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Review

Tools of pathway reconstruction and production of economically relevant plant secondary metabolites in recombinant microorganisms

Clarissa Dziggel, Holger Schäfer and Michael Wink

Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany

Plant secondary metabolites exhibit a variety of biological activities and therefore serve as valuable therapeutics or flavoring compounds. However, the small amounts isolated from plants often cannot meet market demands. This led to the exploration of other, more profitable methods for their production, including plant cell culture systems, chemical synthesis and biotechnological production in microbial hosts. The biotechnological production can be pursued by reconstructing metabolic pathways in selected microbial systems. But due to their complexity, most of these pathways are not completely understood and require the expression of a multitude of genes in a foreign organism. Recently, next generation sequencing data and advances in gene silencing in plants allowed the elucidation of some biosynthetic pathways in more detail. Thus, the de novo production of some natural products, including morphine, strictosidine, artemisinin, taxol® and resveratrol, in extensively engineered microbial hosts has become feasible. This review highlights the reconstruction of these pathways, missing pieces and novel techniques employed.

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1 Introduction

Plants produce a high diversity of low molecular weight compounds, not needed for primary metabolism. For a long time these so called secondary metabolites (SM) were thought to be useless waste compounds produced spontaneously or through unspecific enzymatic reactions. Now it is generally accepted that SM are important for the survival of the plants producing them and that their biosynthesis is a highly regulated process, catalyzed by specific enzymes [1, 2]. Apparently SM have been developed in plant evolution as defense substances against herbivore attack or infection with bacteria, yeasts or fungi. But SM also protect against other plants competing for sunlight, water, and nutrition or function as signaling compounds, antioxidants, UV-protectants or nitrogen storage compounds [3]. This large variety of biological activities can be explained by the enormous structural diversity of SM. According to their biosynthetic origin, SM can be classified into several groups. Some examples are: alkaloids, a structurally broad group of nitrogen-containing SM, which are mainly derived from amino acids like tyrosine, tryptophan, phenylalanine or ornithine. Terpenes, which are derived from C_5 units, such as dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). Phenylpropanoids, including flavonoids, flavones, stilbenes, and anthocyanins, which are synthesized from aromatic amino acids and acetyl-CoA units [2, 4].

Because of their broad spectrum of bioactivities, plant-derived SM are diversely applied as pharmaceuticals, poisons, perfumes or flavoring compounds. But in

Correspondence: Professor Michael Wink, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
E-mail: wink@uni-hd.de

Abbreviations: BIAs, benzylisoquinoline alkaloids; **CRISPR,** clustered regularly interspaced short palindromic repeats; **CYP,** cytochrome P450 enzyme; **DMAPP,** dimethylallyl diphosphate; **FPP,** farnesyl diphosphate; **GGPP,** geranylgeranyl diphosphate; **GPP,** geranyl diphosphate; **IPP,** isopentenyl diphosphate; **MEP,** methylerythritol phosphate pathway; **MIAs,** monoterpene indole alkaloids; **MVA,** mevalonic acid pathway; **SM,** secondary metabolites; **VIGS,** virus induced gene silencing

many cases these valuable compounds are still isolated in very small amounts from plantation grown plants. Different strategies have been applied to improve their production yield in the plant as well as in plant cell cultures, but often without the desired results [5]. For several compounds, a chemical synthesis is possible, but not profitable, so that other ways of their production had to be explored, for example the production in microorganisms like *Escherichia coli* or *Saccharomyces cerevisiae* as reviewed in [6–9]. But the limited knowledge of the biosynthetic pathways of SM has slowed down this new approach [2, 10, 11]. Nevertheless, the investigation of transcriptomes [12], proteomes [13] and metabolomes [14] and gene silencing in plants [15] currently enhances the search for genes and their function in plant metabolic pathways. Very recently complete pathways have been elucidated and in a few cases a de novo synthesis from cheap carbon sources has been achieved in recombinant microbial hosts. The aim of this review is to highlight these pathways concentrating on examples from three classes of SM (terpenoids, alkaloids, phenylpropanoids) and to describe the novel techniques employed.

2 Technical advancements for the heterologous construction and expression of metabolic pathways

During the last 20 years a number of genes, which encode enzymes of secondary metabolism, have been isolated, cloned and expressed successfully (Supporting information, Figs. 1–4 and citations therein). Nevertheless, researchers experience regularly that a protein of interest is produced in low amounts or is inactive in a heterologous expression system and would be grateful for straightforward protocols that guarantee successful expression of just one protein. The expression of multiple enzymes of a metabolic pathway concomitantly in a host cell is even more challenging: the goal is to produce a compound in reasonable amount without interfering with the viability of cells and biomass production. Although there is no "one-size-fits-all"-solution, some technical aspects encountered in the literature of the past 15 years deserve general consideration. In this section we provide an overview of these methods and exemplify their specific use in Section 3.

2.1 Tools to elucidate plant secondary metabolic pathways

Today's knowledge of plant secondary metabolic pathways is usually restricted to model plants and mostly incomplete as many genes have not been identified yet. Recently, high-throughput techniques have provided plant transcriptomes, proteomes and metabolomes. These data sets are used to correlate gene expression with metabolite production, or to search for sequences homologous to proteins with certain catalytic activities. Such approaches result in the identification of candidate genes with potential roles in secondary metabolite pathways [12–14]. In a next step, these candidate genes need to be functionally characterized, e.g. by virus induced gene silencing (VIGS) [15]. VIGS uses one of at least three distinct RNA mediated silencing pathways in plants and leads to post-transcriptional breakdown of target sequences [16]. In this way, for example, a key enzymatic step in morphine biosynthesis was uncovered recently [17–19].

2.2 General adjustments for the heterologous expression of plant genes

Genes can be easily transferred between organisms and can generally be transcribed and translated into proteins due to the universal genetic code. However, the foreign environment may interfere with proper three-dimensional folding and balanced translation speed, which can lead to insoluble and non-functional proteins [20]. Standard procedures for DNA cloning and expression are described in [21] and focus on codon optimization, masking toxicity by protein fusions, and reducing leaky expression. Standard techniques also include truncation of N- or C-termini for better solubility, addition of affinity tags for simplified purification, and initial small scale production for activity screenings. However, beyond these general adjustments, most proteins require individual solutions for their functional expression. Thus, it is advisable to characterize the protein of interest in as much detail as possible, e.g. considering disulfide bridge formation, membrane anchoring, dependency on co-factors, interference with host metabolic pathways, just to name a few. In the studies described in Section 3, *E. coli* and *S. cerevisiae* are predominantly chosen as hosts for the production of plant metabolites. Available tools for the expression of heterologous proteins in these two organisms are covered in reviews with emphasis on vectors, strains, promoters, etc. [22, 23].

2.3 Metabolic engineering

The term metabolic engineering was introduced a quarter century ago to describe the application of recombinant DNA technology for the improvement of metabolic processes in organisms [24, 25]. Nowadays, the optimization of metabolic pathways is approached in various ways. First, one may employ alternative enzymes with the same catalytic function but better performance. With an expanding wealth of next generation sequencing data, certain enzymes of secondary plant metabolism may be found in species other than the originally investigated plant species. These enzymes can be more active or differently regulated, e.g. by feedback inhibition. Thus, all metabolic pathways in microorganisms presented in this

review are not just re-constructed from plants but are composed of genes from various species in order to maximize efficiency. Second, when genes are yet unknown or difficult to express, one can search for enzymes which catalyze similar conversions in related metabolites. On occasion, these enzymes follow a different path but lead to the same outcome, and thus circumvent critical steps in the pathway. For example, Nakagawa et al. [26] used this approach to simplify (R,S)-reticuline production in *E. coli* (Supporting information, Fig. 3b). Third, splitting up long pathways into modules may be beneficial, since they can be expressed and optimized independently. Subsequently, these modules can be combined and further optimized either in the same host or in co-culture. Co-culturing has the additional advantage of distributing the metabolic burden onto different host cells, similar to natural microbial consortia or the compartmentalization found in plant cells [27]. The concept of modularity is exemplified in the de novo synthesis of morphine in yeast [28] and in facilitated flavonoid production in co-cultures of *E. coli* [29]. Last, primary metabolism substrates may not be available in sufficient amount to support secondary metabolite biosynthesis. In this case, genetic modifications of host strains can channel the carbon-flux to the respective pathway, both by strengthening primary substrate production and by limiting substrate consumption in divergent pathways [30]. The shikimate pathway, for example, has been extensively engineered for biosynthesis of aromatic compounds in *E. coli* [31].

2.4 Protein engineering

Recombinant DNA technology allows proteins to be engineered in order to alter their properties, such as stability, substrate specificity, reaction specificity, allosteric regulation, and more [32]. Protein engineering is performed according to either rational design or directed evolution and is usually employed to optimize pathways which contain inefficient steps [33]. Engineering efforts may focus on optimizing a single protein or on physically linking multiple proteins in a pathway. On the one hand, single enzymes may be mutated in order to enhance enzyme productivity or to create novel products [34]. In this way, the substrate repertoire of type III polyketide synthases was expanded to create unnatural polyketide-alkaloid scaffolds [35, 36]. On the other hand, multienzyme complexes and compartmentalization spatially regulate the biosynthesis of plant SM by increasing local metabolite concentrations and efficiently channeling metabolite flux through active sites of enzymes [37]. Thus, artificial clustering of enzymes is pursued by either direct fusion of proteins or by arranging proteins on larger scaffolds [38]. Scaffolds themselves have been suggested to cluster on a higher level, and thus should be designed in order to allow efficient agglomeration [39]. For example, protein fusion led to the de novo synthesis of raspberry ketone in yeast: the authors investigated various fusion combinations and orientations as well as rigid or relaxed linker sequences for the connection of the four enzymes involved [40]. Artificial scaffolds have been used in *E. coli* to express three genes for the synthesis of mevalonate, which serves as a precursor for isoprenoid derived secondary metabolites [41].

2.5 Engineering of cytochrome P450 enzymes

Engineering efforts are regularly encountered with the expression of cytochrome P450 enzymes (CYPs) [42]. CYPs are very important in plant secondary metabolite synthesis, since they catalyze many critical steps like regio- and stereoselective oxygenation, oxidation, C-C coupling, and many more. However, eukaryotic CYPs localize to the ER membrane and require specific reductase partners, both of which can lead to inefficient expression in *E. coli* [43]. When shifting to eukaryotic expression hosts instead, supply shortage of essential haeme cofactors may trigger stress response and low expression levels in *S. cerevisiae* [44]. CYPs have been successfully functionalized either by directing them to the bacterial membrane [45], or by the fusion of a plant CYP with a plant cytochrome reductase for isoflavone synthesis [46], or by a balanced co-expression of CYP and its reductase partner for oxygenated taxane production [11].

2.6 Tool development for DNA cloning and strain engineering

In order to integrate a heterologous pathway in a microbial host without affecting biomass production, one can balance foreign protein production on a genomic, transcriptional and translational level. Gene copy number, promoter strength, and ribosomal binding sites all heavily influence metabolite yield and were adjusted to enhance pinocembrin production in *E. coli* [47]. Xu et al. employed a different strategy by creating artificial promoters instead of using existing ones: hybrid promoters designed to detect and regulate malonyl-CoA levels in vivo in *E. coli* enabled a streamlined production of malonyl-CoA derived compounds [48]. In addition, a fine tuning of pathways may nowadays be achieved by accelerated evolution of host *E. coli* via MAGE (multiplex automated genome engineering) or by genome editing via CRISPR-Cas. MAGE is based on the external addition, electroporation and integration of purposefully designed oligonucleotides into the *E. coli* genome by allelic replacement during DNA replication. Depending on their design, oligonucleotides may lead to gene knock-out or mutations in regulatory sequences for gene expression. Since most steps of this technique can be automated and applied to many genes simultaneously, a very large number of genetically modified strains can be generated and screened for more efficient metabolite production [49]. Clustered regularly interspaced short palindromic repeats (CRISPR) are found

in archaea and bacteria and provide adaptive immunity against phage infections [50]. CRISPR-Cas9 has been developed into a simple tool for target specific genome editing and additional applications like transcription regulation in higher eukaryotes [51]. CRISPR-dCas9 has been successfully employed to optimize expression levels of pathway enzymes for naringenin production in *E. coli* [52]. In addition, CRISPR-Cas9 platforms are currently being established in order to mediate gene silencing in plants [53]. *Agrobacterium*-mediated transformation of a short RNA guide and the Cas9 nuclease are sufficient to cause double strand breaks at a genomic location specified by the RNA guide. These dsDNA breaks are subsequently repaired by non-homologous end joining in an error-prone fashion, which in turn leads to short insertions or deletions and thus gene disruption.

Considering the various options for the engineering of metabolic pathways, researchers are required to put a multitude of different systems to the laboratory test. Transferring all the possible combinations into a laboratory setup is no longer feasible with standard cloning techniques, even though the use of compatible duet vectors (Novagen) allows the expression of multiple constructs in one *E. coli* cell [54]. Thus, synthetic biology relies on newly developed cloning systems such as Gibson assembly [55]. In addition, Golden Gate cloning [56] or its variations BioBrick [57] and BglBrick [58] have been described: in short, carefully designed restriction sites and use of e.g. type IIs restriction enzymes allow the combination of multiple elements in just one reaction vial through iterative cuts and ligations.

3 Biosynthesis of secondary metabolites and pathway reconstruction in microorganisms – case studies

3.1 Terpenes

With more than 40 000 structures, terpenoids represent the largest class of natural products [59]. Many of them show pharmaceutically interesting biological activities like anti-cancer or anti-infectious properties, as for example the sesquiterpene lactone peroxide artemisinin and the diterpene paclitaxel. Artemisinin is isolated from the plant *Artemisia annua* and used as antimalarial drug in artemisinin-based combination therapies [60]. Paclitaxel, an important anticancer drug (taxol®), was first isolated from *Taxus brevifolia* and can be extracted from the bark and needles of different *Taxus* trees [61].

Like all terpenoids, artemisinin and taxol® are synthesized from either the mevalonic acid pathway (MVA: some bacteria, plants, higher eukaryotes) or the methylerythritol phosphate pathway (MEP: plants and most bacteria) derived precursors IPP and DMAPP (Fig. 1). Prenyltransferases use these two C_5 molecules to synthesize linear isoprene precursors like geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). At this point the biosynthetic pathways of artemisinin and taxol® deviate from each other. The sesquiterpene (C_{15}) artemisinin is built from FPP whereas the diterpene (C_{20}) taxol® is synthesized from GGPP [61, 62]. In the next step these linear precursor molecules undergo a cyclisation reaction. In the case of artemisinin, this cyclisation reaction is catalyzed by the amorpha-4,11-diene synthase. The resulting cyclic sesquiterpene amorpha-4,11-diene can be further converted to artemisinic- or dihydroartemisinic acid. Artemisinic acid is produced through three oxidation reactions catalyzed by the cytochrome P450 monooxygenase CYP71AV1 and its various partners (CPR1, CYB5, ADH1 and ALDH1) [63]. The resulting intermediates are artemisinic alcohol and artemisinic aldehyde. Dihydroartemisinic acid, on the other hand, is produced through the formation of dihydroartemisinic aldehyde from artemisinic aldehyde by the action of artemisinic aldehyde double-bond reductase [64]. All enzymes needed for the production of these intermediates have been identified and cloned (Supporting information, Fig. 1), but the last steps of artemisinin biosynthesis remain to be fully elucidated. In plants, dihydroartemisinic acid seems to be converted to artemisinin in a non-enzymatic photooxidation process, whereas artemisinic acid, is thought to be converted to artennuin B [65–67].

Since the last steps to artemisinin production are not yet clear, many attempts have been made to produce the late pathway intermediate artemisinic acid, which can be chemically converted to artemisinin, in commercially relevant amounts. In *S. cerevisiae* the production of artemisinic acid could be improved from mg/L and low g/L titers [68–70] up to 25 g/L [63]. The development of this high-level artemisinic acid production system required many engineering steps. First, the authors employed a previously described yeast strain, in which the mevalonate pathway was engineered to increase production of FPP and to decrease its subtraction for sterol synthesis [70]. Second, the genes necessary for artemisinic acid production (*ADS*, *CYP71AV1*, *CPR1*) were integrated into the yeast system. However, expression of *CYP71AV1* and its cognate reductase *CPR1*, both controlled by strong promoters, resulted in low culture viability. Since poor coupling between CYPs and their CPRs can lead to production of reactive oxygen species, lowering CPR1 expression with a weak promoter increased viability, but also decreased amorphadiene oxidation. In a third and key step, the authors examined an expressed sequence tag library derived from *A. annua* glandular trichomes, and identified additional proteins, namely cytochrome b5 (CYB5) and an alcohol dehydrogenase (ADH1), which both are presumably involved in efficient artemisic acid biosynthesis. Expression of *CYB5* increased production of artemisinic acid, but also resulted in the accumulation of

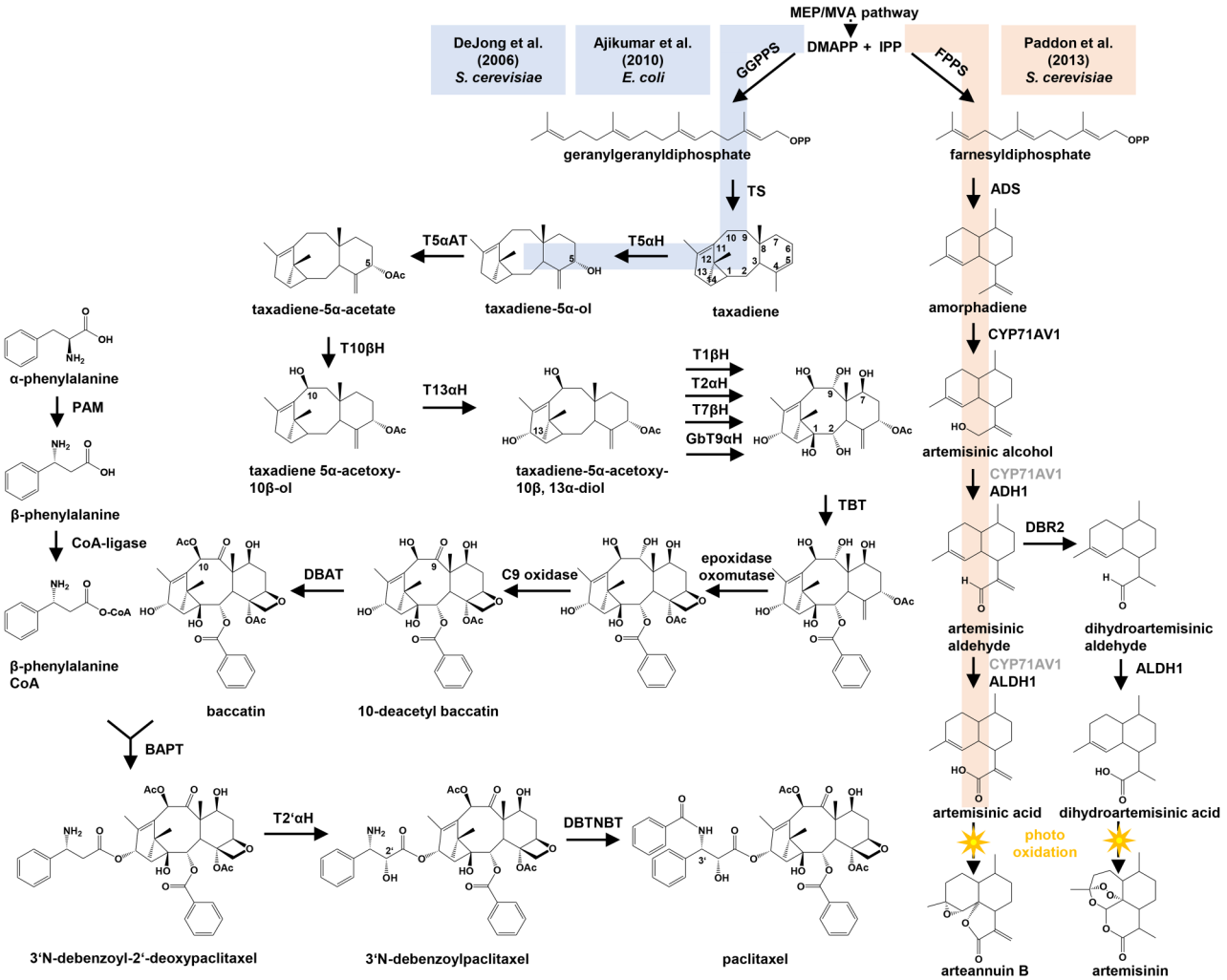

Figure 1. Proposed biosynthetic pathway of paclitaxel and artemisinin. Reconstructions of multi-enzyme parts of the pathway in heterologous microbial hosts are shaded in different colors. For full names of the biosynthetic enzymes and their first heterologous expression in microorganisms see Supporting information.

artemisinic aldehyde, which is potentially toxic. Addition of a previously described artemisinic aldehyde dehydrogenase (*ALDH1*) and *ADH1* limited artemisinic aldehyde accumulation to trace amounts and raised artemisinic acid titers to 8.1 g/L. However, artemisinic acid formed a crystalline, extracellular precipitate. Extractive fermentation using isopropyl myristate oil, and the development of a feedback-controlled ethanol pulse-feed process resulted in the final product titer of 25 g/L.

In the biosynthesis of the diterpene paclitaxel, the cyclisation reaction of GGPP is catalyzed by the enzyme taxadiene synthase. The resulting taxadiene is hydroxylated at various positions (C5, C10, C13, C9, C7 and C2) by cytochrome P450 taxoid-hydroxylases and further modified (e.g. acetylations, benzoylation, epoxide formation, oxidation) to baccatin. The final steps of paclitaxel synthesis include coupling of the C13-side chain to baccatin, a 2'-hydroxylation and another benzoylation. Although most of the enzymes responsible for paclitaxel biosynthesis have been identified and heterologously expressed (Supporting information, Fig. 1), the exact order of the reaction steps is not fully confirmed yet [71–73].

Nevertheless, the reconstruction of early pathway parts in microbial systems has been established. The production of 1 mg/L taxadiene and 25 µg/L taxadiene-5α-ol was reported in *S. cerevisiae* [71]. Later a significant increase of titers up to 1 g/L taxadiene and 58 mg/L taxadiene-5α-ol was achieved in an *E. coli* system, in which a modular approach was used [74]. The upstream module contains eight genes providing IPP and DMAPP, and the downstream module contains two genes for taxadiene synthesis. Next, the expression levels of previously defined bottleneck genes in these modules were modified by using various promoters and by changing plasmid copy numbers. In this way, well balanced expression levels were achieved, which minimized indole induced

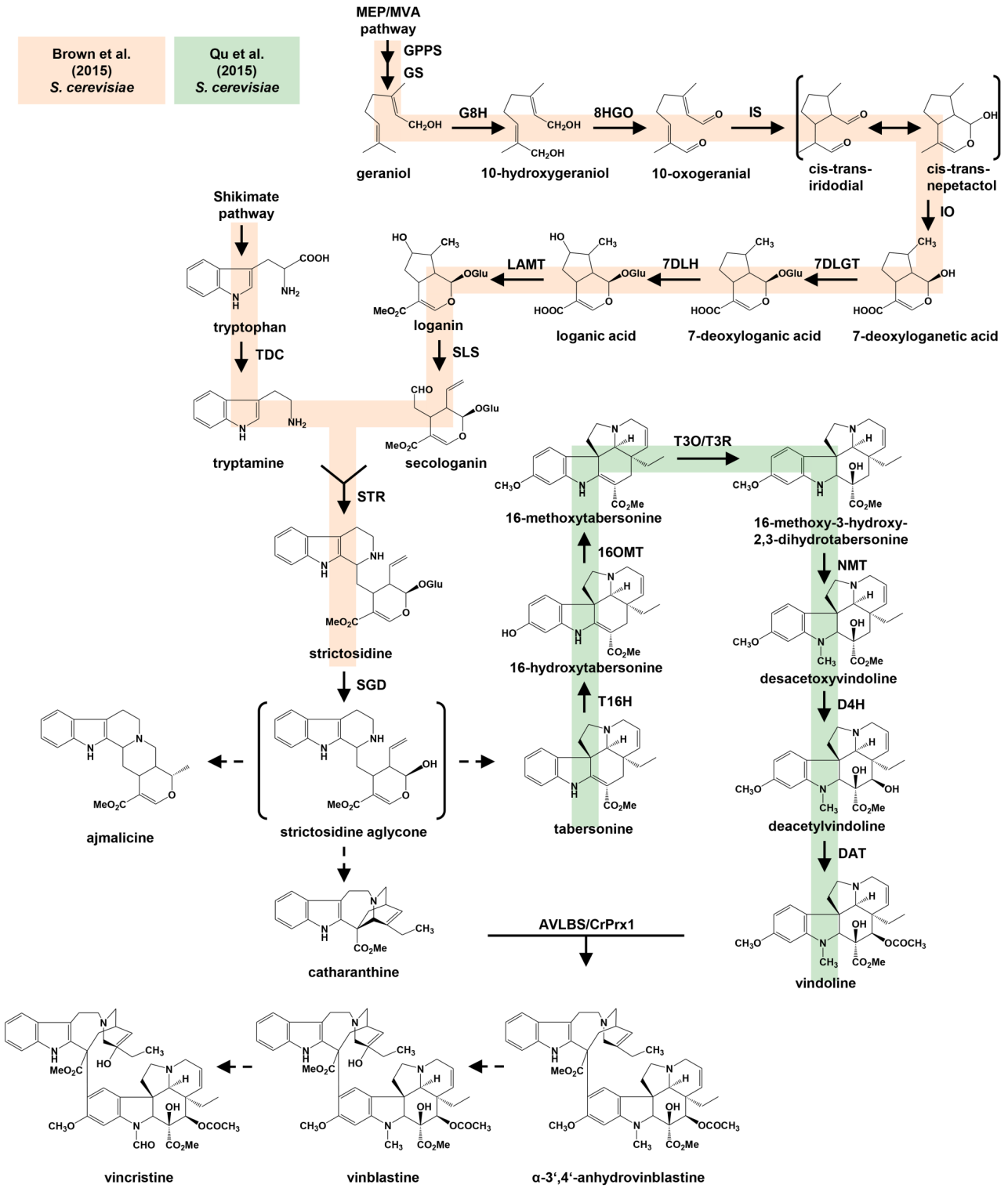

Figure 2. Proposed MIA biosynthetic pathway in *Catharanthus roseus*. Reconstructions of multi-enzyme parts of the pathway in heterologous microbial hosts are shaded in different colors. For full names of the biosynthetic enzymes and their first heterologous expression in microorganisms see Supporting information.

metabolic burden, and raised taxadiene production 15 000-fold compared to unbalanced systems. However, expression of a single additional gene (*CYP725A4* encoding T5αH, taxoid 5α-hydroxylase) disrupted this balance and greatly diminished titers. Six years later and despite extensive CYP engineering, the conversion of taxadiene to taxol® by various CYPs remains challenging [11, 75].

3.2 Monoterpene indole alkaloids (MIAs)

MIAs are found mainly in the plant families of the Apocynaceae, Loganiaceae and Rubiaceae and form a major group (>3000 structures) within the alkaloids [76]. Because of their interesting biological activities, many of them are used in medicine as therapeutic agents. Important examples are the chemotherapeutics vinblastine and vincristine from *Catharanthus roseus* or camptothecin from *Camptotheca acuminata* and its derivatives, irinothecan and topothecan [77, 78].

Most MIAs are derived from the common precursor, strictosidine, or its deglucosylated form (Fig. 2). Strictosidine is formed through a stereo-selective Pictet-Spengler condensation of the glucoiridoid secologanin with the indole tryptamine catalyzed by the enzyme strictosidine synthase [79]. Tryptamine is built from the shikimate pathway derived amino acid tryptophan in a single enzymatic reaction catalyzed by the tryptophan decarboxylase. On the other hand, a series of enzymatic reactions is needed for the biosynthesis of secologanin. In total, it takes seven enzymatic reactions to produce the MEP pathway derived isoprenoid precursors IPP and DMAPP from glyceraldehyde 3-phosphate and pyruvate and ten more reactions to build secologanin from these metabolites [80, 81]. The deglucosylation of strictosidine is catalyzed by the strictosidine-β-D-glucosidase and results in the formation of an unstable aglycon. Through intramolecular Schiff base formation and ring closure this aglycon spontaneously converts to cathenamine and isomers, giving rise to the many different types of MIAs [82].

Today, various biosynthetic genes have been identified and cloned (Supporting information. Fig. 2). Since the secologanin biosynthetic pathway has been elucidated completely, the whole pathway up to strictosidine was successfully reconstructed first in tobacco [81] and later in yeast [83]. In the yeast system production levels up to 0.5 mg/L strictosidine were achieved. For the de novo production of strictosidine in a single yeast strain, Brown et al. integrated 15 plant derived genes, one animal derived gene, and five additional copies of yeast genes; they also deleted three yeast genes. The first engineering step was to increase the supply of IPP and DMAPP precursors, according to existing literature [69, 84, 85]. In short, to boost mevalonate production a truncated, non-feedback-regulated HMG-CoA reductase gene was integrated, along with a second copy of the gene encoding IPP isomerase (*IDI1*). Since IPP is used for both GPP and tRNA synthesis, the GPP level was further enhanced by integrating a second copy of *MAF1*, a negative regulator of tRNA synthesis. The second engineering step enabled the synthesis of free, cytosolic GPP, which does not naturally occur in *S. cerevisiae*. Therefore, the yeast specific farnesyl pyrophosphate synthase (FPPS encoded by *ERG20*) was replaced by a mutated FPPS from *Gallus gallus* with pronounced GPPS activity. In addition, a GPP-specific synthase from *Abies grandis* was integrated as well. Third, the pathway to strictosidine biosynthesis was completed by integration of eleven original *C. roseus* genes encoding enzymes with various cofactor dependencies. Since CYPs rely on electron shuttling from their CPR and in some cases from CYB5, both were integrated from *C. roseus*. Furthermore, two genes (*SAM2* and *ZWF1*) were added to increase S-adenosyl-L-methionine and NADPH availability. However, strictosidine formation was not detected in this system. Geraniol 8-hydroxylase (G8H) was identified as bottleneck by feeding pathway intermediates to the engineered yeast strain. Thus, the key engineering step in this system was the effective hydroxylation of geraniol, which was achieved by integration of three additional, codon optimized copies of *G8H*. In addition, geraniol consumption by esterification and citronellol formation was prevented by deletion of yeast *ATF1* (alcohol acetyl transferase) and *OYE2* (NADPH oxidoreductase), respectively.

While the MIA pathway upstream of strictosidine is well known, the parts downstream of strictosidine deglucosylation remain unclear. For the biosynthesis of catharanthine for example neither enzymes nor genes involved in this pathway could be identified yet [76]. But the identification and heterologous expression of an enzyme converting the strictosidine aglycone to tetrahydroalstonine (tetrahydroalstonine synthase) was reported recently [86], as well as the completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline and its successful assembly in yeast [87].

3.3 Benzylisoquinoline alkaloids (BIAs)

BIAs, a very large, structurally diverse group with more than 2500 defined structures, are found mainly in the families of the Papaveraceae, Ranunculaceae, Berberidaceae and Menispermaceae [88]. Many BIAs possess potent pharmacological activities: morphine (analgesic), codeine (analgesic, antitussive), noscapine (antitussive, potential anticancer drug), papaverine (vasodilator, smooth muscle relaxant) and sanguinarine/berberine (antimicrobials) [89].

All BIAs are derived from a single key intermediate, (S)-norcoclaurine, which is formed through the stereoselective Pictet-Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde, both derived from the amino acid tyrosine (Fig. 3). This condensation reaction is catalyzed by the enzyme (S)-norcoclaurine synthase.

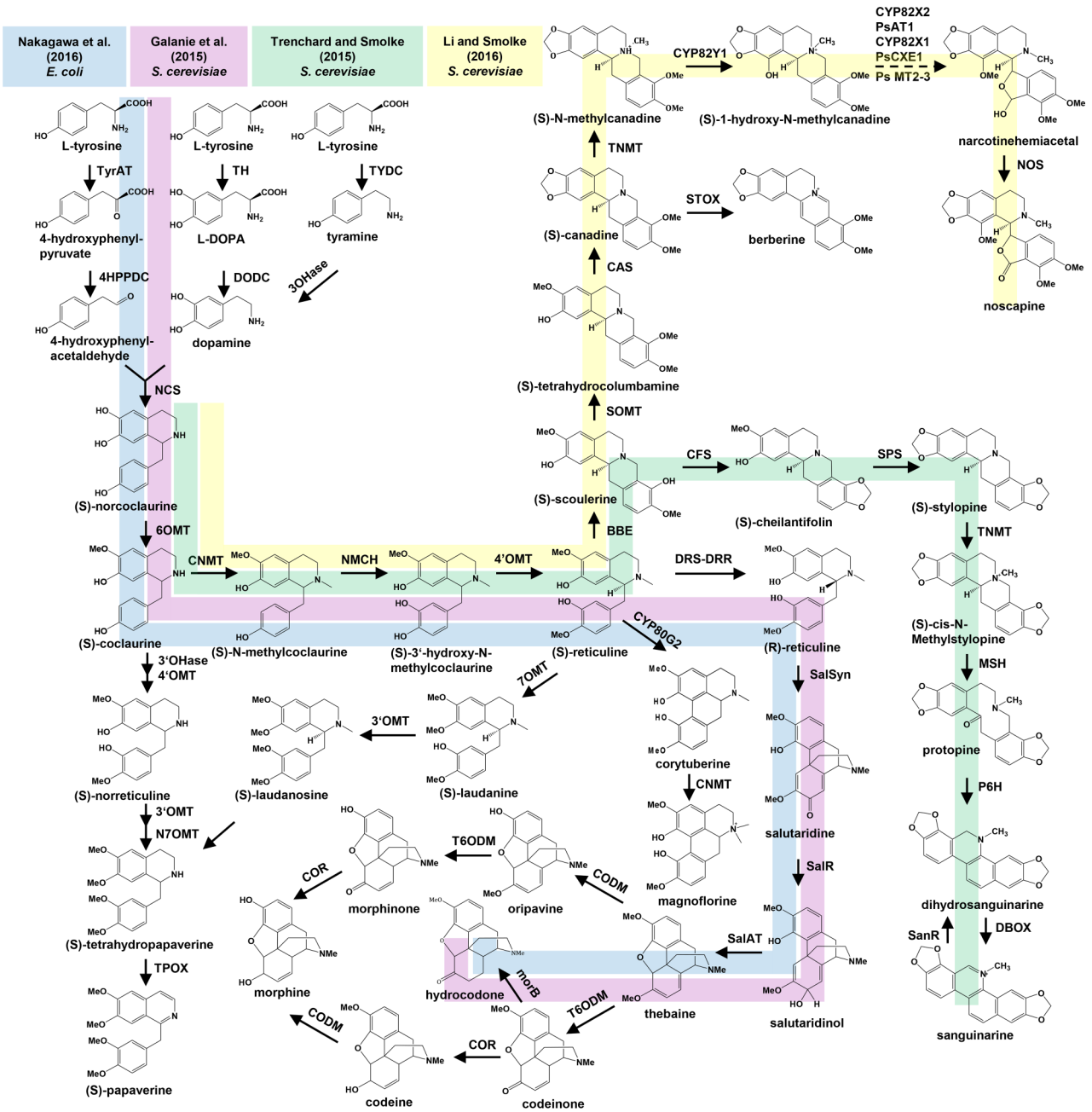

Figure 3. Proposed BIA biosynthetic pathway. Reconstructions of multi-enzyme parts of the pathway in heterologous microbial hosts are shaded in different colors. For full names of the biosynthetic enzymes and their first heterologous expression in microorganisms see Supporting information.

(S)-norcoclaurine is converted to the central pathway intermediate (S)-reticuline in four enzymatic steps, comprising three methylation and one hydroxylation reactions [90, 91]. From (S)-reticuline many different BIA branches can be entered, including the aporphine (e.g. magnoflorine), the benzophenanthridine (e.g. sanguinarine), the morphinan (e.g. morphine), the phthalideisoquinoline (e.g. noscapine) and the protoberberine (e.g. berberine) branch [91, 92]. In contrast to the other BIA branches, synthesis of morphinan branch alkaloids needs further conversion of (S)-reticuline to (R)-reticuline [90].

Overall more than 30 genes have been reported to be involved in BIA biosynthesis [10, 88] and many of them have already been identified, cloned and expressed in a heterologous host (Supporting information, Fig. 3a). In the last years successful reconstructions of multi-enzyme pathway parts in *E. coli* and/or *S. cerevisiae* have been reported. Precursor dependent systems include the pro-

duction of the central intermediate (S)-reticuline (11 mg/L) from dopamine in an *E. coli* system as well as the production of aporphine-type alkaloid magnoflorine (7.2 mg/L) and protoberberine-type alkaloid scoulerine (8.3 mg/L) through a co-culture system of *E. coli* and *S. cerevisiae* [93]. In addition, the synthesis of up to 150 mg/L (R,S)-reticuline in a *S. cerevisiae* system was achieved using (R,S)-norlaudanosoline as a substrate [94]. Furthermore, the production of sanguinarine (80 µg/L) and noscapine (0.6 mg/L) was achieved in *S. cerevisiae* using (R,S)-norlaudanosoline supplementation [92, 95]. Recently, de novo synthesis became feasible and was reported for (S)-reticuline production (46.0 mg/L) from glycerol in *E. coli* [96]. Furthermore, total biosynthesis of opiates from cheaper carbon sources was reported first in yeast and later in *E. coli*, feeding on glucose and glycerol, respectively [26, 28]. The key inventions in both systems share general similarities, but differ in specific solutions. First, in order to split up the pathway encompassing more than 20 genes, both studies describe the use of expression modules, which were optimized separately. For the yeast system, the modules were finally incorporated in a single yeast strain. In contrast, for the *E. coli* system, the modules were established in four separate *E. coli* strains. Second, in each system, one module was dedicated to the overproduction of L-tyrosine by using mutated enzymes resistant to feedback inhibition. Third, both studies included protein engineering steps for the functional expression of salutaridine synthase (SalSyn), a cytochrome P450 enzyme. In the yeast system, the N-terminus was replaced with α-helices from cheilanthifoline synthase, which represents a CYP enzyme homologous to salutaridine synthase, and which had been experienced to express well in yeast. In addition, site directed mutagenesis was used to modify glycosylation patterns for proper incorporation in the ER membrane. In the *E. coli* system the N-terminus of salutaridine synthase was deleted entirely. Last, biosynthesis of morphinan type alkaloids requires (R)-reticuline as intermediate. However, the enzyme, which catalyzes the conversion of (S)- to (R)-reticuline was unknown, until Galanie et al. and two more studies identified this enzyme in *Papaver somniferum* by examining data from gene silencing experiments [17, 19, 28]. The enzyme represents a natural fusion protein of a CYP and an aldo-keto reductase, and is now called either DRS-DRR, or STORR, or REPI. This newly discovered enzyme was used in both systems with mixed results. While (R)-reticuline synthesis was sufficient in yeast, it was inefficient in *E. coli*, despite N-terminal truncation of STORR, and despite supplementation with 5-aminolevulinic acid to boost haeme production. Nevertheless, the substrate (R,S)-tetrahydropapaveroline is converted to (R,S)-reticuline in the *E. coli* system. Thus, with the key invention of using (R,S)-tetrahydropapaveroline as an intermediate, the authors have bypassed this and other critical CYP steps and established an alternative pathway for (R)-reticuline production in *E. coli* (Supporting information, Fig 3b). In comparison on the thebaine level, the *E. coli* system leads to a 300-fold higher thebaine titer (2.1 mg/L) than the yeast based system (6.4 µg/L), presumably due to more efficient expression of *SalSyn*, *SalR* and *SalAT* [26].

In summary, a number of BIA branches have been established in microbial hosts with or without precursor feeding. However, biosynthesis pathways of other BIA branches are not fully elucidated yet, as for example the pathway leading to papaverine, which is still controversially discussed [97, 98].

3.4 Phenylpropanoids

Found throughout the plant kingdom, phenylpropanoids are a large group of phenolic compounds like lignins, stilbenoids and flavonoids with very diverse roles in plant growth, development, protection against herbivores and microbes, and in therapeutic application. These compounds often contain phenolic hydroxyl groups, which can form hydrogen or ionic bonds and thus allow rather non-specific interactions with a variety of cellular macromolecules [3].

Phenylpropanoids are derived from either phenylalanine or tyrosine produced in the shikimate pathway [99]. These aromatic acids are channeled to phenylpropanoid biosynthesis (Fig. 4) by the action of phenylalanine or tyrosine ammonia lyases and converted to a common *p*-coumaroyl-CoA precursor [100]. Together with malonyl-CoA *p*-coumaroyl-CoA undergoes a variety of one step reactions of decarboxylation, condensation and cyclization catalyzed by one of different type III polyketide synthases (e.g. CHS, BAS, STS) [101]. These scaffolds lead to the synthesis of flavonoids, stilbenes, lignans among others [102] and are further modified e.g. by hydroxylation, acylation, glycosylation [103]. Many genes in these pathways have been cloned and functionally characterized (Supporting information, Fig. 4).

The heterologous production of phenylpropanoids in microorganisms started in 2003 with a platform in *E. coli* producing naringenin and pinocembrin, albeit at low titers of 450–750 µg/L and with the need of aromatic acid supplementation [104]. Since then, production titers have been gradually enhanced in various studies, which are extensively reviewed in [105]. Here, we describe the currently best system yielding 421 mg/L naringenin from glucose in *E. coli* [52]. The novelty of this study was to use CRISPR-dCas9 as an effective tool to raise malonyl-CoA availability in an *E. coli* host without interfering with biomass production. Thus, instead of overexpressing genes for malonyl-CoA production, Wu et al. used a fine tuned down regulation of endogenous *E. coli* genes responsible for glycolysis, TCA-cycle and fatty acid biosynthesis, while monitoring both malonyl-CoA and biomass production. First, target genes were identified using guide RNAs and dCas9, which is devoid of nuclease activity and

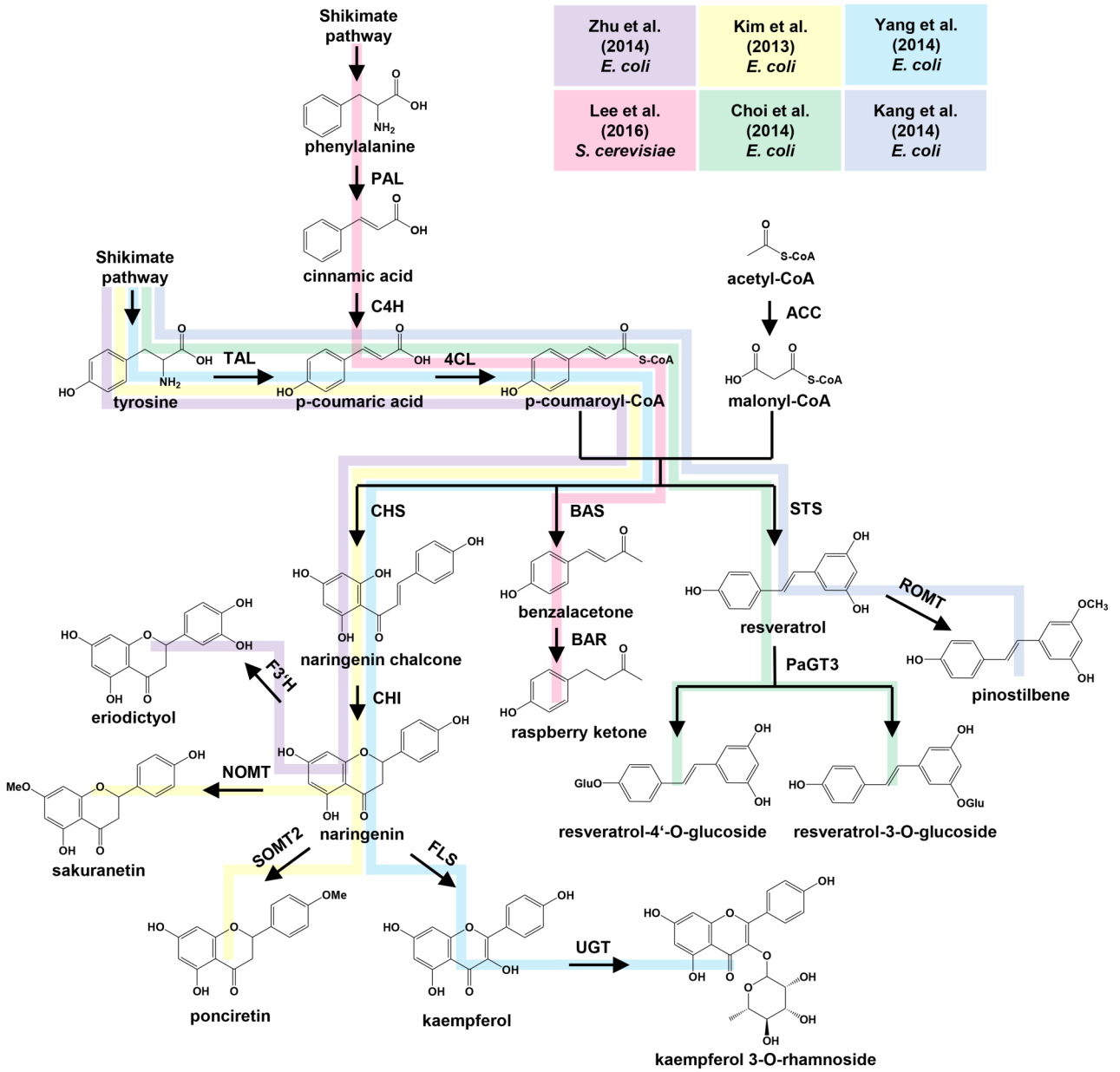

Figure 4. Proposed biosynthetic pathways of selected phenylpropanoids. Reconstructions of multi-enzyme parts of the pathways in heterologous microbial hosts are shaded in different colors. For full names of the biosynthetic enzymes and their first heterologous expression in microorganisms see Supporting information.

blocks transcription. Second, the optimal extent of down regulation was determined by using guide RNAs designed to various locations on the targeted genes. Finally, in an *E. coli* strain additionally transformed with genes for naringenin production, the previously determined genes were simultaneously down regulated in various combinations, and the modified strains were screened for optimal naringenin production.

Soon after naringenin synthesis had been established, various groups started to produce downstream metabolites mostly via biotransformation of precursor substrates. Here, we consider only those approaches that establish a complete de novo pathway using glucose as carbon source. Reviews on precursor feeding and biotransformations are available elsewhere [105–107]. The stilbene resveratrol is accumulated in grape and peanut in response to biotic and abiotic stress [108]. It features antioxidant, anticancer, as well as cardioprotective and neuroprotective activities [109]. Resveratrol also extends life span in various model organisms [110], although this may not be generalized, as we could confirm life span extension in *Caenorhabitis elegans* only in conditions of acute oxida-

tive stress [111]. Resveratrol can be produced from grape juice [112] or glucose in *S. cerevisiae* or *E. coli* [113, 114]. In the *E. coli* system, the genes for resveratrol biosynthesis were added by chromosomal integration. Integration sites were chosen in order to raise tyrosine availability by deletion of *E. coli* genes responsible for tryptophan synthesis and for negative regulation of tyrosine synthesis [114]. The yeast system strengthened the precursor branches of both aromatic acid, and acetyl-CoA synthesis by overexpression of feedback-resistant enzymes [113]. In addition, resveratrol can be modified to raise its stability and bioavailability by methylation [115] or glucosylation [116].

Heterologous flavonoid production is also feasible: eriodyctiol is described as antidiabetic and antinociceptive and is produced in *E. coli* at titers of 5 mg/L from glucose or 107 mg/L from tyrosine [117]. Kaempferol 3-O-rhamnoside is described with antidiabetic and anticancer activities; 57 mg/L are synthesized in engineered *E. coli* [118]. Ponciretin (antimicrobial) and sakuranetin (antimycotic) are produced from glucose in *E. coli* at concentrations of 42.5 mg/L and 40.1 mg/L, respectively [119]. All these studies of flavonoid production in *E. coli* employed engineering steps, which enhance the supply of tyrosine and malonyl-CoA precursors. In contrast, protein fusions (as explained in Section 2.4) allowed the production of 2.8 mg/L raspberry ketone, a flavoring compound, in *S. cerevisiae* [40].

4 Outlook

By far not all plant metabolic pathways have been elucidated and possible therapeutics lie dormant, nevertheless genomics, transcriptomics, proteomics and metabolomics together with gene silencing techniques in plants promise new findings in the near future. A web-based collection of "omics" tools may assist in the mining of the large data sets [120], while adding novel properties by further modifications will expand the repertoire beyond natural occurrence [121]. On the far end facilitation of genome editing of microbial hosts [122] may speed up the process of creating a heterologous production platform for metabolites in an artificial organism, both efficient in yield and balanced for viability. An effective biotechnological production of secondary metabolites, useful for medical and technical applications, will be an important step for a wider acceptance and utilization of SM in the future.

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Michael Wink (Dr. rer. nat.) is a professor of Pharmaceutical Biology at Heidelberg University and director at the Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany. He received his research training in biology and chemistry at the Universities of Bonn, Braunschweig and Munich. In 1988, he became a professor at the University of Mainz and in 1989 at Heidelberg University. His research interests range from plant natural products (identification, biochemistry, pharmacology, biotechnology), medicinal and toxic plants to evolution (phylogeny and phylogeography). He is author of more than 15 books or monographs and author/coauthor of more than 650 refereed publications.

Holger Schäfer (Dr. rer. nat.) studied biology at the University of Darmstadt and obtained his doctorate in 2000 at the University of Heidelberg. He is working as a research assistant at the Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany. His research interests are in molecular biology with a focus on the recombinant production of proteins and natural products.

Clarissa Dziggel (Dipl. Biol.) studied biology at the RWTH Aachen University. Since 2012, she is working on her PhD at the Institute of Pharmacy and Molecular Biotechnology, Heidelberg, in the research group of professor Wink. Her research focuses on the recombinant production of enzymes involved in natural product synthesis and their enzymatic products.

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