Phenylpropanoids and phenylpropanoid-derived phenolic compounds such as flavonoids, anthocyanins, or stilbenes are secondary plant metabolites which serve as pigments and scent compounds or provide protection against environmental stress. Due to their antioxidant properties they also have been widely recognized for their benefit on human health. Traditionally, such compounds are extracted from their natural plant sources, but this approach is limited by low abundance and environmental, seasonal as well as regional variations in yield. Chemical synthesis is not a true alternative for the large scale production of more complex phenylpropanoid-derived substances since chemical synthesis becomes commercially unfeasible as the structural complexity of these plant natural products increases. In the last years, many biosynthetic pathways for plant natural products have been elucidated through the advancements in DNA sequencing technologies. In combination with new recombinant DNA technologies this technical progress opens the door toward the functional integration of full biosynthetic pathways for the synthesis of phenylpropanoids and phenylpropanoid-derived compounds in microorganisms. We believe that this approach has great potential to provide sufficient quantities of the desired plant natural product from cheap and renewable resources. This commentary highlights recent advances in the microbial production of phenylpropanoid-derived compounds with an emphasis on flavonoids and stilbenes.

Introduction

Phenylpropanoids (PPs) and PI-derived compounds constitute the major source of plant phenolic compounds, which have found numerous applications in food industries as colorants, fragrances and flavoring agents. However, today their health promoting qualities are attracting most of the attention. Many studies suggest that various phenylpropanoid-derived compounds possess health-protecting effects against cardiovascular diseases, cancer, diabetes, and Alzheimer disease. Not surprisingly, the pharmaceutical industry has already reaped the benefits of the positive health effects of PP-derived compounds and other (plant) natural products. In the area of cancer treatment alone, more than 48% of all novel structures approved as therapeutic agents from around the 1940s to date, were natural products or direct derivatives thereof. Considering the vast chemical diversity of natural products, the percentage of PI-derived compounds and other (plant) natural products among newly discovered drugs is expected to increase in the future. With an increasing demand comes the need for efficient production processes to provide the required quantities for industrial or clinical applications. PPs can be extracted from their native plant sources, but they usually account for less than one percent dry weight of the plant only. This strategy is also limited by slow growth of plants, environmental and regional factors affecting overall yields and difficult separation from structurally similar compounds during purification.

Putting bugs to the blush

Metabolic engineering for phenylpropanoid-derived products in microorganisms

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Although progress has been made in the metabolic engineering of plants for increased synthesis of PPs in the last years, their application as production hosts is still limited.10,11 Historically, total or semi-synthetic approaches have been used to meet the demand,13 but the structural complexity of PPs and PP-derived compounds, often possessing multiple chiral centers and functional groups, requires a large number of separate steps during chemical synthesis. Typically, long synthetic routes dramatically decrease the overall yield, while the amount of resources consumed and the number of unwanted products formed increase.10 In contrast, a recent example shows that plant natural product synthesis with plant cell cultures might prove a promising alternative to isolation and chemical synthesis: production of Taxol, an anticancer agent originally isolated from the bark of the pacific yew tree (Taxus brevifolia), is currently produced in plant cell cultures of T. chinensis, reducing the production costs by 80% and 20%, when compared with isolation from T. chinensis and semisynthetic production respectively.13 However, application of plant cell cultures is limited by slow growth of the cell cultures, low and variable product yields, difficult scale-up, and complicated downstream processing.13 In contrast, microbial production of PPs would alleviate many of the aforementioned shortcomings. Well studied microorganisms such as Escherichia coli, Saccharomyces cerevisiae, Corynebacterium glutamicum, Bacillus subtilis, or Pseudomonas putida found already various large-scale applications for the production of numerous compounds in the food, chemical and pharmaceutical industries.14,15 Microorganisms have the advantage of high growth rates (and thus short production times) and readily scalable cultivation and production technologies. Furthermore, microbial production avoids the use of organic solvents, heavy metals, and strong acids or bases making processes more environmentally friendly as compared with chemical production. The main advantage of microorganisms is the availability of extensive molecular tools for their genetic manipulation allowing heterologous expression of whole biosynthetic pathways and the modification of metabolic profiles according to production conditions. In addition, microbial hosts usually provide a clean genetic background for the production of heterologous compounds, as they generally do not possess any competing pathways. As a result, PPs and PP-derived compounds would be synthesized as chemically distinct compounds, which simplifies product purification in comparison to purification from plants or plant cell cultures.14

**General Metabolic Engineering Strategies for the Microbial Production of Phenylpropanoid-Derived Natural Products**

Genetic information of more than 70,000 plants has become available in the last years (http://www.plantgdb.org) and many advances in the field of DNA sequencing and bioinformatics have led to increased knowledge of biosynthetic pathways for secondary metabolites in plants. Identification of genes and enzymes involved in biosynthetic pathways for PP-derived compounds and the development of molecular strategies for the concerted heterologous expression of multiple genes have stimulated rational metabolic engineering of microorganisms for the production of such compounds. In order to improve product yields, many genetic strategies can...
be used that optimize the expression of heterologous genes and reduce the metabolic flux toward the production of the desired metabolites (Fig. 1). An important decision is the microbial host system, which serves as a chassis for the expression of the heterologous genes. The organism should be genetically accessible to allow the construction of tailor-made recombinant and the properties tailored for production purposes. For the expression of correctly folded enzymes with appropriate kinetic parameters, enzymes can be expressed either from their respective native gene sequence or from a custom-made gene sequence, which has been adapted to the codon usage pattern of the respective microbial host. Protein engineering can be a powerful approach in case individual enzyme parameters turn out to be limiting for the overall flux through the heterologous pathway.13 Further optimization can be achieved through fine-tuning of individual promoters (constitutive or controlled induction of gene expression) and promoter strength.13 Alternatively, all or selected genes of the new pathway can be expressed as operon under the control of a single promoter. When using native regulatory elements in the constructed biosynthetic pathway, expression of regulators to stimulate the expression of the pathway genes is also a viable option.13 In addition, one has to decide whether the heterologous pathway should be expressed plasmid-based or integrated into the host genome. The latter is usually desired in order to develop a stable production strain.13 The metabolic environment in the host organism can also be optimized for PP production by increasing precursor- and co-factor supply.13 Furthermore, blocking or minimizing the flux through microbial pathways that also utilize or degrade intermediates or products of the heterologous pathway can result in improved product titers.13,13 Last but not least, improving product export can be crucial during strain optimization to keep the intracellular product concentrations below cytotoxic levels and to increase the flux through the heterologous pathway.12 Not surprisingly, almost all of the best performing microbial production strains for the synthesis of PPs and PP-derived compounds were constructed by combining several strategies.

**The Phenylpropanoid Pathway**

Like all natural products, PPs and derivatives thereof are synthesized from a small subset of primary metabolites provided by the central metabolism. The aromatic precursor trans-cinnamic acid is then converted to trans-coumaric acid, yielding a chalcone (CHS) that serves as a substrate for the production of a wide variety of flavonoids. The pathway is initiated with the conversion of phenylalanine to trans-cinnamic acid by a cinnamate 4-hydroxylase (C4H). The amino group of trans-cinnamic acid is then converted to trans-coumaric acid, which is subsequently converted to trans-coumaric acid by a chalcone synthase (CHS). Subsequently, both acids can be modified by reduction, O-methylation and/or aromatic hydroxylation to give rise to precursors for flavonoids, stilbenoids, cucumarioids, and lignans.

**Flavonoids**

Flavonoids play important roles in plant pigmentation, fertility and plant defense against UV exposure and pathogen attack.1 The core structure of the flavonoid skeleton consists of two aromatic C6 rings interconnected by a three carbon heterocyclic ring. The substitution pattern of this central ring further subdivides flavonoids into flavones, flavonols, flavonones, iso-flavones, anthocyanins, flavanoids, and flavanols.2 The structure of the flavonoid backbone can be further modified by methylation, acetylation, and C- and O-glycosylations. The resulting product diversity gives rise to the different classes of flavonoids.

The heterologous production of flavonoids in bacteria was first described by Hwang et al. in 2003.24 The heterologous production of naringenin and pinocembrin in E. coli BL21 (DE3) was achieved by placing the three genes encoding a PAL, a 4CL, and a CHS under control of the inducible T7 promoter. Optimization of culture conditions by increasing the levels of the trans-phenylalanine encoding for the acetyl-CoA carboxylase, the succinyl-CoA synthetase (both TCA-cycle) to channel the metabolic flux toward malonyl-CoA. Deletion of the genes for a fumarate hydratase and the β-subunit of the succinyl-CoA synthase (both TCA-cycle) and overexpression of the genes encoding for the acetyl-CoA carboxylase, methoxylation, acetylation, and C- and O-glycosylations give rise to the different classes of flavonoids. The heterologous production of flavonoids in bacteria was first described by Hwang et al. in 2003.24 The heterologous production of naringenin and pinocembrin in E. coli BL21 (DE3) was achieved by placing the three genes encoding a PAL, a 4CL, and a CHS under control of the inducible T7 promoter. Optimization of culture conditions by increasing the levels of the trans-phenylalanine encoding for the acetyl-CoA carboxylase, the succinyl-CoA synthetase (both TCA-cycle) to channel the metabolic flux toward malonyl-CoA. Deletion of the genes for a fumarate hydratase and the β-subunit of the succinyl-CoA synthase (both TCA-cycle) and overexpression of the genes encoding for the acetyl-CoA carboxylase,
phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and the pyruvate dehydrogenase complex resulted in an E. coli strain exhibiting a 4-fold increase in intracellular malonyl-CoA levels and accumulating up to 474 mg/L naringenin.28 Other important co-substrates/co-factors for the synthesis of flavonoids are UDP-glucose for the synthesis of anthocyanins and NADPH for the biosynthesis of leucoanthocyanidins and (-)-catechins.29,30 Increased intracellular UDP-glucose levels could be achieved by overexpressing the nucleoside diphosphate kinase.

Figure 2. Schematic overview of biosynthetic pathways leading to various phenylpropanoid-derived compounds in plants. Starting either from L-phenylalanine or L-tyrosine, different phenylpropanoids (p-coumaric-, caffeic-, ferulic-, sinapic-, or trans-cinnamic acid) are formed. Phenylpropanoids are precursors of coumarins (green box), stilbenes (blue box), flavonoids (orange box), and lignans (red box). Abbreviations: 4CL, 4-coumaryl-CoA ligase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamyl-CoA reductase; CHI, chalcone isomerase; CHS, chalcone synthase; DIR, dirigent protein; LAC, laccase; MON, cytochrome P450 monooxygenase; OMT, O-methyltransferase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; TAL, tyrosine ammonia lyase.
involved in the biosynthesis of UTP from supplemented sucrose acid and by bidirec-
ting a competing pathway through dele-
tion of the gene for the UDP glucose dehydrogenase retreating UDP-glucose to UDPglucuronate. In combination with inhibition of fatty acid synthesis by addition of cerulenin and heterologous expression of a malonate assimila-
tion pathway of Escherichia coli, anthocyanin titers of up to 113 mg/L of pelargonidin 3-O-glucoside could be achieved with E. coli. The importance of other metabolic pathways drawing off precursors and intermediates became also obvious during the heterologous produc-
tion of the flavonoid 7-O-methyl aroma-
dendrin (7-OMA) from p-coumaric acid in E. coli. Detailed analysis of the pro-
duction strains showed that the 7-OMA concentration did not correspond to the precursor consumption, indicating that p-coumaric acid is degraded by E. coli. Indeed, previous studies showed that E. coli is able to utilize aromatic acids as sole carbon source via several degradation pathways. Thus, deletion of these par-
ticular metabolic routes represent promis-
ting targets for metabolic engineering to improve flavonoid production in E. coli.

Stilbenes

Resveratrol, probably the best known stil-
benes, is believed to prevent cardiovascular diseases and may also provide protection against certain types of cancer, diabe-
tes, and neurodegenerative diseases such as Alzheimer disease. These properties make resveratrol an interesting candidate for various applications in the pharma-
aceutical- and food industry. Similar to the biosynthetic pathway to flavonoids, the pathway to stilbenes branches off from the phenylpropanoid pathway at the stage of the CoA-activated phenylpropanoids (Fig. 2). Stilbene synthases (STS), which are also type III polyketide synthases like the CHS, catalyze the formation of the same tetraketide intermediate, but follow a dif-
ferent cyclization mechanism. A STS cyclizes the tetraketide intermediate via an intramolecular C2 → C7 cyclization reaction, whereas a CHS follows an intramole-
ular C6 → C1 Claissen condensation (Fig. 3). Microbial production of resveratrol was first reported in S. cerevisiae, through trans-activation of a 4CL (Populus trichocarpa × Populus deltoids) and a STS from grapevine (Vitis vinifera), resulting in 1.45 μg/L of resveratrol start-
ing from p-coumaric acid. For optimiz-
ing the conversion of p-coumaric acid to resveratrol, Zhang et al. employed a fusion protein of 4CL (A. thaliana) and STS (V. vinifera) in E. coli, employing 4CL and STS from different plant sources (Nicotiana tabacum or A. thaliana and Anacardium hypogaea or V. vinif-
era, respectively). Recently a more system-
atic study was published with respect to the optimization of resveratrol production from p-coumaric acid. Two different E. coli strains (BL21 Star and BW27784), different promoters (the inducible T7 promoter and the constitutive promoter of the glyceraldehyde-3-phosphate dehy-
drogenase gene [P_gad]) as well as different en-
zyme gene combinations of a library of two 4CLs and eight STSs were systematically evaluated for improved resveratrol titer. The best performing strain was an E. coli BW27784 strain expressing the 4CL from A. thaliana and the STS from V. vinifera in a bicistronic operon on a pUC plasmid. Production with this strain was improved by inhibiting fatty acid biosynthesis to increase the malonyl-CoA availability to final titers of 2.4 g/L resveratrol. However, final resveratrol titer without precursor feeding and functional expression of a TAL are still quite low (1 to 2 mg/L).

Other Phenylpropanoid-Derived Natural Products

Coumarins, derived from trans-cinnamic acid or p-coumaric acid (Fig. 2) also constitute an important class of active pharmacological substances with pos-
sible applications ranging from analgesic to HIV-therapy. Heterologous synthesis of coumarins has not been reported yet. This can be attributed to insufficient knowledge of the biosynthetic pathways leading to coumarins, which comprise the activity of many enzymes that are dif-
ficult to express such as cytochrome P450 monooxygenases or O-methyltransferases of unknown identity. Further, the introduction of the CoA-
activated (hydroxy)-cinnamic acids in the phenylpropanoid pathway finally leads to the formation of their respective alco-
hol (monoilignol) (Fig. 2). Monolignols are precursors of lignin, which confers mechanical strength to plant cell walls and of the structurally diverse group of lignans. Lignans are also known as dili-
gnols because they are formed through the oxidative dimerization of two mono-
lignol units. Dimerization is initiated by laccases or peroxidases forming free radical intermediates, but the patterns of dimerization are controlled by so-called dirigent proteins (DIRs). DIRs capture and orientate the free radicals in such a way as to enable the formation of only one specific dimer. The guiding role of DIRs is unmistakable as many unspec-
cific coupling products are formed in their absence. Due to the complex glyco-
sylation patterns of the DIRs, their func-
tional expression has been restricted to cell cultures until now. Similar to cou-
marins, knowledge about the biosynthetic pathways leading to complicated lignin structures such as justicin B (Fig. 2) is incomplete. In particular, the unknown identity of enzymes involved in further dilignol modifications (cytochrome P450 monooxygenases, dioxygenases, peroxi-
dases, and O-methyltransferases) have rendered the production of lignins with microorganisms impossible to date. Heterologous expression of biosynthetic pathways in microorganisms can also be used for the production of unnatural bio-
active compounds. This field of research, often referred to as combinatorial bio-
synthesis, gives access to novel and com-
plex compounds. Supplementation of unnatural precursors and/or application of additional enzymes (e.g., hydroxylat-
ing, methylation, prenylating enzymes) are the two basic strategies for creating novel bioactive compounds. The relatively broad
substrate specificity of many plant biosynthetic enzymes enables the conversion of non-natural precursors. For example, Katsuyama and coworkers were able to produce 36 unnatural flavonoids and stilbenes by feeding non-natural carboxylic acids to an E. coli strain equipped with a heterologous phenylpropanoid pathway.

**Conclusion and Outlook**

Metabolic engineering of microorganisms has already proven to be a successful approach to obtain access to phenylpropanoid derived compounds, namely flavonoids and stilbenes. Currently, coumarins and lignans cannot be produced by microorganisms, but considering the dramatic proceedings in DNA sequencing technologies and bioinformatics for the analysis and annotation of (plant) DNA sequences this is expected to change in the future. Additionally, recent developments in the microbial expression of complex eukaryotic enzymes such as plant cytochrome P450 monooxygenases have shown that (rational) protein engineering is a promising strategy for adapting enzymes to the host environment. Furthermore, adaptation of the host metabolism with respect to precursor and/or co-factor supply has proven its value toward increasing product titers. Future fine-tuning of the host metabolism in relation to the heterologous pathway is expected to enhance product titers even more. As the number of genes in heterologously expressed pathways increases, fast cloning technologies for single genes and synthetic operons will become more important. Future technologies should also enable the fine-tuning of gene expression in order to accomplish
an optimal flux through the heterologous pathway for optimal productivity titers and minimized accumulation of toxic intermediates. In addition to classical strategies such as varying the gene copy number and promoter type, a likely advantage of genome-minimized regions can also be employed to control the relative amounts of individual enzymes on the translational level. Instead of using an existing host in

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which the heterologous pathway has to be integrated and host and pathway have to be reciprocally adapted, future biotechnologists may directly employ a synthetic producer with a tailor-made metabolic net work. Another option is to create a metabolic network with A2 in the heterologous product synthesis. There are still a multitude of fives to pull in the meta bolic engineering of microorganisms and undoubtedly much more will be uncovered with the aid of network and/or metabolic modeling tools, which will unquestionably increase the number of phytochemicals derived plant (non-)natural products that can be produced on a commercially viable scale.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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