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Metabolic engineering of microorganisms for the synthesis of plant natural products

Jan Marienhagen*, Michael Bott

Institut für Bio- und Geowissenschaften, IBG-1: Biotechnologie, Forschungszentrum Jülich, D-52425 Jülich, Germany

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ABSTRACT

Of more than 200,000 plant natural products known to date, many demonstrate important pharmacological activities or are of biotechnological significance. However, isolation from natural sources is usually limited by low abundance and environmental, seasonal as well as regional variation, whereas total chemical synthesis is typically commercially unfeasible considering the complex structures of most plant natural products. With advances in DNA sequencing and recombinant DNA technology many of the biosynthetic pathways responsible for the production of these valuable compounds have been elucidated, offering the opportunity of a functional integration of biosynthetic pathways in suitable microorganisms. This approach offers promise to provide sufficient quantities of the desired plant natural products from inexpensive renewable resources. This review covers recent advancements in the metabolic engineering of microorganisms for the production of plant natural products such as isoprenoids, phenylpropanoids and alkaloids, and highlights general approaches and strategies to gain access to the rich biochemical diversity of plants by employing the biosynthetic power of microorganisms.

1. Introduction

Plant secondary metabolites are not directly involved in growth, development or reproduction of plants, but have important functions in plant defense and signaling, or serve as pigments or fragrances. Unlike primary metabolites, which are mostly the same across the broad spectrum of all living organisms, plant secondary metabolites can vary from species to species and comprise a diverse array of complex chemical structures.

Since time immemorial, plant natural products (PNPs) such as isoprenoids, alkaloids and flavonoids, which are all plant secondary metabolites have been used by humans as colorants, flavors and fragrances. Due to the scarce availability and high price of natural products, synthetic compounds have gained more and more importance in the food industry in the last decades. However, nowadays customers demand natural products as a consequence of proven toxicological effects of some synthetic compounds (Sowbhagya and Chitra, 2010). Usually, PNPs have no nutritional value, but a diet rich in PNPs can boost the immune system (Licciardi and Underwood, 2011) or protect the human body from free radicals and are thus believed to prevent or suppress carcinogenesis (Guo et al., 2009; Tan et al., 2011). Ever since, PNPs were also of great importance as pharmaceutical drugs. In the last decades, PNPs were heavily exploited in drug discovery by high-throughput screening (HTS) of natural product libraries (Koehn and Carter, 2005) and almost half of all pharmaceuticals brought to the market from 1981 to 2006 were (plant) natural products or directly derived thereof (usually by semisynthetic modifications) (Newman and Cragg, 2007). This trend continues as more recent reports show, that 19 new (plant) natural product-based pharmaceutical drugs were approved for marketing worldwide in the last five years (Mishra and Tiwari, 2011). These numbers are noteworthy, especially when considering that pharmaceutical companies nowadays favor robotic HTS of vast libraries of synthetic molecules, which are generated by combinatorial chemistry (Newman, 2008). However, due to the limited chemical diversity and structural complexity of such synthetic libraries, as well as great success of natural product-derived

* Corresponding author. Tel.: +49 2461 61 2843; fax: +49 2461 61 2710.
E-mail address: j.marienhagen@fz-juelich.de (J. Marienhagen).

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drugs on the market in the last years, screening of untapped biological resources for new natural products is expected to be continued in the future (Li and Vederas, 2009).

Once a potent PNP is discovered, the ability to produce enough material for industrial or clinical applications becomes the main limiting factor. In the last years significant work has been made on improving PNP yields in native plant sources (Wu and Chappell, 2008) and also combinatorial biosynthesis in plants has made great leaps forward (Pollier et al., 2011). Despite all progress, final extraction from native plant sources is often problematic since PNPs tend to accumulate at low quantities over long growth periods. Furthermore, purification of the desired products requires separation from a multitude of often structurally very similar compounds and overall yields are also subject to environmental and regional factors (Chemler and Koffas, 2008). Recently, plant cell cultures of innately undifferentiated cambial meristematic cells from *Taxus cuspidata* proved to be promising for the production of several PNPs (Lee et al., 2010). Nevertheless, application of plant cell cultures is limited due to inconsistent performance and economic constraints associated with slow growth and low product yield (Kolewe et al., 2008). Total chemical synthesis or semisynthesis from isolated precursors poses many challenges for chemists and has little practical value for the production of complex PNPs with multiple chiral centers and labile connectivities (Pickens et al., 2011). Indeed, elegant methodologies for the synthesis of complex PNPs exist, but with an increasing number of separate steps, synthetic routes become impractical as the overall yield decreases, whereas the amount of resources consumed and (toxic) waste accumulated increase (Newhouse et al., 2009).

Microbial production of PNPs is a promising alternative with several advantages in comparison to above-mentioned approaches. Since many decades microorganisms have been increasingly used to produce a broad range of value-added compounds with numerous applications in the food, chemical and pharmaceutical industries. Important examples of these compounds include many (proteinogenic) amino acids, organic acids, vitamins, fine-chemicals, biofuels as well as a large number of pharmaceutical drugs (Becker and Wittmann, 2011; Du et al., 2011; Wendisch et al., 2006). Microbial production has the advantage of being much more environmentally friendly compared to chemical synthesis, since it avoids the use of organic solvents, heavy metals and strong acids or bases. Furthermore, in contrast to natural product synthesis in plants and plant cell cultures, microbial production based on renewable feed stocks is inexpensive and the rapid growth of microorganisms allows short production times. Unlike traditional synthetic chemistry-based routes, microbial fermentations are readily scalable from the lab bench to industrial-sized fermenters of several hundred cubic meters. In addition, the genetic accessibility of industrially relevant microorganisms such as *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, *Pseudomonas putida* or *Saccharomyces cerevisiae* allows construction of tailor-made recombinant strains by metabolic engineering to modify metabolic profiles according to individual production purposes. This includes also the heterologous expression of whole metabolic pathways of plants in microorganisms to confer the capability for PNP synthesis. Since recombinant microorganisms are usually void of competing pathways to the heterologously expressed pathway from plants, desired PNPs are typically made in the cell as chemically distinct substances (Chemler and Koffas, 2008). This in turn simplifies downstream processing in comparison to product purification from plants or plant cell cultures.

This review summarizes the recent advances in metabolic engineering of microorganisms for the synthesis of PNPs with a focus on isoprenoids, phenylpropanoids and alkaloids as well as the metabolic engineering strategies leading to PNP-producing microorganisms. Although many metabolic engineering approaches are PNP-specific, we highlight general challenges and technological strategies for microbial PNP synthesis and give an outlook on potential future developments of this research field.

2. Plant natural product synthesis in microorganisms

Considering that more than 200,000 PNPs are known (Osbourn and Lanzotti, 2009), it is astonishing that PNPs are derived from only a handful of primary metabolites formed in few central metabolic pathways (Fig. 1). Most of these chemically diverse compounds fall into three classes of natural products: firstly, isoprenoids including sterols and carotenoids; secondly, phenylpropanoids and phenylpropanoid-derived compounds such as flavonoids, stilbenes and lignans; and thirdly alkaloids as nitrogen containing PNPs. Other PNPs such as polyketides, sulfur- and nitrogen containing glucosinolates, or polyacetylenes as fatty acid-derived natural products are not covered by this review. Readers, who are interested these PNPs are referred to other recent reviews and research articles (Gao et al., 2010; Minto and Blacklock, 2008; Moldrup et al., 2011; Niraula et al., 2010; Sonderby et al., 2010).

2.1. Isoprenoids

With more than 40,000 known structures, including sterols and carotenoids, isoprenoids (also known as terpenoids), represent the largest class of PNPs (Bohlmann and Keeling, 2008). Many carotenoids and monoterpenes such as menthol, pinene or limonene for the cosmetic-, fragrance- and food industry are synthesized by plants in larger quantities and thus can be produced and purified rather simply from plants (Namitha and Negi, 2010; Schewe et al., 2009). However, many isoprenoids with significant pharmaceutical importance, most notable examples being the antimalarial drug artemisinin and the anticancer drug paclitaxel (Fig. 2), have a low abundance in plants. All isoprenoids originate from the five-carbon building blocks isopentenyl-pyrophosphate (IPP) and its isomer dimethylallyl-pyrophosphate (DMPP), both derived from either the mevalonate (MEV) pathway, employed in many prokaryotes and in the cytosol of higher plants, or the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway, which is used in most prokaryotes, all eukaryotes and the chloroplasts of higher plants (Fig. 1) (Kuzuyama and Seto, 2003; Rohmer et al., 1993). The nomenclature for the latter pathway was confusing as several other names, such as DXP-, DOXP-, Rohmer-, or non-mevalonate pathway have been used in literature. Only recently, the pathway was officially named the "MEP pathway", according to the first committed precursor in the biosynthetic route of *E. coli* (Phillips et al., 2008). Condensation of the two C_5-pyrophosphates and larger IPP- and DMAPP derived building blocks such as farnesyl pyrophosphate (FPP, C_{10}), geranyl pyrophosphate (GPP, C_{20}) and geranylgeranyl pyrophosphate (GGPP, C_{40}) give access to monoterpenes (C_{10}, e.g. menthol), sesquiterpenes (C_{15}, e.g. artemisinin), diterpenes (C_{20}, e.g. paclitaxel), triterpens (C_{30}, e.g. steroids), and tetraterpenes (C_{40}, carotenoids) (Fig. 2). All organisms are able to synthesize isoprenoid structures and hence can provide IPP, DMAPP and other longer isoprenoid backbones as precursors (Hunter, 2007). However, only a few microorganisms such as *E. coli* and *S. cerevisiae* have been engineered for production of plant isoprenoids. These microbes share the advantages of having been studied over decades, and thus their physiology, biochemistry, genome, transcriptome, proteome and metabolome is already well understood (Tyo et al., 2007). This knowledge as well as long lasting experience in industrial production of other small metabolites and proteins with both microorganisms make *E. coli* and *S. cerevisiae* excellent hosts or "platform organisms" for the production of isoprenoids as well as other (plant) natural products.

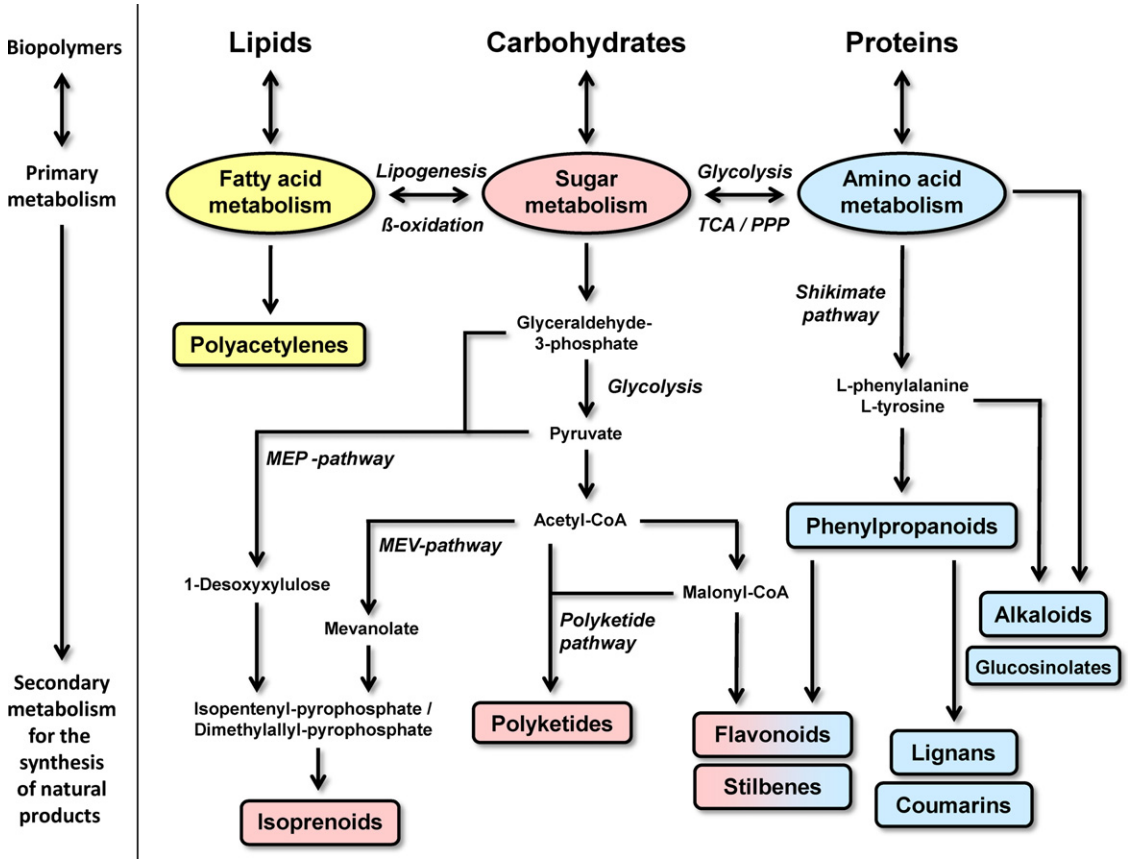

Fig. 1. Schematic overview of biosynthetic routes and precursors of the primary metabolism leading to the major classes of the plant natural products (PNPs). Individual modules of the primary metabolism and the PNP classes are color-coded to illustrate their respective origin from fatty acid, sugar or amino acid metabolism.

Isoprene, the simplest member of the isoprenoids is emitted from plants as volatile compound (Vickers et al., 2011) and represents an important feedstock in the synthetic chemistry industry. Here isoprene is used for the production of rubber, pesticides, synthetic oil additives and biofuels (Ducat et al., 2011). Currently, isoprene is produced from petroleum distillate as a byproduct of the oil refining process, but increasing oil prices and growing interest in sustainable production processes resulted in metabolic engineering efforts for isoprene production with microorganisms. Heterologous expression of a *Pueraria montana* (kudzu) isoprene synthase gene, catalyzing the conversion of DMAPP to isoprene in the cyanobacterium *Synechocystis* sp. PCC6803, enabled photosynthetic isoprene generation from CO_2 and H_2O (Lindberg et al., 2010). GC analysis of head space gases in sealed *Synechocystis* cultures revealed that 50 mg isoprene per gram dry cell weight per day could be achieved. In 2011, Zhao et al. constructed a short synthetic pathway for isoprene production in *E. coli* by introducing an isoprene synthase gene from *Populus nigra* (black poplar) (Zhao et al., 2011). Simultaneous heterologous expression of two genes from *B. subtilis* encoding for the first two enzymes of the MEP pathway finally resulted in the accumulation of 314 mg/L isoprene in fed-batch cultivation experiments. More recently, expression of an isoprene synthase from a poplar (*Populus alba*) along with the MVA pathway from *S. cerevisiae* in *E. coli* yielded up to 532 mg/L isoprene under fed-batch fermentation conditions (Yang et al., 2012). Currently, Genencor is working on the development of a reliable, high-efficiency fermentation-based process for isoprene, for example to produce rubber tires in a collaboration with The Goodyear Tire & Rubber Company (Singh, 2011).

The antimalarial drug artemisinin is a sesquiterpenoid lactone endoperoxide, extracted from *Artemisia annua* (Fig. 2). Many chemical strategies from various precursors for a total synthesis of artemisinin and its derivatives exist, but all require at least 8 steps and provide yields of less than 10% (Covello, 2008). A first step towards a microbial production of the immediate precursor artemisinic acid, which can easily be chemically converted to artemisinin, was made in 2006. In this study, an amorpha-4,11-diene synthase (ADS) gene from *A. annua* was heterologously expressed in *S. cerevisiae* to synthesize the intermediate amorpha-4,11-diene via cyclization of FPP (Lindahl et al., 2006). However, only low yields were reported (600 μg/L in 16 days batch cultivation). Subsequently, optimization of FPP precursor supply from the MEV pathway at the intersection of primary and secondary metabolism proved to be important for boosting amorpha-4,11-diene production (Ro et al., 2006). In the same manuscript, identification and expression of amorphadiene oxidase, a cytochrome P450 monooxygenase (AMO), along with the respective reductase (CPR) from *A. annua* allowed the production of artemisinic acid in significant amounts of up to 100 mg/L in yeast. Artemisinic acid production in yeast was further improved to reach 250 mg/L in shake flasks and 1 g/L in bioreactors by modulating the selection markers and the culture compositions (Ro et al., 2008). In the context of this work, global transcriptional analysis by yeast microarrays demonstrated that the expression of drug-resistance genes is induced as response to elevated artemisinic acid levels. More recently, a novel high-density fed-batch fermentation process was developed for the strain of Ro et al. (2006) yielding 2.5 g/L artemisinic acid with galactose as sole carbon source (Lenihan et al., 2008).

Alternatively, Keasling and co-workers reconstituted and optimized the MEV pathway of *S. cerevisiae* in *E. coli* (Martin et al., 2003), an approach which was later picked up for the production of

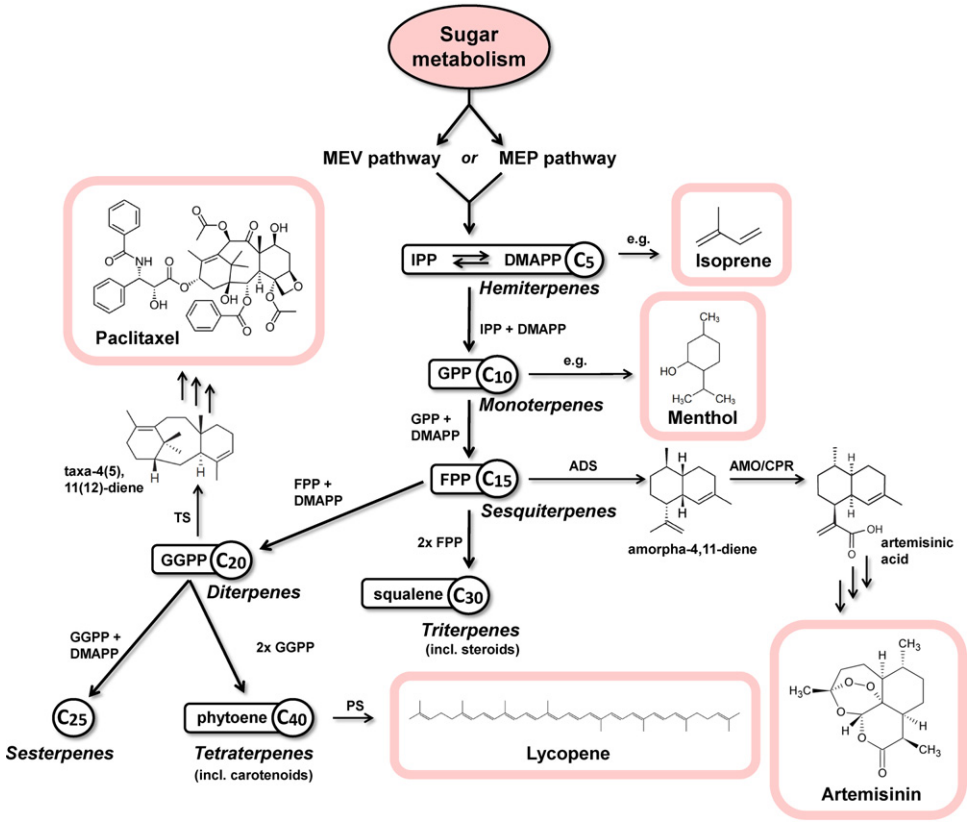

Fig. 2. Schematic representation of isoprenoid biosynthesis from isopentenyl-pyrophosphate (IPP) and dimethylallyl-pyrophosphate (DMAPP) to important precursors of various plant isoprenoids. *Abbreviations*: ADS, amorphadiene synthase; AMO, amorphadiene oxidase; CPR, cytochrome P450 reductase; FPP, farnesyl-pyrophosphate; GPP, geranyl-pyrophosphate; GGPP, geranylgeranyl-pyrophosphate; PS, phytoene desaturase; TS, taxadiene synthase.

isoprene with *E. coli* as discussed above (Yang et al., 2012). Balancing of the carbon flux through the heterologous MEV pathway in *E. coli* by altering individual gene expression minimized growth inhibition due to accumulation of the toxic 3-hydroxy-3-methylglutaryl-CoA intermediate and resulted in a further increase in isoprenoid production (Pitera et al., 2007). However, for the three-step oxidation of amorpha-4,11-diene to the artemisinin precursor artemisinic acid using the *E. coli* platform, functional heterologous expression of AMO and CPR is necessary. This was finally achieved by expression of the codon-optimized AMO and CPR genes from *A. annua* (Chang et al., 2007). Protein engineering efforts to replace the N-terminal membrane anchor of AMO with sequences from other P450 enzymes and optimized culturing conditions, were the key for production of more than 100 mg/L artemisinic acid with *E. coli*. In another protein engineering campaign, Keasling and coworkers used the long-chain fatty acid hydroxylase $P450_{BM3}$ of *Bacillus megaterium* and rationally engineered it to bind and oxidize amorpha-4,11-diene to artemisinic-11S,12-epoxide (Dietrich et al., 2009). This non-natural epoxide can serve as an intermediate in a novel semi-biosynthetic route towards artemisinin, as the chemical epoxide cleavage yielding dihydroartemisinic alcohol avoids the need for the difficult regioselective reduction of artemisinic acid to form dihydroartemisinic acid, as it is required when using AMO/CPR. An engineered *E. coli* strain expressing this $P450_{BM3}$-variant accumulated the epoxide to titers of 250 mg/L. Production of the precursor amorpha-4,11-diene with *E. coli* was further improved by replacing the second and third enzyme of the yeast-derived MEV pathway in *E. coli* with equivalent genes from *Staphylococcus aureus*, which more than doubled amorpha-4,11-diene titers (Tsuruta et al., 2009). Cultivation of the improved strain under carbon and nitrogen restriction finally yielded an average titer of 27.4 g/L amorpha-4,11-diene.

Currently, engineered transgenic plant lines of *A. annua* produce artemisinin up to 1.73 mg per gram dry weight (Alam and Abdin, 2011). Although engineered *E. coli* and *S. cerevisiae* strains are already capable of producing the semisynthetic artemisinin precursors amorpha-4,11-diene or artemisinic acid at high titers, further yield optimization and industrial scale-up will be required to raise artemisinic acid production to a level high enough to significantly reduce prices towards desired 100 US$/kg artemisinin or less (Covello, 2008). Very recently, Lévesque and Seeberger published the setup for a continuous-flow process that cheaply converts dihydroartemisinic acid into artemisinin (Levesque and Seeberger, 2012). This three-step reaction sequence includes a photochemically induced oxidation of dihydroartemisinic acid with singlet oxygen and is estimated to produce up to 200 g artemisinin per day. Combined with the microbial production of artemisinin precursors, this process could help to meet the ever-growing demand for artemisinin, especially in developing countries.

The diterpenoid paclitaxel (or taxol) (Fig. 2) as well as other structurally related analogs, originally isolated from the Pacific yew tree (*Taxus brevifolia*), have been approved for the treatment of ovaria, lung and breast cancer (Croteau et al., 2006). Due to only limited supply from the original source, paclitaxel is nowadays largely produced by *Taxus* cell culture methods (Roberts, 2007) or by semisynthetic means (Kingston, 2000). However, low productivity of cell cultures as well as potency and commercial success of paclitaxel aroused great interest in the microbial production of this diterpenoid. Compared to artemisinin, the biosynthetic route to paclitaxel is much more complex since half of the proposed

19 enzymatic steps are considered to be catalyzed by cytochrome P450 monooxygenases, not necessarily operating in a linear fashion (Kaspera and Croteau, 2006). The intermediate targeted for microbial production was taxa-4(5),11(12)-diene. Introduction of the genes for the geranylgeranyl pyrophosphate synthase (GGPPS) and the taxadiene synthase (TS) from *Taxus chinensis* established the early part of the paclitaxel pathway from IPP and DMAPP over GGPP to taxa-4(5),11(12)-diene in yeast (Engels et al., 2008) (Fig. 2). Again, elevation of precursor levels proved to be an efficient strategy to boost overall product titers. In yeast IPP and DMAPP are mostly used for steroid biosynthesis, and thus the MVA pathway is subject to feedback regulation, with the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase as the major regulatory target. N-terminal truncation of HMG-CoA reductase abolished feedback inhibition and expression of a codon-optimized TS from *T. chinensis* in combination with a GGPPS from the archaeon *Sulfolobus acidocaldarius* resulted in the accumulation of 8.7 mg/L taxa-4(5),11(12)-diene in shake flasks after 48 h of cultivation. More recently, Stephanopolous and co-workers abandoned the basic assumption that individual rational metabolic engineering efforts in the paclitaxel pathway are additive and shifted towards a combinatorial approach for pathway optimization (Ajikumar et al., 2010). This "multivariate-modular approach" to pathway engineering also addresses difficult to predict effects such as toxicity of intermediates, competing pathways or adverse cellular effects of plasmids employed for expression (plasmid-born metabolic burden), but requires screening of a larger number of genetically modified strains. For taxa-4(5),11(12)-diene production in *E. coli*, the pathway was partitioned into two separate modules consisting of the MEV pathway upstream of IPP and the taxa-4(5),11(12)-diene pathway downstream of IPP (Fig. 2). The promoter strength of individual genes (four of eight genes in the upstream module, both genes of the downstream module) and the copy number of plasmids was simultaneously varied. Subsequent screening of 32 strains for an improved product yield resulted in maximized product concentration and minimized accumulation of the toxic intermediate indole by balancing both modules against each other. In the course of these experiments, it became also obvious that a chromosomal integration of modules is advantageous with regard to the cell's metabolic burden caused by plasmid-borne gene expression. Efforts finally succeeded in increasing the taxa-4(5),11(12)-diene titer to approximately 1 g/L in fed-batch cultivations with glycerol as sole carbon source. This focused combinatorial approach has the potential of becoming a general strategy for engineering microorganisms for the production of other PNPs or any other metabolite. If high-throughput screens are available for the natural product in questions (which is not the case for taxa-4(5),11(12)-diene or any intermediate), this focused approach could be extended to a non-focused strategy. Such an extension would require screening of many more strains since more parameters could be varied simultaneously, but higher productivities can be expected.

The first P450-catalyzed oxygenation of taxa-4(5),11(12)-diene at the C5-position was also tackled by Stephanopolous and co-workers (Ajikumar et al., 2010). A previously discovered cytochrome P450 taxadiene 5α-hydroxylase (Jennewein et al., 2004) was fused with its corresponding reductase and engineered into the heterologous pathway of the best taxa-4(5),11(12)-diene-producing *E. coli* strain to extend the pathway by one step to taxa-4(20),11(12)-dien-5α-ol. The oxygenation was successful, but the overall productivity was significantly reduced with a concomitant increase in growth-inhibiting indole formation. The authors argued that the introduction of an additional medium copy plasmid bearing the gene for the engineered P450/CPR fusion-protein disturbed the carefully engineered balance in the upstream and downstream pathway. Very recently, a transcriptomics study revealed significant differences in pyruvate metabolism between the K- and B-lineages of *E. coli*, both engineered for the production of taxa-4(5),11(12)-diene (Boghigian et al., 2012). In the B-derivative, producing roughly 2.5-fold less taxa-4(5),11(12)-diene in comparison to the K-derivative, pyruvate kinase I (*pykF*) and phosphoenolpyruvate carboxykinase (*pck*) were upregulated, indicating that pyruvate is being produced from both phosphoenolpyruvate and oxaloacetate. Because pyruvate is one of the direct precursors (along with glyceraldehyde-3-phosphate) for isoprenoid synthesis through the MEP pathway, increasing pyruvate flux from this pathway by upregulation of *pykF* and *pck* is a possible explanation for the increased productivity of the K-lineage of *E. coli*. These findings highlight, that application of global transcript profiling can be used to reveal important differences between PNP-producing host cell metabolisms and thus provide clues for future metabolic engineering efforts to improve PNP titers.

Carotenoids are tetraterpenes, which are initially formed by condensation of two molecules of GGPP to yield phytoene as the first committed step of carotenoid synthesis (Fig. 2). In general, carotenoids are produced by all chlorophyll-containing photosynthetic organisms, many bacteria and some fungal species. Animals do not synthesize carotenoids de novo, but take them up with their diet and use carotenoids as pigments, as it is the case for salmon, shrimps and flamingos for example. Carotenoids such as β-carotene, lutein, lycopene, α-carotene and astaxanthin serve a number of industrial uses, ranging from food colorants and feed supplements to cosmetics (Chemler and Koffas, 2008). In addition, carotenoids have gained increasing attention due to their antioxidative properties, making them interesting as potential anticancer agents (Bhuvaneswari and Nagini, 2005). Although, compared to artemisinin or paclitaxel, being synthesized in larger amounts by their respective natural producers, carotenoids have been subject to extensive metabolic engineering efforts for production in microorganisms. This can be attributed to the high industrial demand, but also to convenient colorimetric screening properties which made carotenoids model products for the development of many new molecular tools for pathway optimization and enzyme engineering (Klein-Marcuschamer et al., 2007). Notably, in the case of carotenoids, engineering efforts were not limited to *E. coli* and *S. cerevisiae*, probably because other genetically accessible microorganisms such as the microalga *Haematococcus pluvialis* or the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) already accumulate high carotenoid levels in nature (Schmidt et al., 2011). Since aspects of metabolic engineering for the microbial production of many different carotenoids have been reviewed previously (Das et al., 2007; Klein-Marcuschamer et al., 2007), we focus on recent advancements for the production of lycopene only.

Lycopene is a non-provitamin A carotenoid that is responsible for the red to pink colors seen in tomatoes, pink grapefruits, and other foods. It has a reactive oxygen species (ROS) scavenging activity and is believed to induce enzymes of the cellular antioxidant defense system (Kelkel et al., 2011). The red-colored lycopene is directly derived from the colorless phytoene by a phytoene desaturase-catalyzed introduction of four double bonds (Fig. 2). Cyclization of lycopene leads either to the formation of β-carotene and its derivative xanthophylls (e.g. β-cryptoxanthin or zeaxanthin) or α-carotene and lutein. In addition, C_{50}-carotenoids, such as sarcinaxanthin of *Micrococcus luteus* or decaprenoxanthin of *C. glutamicum* are also derived from lycopene (Krubasik et al., 2001; Netzer et al., 2010). Thus, lycopene is not only a product of biotechnological relevance, but also an important intermediate for biosynthesis of other carotenoids. Screening of genomic libraries for improved lycopene production in the genetic background of an engineered *E. coli* strain revealed several novel target genes which proved to be useful for boosting lycopene production (Jin and Stephanopoulos, 2007). Based on these findings, 40 strains

were constructed in a systematic and combinatorial approach, comprising simultaneous knockout and overexpression of multiple gene targets. Screening of this "metabolic landscape" finally yielded a strain which accumulated 16 mg/g CDW of lycopene within 24 h in a batch shake flask (5 g/L glucose, M9 minimal medium). These results show that combinatorial approaches that allow the introduction of more than one metabolic perturbation lead to increased product titers in shorter time-frames. In a similar approach, random transposon mutagenesis was employed to identify combinatorial gene knockout targets in the background of eight strains bearing deletions or chromosome-based overexpression of known target genes (Alper and Stephanopoulos, 2008). Collectively, of 800,000 mutants screened, 290 were selected for further characterization. Visualization of the complex metabolic landscape showed that many paths to increased lycopene production are possible. Hence, during combinatorial screening, many strain variants should be retained when conducting an iterative search strategy. This strategy mimics protein engineering where better performing protein variants are pooled instead of selecting only the best performing mutein. Choi et al. followed a different approach and reported an in silico method which scans all metabolic fluxes in a metabolic model of *E. coli* to identify gene amplification targets for an enhanced production of lycopene (Choi et al., 2010). This strategy called *Flux Scanning based on Enforced Objective Flux* (FSEOF), not surprisingly, suggested increasing the flux through the upper glycolytic pathway to glyceraldehyde-3-phosphate (G3P) and the fluxes through the MEP- and lycopene biosynthetic pathway. Furthermore, FSEOF suggested increasing the flux into the citric acid cycle for the generation of NADH, NADPH and ATP, required for the synthesis of IPP and DMPP via the MEP pathway. Finally, wet-lab experiments overexpressing selected genes confirmed the predictions by increased lycopene titers. FSEOF is only one example of the increasing number of systemic approaches to strain improvement (Wiechert and Noack, 2011). Another issue tackled in the course of metabolic engineering of *E. coli* for lycopene production is the limited genetically stable expression of heterologous genes or whole pathways from plasmids. If such genes are plasmid-borne, displacement of productive plasmids with mutant nonproductive ones can confer growth advantages, since recombinant pathways in general place heavy metabolic burdens on the cell. Thus, allele segregation can lead to a slow loss of productivity within ~500 generations (Tyo et al., 2009). Tyo et al. developed the *Chemically Inducible Chromosomal Evolution* (CIChE) method, a chromosomal high gene copy expression system for engineering *E. coli* (Tyo et al., 2009). With CIChE, chromosomes with approximately 40 stable and consecutive copies of a recombinant pathway can be constructed. In a CIChE-constructed lycopene strain the increased yield relied on a high, fixed gene copy number, implying that the copy number may be as important as manipulation of metabolic networks. *Multiplex Automated Genome Engineering* (MAGE) is a combinatorial engineering approach which has demonstrated that a large number of chromosomally encoded genes for lycopene-synthesis can be targeted for modification at the same time (Wang et al., 2009). By MAGE genetic diversity can be introduced simultaneously at several predefined genetic loci in the chromosome via successive rounds of oligonucleotide-mediated allelic replacement. This combinatorial strategy was tested by targeting simultaneously 24 endogenous genes, previously shown to influence lycopene production in *E. coli*. An oligonucleotide for each gene was designed that randomized its Shine–Dalgarno sequence. After generating a library of variants by transformation with the randomized oligonucleotide mix for all 24 genes, screening of a small subset of variants resulted in variants with 5-fold increased lycopene production.

Due to their high-energy content and physicochemical properties similar to petroleum-derived liquid fuels, some isoprenoids gained attention as potential biofuels (Rude and Schirmer, 2009). Very recently, FPP-overproducing platforms based on *E. coli* and *S. cerevisiae* were used to overproduce the sesquiterpene bisabolene (Peralta-Yahya et al., 2011). Bisabolene is the immediate precursor of bisabolane, which has similar properties as D2 diesel and thus represents a biosynthetic alternative to petroleum-derived fuels. In *E. coli*, bisabolene synthase screening and codon-optimization followed by metabolic engineering of the heterologous MEV-pathway led to bisabolene titers of more than 900 mg/L. Similar yields could be obtained with *S. cerevisiae*. However, further metabolic engineering and process engineering efforts are needed to improve the platform strains and decrease production costs, because at an estimated price of ~$ 6 per gallon, bisabolane is currently much more expensive than D2 diesel.

2.2. Phenylpropanoids

Phenylpropanoids, synthesized from the amino acids L-phenylalanine and L-tyrosine, comprise a diverse array of compounds and constitute the major source of phenolic PNPs (Fig. 3). Phenylpropanoids and phenylpropanoid-derived products provide protection from ultraviolet light, defend against pathogens and herbivores, and serve plants as floral pigments and scent compounds (Vogt, 2010). Extraordinary antioxidant, antiviral, antibacterial and anticancer activities of many phenylpropanoids, especially flavonoids and stilbenes, aroused significant interest in the health-protecting effects of these compounds (Clere et al., 2011; Gresele et al., 2011). The name "phenylpropanoid" originates from the aromatic phenyl group and the three-carbon propene tail of cinnamic acid or *p*-coumaric acid. This core structure is directly derived from L-phenylalanine or L-tyrosine in the first committed step of phenylpropanoid biosynthesis via non-oxidative elimination of the amino group by a phenylalanine ammonia-lyase (PAL) or a tyrosine ammonia-lyase (TAL), respectively (Fig. 3). Subsequently, the phenylpropanoid core can be extensively modified by reduction, aromatic hydroxylation and *O*-methylation to give access to various phenylpropanoids, which in turn are precursors for flavonoids, coumarins, lignans and stilbenes.

Entry to the phenylpropanoid pathway from L-phenylalanine requires cloning and heterologous expression of PAL to synthesize cinnamic acid and an additional cinnamate 4-hydroxylase in C4H to convert cinnamic acid to *p*-coumaric acid (Fig. 3). Successful expression of PAL and C4H and synthesis of *p*-coumaric acid from L-phenylalanine was first demonstrated in *S. cerevisiae* by Ro and Douglas (2004). Alternatively, heterologous expression of a TAL from *Rhodotorula glutinis* in *E. coli* and *S. cerevisiae* resulted in the direct synthesis of *p*-coumaric acid from L-tyrosine in a single step (Vannelli et al., 2007). In the latter case, coexpression of a *p*-coumaric acid decarboxylase (DEC) from *Lactobacillus plantarum* yielded a strain able to produce up to 0.4 g/L p-hydroxystyrene (pHS) from *p*-coumaric acid, a chemical monomer important for the production of numerous industrial polymers (Qi et al., 2007) (Fig. 3). However, the maximum concentration of pHS was limited due to the toxicity of the product to the *E. coli* host. This product toxicity was overcome by the application of a two-phase water-decanol fed-batch cultivation setup for an engineered solvent-tolerant *P. putida* S12 strain (Verhoef et al., 2009). During fermentation of this strain which harboured genes for the bifunctional PAL/TAL enzyme from *Rhodosporidium toruloides* and the DEC from *L. plantarum*, 1.2 mM pHS accumulated in the water phase, while a final concentration of 147 mM (17.6 g/L) pHS was obtained in the 1-decanol phase. In plants, cinnamic acid and *p*-coumaric acid are precursors for the biosynthesis of coumarins, a class of PNPs which comprises many fragrances or precursors for the pharmaceutical industry (Fig. 3) (Wu et al., 2009). However, due to the limited knowledge of the biosynthetic pathways and numerous unknown cytochrome P450 monooxygenases and *O*-methyltransferases presumably involved

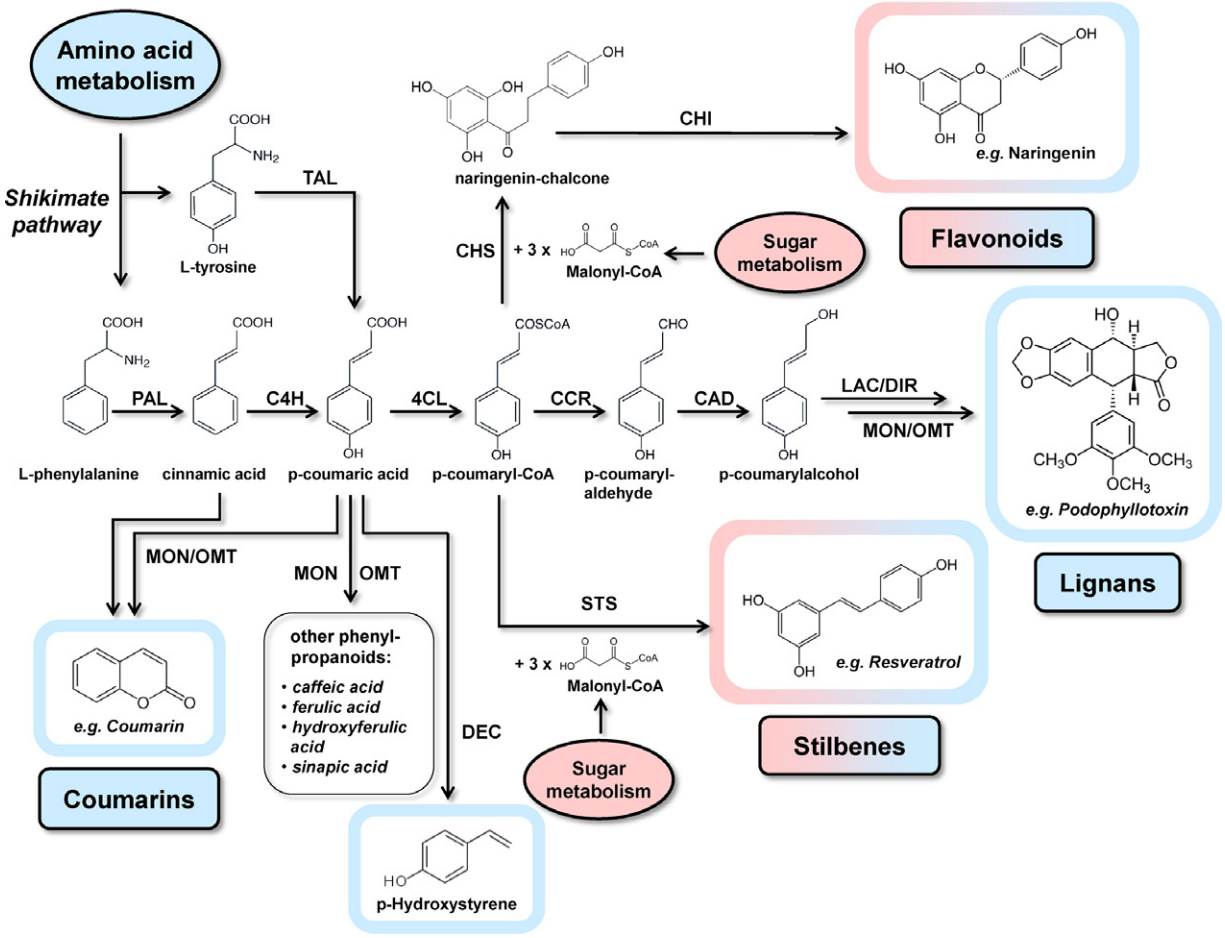

Fig. 3. Schematic overview of biosynthetic routes leading to various phenylpropanoid-derived plant natural products. Starting from either L-phenylalanine or L-tyrosine, different phenylpropanoids (*p*-coumaric acid, caffeic acid, ferulic acid, hydroxyferulic acid, sinapic acid) are formed. Phenylpropanoids are either direct precursors of lignans and coumarins or become part of the stilbenes and flavonoids, which are synthesized via a hybrid phenylpropanoid/polyketid-pathway requiring malonyl-CoA. *Abbreviations*: 4CL, 4-coumaryl-CoA ligase; C4H, cinnamic acid 4-hydrolase (cytochrome P450 monooxygenase); CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamyl-CoA reductase; CHI, chalcone synthase; CHS, chalcone synthase; DEC, p-hydroxystyrene decarboxylase; DIR, dirigent protein; LAC, laccase; MON, cytochrome P450 monooxygenase; OMT, *O*-Methyltransferase; PAL, phenylalanine ammonia-lyase; STS, stilbene synthase; TAL, tyrosine ammonia-lyase.

in the biosynthesis, coumarins have not been exploited for heterologous synthesis in microorganisms yet.

p-coumaryl-CoA, the coenzyme A (CoA) activated form of *p*-coumaric acid synthesized by a 4-coumaroyl CoA ligase (4CL), and other CoA-linked phenylpropanoids are the direct precursors of the large groups of flavonoids and stilbenes (Fig. 3). Chalcone synthases (CHS) and stilbene synthases (STS), both type III polyketide synthases, catalyze the iterative condensation of three acetyl units (derived from malonyl-CoA) to the CoA-linked starter molecule. Subsequently, this chain extension is followed by cyclization of the linear intermediate catalyzed by the same enzymes. Alternative intramolecular cyclization patterns lead to the different product scaffolds of chalcones and stilbenes (Austin et al., 2004). Chalcones are converted by chalcone isomerases (CHI) in a ring closing step to form the core flavonoid structure, which is composed of two benzene rings linked by a heterocyclic pyran or pyrone ring. Chemical diversification as a result of a multitude of possible chemical modifications, such as hydroxylations, methylations, methoxylations, acylations, *C*- and *O*-glycosylations further subdivides flavonoids into flavonols, flavones, flavanones, isoflavones, anthocyanins and catechins.

Since 2003, scientists are intensively exploring heterologous production of various plant derived flavonoids in microorganisms (Wang et al., 2011a). Recently, an economical process without feeding precursor amino acids for microbial production of naringenin from glucose was developed (Santos et al., 2011). This was achieved by introducing a heterologous pathway, consisting of a CHS and codon-optimized TAL, 4CL and CHI, into *E. coli* strains which were engineered for L-tyrosine production. Such strains were able to produce 29 mg/L naringenin from glucose and up to 84 mg/L naringenin from glucose when enzymes involved in fatty acid biosynthesis were additionally suppressed. The increase in naringenin titers hints at malonyl-CoA limitation during naringenin synthesis, since decreased fatty acid biosynthesis resulted in higher malonyl-CoA availability, which in turn boosted flavonoid synthesis. This observation confirms previous metabolic engineering efforts where increased intracellular malonyl-CoA pools turned out to be the key for improved flavonoid production (Fowler et al., 2009; Leonard et al., 2007; Miyahisa et al., 2005; Zha et al., 2009). In addition, an integrated computational and experimental approach aimed at improving the intracellular availability of malonyl-CoA in *E. coli* for improved naringenin production (Xu et al., 2011). The computational approach proposed a set of genetic interventions to up-regulate glycolytic reactions and channel flux towards malonyl-CoA in combination with a down-regulation of the tricarboxylic acid cycle (TCA cycle) to reduce the drain of carbon towards TCA cycle products. Combinatorial construction of *E. coli* strains, subsequent cultivation and determination of extracellular naringenin levels finally identified a variant that exhibited a 4-fold increase in the levels of intracellular malonyl-CoA compared to the wild type

strain, and produces 474 mg/L naringenin in a lab-scale fermentation process. Similar to malonyl-CoA, NADPH supply can also be an important limitation in the engineering of flavonoid biosynthesis as it could be demonstrated during metabolic engineering of *E. coli* for the production of leucoanthocyanidins and (+)-catechins (Chemler et al., 2010). Biosynthesis of these compounds requires the activity of NADPH-dependent reductases. Chemler et al. employed a stoichiometry-based modeling approach to identify combinations of gene knockouts that should improve intracellular NADPH availability and enhance flavonoid production. The best engineered strain, carrying deletions of phosphoglucose isomerase, phosphoenolpyruvate carboxylase and phospholipase, accumulated up to 817 mg/L of leucocyanidin and 39 mg/L (+)-catechin from glucose, corresponding to a 4-fold and 2-fold increase, respectively. Besides anti-inflammatory activity and anticancer activity, the flavonoid 7-*O*-methyl aromadendrin (7-OMA) has also been reported to stimulate insulin-mediated glucose uptake, implying that this compound is a potential candidate to improve the diabetic conditions in type 2 diabetes mellitus (Zhang et al., 2010). In 2011, Malla et al. engineered *E. coli* to employ various plant biosynthetic pathways for the production of 7-OMA from its precursor *p*-coumaric acid (Malla et al., 2012). Reconstruction of the pathway was achieved by implementing pathways for acetate assimilation and malonyl-CoA synthesis through heterologous expression of the acyl-CoA carboxylase α and β subunits, the biotin ligase and the acetyl-CoA synthetase from *Nocardia farcinica* for an increased precursor supply. In addition, genes for the 4CL from *Petroselinum crispum*, the CHS from *Petunia hybrid* and the CHI from *Medicago sativa* were expressed to first yield naringenin. This compound was then hydroxylated by a flavanone-3-hydroxylase (*Arabidopsis thaliana*) and methylated to produce 7-OMA in the presence of a 7-*O*-methyltransferase from *Streptomyces avermitilis*. Overexpression of the three genes for flavanone biosynthesis and two genes for flavanone modification, along with malonyl-CoA overproduction (four genes) yielded 2.7 mg/L 7-OMA upon supplementation with 500 μM *p*-coumaric acid in 24 h. In the same time-frame a strain expressing only the flavanone modification enzymes produced 30 mg/L 7-OMA from 500 μM naringenin. Interestingly, the production level of 7-OMA starting from *p*-coumaric acid is very low compared to the consumption of the precursor, indicating degradation of *p*-coumaric acid. Indeed, it was previously reported, that *E. coli* can employ various degradation pathways to utilize aromatic acids as sole carbon sources (Diaz et al., 2001). Hence, inactivation of these pathways could possibly improve stability of aromatic acids, including *p*-coumaric acid, and increase flavonoid production with this microorganism in the future.

In contrast to the long standing interest to produce flavonoids in microorganisms, microbial stilbene production has not received so much attention. However, in the last years the stilbene *trans*-resveratrol became renowned for its ability to prolong the lifespan of *S. cerevisiae*, *Drosophila melanogaster* (fruit-fly), *Caenorhabditis elegans* (round-worm) and the short-lived fish *Nothobranchius furzeri* (turquoise killifish) by activating sirtuin deacetylases (Fig. 3) (Knutson and Leeuwenburgh, 2008). Furthermore, *trans*-resveratrol has been hypothesized to play a role in the prevention of cardiovascular diseases and may also provide some protection against certain types of cancer and diabetes (Kiselev, 2011). Therefore, it is no surprise that these findings stimulated efforts to produce this stilbene heterologously in microorganisms for numerous purposes in pharmaceutical and food industries. The first report of the reconstruction of a biochemical pathway in a heterologous host to produce resveratrol dates back to 2003, when a 4CL from a hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) and a stilbene synthase (STS) from grapevine (*Vitis vinifera*) were introduced in *S. cerevisiae* to produce trace amounts of 1.45 μg/L resveratrol from the precursor *p*-coumaric acid (Fig. 3) (Becker et al., 2003). Another study explored the possible application of a 4CL::STS fusion protein (4CL, *A. thaliana*; STS, *Vitis Vinifera*) to increase resveratrol yields (Zhang et al., 2006). This 4CL::STS fusion, with both enzymes only separated by a three amino acid linker (Gly-Ser-Gly), synthesized eight times more resveratrol from *p*-coumaric acid compared to a strain expressing each enzyme individually (5.15 mg/L and 0.65 mg/L resveratrol, respectively). According to the authors, this increase in metabolic efficiency could be explained either by channeling intermediates between enzymes or by localizing two active sites in close proximity. Subsequent studies also explored stilbene production in *E. coli*, employing 4CLs and STSs from various plant sources, but always the precursor *p*-coumaric acid or other phenylpropanoic acids were provided (Beekwilder et al., 2006; Watts et al., 2006). In a more recent study, an attempt was made to functionally express a codon-optimized gene encoding the TAL from *Rhodobacter sphaeroides* in *S. cerevisiae*, but only low yields of resveratrol (1.06 mg/L) could be obtained without feeding any precursors (Wang et al., 2011b). In the same year, two different *E. coli* strains (BL21 Star and BW27784), two different promoters and a library of two 4CLs and nine STSs in different combinations were systematically examined to maximize resveratrol production from *p*-coumaric acid in *E. coli* (Lim et al., 2011). With an additional inhibition of fatty acid biosynthesis to improve malonyl-CoA availability for resveratrol synthesis, the best performing strain (*E. coli* BW27784, harboring the STS gene from *V. vinifera* and the 4CL gene from *A. thaliana* organized as a bicistronic operon on a pUC plasmid) accumulated 2.39 mg/L resveratrol starting from 2.46 mg/L *p*-coumaric acid in shake-flask fermentations in a 24 h period. This systematic study shows that the development of efficient production platforms for PNPs requires identification and engineering of enzymes, design of stable expression constructs, proper host selection in addition to an understanding of components on the molecular level. The "*screening approach*", starting from several components in a multitude of combinations for identifying and simultaneously eliminating bottlenecks during product synthesis, could be a promising strategy for the development of any PNP-production platform in the future. It furthermore reaffirms the "multivariate-modular approach" developed by Ajikumar and co-workers as powerful-screening approach towards improved taxa-4(5),11(12)-diene in *E. coli* (Ajikumar et al., 2010).

Since many biosynthetic enzymes involved in PNP synthesis exhibit relatively broad substrate specificity (Schwab, 2003), providing the flavonoid pathways with unusual precursors can result in the generation of new non-natural flavonoids and stilbenes (Horinouchi, 2008). However, engineering of microorganisms for the combinatorial biosynthesis of such non-natural phenylpropanoid-derived compounds would go beyond the scope of this review. Readers interested in this particular topic are referred to a recent review (Horinouchi, 2009).

Two subsequent reductions of CoA-activated phenylpropanoids such as *p*-coumaryl-CoA, coniferyl-CoA and sinapyl-CoA lead to their respective alcohols (monolignols) (Fig. 3). These monolignols represent not only the core building blocks of lignin as integral part of secondary plant cell walls (Neutelings, 2011) but are also precursors for the structurally very diverse class of lignans (Korkina et al., 2011). Derived from the oxidative dimerization of two monolignol units, and thus also termed dilignols, lignans have found many applications in medicine, such as podophyllotoxin (Fig. 3) in venereal wart treatment, or its semisynthetic derivatives (e.g. etoposide or teniposide) in cancer therapies (Ionkova, 2011; Lv and Xu, 2011). The oxidative dimerization is initiated by free-radical intermediate-forming laccases or peroxidases, but the final linkage pattern of the two phenylpropanol units is largely controlled by so-called dirigent proteins (DIRs) (Davin and Lewis, 2005). These DIRs capture and orientate the free radicals in such a way as to enable the formation of only one specific dilignol, whereas absence

of DIRs results in many non-specific coupling products. However, until now, expression of functional DIRs is restricted to cell cultures because of the complex glycosylation patterns of these proteins (Kim et al., 2002; Pickel et al., 2010). Furthermore, limited knowledge of the enzymes involved in further dilignol modification steps, namely difficult to express cytochrome P450 monooxygenases, dioxygenases, peroxidases and *O*-methyltransferases (Hemmati et al., 2010), has rendered attempts to produce lignans with microorganisms impossible to date.

2.3. Alkaloids

Alkaloids are nitrogen-containing, low-molecular-weight compounds, which are usually derived from amino acids such as L-histidine, L-phenylalanine, L-tyrosine, L-tryptophan, L-lysine and L-ornithine (except steroidal alkaloids) and found primarily in plants (Fig. 1). Approximately 12,000 different alkaloid structures are known, many of which serve as defense agents in plants against pathogens and herbivores (Julsing et al., 2006). Alkaloids are also known for their medicinal uses: morphine and codeine as analgesics; (+)-tubocurarine as a muscle relaxant and vincristine, vinblastine or camptothecin as anticancer agents to name only a few. Morphine, codein and (+)-tubocurarine are benzylisoquinoline alkaloids (BIAs), one of the largest and most structurally diverse family of alkaloids, which comprises approximately 2500 member compounds (Keasling, 2008). Production of BIAs was limited to plant cell and tissue cultures, since BIAs are among the most potent but complex L-tyrosine-derived alkaloids with rather long biosynthetic pathways in nature (more than 17 enzymes for morphine) (Julsing et al., 2006). However, the fact that the BIA-pathway intermediates thebaine and reticuline represent important precursors for synthetically derived analgesics or antimalarial and anticancer drugs raised interest in the reconstitution of the BIA pathway in microorganisms. In 2008, Minami et al. reconstructed a BIA pathway in *E. coli*, which consisted of monoamine oxidase (MAO) from *M. luteus* and four genes from *Coptis japonica* (goldthread) to produce up to 55 mg/L (*S*)-reticuline from 5 mM dopamine (Fig. 4) (Minami et al., 2008). However, the BIA pathway deviated from the natural pathway since the activity of the prokaryotic monoamine oxidase rendered the tedious expression of a cytochrome P450 monooxygenase (CYP80B1) and its corresponding reductase in *E. coli* unnecessary (Fig. 4). *S. cerevisiae* strains expressing either the cytochrome P450 monooxygenase CYP80G2 or the berberine bridge enzyme (BBE) were cocultured with these *E. coli* cells producing (*S*)-reticuline from dopamine to successfully synthesize magnoflorine or scoulerine, respectively from (*S*)-reticuline. Final yields of magnoflorine and scoulerine were 7.2 and 8.3 mg/L in the co-culture medium. Although coculture systems can be used to synthesize metabolites in microbial hosts, such systems face limitations in metabolite transport across the cell membranes of the microorganisms as well as challenges in fermentation scale-up. In another approach *S. cerevisiae* alone was engineered to produce both enantiomers of reticuline from the unnatural supplemented precursor (*R*,*S*)-norlaudanosoline in three steps (Hawkins and Smolke, 2008). Product titers of up to 150 mg/L were achieved by evaluating different combinations of three recombinant enzymes from *Thalictrum flavum* (yellow meadow-rue) and *Papaver somniferum* (opium poppy) and optimized enzyme expression levels. In addition, yeast strains producing (*R*,*S*)-reticuline were further engineered to produce intermediate BIAs along two branches from reticuline: the sanguinarine/berberine branch and the morphinan branch. Expression of three downstream enzymes from *T. flavum* and *P. somniferum* and a reductase partner from *A. thaliana* allowed the synthesis of scoulerine, tetrahydrocolumbamine and tetrahydroberberine from (*S*)-reticuline. In addition, expression of a human P450 enzyme and its native reductase resulted in the synthesis of the morphine precursor salutaridine from the R enantiomer of reticuline.

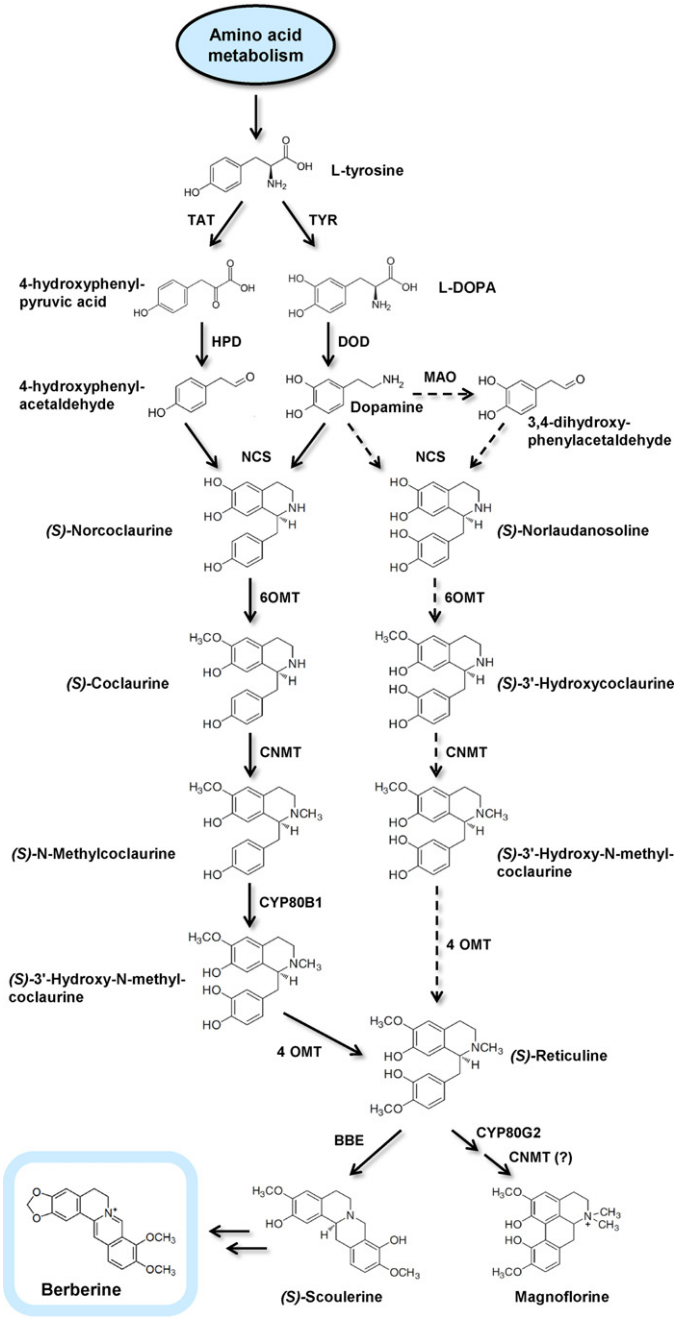

Fig. 4. Overview of the native benzylisoquinoline (BIA) pathway in plants and the synthetic BIA pathway constructed in *Escherichia coli*. Introduction of a monoamine oxidase (MAO) into the synthetic pathway circumvents the tedious expression of the cytochrome P450 monooxygenase CYP80B1 and its reductase. Reactions of the native plant pathway are indicated by solid arrows, dashed arrows indicate reactions solely being part of the synthetic pathway in *E. coli*. *Abbreviations*: 4′OMT, 3′-hydroxy-N-methylcoclaurine 4′-*O*-methyltrans-ferase; 6OMT, norcoclaurine 6-*O*-methyltransferase; BBE, berberine bridge enzyme; CYP80G2, cytochrome P450 monooxygenase; CNMT, coclaurine N-methyltransferase; DOD, L-DOPA decarboxylase; HPD, 4-hydroxyphenylpyruvate decarboxylase; NCS, norcoclaurine synthetase; TAT, tyrosine aminotransferase; TYR, tyrosinase.

Both approaches for microbial alkaloid production are, compared to the microbial synthesis of isoprenoids and phenylpropanoids, not economical, because they rely on the supplementation of expensive intermediate metabolites. More recently, a tailor-made alkaloid biosynthetic pathway from L-tyrosine was

constructed in L-tyrosine over-producing *E. coli* cells to produce (*S*)-reticuline from glycerol (Nakagawa et al., 2011). Key to success was the introduction of a tyrosinase (TYR) and its adaptor protein from *Ralstonia solanacearum* to convert L-tyrosine to L-DOPA, and a L-DOPA decarboxylase (DOD) from *P. putida* to decarboxylate L-DOPA to the BIA precursor dopamine (Fig. 4). This short pathway closed the gap between L-tyrosine and the synthetic pathway to (*S*)-reticuline via the MAO from *M. luteus* previously established by the same group (Minami et al., 2008). This engineered *E. coli* strain has one gene knockout and carries four overexpressed genes on a plasmid to turn *E. coli* into an L-tyrosine overproducer. Additionally, this strain bears the synthetic pathway from L-tyrosine to (*S*)-reticuline, which is constituted of eight genes originating from four different organisms. The recombinant strain could produce up to 46.0 mg/L of the BIA intermediate (*S*)-reticuline from 15 g glycerol in a jar fermenter within three days, suggesting that the fermentation platform would enable low-cost production of many different alkaloids. The amount of (*S*)-reticuline produced with this engineered *E. coli* strain already exceeds the amount that can be purified from plants, nevertheless the conversion efficiency from glycerol is only 0.15%. However, the recently published optimization of (*S*)-reticuline production with the same *E. coli* strain in shake flask cultures instead of the previously employed jar fermenter setup will allow parallel cultivation of many engineered strains to speed up the strain development for microbial alkaloid production (Nakagawa et al., 2012).

3. Concluding remarks and outlook

The growing number of successful examples for microbial synthesis of PNPs shows that metabolic engineering of microorganisms for PNP production is a powerful approach to gain access to bioactive compounds which accumulate only at low quantities in their naturally producing plant or are too complex to be synthesized by total chemical synthesis. Especially advances in the knowledge of the biosynthetic pathways in plants and identification of the respective genes and enzymes involved have driven the rational development of high-producing strains in the last decade. However, as is already evident in the most recent literature, future successful endeavors will not only require the functional integration of complete or partial heterologous pathways into the respective microbial host, but also the adaptation of the cellular context of the host organism to optimize product titers. Thus, understanding and engineering at a systems level will be important to fine-tune the metabolic interplay between host metabolism and the respective heterologous pathway.

Currently, *E. coli* and *S. cerevisiae* are employed for the microbial synthesis of almost all PNPs. However, with the availability of new molecular tools and affordable and fast genome-sequencing technologies, new platform organisms with different properties are emerging. For example, due to their extraordinary metabolic versatility, aspergilli are used for the production of a variety of products such as organic acids, pharmaceuticals and proteins. In contrast to bacteria and yeast *Aspergillus* can tolerate high temperatures (10–50 °C), a wide range of pH (2–11) and salinity (0–34%), and utilize diverse biopolymers such as starch, (hemi-)cellulose, pectin or xylan (Meyer et al., 2011). Several genome sequences of industrially relevant species as well as tools for their genetic manipulation are available, making *Aspergillus* species promising organisms for the production of selected PNPs.

Despite all advances, up to now several groups of PNPs such as coumarins, lignans or certain alkaloid families, cannot be produced by microorganisms. This is mostly due to long biosynthetic pathways with many steps which are catalyzed by enzymes of unknown identity (Leonard et al., 2009). In the last years, genetic information of more than 70,000 plants became available and eight plant genome sequences have been fully determined today (http://www.plantgdb.org; http://www.ncbi.nlm.nih.gov/genomes/PLANTS/) (Duvick et al., 2008). Considering the dramatic proceedings in DNA sequencing technologies in terms of accuracy, speed and costs, as well as more advanced bioinformatic algorithms for sequence analysis and annotation, one can expect that much more genetic information for gene mining will become available in the near future.

Recent examples show that plant enzymes difficult to express in microbes such as cytochrome P450 monooxygenases or *O*-methyltransferases can be successful engineered by rational design or evolutionary strategies to be functional in microbial hosts. Advances in methodologies for generating high-quality libraries of enzyme variants (Shivange et al., 2009) and novel HTS technologies (Yang and Withers, 2009) will lead to an accelerated engineering of enzymes for heterologous PNP synthesis. When all genes coding for the enzymes of a particular pathway are available, rapid assembly of (multicistronic) genetic constructs is the next step towards the microbial synthesis of a PNP. In recent years several new methods for rapid cloning of single genes and synthetic operons have become available to speed up the construction of synthetic pathways (Blanusa et al., 2010; Shao and Zhao, 2009). However, since metabolic intermediates or the enzymes themselves can be cytotoxic, fine-tuning of gene expression is important to achieve an optimal flow through the heterologous pathway and optimized product titers. In addition to varying the copy number of individual genes, the ability to control the relative amounts of individual enzymes at the transcriptional and translational levels is a promising strategy to fine-tune metabolic pathways. Promoter libraries have been developed for both platform organisms *E. coli* (Alper et al., 2005) and *S. cerevisiae* (Nevoigt et al., 2006) to confer a range of expression levels to different genes. Since genes in prokaryotic hosts are often arranged as an operon under the control of a single promoter, a library of tunable intergenic regions that control expression levels using differences in mRNA secondary structures, RNase cleavage sites, and ribosome binding site-sequestering sequences can be helpful to control relative amounts of individual enzymes on the translational level (Pfleger et al., 2006). More recently, a method for predictable control of protein expression based on the design of Shine–Dalgarno sequences was also developed (Salis et al., 2009) to aid in the design of genetic constructs for heterologous gene expression.

Innovative synthetic biology approaches for pathway and genome engineering are expected to play an increasingly important role for microbial PNP synthesis. For example, instead of integrating a heterologous pathway into an existing microorganism and reciprocal adaptation of host and pathways, future biotechnologists may directly create the synthetic producer host with a tailor-made metabolic network. Recent work on the synthesis, assembly and cloning of complete prokaryotic genomes shows that synthetic genome engineering may become reality in the future (Lartigue et al., 2009). In contrast to this bottom-up approach, construction of genome-minimized hosts (top-down) currently seems to be a more feasible approach. The idea is that by deleting nonessential genes one can optimize PNP synthesis by redirecting cellular resources toward only those pathways that are necessary for growth and PNP biosynthesis. Besides a few other microorganisms, *E. coli* has been the main target of genome minimization efforts, which have resulted in strains with a genome size reduced by up to 15% (Posfai et al., 2006). These strains show no growth deficiencies, but unanticipated beneficial properties: high electroporation efficiency and accurate propagation of recombinant genes and plasmids that were unstable in other strains.

With the application and integration of the latest tools and strategies in systems biology, protein engineering, process

engineering, high-throughput technologies for cultivation, screening and analytics, metabolic engineering of microorganisms for PNP synthesis is expected to become increasingly powerful. Together with the advances in the field of synthetic biology, future metabolic engineering efforts of microorganisms will greatly expand the number of PNPs that can be produced economically in sufficient amounts.

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