more complex and include more than single gene expression or expression of various endogenous enzymes from the MVA or MEP pathway. Expression of heterologous proteins or even complete heterologous pathways is a currently employed strategy (Rodríguez-Sáiz et al. 2007; Arsenault et al. 2008; Engels et al. 2008). Such an approach is described by the introduction of the MVA pathway from *S. cerevisiae* into *E. coli*, thereby obtaining 6.5-fold elevated production (112.2 mg/l) of amorphadiene (AMD) compared to amorphadiene synthase (ADS) containing *E. coli* (Martin et al. 2003). While expressing heterologous genes or pathways for overproduction of isoprenoids is highly desired, a serious risk is given by host-specific downregulation of the heterologous biosynthetic genes. There are examples where the engineered pathway was tuned suboptimal, on purpose, for maximal production. Pitera et al. (2007) gave a good example that the heterologous MVA pathway engineered in *E. coli*, which normally harbors solely the MEP pathway (Table 1), accumulated products of certain enzymes inducing growth inhibition or becoming toxic for the host. Only by tuning the gene expression did these bottlenecks of isoprenoid production became acceptable and relieved the induced growth inhibition. This was accomplished by monitoring the specific metabolic profiles of well-growing and growth-limited mutants. By titration of gene expression and analysis of metabolite production, HMG-CoA accumulation appeared as one of the reasons of growth inhibition. After introduction of the tHMGR, the rate of biosynthesis was increased, and growth was restored by approaching the growth rate of the wild type (Pitera et al. 2007).

Bottlenecks within pathways are natural and serve as control points within a native organism to regulate resource utilization and production of metabolites. Metabolic engineering strives to elevate these bottlenecks by adapting expression levels of native or heterologous proteins and inherently production rates and levels of metabolites. A way to empirically search for optimal tuning of genes and hence elevating bottlenecks in a pathway involves the creation of a library from the intergenic regions containing various

mRNA secondary structures, RNase cleavage sites, and ribosomal binding site sequestering sequences (Pfleger et al. 2006). Another parameter is the promoter, which has a significant influence on product yield, as described in a case of chromosomal replacement of endogenous promoter with the exogenous strong bacteriophage T5 promoter in *E. coli* (Yuan et al. 2006).

Parallel usage of transcriptomics, proteomics, and metabolomics becomes more often a choice of strategy to know the circuit before engineering it (Mercke et al. 2004; Ghassemian et al. 2006; Rischer et al. 2006). After engineering a pathway, it is desirable to analyze the metabolic profile to be able to compare before–after situations and detect effects on the pathway originating from more distant networks. The ultimate goal is to be able to predict which steps need to be engineered to maximize a metabolic flux through a desired pathway. This can be done by comprehensive $^{13}$C-glucose labeling studies in which the distribution of this label among the metabolites is calculated based on the known biochemistry of the participating pathways and, at the same time, keeping an account for general cofactors such as ATP, NADH, and NADPH (Suthers et al. 2007). Another technique, apart from standard analytical methods such as NMR, GC–MS, and HPLC, to detect higher carbon flux through a pathway is the use of biological sensors (Pfleger et al. 2007). The biosensor system is based on a strain where the MEP pathway has been permanently disrupted by deleting an early gene in the pathway. Gene disruption makes the cell auxotroph for isoprenoids due to lack of production of IPP and DMAPP (Pfleger et al. 2007). As a consequence, this strain can produce isoprenoid building blocks only from an engineered partial MVA pathway containing the enzymes downstream of HMGR or when MVA is supplemented to the media. The strain has further been engineered to express green fluorescent protein (GFP) constitutively, for real-time growth rate detection (Pfleger et al. 2007). As a result, the auxotrophic strain can be cocultivated with a second strain, producing mevalonate, to survive. The growing bacteria will give a fluorescent signal, which is correlated to the levels of mevalonic acid. The use of auxotrophic strains also gives way for plasmid stabilization in microorganisms like *E. coli*. An example is the cloning of a heterologous MVA pathway in an *E. coli* knockout, where the last step responsible for IPP production was deleted and rescued by a similar gene on the plasmid. By using an auxotrophic strain and the plasmid to rescue the IPP production, a higher plasmid stability was established (Kroll et al. 2009).

Predictions on metabolic fluxes have successfully been performed to identify target genes for knockout to increase lycopene production in *E. coli* (Alper et al. 2005). This approach has led to the construction of a knockout variant that produces 40% more lycopene compared to the parental strain. A recent approach to obtain maximum production levels was not only relying on single knockout or overexpression of single genes but performing a multidimensional target search by combining knockout and overexpression targets (Jin and Stephanopoulos 2007). A multidimensional search was performed by combining several one-dimensional search programs. Hereby, it becomes possible to screen in silico combinations of knockouts and overexpression patterns that would not have been detected by the use of solely a one-dimensional search. Based on initial selection of mutant candidates from in silico predictions, 40 mutants were constructed and screened for optimized carotenoids production, and one was identified to be superior and able to produce 16 mg of carotenoids per gram of cells in 24 h while grown in minimal media with glucose. Recombinant carotenoids production is a comparatively simple system to validate the use of in silico prediction models, as they can be detected easily by spectroscopy because of their typical yellow to red color (Klein-Marcuschamer et al. 2007).

Out of simplicity reasons, most engineering efforts have focused on the primary pathway to optimize carotenoid production and neglected parallel or distant pathways competing for precursors and cofactors. System biology opens the possibility to handle the interaction between biological networks at transcriptomic and at metabolomic levels. A good example is the work of Papon et al. (2005), looking outside the MVA and MEP biosynthetic pathways for nondirect regulation of IPP production. Here, supplementation of cytokinin and ethylene had a positive effect on the elevated expression level of three genes from the MEP pathway in *Catharanthus roseus* cell suspensions (Papon et al. 2005). In yeast, redirection of the carbon flux from competing pathways, such as sterols, into the desired production of terpenoids has proven to be most fruitful. By downregulating ERG9, a squalene synthase in *S. cerevisiae*, the production of the sesquiterpenoid AMD was increased fivefold compared to its control expressing only ADS (Paradise et al. 2008).

A novel strategy is introduced by the engineered increase of pathway competition, rather than to reduce this competition. The main idea is to overexpress an enzyme situated at a branching point in the biosynthetic network. One interesting branching enzyme is the acetaldehyde dehydrogenase in yeast competing for cytosolic acetaldehyde versus transport and use in mitochondria. With additional overexpression of a heterologous cytosolic acetyl-CoA synthase and the ADS, a metabolic sink was created to drive the carbon flux into the MVA pathway and produce AMD (Shiba et al. 2007).

High yields of carotenoids in cauliflower (*Brassica oleracea* L. var. botrytis) were accomplished when a metabolic sink was combined with overexpression of the carotenoid biosynthetic enzymes (Li and Van Eck 2007). In

plants, carotenoids are synthesized in the membranes of plastid and accumulated in chromoplasts which can store carotenoids with tremendous efficacy and act as a metabolic sink preventing possible negative feedback by the carotenoids on the biosynthetic pathway. By overexpression of *Or*, a gene involved in differentiation of plastids into chromoplasts, a five times increase in concentration of β-carotene was obtained in a heterozygous mutant and eight times increase in a homozygous mutant. An interesting fact is that *Or* did not affect the expression levels of carotenoid or MEP pathway biosynthetic genes (Li et al. 2001; Lu et al. 2006). However, when making use of organelles as production sites, it is important to consider the adverse effect of depletion of common metabolites that cannot be transported across membranes. As an example, depletion of acetyl-CoA in tobacco mitochondria, by the introduction and the expression of an acetyl-CoA hydrolase, resulted in a growth-affected phenotype (Bender-Machado et al. 2004). An opposite example is a case study where *E. coli* was engineered for carotenoid production with a heterologous MVA pathway supplemented with extra mevalonate. Carotenoid production resulted in a yield of 503 mg/l which is the highest level reported so far in *E. coli* (Yoon et al. 2007).

## Engineering the secondary terpenoid metabolism

In contrast to the primary metabolism, no standard biological components exist which would fit for all different secondary pathways. Secondary metabolite routes are per definition species specific, and therefore we have to expect a high diversity in structures between organisms in the terpenoid biosynthesis (Fischbach and Clardy 2007). Artemisinin is an excellent example since *Artemisia annua* produces low amounts of artemisinin, while its relatives *Artemisia absinthium* (Van Nieuwerburgh et al. 2006) and *Artemisia afra* (Van der Kooy et al. 2008) have no artemisinin at all. The properties of many terpenoids make them interesting for recombinant production, although many pathways are yet too complex or enzymes unknown; for this reason, it was chosen to discuss three case studies: artemisinin, paclitaxel, and astaxanthin.

### Case study: artemisinin

Artemisinin is a sesquiterpenoid lactone endoperoxide extracted from *A. annua* L. (Asteraceae; known as sweet wormwood). The activity of artemisinin against malaria parasite *P. falciparum* has been well established. In addition, it has been proven that artemisinin acts by selective inhibition of the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA). The same study showed that artemisinin distributes to a change in membranous structures in the cytoplasm of parasites inside erythrocytes (Eckstein-Ludwig et al. 2003). Examples of derivatives of artemisinin include artemether and artemisone, which are on the market or currently under development (Haynes et al. 2006).

The low production yield of artemisinin from *A. annua* (0.01–1% of dry weight) has led to difficulties in managing the demands while offering an acceptable price for most patients (Mutabingwa 2005; Liu et al. 2006). The wish to improve the overall supply of artemisinin at reduced market price has encouraged interest in molecular biology and biochemistry of artemisinin biosynthesis (Fig. 2; Bouwmeester et al. 1999; Mercke et al. 2000; Bertea et al. 2005; Ro et al. 2006; Covello et al. 2007). A recent study demonstrated that both the cytosolic MVA pathway and the plastidic MEP pathway might be involved in the production

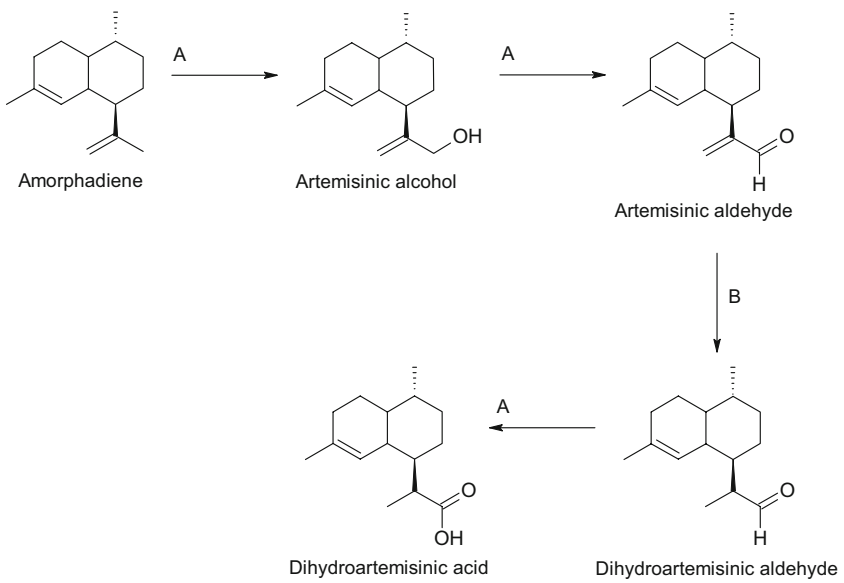


**Fig. 2** General overview of the artemisinin pathway from amorphadiene to dihydroartemisinin, according to Zhang et al. (2008).The enzymes are: *A* = cyp71AV1 and *B* = Dbr2

of IPP for artemisinin biosynthesis (Towler and Weathers 2007). The biosynthesis starts with the cyclization of FPP to AMD by ADS (Wallaart et al. 2001; Kim et al. 2006; Picaud et al. 2006). Subsequent enzymatic steps can possibly be performed in different orders. The main likely conversion scheme *in planta* for production of artemisinin include the oxidation of AMD to artemisinic alcohol followed by a further oxidation at C12 from alcohol to aldehyde by the cytochrome P450 CYP71AV1. In the next step, the carbon double bond at C11, 13 is reduced forming dihydroartemisinic aldehyde by artemisinic aldehyde reductase, followed by conversion of dihydroartemisinic aldehyde to dihydroartemisinic acid by dihydroartemisinic aldehyde dehydrogenase. The formation of 1,2,4-trioxane moiety is not fully understood yet and is suggested to occur in a nonenzymatic manner (Ro et al. 2006; Covello et al. 2007; Rydén and Kayser 2007). Identification of these enzymes is important towards the production of artemisinin in heterologous systems. The applicability of this strategy was proven when the two genes ADS and cytochrome P450 CYP71AV1 were expressed in yeast. The transgenic yeast produced more artemisinic acid in contrast to relative plant biomass (4.5% dry weight in yeast compared to 1.9% artemisinic acid, and 0.16% artemisinin in *A. annua*) and in a shorter time period (4–5 days for yeast versus several months for *A. annua*; Ro et al. 2006). When these two enzymes were coexpressed in yeast together with artemisinic aldehyde reductase (*Dbr2*), dihydroartemisinic acid was produced at a level of 15.7 (±1.4)-mg/l culture and the related artemisinic acid was produced at levels of 11.8 mg/l (Zhang et al. 2008). The production level of artemisinic acid was reduced as compared to the mutant without the artemisinic aldehyde reductase (29.4 mg/l), due to production of dihydroartemisinic acid. This is preferred because it is converted towards artemisinin more easily and cheaply.

Today, artemisinin production has also been improved by means of genetic transformation of *A. annua* plants (Liu et al. 2006). Cotton FPP synthase was transferred into *A. annua*, and the transgenic plant showed an artemisinin production of 8–10 mg/g dry weight, which is twofold or threefold higher than the wild type (Chen et al. 2000). In addition, the overexpression of the IPP transferase gene in *A. annua* increased artemisinin content by 30–70% in contrast to its control (Geng et al. 2001).

## Case study: paclitaxel

Paclitaxel is a well-known example of a clinical important natural product with dramatic problems of supply. Paclitaxel is isolated from the bark of the Pacific yew (*Taxus brevifolia*), which is a rare and slowly growing tree. In addition, the amount of the active constituent produced in the tree is about 1 mg per 750 kg of bark (Huang et al. 2001), and its harvest is destructive for the tree itself. An alternative production system is *Taxus* cell culture, which showed increased paclitaxel production after methyl jasmonate induction (Yukimune et al. 2000). Furthermore, fungi including endophytes have been found and published to produce taxanes, including paclitaxel, in amounts of 500-mg/l media (Zhao et al. 2008).

The biosynthesis (Fig. 3) of paclitaxel has been studied intensively, and some of the enzymes involved in the pathway have been elucidated (Jennewein et al. 2004; Nims et al. 2006) like TXDS, taxadien-5α-ol-*O*-acetyltransferase, cytochrome P450 taxadienyl acetate 10β-hydroxylase, 10-deacetylbaccatin III-10β-*O*-acetyltransferase, taxane 2α-*O*-benzoyltransferase, taxadiene 5α-hydroxylase, and taxoid 13α-hydroxylase (Dejong et al. 2006). The remaining enzymes include at least seven additional hydroxylases steps for oxidation of the taxane ring (at C1, C2, C5, C7, C9, and C13), the phenylpropanoid side chain, an oxidase for the formation of the C9 carbonyl function, the C13-*O*-phenylisoserinyl acyl-transferase, the side chain *N*-benzoyltransferase as well as enzymes responsible for the catalysis of the oxetane D-ring of paclitaxel (Walker and Croteau 2001). A functional expression of the TXDS was achieved in *A. thaliana* seedlings and leaves (Besumbes et al. 2004). In the transgenic plant leaves, expressing the gene for TXDS under an inducible promoter, the production of TXD reached 600 ng/gram of dry weight. These experiments show that significant increases in the production of TXD can be accomplished by selecting temporal and spatial coordinates of TXDS expression, establishing a platform technology for future metabolic engineering of taxanes in crop plants (Besumbes et al. 2004). The identification of alternative enzymes coming from other organisms can represent an additional advantage in the production of paclitaxel precursors. When Pacific yew extracts were treated with three enzymes originating from *Nocardioides albus*, *Nocardioides luteus*, and *Moraxella* spp., a C-13 taxolase, a C-10 deacetylase, and a xylosidase, respectively, the mix of taxanes was converted primarily to 10-deacetylbaccatin III, and the amount of this precursor was increased by four to 24 times (Patel 1998).

The first expression of the partial paclitaxel pathway up to taxadiene was conducted by Huang et al. (2001). A major drawback was the limited expression of cytochrome P450 in *E. coli*, reason why the authors have used *S. cerevisiae* for subsequent studies. In 2006, Dejong et al. (2006) expressed the first eight known genes from paclitaxel pathway (GGPP synthase included) in *S. cerevisiae*. More recently, the production of the early precursor baccatin III of paclitaxel was 0.7 mg/l up to 1 mg/l of medium. The downstream products could not be measured, indicating that the bottleneck was encountered at the 5α-hydroxylase level (Dejong et al. 2006). At the moment, the production of TXD

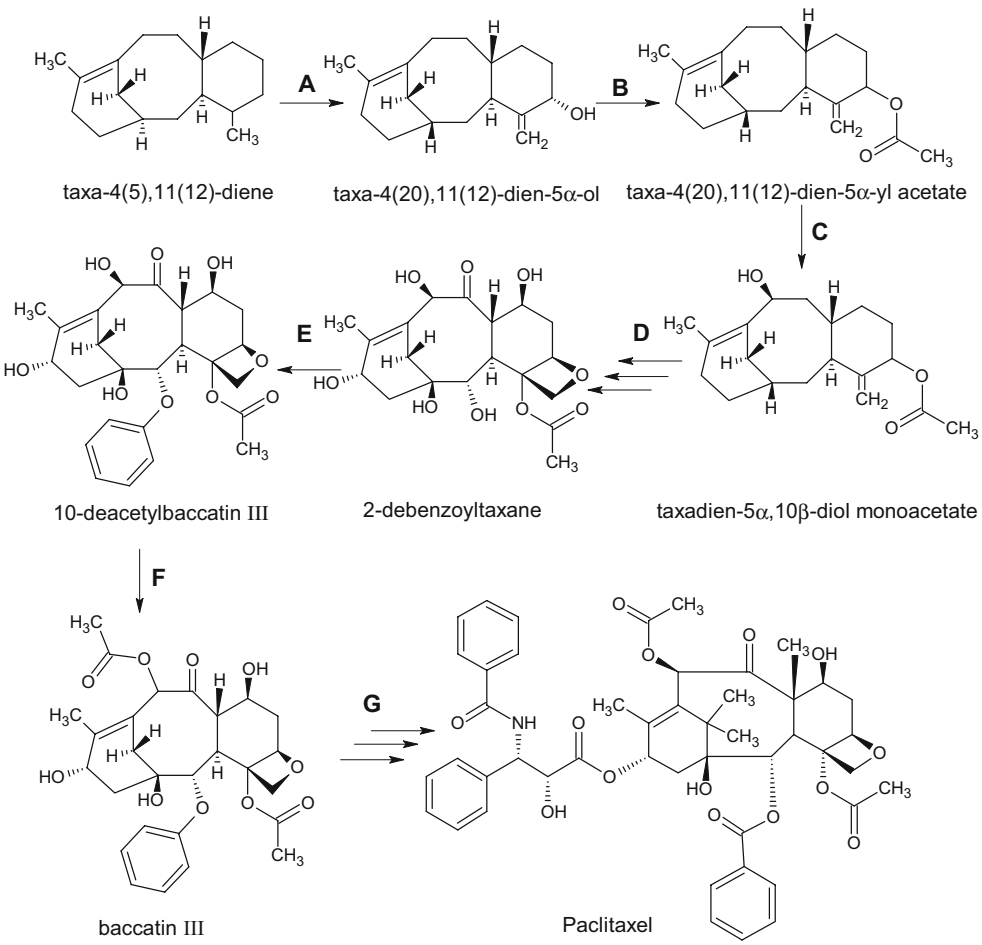


**Fig. 3** General overview of the paclitaxel pathway starting from taxadiene according to Walker and Croteau (2001). Several enzymes (*A*, *B*, *C*, *E*, and *F*) are known to function within this pathway; however, some important enzymatic (*D* and *G*) steps remain unknown. Known enzymes are: *A* = P450 taxadiene 5α-hydrolase, *B* = taxa-4(20), 11 (12)-dien-5α-ol-*O*-acetyltransferase, *C* = P450 taxane 10β-hydrolase, *E* = taxane 2α-*O*-benzoyltransferase, *F* = 10-deacetylbaccatin III-10-*O*-acetyltransferase

in *S. cerevisiae* has been increased further to 8.7 mg/l by the introduction of GGPP synthase originating from *Sulfolobus acidocaldarius* (Engels et al. 2008).

## Case study: astaxanthin

Carotenoids are a class of tetraterpenoid pigments that are often associated with flowers and fruits and are known as potent antioxidants (McNulty et al. 2008). All chlorophyll-containing photosynthetic organisms produce carotenoids, as do red yeasts and microalgae. They serve a number of industrial uses ranging from coloring agents for agriculture, food, and cosmetics to precursors of vitamin A in humans (Yeum and Russell 2002) and animals (Barkovich and Liao 2001) and feed supplements for poultry and fish (Rajasingh et al. 2006). They are also applied in prevention of macular degeneration (Landrum and Bone 2001; Bone et al. 2007) and suggested to be useful as anticancer agents (Bhuvaneswari and Nagini 2005). Within the plant, carotenoids are synthesized inside the plastids. Some examples of carotenoid compounds are β-carotene, lutein, lycopene, α-carotene, β-cryptoxanthin, and astaxanthin (Faulks and Southon 2005).

Astaxanthin is an oxygenated carotenoid with valuable industrial applications. It is commonly used as food additive in aquaculture to obtain the desired degree of pigmentation of flesh from salmon and trout allowing successful marketing. Furthermore, when astaxanthin was applied as a nutriceutical, several positive actions on degenerative diseases have been reported (Mayne et al. 1996; Chew et al. 1999; Wang et al. 2000). Astaxanthin is formed via the isoprene pathway: IPP is isomerized to DMAPP and proceeds on to GGPP via the enzyme GGPP synthase; subsequently, two GGPP molecules are condensed to form the colorless carotenoid phytoene. By dehydrogenation and two cyclization reactions, the precursor phytoene is converted into β-carotene. Finally, β-carotene is oxidized to yield astaxanthin. The existence of a monocyclic carotenoid biosynthetic pathway in *X. dendrorhous* was proposed by An et al. (1999). The monocyclic pathway diverges from the dicyclic pathway at neurosporene and proceeds through β-zeacarotene, γ-carotene, torulene, and 3-hydroxy-3′,4′-didehydro-β,-ψ-carotene-4-one to the end product 3,3′-dihydroxy-β,-ψ-carotene- 4,4′-dione (Visser et al. 2003).

The astaxanthin content of wild-type strains of *X. dendrorhous* is approximately 200–400 μg/g of dry yeast. This yeast can be easily adapted to fermentation in bioreactors, with reduced costs for expensive media. The level of astaxanthin in *X. dendrorhous* has to be increased

by a factor 50 to become competitive with industrial chemically synthesized astaxanthin. Natural astaxanthin from the alga *H. pluvialis* and likely also from *X. dendrorhous* is produced to supply markets. However, the demand for natural astaxanthin and consequently the astaxanthin price and competition position versus synthetic astaxanthin might increase in the near future (Guerin et al. 2003). Therefore, research has been focused on the improvement of astaxanthin production. One approach is the optimization of culture media and fermentation conditions. In general, the improvement of the astaxanthin levels by fermentation is not satisfactory for commercial exploitation. Another approach is the isolation of astaxanthin hyperproducing strains and/or carotenoid biosynthetic mutants. This is achieved by using random mutagenesis or by the manipulation of the expression of one or more astaxanthin-biosynthetic genes or regulatory genes in *X. dendrorhous* (Visser et al. 2003).

Astaxanthin is also produced in plants, like tobacco and potato (Mann et al. 2000; Gerjets and Sandmann 2006). For the expression in potato, the ketolase gene *crtO* from the *Cyanobacterium synechocystis* 6803 was transferred to plants giving an astaxanthin production of 1.8 μg/g of dry weight (Gerjets and Sandmann 2006). Transformation of tobacco leaves was performed using the gene *crtO* from the alga *H. pluvialis*, which encodes a β-carotene ketolase. In this case, the yield of astaxanthin was up to 83 μg/g fresh weight (Mann et al. 2000).

## Perspectives

Synthetic biology and inverse metabolic engineering have been welcomed as new approaches to simplify engineering of biological systems, here with the focus of isoprenoid pathways. The assembly of the primary and secondary pathways for more than 25,000 known terpenoids seems to be unrealistic today because of the structural complexity and diversity. But first approaches for complex structures, like paclitaxel and artemisinin, show that disassembling, construction, and rearrangement of DNA, based on promoter and encoding sequences, are possible and work right up for five genes. Challenges to be faced are to break down complex cellular networks in organisms not yet understood, to redesign intracellular networks to keep host systems balanced, and to trigger it for optimal production. In nature, there is a high variety of mostly unexplored microorganisms. Screening of terpenoid producers and gene discovery will help to identify more suitable "platform" organisms in the future. One strategy must be to look for microorganisms which express a natural product pattern as close as possible to the desired target structure. The main disadvantage is that these microorganisms lack genetic characterization, and the construction of suitable vectors with the regulating sequences remains a major problem. But it is a matter of time and the price of genome sequencing will decrease and become affordable for a routine lab procedure in a metabolic engineering lab. The combinatorial biosynthesis has several advantages in comparison to natural products isolation from known hosts or conventional organic synthesis. As discussed for paclitaxel, the natural product is not sufficiently available from *Taxus* species forcing to face the problem of deforestation and extinction of the species in Northern parts of China, India, and the Himalaya.

In the future, metabolic engineering will provide a third alternative to plant cell culture and chemistry to cover the high demand of this pharmaceutical drug for a reasonable price. But pathway engineering will provide more benefits compared to traditional approaches: stereochemically complex natural products like artemisinin, paclitaxel, sarcodictyin, eleuthoside can hardly be synthesized by conventional synthetic chemistry. This approach still needs to be adapted to minimize unwanted side products in the synthesis. A biosynthetic production can be performed at room temperature, under buffer media conditions and under atmosphere pressure. This is called white biotechnology and supports the transition from classical "dirty" organic chemistry. It is gaining enthusiasm worldwide, where companies strongly associated with chemistry are investing in alternatives based on evolved proteins to improve industrial processes and create novel compounds (Ernst&Young 2007). Upcoming white biotechnology will benefit extremely from synthetic biology and metabolic engineering and change the way to synthesize even small terpenoids.

But open questions still have to be answered. Can all complex terpenoid pathways like paclitaxel be transferred to engineered microorganisms? How to design more standardized parts for a uniformed system? How to redesign the cellular network of the host? Answers to these questions need an interdisciplinary approach. The advances in protein design, like directed evolution and construction of synthetic genes, will bring up optimized proteins adapted to the host environment. Bioinformatics, structural biology, and system biotechnology will make it possible to predict essential pathways and create standard methods for construction of enzymes and promoters to target enzyme activity where needed in the cell. Unfortunately, in the near future, it will not be realistic to buy a minimal cell out of the catalog to design a pathway of interest. However, new genetic tools for engineering will allow constructing a new generation of microorganisms that function as programmable biosynthesis machines.

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