Review

Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes

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Plant natural products derived from phenylalanine and the phenylpropanoid pathways are impressive in their chemical diversity and are the result of plant evolution, which has selected for the acquisition of large repertoires of pigments, structural and defensive compounds, all derived from a phenylpropanoid backbone *via* the plant-specific phenylpropanoid pathway. These compounds are important in plant growth, development and responses to environmental stresses and thus can have large impacts on agricultural productivity. While plant-based medicines containing phenylpropanoid-derived active components have long been used by humans, the benefits of specific flavonoids and other phenylpropanoid-derived compounds to human health and their potential for long-term health benefits have only been recognized more recently. In this part of the review, we discuss in detail the recent strategies and achievements used in the reconstruction of multienzyme pathways in plants and microbes in an effort to be able to attain higher amounts of the desired flavonoids and stilbenoids exploiting their beneficial properties as analyzed extensively in Part I of this review [1].

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1 Biotechnology of flavonoids and stilbenoids – reconstruction of multienzyme pathways in plants

With increase in the understanding and documentation of the biological properties and, in many cases, potential beneficial effects of flavonoids and stilbenoids (as thoroughly discussed in Part I of this review [1]), there is increasing interest in the engineering of their metabolism. As reviewed in ref. [1], their apparent pharmacological properties could explain why, whenever consumed regularly in the human and in animal diet, these compounds show pleiotropic health-promoting and disease-preventing activities [2]. However, only a few of these bioactive compounds are produced commercially as pure compounds in small quantities and often in low yields and in unreliable quality and bioactivity [3]. An obvious approach toward this end is to exploit the structural genes encoding enzymes of the general phenylpropanoid and particularly flavonoid and stilbenoid metabolism, whose coordinate expression leads to flavonoid or stilbenoid pro-

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Abbreviations: CPR, cytochrome P450 reductase; **FL6H,** flavonol 6-hydroxylase

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duction. As pointed out by various research groups [4–6], genomics approaches in diverse plant species promise to yield new structural and regulatory genes for flavonoid biosynthesis, helping to complete the cast of players for biotechnological intervention and to link flavonoid metabolism to the broader network of plant metabolism. A key point to be kept in mind is that during *in planta* flavonoid biosynthesis, structural genes, transcription factors, and circadian-controlled transcription factors work together [7].

During land plant evolution, enzymes for the general phenylpropanoid and flavonoid pathways were likely recruited from those involved in primary metabolism [8]. Support for this comes from the fact that the majority of enzymes of flavonoid biosynthesis are members of three classes of enzymes found in all organisms (a detailed full name description of nearly all known enzymes involved in the phenylpropanoid metabolic pathway is given in Part I of this review [1] (Table 1 and Fig. 3 of that paper)): the 2-oxoglutarate-dependent dioxygenases (ODD) (ANS, F3H, FL6H, FLS, FNSI), NADPH-dependent reductases (ANR, DFR, FNR, IFR, LAR, VR) and cytochrome P450 monooxygenases (C3H, C4H, CH3H, F2H, F5H, F6H, F3′H, F3′5′H, FNSII, IFS, I2′H). CHS and CHI, on the other hand, appear to have a more limited ancestry. CHI, in particular, appears to be unique to plants in both sequence and 3-D structure [9], while CHS is a member of the plant polyketide synthase superfamily, which also includes stilbene synthase [10]. Thus, CHS and related enzymes may have been recruited from such ancestral enzymes for natural product biosynthesis. Finally, with respect to potential metabolic engineering approaches, it should be recognized that flavonoid biosynthetic enzymes might function in multienzyme complexes, *i.e.*, the metabolons [11]. These may be important in the efficient channeling of carbon into specific branch pathways, since there is strong competition for substrates at the branch points of pathways, the intermediates are highly reactive and potentially toxic, and there is a need for quick response to external and internal signals that control the production of flavonoids [5].

1.1 Transcription factors as tools for pathway manipulation

The structural genes encoding enzymes for the biosynthesis of flavonoids, stilbenes, and other phenylpropanoids are at the end points of regulatory pathways that control their spatiotemporal patterns of expression, and thus phenylpropanoid natural product accumulation. Specific transcription factors that modulate the activity of RNA polymerase II at target promoters are important regulators of the temporal and spatial expressions of these structural genes, and are thus potentially important tools for manipulating these multienzyme pathways. For example, the organ- and tissue-specific regulations of anthocyanin biosynthesis is well known to be controlled by specific transcription factors, which are structurally and functionally well conserved between species, and which lead to the coordinate activation of multiple structural genes in this pathway [12, 13].

From the study of the well-documented mechanisms of action of various transcription factors [12–14], four general conclusions about how transcription factors may be exploited in the metabolic engineering of flavonoid and phenylpropanoid metabolism are drawn: (i) transcription factors typically control the expression of multiple genes encoding enzymes in a given pathway, allowing efficient manipulation of multienzyme pathways, (ii) ectopic expression of specific transcription factors can be used as a tool for redirecting metabolic differentiation of the cells, (iii) pathway-specific transcription factors could be used to modulate the production of specific secondary metabolites and (iv) inactivation of transcriptional repressors could be used to derepress metabolic channeling into a pathway leading to a specific metabolite.

A large number of transcription factors involved in plant flavonoid biosynthesis are known and details of the most recent biotechnological advances using genes encoding these factors are shown in Table 1. Initial work on the genetic and molecular regulation of flavonoid multienzyme pathways and subsequent manipulation of flavonoid biosynthesis targeted maize and *Antirrhinum* transcription factors, work that was aided by the variety of anthocyanin pigmentation mutants available (Table 1, [15, 16]). A primary target was the use of such transcription factors to alter flower color by manipulating anthocyanin pigment biosynthesis. Since then, research has been expanded to identify regulatory genes in many evolutionarily distant species [17–20]. Very well-known regulatory genes are *C1* and *R* that induce anthocyanin production in maize (reviewed in refs. [21, 22]). Later attempts to improve the flavonoid profiles with respect to nutritional aspects have followed [18, 22, 23].

The ability of *C1/R* genes to promote flavonoid biosynthesis [3] was used to produce maize cells that were able to produce a health-promoting isoflavone. When a model cell system was employed (maize Black Mexican Sweet cells), cells transformed with *IFS* could not produce genistein unless a construct that harbored a fusion of the *C1* and *R* transcription factors (*CRC* construct) was cotransformed [17]. The activation of the isoflavonoid branch (see Fig. 3 in Part I of this review [1]) by the *CRC* construct was used to increase the production of isoflavones in soy [18]. Similarly, the expression of both the *C1* and *Lc* transcription factor genes in tomato [24] was sufficient for the upregulation of the flavonoid pathway in the tomato fruit, when the expression of *Lc* alone in tomato also increased anthocyanin content [16].

The *PAP1 MYB*-related transcription factor from *Arabidopsis thaliana* promotes anthocyanin biosynthesis [14, 16, 19, 25] by the activation of the initial genes of the flavonoid pathway, although the expression of another

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Table 1. Biotechnology of transcription factors

Flavonoid target	Plant gene (plant donor)	Transcription factor type	Engineered plant	Positive (+) or negative (–) gene regulation	Metabolic effects (reference)
Kaempferol, naringenin	*C1*, *LC* transcription factors *(Zea mays)*	*Myb/Myc*	*L. esculentum*	(+)*PAL*, (+)*CHS*, (+)*F3H*, (+)*FLS*, (+)*DFR*, (+)*ANS*, (+)*F3GT*, (+)*A3RT*, (+)*GST*	High-flavonol tomatoes (mainly Kaempferol, lesser effect for naringenin) resulting from the heterologous expression of the maize transcription factor genes *LC* and *C1* [24]
Genistein, daidzein	*IFS*, *CHR* (*Glycine max*), *C1/R1* transcription factors fusion (*Z. mays*)	*Myb/Myc* *Z. mays*	*N. tabacum*,	(+)*PAL*, (+)*CHS*, (+)*CHI*, (+)*F3H*, (+)*DFR*, (+)*F3GT*, (+)*GST*	Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues [17]
Genistein, daidzein	*IFS* (*G. max*), *C1/R1* transcription factors fusion (*Z. mays*)	*Myb/Myc*	*G. max*	(+)*PAL*, (+)*CHS*, (+)*CHI*, (+)*F3H*, (+)*DFR*, (+)*F3GT*, (+)GST	Metabolic engineering to increase isoflavone biosynthesis in soybean seed [18]
Anthocyanins, flavones	*P1* transcription factor (*Z. mays*)	*Myb*	*Z. mays*	(+)*PAL*, (+)*CHS*, (+)*DFR*	Increased anthocyanins and flavone levels [102, 103]
Anthocyanins	*MYB10* transcription factor (*M. domestica*)	*Myb*	*N. tabacum*, *M. domestica*	Orthologue to *PAP1* (+)*F3H*, (+)*F3′H*, (+)*F3′5′H*, (+)*DFR*, (+)*ANS*, (+)*F3GT*	Increased levels of anthocyanins in leaves [22]
3-Rutinoside-5-glucoside of delphinidin, petunidin, and malvidin	*ANT1* overexpression (*L. esculentum)*	*Myb*	*L. esculentum*	(+)*CHS*, (+)*CHI*, (+)*F3H*, (+)*DFR*, (+)*F3GT*, (+)*A5GT* (+)*GST*	Increase in anthocyanin content [27]
Anthocyanins	*Del* (*Delila*) transcription factor (*Antirrhinum*)	*Myc*	*L. esculentum*	(+)*F3H*, (+)*DFR*, (+)*F3GT*, (+)*CHS*	Enhanced anthocyanin pigmentation [29]
Anthocyanins	*Lc* transcription activator (*Z. mays*)	*Myc*	*L. esculentum*	(+)*PAL*, (+)*C4H*, (+)*FLS*, (+)*CHS*, (+)*CHI*, (+)*F3H*, (+)*F3′H*, (+)*F3′5′H*, (+)*DFR*, (+)*ANS*, (+)*F3GT*, (+)*A3RT*, Orthologue to *DEL*	Enhanced pigmentation under high light conditions [16, 104]
Anthocyanins	*MYC-RP*, *MYC-GP* (*P. frutescens*)	*Myc*	*L. esculentum*	(+)*CHS*, (+)*F3H*, (+)*DFR*, (+)*F3GT*	Increase in anthocyanin content [31]
Anthocyanidins, proantho-cyanidins	*ANR* (*Medicago truncatula*), *PAP1* transcription factor (*A. thaliana*)	*Myb*	*N. tabacum*	(+)*F3H*, (+)*F3′H*, (+)*F3′5′H*, (+)*DFR*, (+)*ANS*, (+)*F3GT*	Introduction of proanthocyanidins into nonproducing plant for conversion of anthocyanidin into (epi)-flavan-3-ol [20]
Proantho-cyanidinsfactors	*TT2*, *PAP1* transcription (*A. thaliana*)	*Myb*	*A. thaliana*	(+)*PAL*, (+)*CHS*, (+)*DFR*, (+)*GST*	Ectopic accumulation of pro-anthocyanins with the coexpression of a positive regulator of anthocyanin biosynthesis with *TT2 MYB* transcription factor [19]

A partial list of metabolic engineering results achieved using transcriptional factors known to regulate flavonoid biosynthesis, along with the description of the metabolic phenotype.

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WD40 signaling protein is also needed [25, 26]. *PAP1* along with an anthocyanidin reductase can be used to transform forage species to divert metabolism away from the production of proanthocyanidins, in order to reduce the negative impacts of bloat [20]. Furthermore, orthologs of *PAP1* from *Malus domestica* (*MdMYB10*) [22] and *Lycopersicon esculentum* (*ANT1*) [27] resulted in strong phenotypes due to high accumulation of anthocyanins. Another *MYB*-related transcription factor from maize, *P1*, regulates the branch of flavonoid biosynthesis that leads to red phlobaphene pigments (not shown in Table 1). The importance of the latter is illustrated by its use in generating maize plants with increased production of maysin, a flavone glycoside conferring natural resistance against corn earworm [28].

The *Antirrhinum DEL* (*Delila*) transcription factor (Table 1) that shares similarity with *myc*-type factors of maize is required for anthocyanin biosynthesis in the corolla tube and other tissues of the *Antirrhinum* flower [29, 30], illustrating the existence of evolutionarily conserved regulatory mechanisms in maize and *Antirrhinum* [30]. Two apparently orthologous genes from *Perilla frutescens*, *MYB-RP* and *MYB-GP* (Table 1), show similar expression profiles, and when expressed in tobacco and in tomato increase the anthocyanin content of tobacco flowers and vegetative tissues and flowers in tomato [31].

Another group of regulatory genes with great biotechnological potential are homologs of the *Arabidopsis DET1* gene (*HP-2* in tomato, Table 1), negative regulators of anthocyanin biosynthesis [32]. Downregulation of *HP-2* expression by RNAi technology resulted in increased amounts of flavonoids and carotenoids [23, 33].

1.2 Agricultural and forest biotechnology

Advancements in plant biochemistry, molecular biology, structural biology and genomics have fueled research interest and investment in plant metabolic engineering approaches to agricultural crop improvement [34, 35]. A number of approaches to alter *in planta* gene expression have applications to pathway engineering. These include "activation tagging" to produce ectopic dominant mutations by overexpression of endogenous genes [27], introduction of novel biosynthetic activities from other organisms to increase levels of a rate-limiting biosynthetic enzyme, inhibition of gene expression by RNA silencing, antisense or sense inhibition [36], and up- or downregulation of entire pathways using regulatory factors [24, 37, 38].

Plant metabolic engineering approaches applied to flavonoid and phenylpropanoid pathways could help to meet the demands of society for a more environmentally benign agriculture with less reliance on agrochemicals for crop protection [39] or even to improve human and animal health through altering flavonoid and phenylpropanoid metabolic pathways in plant-based foods for the production of so-called "nutraceutical" metabolites with potential human health benefits, as discussed above [6, 36, 40].

Several of the approaches described above are highlighted in Table 2 that provides a list of recent successful case studies for the metabolic engineering of flavonoids and stilbenoids. Table 2 mainly focuses on metabolic improvements concerning increases in the biosynthesis of stilbenoids and flavonoids in some plant edible parts (tomato fruit, potato tuber) and food crop plants (rice, alfalfa, flax, oil rapeseed, *etc*.). The biotechnological manipulation of flower color and attempts at novel flower color creations have been reviewed recently by Forkmann and Martens [4].

Specific examples of metabolic engineering in agriculture pertain mostly to isoflavonoids. As mentioned above, some members of isoflavonoids are important antimicrobial phytoalexins/phytoanticipins in both forage and seed crop legumes [41]. Biotechnology strategies for improving the efficacy of such antimicrobial agents may include genetic modifications to increase the speed and levels of their accumulation [42–44].

Furthermore, it has been proposed that increased lipophilicity of phytoalexins/phytoanticipins candidates can increase antifungal activity; thus prenylated isoflavonoids are strongly antimicrobial, whereas their nonprenylated precursors have little or no antimicrobial activity [39]. Genomic and biochemical strategies for discovering new plant and microbial [45] prenyltransferases active against flavonoids may provide tools for increasing the proportion of prenylated isoflavonoids. Interestingly, prenylated flavonoids have much greater bioactivity as phytoestrogens [46], suggesting that genetic modification of food crops to contain higher levels of prenylated flavonoids may also enhance the human diet [47].

Many species of wild plants are prominent in their uses as traditional medicines. For example, *Saussurea involucrata*, a herb that grows at high altitudes, is well known in traditional Chinese medicine for its effects in promoting blood circulation and for its anti-inflammation and analgesic activity. Among compounds that have been found to possess such beneficial effects is the flavone apigenin. An *in vitro* cell culture system for *S. involucrata* with sufficient yields or apigenin could protect this species from disappearing [48] due to overharvesting as well as providing a simple method for overproducing this highly active compound. *Agrobacterium rhizogenes* was used to transform *S. involucrata* and overexpress *CHI*, producing a key enzyme in flavonoid biosynthesis thus resulting in the increased production of flavonoids in general (up to four-fold) and in increased production of apigenin in particular (up to 12-fold) [48].

An earlier report of genetic modification of plants to overproduce flavonoids beneficial for the human health was the increased flavonoid content of vegetables generated using a *Petunia hybrida CHI* gene with its promoter and terminating sequences [49]. This gene was used to

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Table 2. General plant biotechnology applications that concern metabolic engineering of a certain metabolic flavonoid or stilbenoid target, along with the description of the obtained metabolic effect

General plant biotechnology applications Metabolic target	Plant gene donors (target gene(s))	Engineered plant	Metabolic effects (reference)
Stilbenoids			
Resveratrol	*Vitis vinifera* (*RS*)	*Brassica napus*	Increased resveratrol glucosides production in seeds [105]
Resveratrol	*Arachis hypogaea* (*RS*)	*Rehmannia glutinosa*	Resveratrol aglycones and glucosides production [106]
Resveratrol	*V. vinifera* (*RS*)	*L. esculentum*	Resveratrol aglycones and glucosides production [107]
Resveratrol	*Parthenocissus henryana* (*RS*)	*Lactuca sativa*	Resveratrol accumulation in leaves [108]
Resveratrol, piceid	*P. hybrida (CHS, CHI), Medicago sativa (CHR), Gerbera (FNSII)*	*L. esculentum*	Stilbenoids production in non-producing fruit [109]
Flavonoids			
Apigenin	*Saussurea medusa* (*CHI*)	*S. involucrata*	Overproduction of apigenin through *CHI* overexpression. *CHI* catalysed the conversion of chalcone into naringenin a prerequisite for apigenin production [48]
Kaempferol glycoside, quercetin glycoside	*P. hybrida* (*CHI*)	*L. esculentum*	Production of increased levels of kaempferol and quercetin glycosides [49]
Naringenin, kaempferol, rutin, quercetin	*L. esculentum* (*CHS*, *CHI*, *FLS*)	*L. esculentum*	Increase in total fruit flavonols was achieved through ectopic expression of chalcone isomerase chalcone synthase and flavonol synthase [110]
Chalcones (butein, isoliquiritigenin), flavones (luteolin), flavonols (rutin, quercetin glucosides, kempferol)	*P. hybrida (CHS, CHI), M. sativa (CHR), Gerbera (FNSII)*	*L. esculentum*	Novel flavonoids produced in nonproducing fruit [109]
Genistein	*G. max* (*IFS*)	*A. thaliana*	Expression of isoflavone synthase for the biosynthesis of genistein [50]
Genistein	*G. max* (*IFS*), *N. tabacum* (*F3H*), *Astragalus membranaceus* (*PAL*)	*N. tabacum*, *L. sativa*, *P. hybrida*	Genistein production [111]
Genistein	*G. max* (*IFS*)	*Oryza sativa*	Genistein production in rice plants that over-express IFS modulate *nod* gene expression in various *Rhizobia* [112]
Genistein	*G. max* (*IFS*)	*A. thaliana*	Production of genistein from *A. thaliana* and competition with pathway components that lead to flavonol synthesis [113]
Glucosides of genistein, biochanin A, pratensein	*M. truncatula* (*IFS*)	*M. sativa*	Differential tissue and environment-specific effects in production of Genistein, Biochanin A, and Pratensein glucosides, as other flavonoids, due to *IFS* transgene expression [114].
Neohesperidin	*Citrus maxima* (*F7RT*)	*N. tabacum*	Metabolic engineering of plant cells for bio-transformation of hesperedin into neohes-peridin [115]

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Table 2. Continued

General plant biotechnology applications Metabolic target	Plant gene donors (target gene(s))	Engineered plant	Metabolic effects (reference)
Aureusidin 6-*O*-glucoside	*Antirrhinum majus* (*4′CGT*, *AS*)	*Torenia hybrida*	Yellow flowers generated by the aureusidin 6-*O*-glucoside synthesis [116]
Anthocyanins	*P. hybrida* (*CHS*, *CHI*, *DFR*)	*Solanum tuberosum*	Altered anthocyanin levels in potato tubers [117]
Anthocyanidins	*Gerbera* hybrid, *Matthiola incana*, *Callistephus chinensis*, *Dianthus caryophyllus*, *L. esculentum*, *Rosa hybrida* (*DFR*)	*S. cerevisiae*, *N. tabacum*	Several cDNA clones encoding *DFR* have been isolated from different plant species for the heterologous expression and anthocyanidins production [101]
Anthocyanins	*Solanum sogarandinum* (*A5GT*)	*S. tuberosum*	Ectopic expression of *A5GT* that confers 2-fold increase in resistance against *Erwinia carotovora* with simultaneously increase in organoleptic characteristics [118]
Quercetin, proanthocyanins, anthocyanins	*O. sativa* (*ANS*)	*O. sativa*	Overexpression of anthocyanidin synthase accumulates a mixture of flavonoids [119]
Lignans, total phenolics	*P. hybrida* (*CHS*, *CHI*, *DFR*)	*Linum usitatissimum*	Increase in total phenolic antioxidant levels, including slight increase (12–14%) in lignan content [120]

transform tomato (*L. esculentum*) and although the resulting flavonols were mainly glycosylated, the modified tomatoes contained significantly increased levels of quercetin glycosides and smaller but still substantial increases in kaempferol glycosides in fruit peel (up to 78-fold elevated concentration of flavonols).

In another example of the potential for metabolic engineering of plants for improved nutrition, an *IFS* gene from soybean was expressed in *A. thaliana*, a plant that does not naturally produce genistein. Genistein was readily detected in the transgenic plants [50], indicating that there is the possibility for enhancing flavonoid content of foods by the heterologous expression of nonlineage-specific flavonoid biosynthetic genes.

Phenolic compounds including flavonoids are abundant in the foliage of many tree species and appear to play important roles against insect herbivory, a major problem for plantation forestry. For example in *Populus* (poplars and aspens), phenylpropanoid-derived phenolic glycosides and condensed tannins may constitute up to 35% of foliar dry weight [51]. The rapid induction of *DFR* gene expression and condensed tannin accumulation in aspen in response to insect herbivory suggests that these compounds are important in defense against herbivors in *Populus* [52], supported by insect herbivory-induced activation of flavonoid metabolism in *Populus* as assayed by microarray expression profiling [53]. *Populus* is the first tree to have its complete genome sequenced [54]. Recent annotations of the complete set of *Populus* flavonoid biosynthetic genes were found to be involved in the biosynthesis of condensed tannins [51], and identification of potential regulatory and signaling genes may control herbivory-induced condensed tannin biosynthesis [53]. This raises the possibility of enhancing defenses against insect attack in *Populus* plantations by the selection of genotypes with the alleles that condition more rapid and intense condensed tannin accumulation in response to herbivory, or by genetic modification using entirely endogenous *Populus* genes to achieve the same goal. In conifers, too, insect herbivore leads to rapid activation of phenylpropanoid and flavonoid metabolism [55]. While potential roles for flavonoids as part of conifer chemical defenses have not yet been experimentally established, these results suggest this possibility and thus the potential for biotechnological intervention to enhance conifer defenses against devastating insect infestations. This work may be aided by the characterization of pine [56] and spruce [57, 58] *MYB* transcription factors that, analogous to angiosperm *MYB* genes discussed above, regulate different aspects of phenolic metabolism in conifers.

2 Reconstruction of multienzyme pathways of flavonoids and stilbenoids in microbes

Both prokaryotic and eukaryotic microbes such as *Escherichia coli* and *Saccharomyces cerevisiae* are widely

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used as model systems and for the expression of genes that are able to convert fed precursors or endogenously produced substrates into valuable end products. Several compounds with high additive value such as isoprenoids and polyketides [59, 60], flavors and fragrances [61], terpenoids [62], and of course plant-derived flavonoids could be the end products of such bioconversions in microbes, because the interactions between proteins are easily studied and manipulated and the overproduction of either endogenous or heterologous proteins is easily carried out. Thus, these systems have great potential for heterologous production of bioactive flavonoid compounds.

The first studies on microbial expression of plant-derived phenylpropanoid and flavonoid enzymes had their main objectives of assaying the activity of specific enzymes such as PAL [63], C4H [64], 4CL [65], CHS [66], CHI [67], IFS [50, 68], I2′H [69], F2H [70], F3′H [71], F6H [72, 73], ANS [74], IOMT [75], VR [68], and testing the potential for the biotransformation of precursor compounds harnessing the metabolic power of microbial hosts. These first tests were positive and gave birth to the term "metabolic pathway engineering" leading to current work on the introduction of multienzymic systems into microbial cells for the purpose of producing specific plant-derived secondary metabolites. Although in 2002 Verpoorte [76] stated that it would be possible to engineer only short biosynthetic pathways, the potential of using microbes to reconstitute whole metabolic pathways was illustrated by the subsequent work of Hauf *et al*. [77] who simultaneously expressed seven glycolytic enzymes.

Several of the model microbial systems with successful metabolic engineering achievements of flavonoids and stilbenoids for both prokaryotic (Section 2.1) and eukaryotic systems (Section 2.2) are given in Tables 3 and 4, respectively. Some key examples of these cases are discussed below.

2.1 Prokaryotes

In the first report of the production of compounds with medicinal interest in engineered *E. coli* strains, Watts *et al.* [78] overexpressed the production of the stilbenes resveratrol and piceatannol under appropriate culturing conditions. Strains were generated that expressed plant *4CL* and *STS* genes that were capable of producing resveratrol or piceatannol when supplied with either *p*-coumaric acid or caffeic acid as precursor molecules. The yield of resveratrol in the transformed strains was extremely high (about 100 μg/mL culture) and this, along with the fact that no codon optimization was necessary, highlights the potential for prokaryotes as hosts for heterologous flavonoid production.

An earlier attempt to reconstitute the whole biosynthetic pathway in order to produce the flavanone naringenin in bacteria, starting with PAL, was not successful [79]. In that work, expressions of *A. thaliana* genes encoding *PAL*, *C4H*, *4CL* and *CHS*, the first four enzymes required in sequence for naringenin biosynthesis, were introduced into an *E. coli* host strain. However, since the cytochrome P450 enzyme C4H was not functional in the bacterial host, this effort was unsuccessful. To overcome this problem, a *Rhodobacter capsulatus* gene encoding tyrosine ammonia-lyase (TAL), capable of converting tyrosine into *p*-coumaric acid (the product of C4H), was used, and made possible the production of naringenin (up to about 21 μg/mL cultivation in 48 h).

Several research groups have begun to "program" bacterial hosts to produce more complex secondary metabolites that are the end products of the coordinated action of several enzymes. A project with no direct metabolite production but with great impact on phenylpropanoid pathway reconstitution in heterologous organisms was reported by Hotze *et al*. [80], who developed a strategy for analyzing the activities encoded by the rapidly increasing number of plant cytochrome P450 sequences. They found that the expression of a chimeric protein consisting of a truncated C4H domain and a truncated cytochrome P450 reductase (CPR) domain linked with a Ser-Thr-Ser-Ser-Gly linker has the potential to allow functional expression of *C4H* in a bacterial host. Such an approach could be used for the engineering of different branches of the phenylpropanoid system in host organisms that either do not have the necessary CPR, or have low levels of *CPR* expression. For example, it is obvious that yeast's CPR is not able to support the expression of high C4H activities, so when high levels of C4H functionality are required, *C4H* expression should be coupled with *CPR* expression [81].

Leonard *et al.* [82] reported the production of naringenin, eriodictyol, dihydrokaempferol, dihydroquercetin, kaempferol and quercetin by the coexpression up to eight genes in the flavonoid metabolic pathway in *E. coli*, when the strains were provided the precursor *p*-coumaric acid. In this study, two proteins were linked *via* a Gly-Ser-Thr linker in a way such that their activities were not lost, allowing the expression of two enzymes under the control of just one promoter. They also managed to optimize the bacterial growth conditions for metabolite production. They suggested that the generation of quercetin was better achieved using low copy number plasmids because of the enhanced stability of the expression, and that growth media could affect the quantity of flavonol production.

It was little earlier that Yan *et al*. [83] proposed for the first time a strategy for synthesizing plant anthocyanins in a bacterial host. Following classical biotechnology protocols, they managed to produce 3-*O*-glucosides of pelargonidin and cyanidin by the expression of *F3H*, *DFR*, *ANS*, and of a *F3GT* in *E. coli*. Anthocyanins were produced at a low concentration (5.6 μg/L) with several byproducts such as dihydrokaempferol, dihydroquercetin, kaempferol, quercetin at relatively higher levels (5.56, 8.8, 0.44, and 0.444 mg/L, respectively). The higher levels of

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Table 3. Prokaryotic microbial biotechnology applications employing metabolic engineering of a certain metabolic flavonoid or stilbenoid target, in order to lead the bacterial strains to obtain a certain metabolic effect (as exists in plants) produced by the microbes

Prokaryotic microbial biotechnology applications		
Flavonoid target	**Plant gene donors (target genes)**	**Metabolic effects (reference)**
p-Coumarate	*C. roseus (C4H, CPR)*	Coupling of *C4H* with *CPR* for *p*-coumarate production [80]
p-Coumarate	*Rhodotorula glutinis* (*PAL*), *Helianthus tuberosus* (*C4H*, *CPR*)	Production of *p*-coumarate *E. coli* and *S. cerevisiae* [121]
Resveratrol, piceatannol	*A. thaliana* (*4CL*), *A. hypogaea* (*RS*)	*p*-Coumaric acid and caffeic acid conversion to resveratrol or piceatannol [78]
Naringenin	*TAL* from *R. capsulatus* (*TAL*), *A. thaliana (PAL*, *C4H*, *4CL*, *CHS*)	Overproduction of naringenin [79]
Naringenin, eriodictyol, dihydro-kaempferol, dihydroquercetin, kaempferol, quercetin	*Petroselinum crispum* (*4CL*), *M. domestica* (*F3H*), *A. thaliana* (*FLS*), *P. hybrida* (*CHS*, *CHI*), *C. roseus* (*F3′5′H*, *CPR*)	Production of flavonols by the expression of protein fusions [82]
Apigenin, luteolin	*P. hybrida* (*FNSI*)	Flavone synthase expression allows the biosynthesis of flavone derivatives in *E. coli* [122]
Naringenin, pinocembrin	*R. rubra* (*PAL*), *S. coelicolor* (*4CL*), *G. echinata* (*CHS*)	Naringenin and pinocembrin production using alternative molecular strategies in bacteria [123]
Pelargonidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside	*M. domestica* (*F3H*, *ANS*), *Anthurium andraeanum* (*DFR*), *P. hybrida* (*F3GT*)	Conversion of naringenin or eriodictyol to the corresponding 3-*O*-glucosylated form [83]

these byproducts could be either due to region-specific activities of the implicated enzymes, suggesting that they can participate in reactions with several substrates, or due to lower catalytic efficiencies of the intermediate steps.

2.2 Eukaryotes

The yeast *S. cerevisiae* has also been used to express flavonoid biosynthetic genes. Along with the fact that it has similar compartments with plant cells, *S. cerevisiae* has the advantage of permitting post-translational modifications of eukaryotic proteins. Additionally, yeast has an endomembrane system (*e.g.*, endoplasmic reticulum), into which plant P450 enzymes such as C4H are typically embedded [84], together with their CPR partners [85]. A general model for the spatial positioning of the enzymes implicated in anthocyanin (flavonoids in general) biosynthesis is discussed by Jaakola [86] and Winkel [87].

When Ralston *et al.* [88] expressed several *CHI* genes in yeast and added chalcones to the culture media, the chalcones were rapidly converted to flavanones with different efficiencies depending on the type of CHI. This indicates that yeast can take up chalcone across the plasma membrane, a fact that could be used for the production of several naringenin derivatives. When the chalcone substrates were added to the culture media of yeast cotransformed with *IFS* and *CHI* genes, the chalcones were first converted to flavanones and subsequently to 2-hydroxyisoflavanones and isoflavones by the action of IFS. On the other hand, the coexpression of *CHI* with *F3H* or *FNSII* in yeast cells resulted in the synthesis of dihydroflavonols or flavones, respectively, when chalcones were used as substrates. This shows that naringenin can be used as a substrate not only by IFS but also by F3H and FNSII. When the *CHI* genes were coexpressed with *IFS*, chalcone substrates were converted to flavanone and then to isoflavones as a result of the coordinate activity of both enzymes.

A similar result was observed when a 2-hydroxyisoflavone synthase (*IFS*) gene from *Glycyrrhiza echinata* was expressed into *S. cerevisiae*, where the enzyme was targeted to microsomes [89]. This strain was capable of transforming the 5-deoxyflavanone liquiritigenin and flavanone naringenin to daidzein and genistein, respectively. An almost identical result was obtained by expression of an isoflavone synthase gene from *Trifolium pratense* in *S. cerevisiae*, allowing the biotransformation of 7-hydroxyflavanone, 5,7-dihydroxyflavanone, 7,4′-dihydroxyflavanone (liquiritigenin), and 5,7,4′-trihydroxyflavanone (naringenin) to the corresponding isoflavones [90]. Moreover, it was shown that the co-expression of a CPR [88] increased the biotransformation of naringenin to genistein [91].

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Table 4. Eukaryotic microbial biotechnology applications employing metabolic engineering of a certain metabolic flavonoid or stilbenoid target, in order to lead the bacterial strains to obtain a certain metabolic effect (as exists in plants) produced by the microbes

Eukaryotic microbial biotechnology applications			
Flavonoid target	**Plant gene donors (target genes)**	**Engineered organisms**	**Metabolic effects (reference)**
p-Coumarate	*R. glutinis* (*PAL*), *H. tuberosus* (*C4H*, *CPR*)	*S. cerevisiae*	Production of *p*-coumarate in *S. cerevisiae* [121]
Resveratrol	*Populus trichocarpa x P. deltoides* (*4CL*), *V. vinifera* (*RS*)	*S. cerevisiae*	Engineering of *S. cerevisiae* for the synthesis of resveratrol [94]
Resveratrol	*Rhodobacter sphaeroides* (*TAL*), *A. thaliana* (*4CL*), *V. Vinifera* (*RS*)	*S. cerevisiae*	Using protein fusions to engineer resveratrol biosynthesis in yeast and mammalian cells [124]
Resveratrol	*N. tabacum* (*4CL*), *V. vinifera* (*RS*)	*S. cerevisiae*	Production of resveratrol in *S. cerevisiae* [125]
Naringenin, pinocembrin	*Rhodosporidium toruloides* (*PAL*), *A. thaliana* (4CL), *Hypericum androsaemum* (*CHS*)	*S. cerevisiae*	Production of naringenin and pinocembrin in *S. cerevisiae* [98]
Naringenin, apigenin, dihydrokaempferol	*G. max* (*CHI*, *IFS*), *Gerbera hybrida* (*FNSII*), *G. max* (*F3H*)	*S. cerevisiae*	Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast [88]
Genistein, daidzein	*G. echinata* (*IFS*)	*S. cerevisiae*	Transformation of liquiritigenin and naringenin to daidzein and genistein, respectively [89]
Genistein, daidzein	*Trifolium pratense* (*IFS*)	*S. cerevisiae*	Transformation of liquiritigenin, naringenin, 7-hydroxyflavanone, and 5,7-dihydroxyflavanone to the corresponding isoflavones [91]
Genistein	*G. max* (*IFS*)	*S. cerevisiae, A. thaliana*	Expression of isoflavone synthase for the biosynthesis of genistein [50]
Apigenin, luteolin, 7,4′-dihydroxyflavone	*Gerbera* (*FNSII*)	*S. cerevisiae*	Cloning and expression of *FNSII* in yeast cells [92]
p-Coumarate	*Populus kitakamiensis* (*PAL*), *Populus trichocarpa* x *P. deltoides* (*C4H*, *CPR*)	*S. cerevisiae*	Construction of yeast strains that transforms endogenously produced phenylalanine into *p*-coumarate [93]
Genistein, daidzein	*G. max* (*IFS*), *M. sativa* (*CHI*)	*S. cerevisiae*	Genistein or daidzein production with an artificial bifunctional enzyme [126]
Eriodictyol, dihydroquercetin	*A. thaliana* (*F3′H*)	*S. cerevisiae*	F3′H expression into yeast cells led to conversion of naringenin or dihydrokaempferol into eriodictyol or dihydroquercetin [71]
Quercetin	*P. hybrida* (*FLS*)	*S. cerevisiae*	Production of quercetin by conversion of dihydroquercetin [127]
Methylated flavonols	*Chrysosplenium americanum* (*FL6H*)	*Pichia pastoris*	*FL6H* protein hydroxylates partially methylated flavonols at the 6 position of their A-ring [72]
Naringenin, pinocembrin	*A. thaliana* (*C4H*), *P. crispum* (*4CL*), *P. hybrida* (*CHS*, *CHI*)	*S. cerevisiae*	Yeast strain produced the flavanones naringenin and pinocembrin when fed with phenylpropanoid acids [99]
Resveratrol	*Populus* (*PAL*), *G. max* (*C4H*, *4CL*), *V. vinifera* (*RS*)	*S. cerevisiae*	Engineering of *S. cerevisiae* for the synthesis of resveratrol (Trantas and Ververidis, unpublished data)
Genistein	*Populus* (*PAL*), *G. max* (*C4H*, *4CL*, *CHS*, *CHI*, *IFS*)	*S. cerevisiae*	Engineering of *S. cerevisiae* for the synthesis of genistein (Trantas and Ververidis, unpublished data)
Kaempferol	*Populus* (*PAL*), *G. max* (*C4H*, *4CL*, *CHS*, *CHI*, *F3H*), *S. tuberosum* (*FLS*)	*S. cerevisiae*	Engineering of *S. cerevisiae* for the synthesis of kaempferol (Trantas and Ververidis, unpublished data)
Quercetin	*Populus* (*PAL*), *G. max* (*C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3′H*), *S. tuberosum* (*FLS*)	*S. cerevisiae*	Engineering of *S. cerevisiae* for the synthesis of quercetin (Trantas and Ververidis, unpublished data)

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Yeast also provides a useful system to evaluate enzyme activities. For example, legume IFS enzymes together with a P450 reductase can convert liquiritigenin and naringenin to daidzein and genistein, respectively. In a yeast expression system, legume IFS enzyme carried out these conversions, although with different efficacies [50]. Interestingly, IFS genes cloned from a divergent group of species show very high protein product similarities between them; even IFS from sugar beet (*Beta vulgaris*), a nonlegume species, shows great protein similarity, suggesting stringent stuctural requirements for protein catalytic activity. As mentioned above, when IFS from soybean expressed in *A. thaliana*, a non-genistein producing plant, genistein was detected, indicating that there is the possibility for enhancing flavonoid content of foods derived from food plants by metabolic engineering practices [50].

Two types of flavone synthases have been cloned up to now: one from parsley (*Petroselinum hortense*) and the other from a *Gerbera* hybrid (FNSI and FNSII, respectively). Heterologous expression of the *FNSII* gene cloned from *Gerbera* enables the control of the synthesis of several flavones depending on the substrate specificity. When naringenin is used as a substrate it is biotransformed to apigenin and when eriodictyol or liquiritigenin are used as substrates they are biotransformed to luteolin or 7,4′-dihydroxyflavone, respectively [92].

The work by Ro and Douglas [93] suggests that it should be possible to engineer yeast strains that accumulate flavonoids, starting with phenylalanine, and proceeding through general phenylpropanoid metabolism to naringenin and beyond. They constructed yeast strains that were capable of transforming endogenously produced phenylalanine into *p*-coumarate. This was accomplished by using two plasmid vectors that can be stably propagated through mitotic partitioning. One vector expressed the gene encoding an isoform of *PAL*, cloned from *Populus*, and the other contained the *Populus C4H* and *CPR* genes. All genes were under the control of galactose-inducible promoters. The results showed that the strong accumulation of phenylpropanoid product, *p*-coumarate, from endogenous phenylalanine was possible by the co-expression of *PAL* and *C4H*. The authors suggested that C4H controls metabolic flux and channels phenylalanine derived from the shikimate pathway into phenylpropanoid metabolism, with the aid of PAL which produces cinnamic acid, the substrate for C4H. Interestingly, co-cultivation of separate strains expressing PAL and C4H/CPR also resulted in *p*-coumarate production, although at lower concentrations. This finding shows that no physical interaction of PAL with C4H is required for the biotransformation of phenylalanine into *p*-coumarate. Furthermore, transformed strains efficiently secreted *p*-coumarate into the culture media, illustrating that such a technique could be used as a biological factory for producing *p*-coumarate. This work opens the door to generating further strains that channel *p*-coumarate into flavonoid biosynthesis.

Given the proven health benefits of phytoestrogens (see Part I of this review [1]), addition of such compounds in selected beverages or foods, through the use of heterologous genes introduced into microbes, could enhance dietary benefits. Becker *et al.* [94] succeeded in enhancing the production of the stilbene resveratrol by making use of specifically transformed yeast strains. In this work, grape must was fermented with a yeast strain harboring two plasmids, one carrying a *4CL* gene from poplar and the other carrying resveratrol synthase (*RS*) gene from grape [94]. Transformed yeast strains were capable of converting *p*-coumaric acid from the medium into resveratrol glycosides. However, resveratrol glycosides were not synthesized at high concentrations (0.5–1.5 μg/L culture), glycosides were synthesized instead of aglycons, and resveratrol was not released into the medium. While this strategy requires optimization, it may provide a method for enhancing resveratrol content in fermented beverages [95, 96].

Several plant genes encoding flavonoid biosynthetic enzymes have been functionally expressed as individual enzymes in yeast, suggesting that they will be useful in the metabolic engineering of yeast as well as plants. Tian and Dixon [97] have tried to optimize the manipulation of plant secondary metabolism in yeast and plants for the production of daidzein or genistein and their glycosylated derivatives. The activities of CHI (to cyclize chalcones) and IFS (to convert resulting flavanones into isoflavanones, which are rapidly converted into isoflavones) were combined in a single functional chimeric gene. This construct containing a single *CHI/IFS* ORF with the two proteins linked by a tripeptide Gly-Ser-Gly bridge between the two proteins confers additional flexibility to the linkage without interfering with function. The rational designing of that bifunctional enzyme efficiently converted chalcones into isoflavones both in yeast and in plant cells [97]. This approach should facilitate the engineering of complex metabolic pathways in plants and yeast.

Analysis of *tt7* mutant of *A. thaliana* showed that the *TT7* gene is required for flavonoid 3′-hydroxylase activity. Furthermore, Schoenbohm *et al.* [71] proved that TT7 is actually the P450 monooxygenase F3′H that catalyzes the 3′-hydroxylation of the flavonoid B-ring to the 3′,4′-hydroxylated state. When expressed into yeast cells, the enzyme was capable of bioconverting naringenin or dihydrokaempferol into eriodictyol or dihydroquercetin, respectively [71]. This enzyme should be useful for flavonoid engineering both in yeast and in plants.

The aromatic hydroxylation at position 6 of methylated flavonols is of particular interest, since it is catalyzed by the FL6H [72], which is an ODD enzyme, rather than a cytochrome P450-dependent monooxygenase (see biosynthetic pathways in Fig. 3 of Part I of this review [1]). A cytochrome P450 enzyme named flavonoid 6 hydroxy-

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lase (F6H, see also Fig. 3 of Part I of this review [1]), which most probably came through different evolutionary routes, is capable of hydroxylating naringenin at 6 position producing 6-hydroxylated bioactive compounds [73]. Recent work by Anzellotti and Ibrahim [72] showed that an N-terminally truncated FL6H is capable of hydroxylating partially methylated flavonols at the C-6 position of their A-ring. The engineered enzyme was expressed in *Pichia pastoris* where the function of the enzyme was confirmed.

In two other studies, flavanones were produced in *S. cerevisiae* using slightly different approaches. Jiang *et al*. [98] used a full metabolic pathway with PAL as the leading enzyme followed by 4CL and CHS while Yan *et al*. [99] reconstructed the flavanone producing pathway starting with C4H, followed by 4CL, CHS and CHI [99]. In the first study, a PAL enzyme was used that also had TAL activity, thus allowing the conversion of tyrosine directly into *p*-coumaric acid without the need for PAL and 4CL activities. They also exploited the ability of naringenin or pinocembrin chalcone to spontaneously convert into the corresponding flavanones without the expression of any *CHI*. On the other hand, Yan *et al*. constructed a system which was fed with precursor phenylpropanoid acids. The results showed that the second approach, which is judged as more rational than the first, is more efficient in producing flavanones (up to 28 mg/L naringenin, compared to 10 mg/L obtained with the first approach).

In an attempt to compare the activities of FNSI over FNSII in the production of flavones, Leonard *et al*. [100] also checked whether the yeast endogenous CPR enzyme is capable of supporting the P450 enzymes by providing them with reduced equivalents. The latter was compared with a plant-derived *CPR* cloned from *Catharanthus roseus*. Their findings led them to conclude that the endogenous CPR activity is enough for the optimum activity of P450 enzymes. Nevertheless, there is other evidence that using heterologously expressed *CPRs* from other species leads to increased P450 enzyme activity that could lead to increased levels of secondary metabolite production [85]. Important in the field of heterologous flavonoid production is also their finding that certain carbon sources (*e.g*., raffinose instead of glucose as a pre-induction substrate and acetate as a sole carbon source) dramatically increased the levels of flavonoids produced [100].

Finally, DFR is an enzyme that initiates the biosynthesis of leucoanthocyanidins, water-soluble compounds that are responsible for a plethora of colors. *DFR* genes from six different species were cloned and functionally expressed in *S. cerevisiae*, *E. coli*, and protoplasts of *Nicotiana tabacum* [101]. Protein extracts from yeast strains or protoplasts from *N. tabacum* expressing *DFR* genes exogenously supplemented with various flavanonols (also named dihydroflavonols) were capable of converting them into their corresponding leucoanthocyanidins (see Fig. 3 of Part I [1] of this review for details). The authors were able to show that the heterologously expressed *DFRs* did not appear to have a distinct flavanone 4-reductase action, being able to catalyze the reduction of flavanones into flavan-4-ols [101].

3 Conclusions

Advances in the last decade have shown that metabolic engineering in plants for agricultural crop improvement is feasible and perhaps society will soon be rewarded for this investment. Phenylpropanoid-derived natural products evolved in parallel with plants and their role in every day plant life was to protect them from environmental biotic and abiotic stresses. It is long known that these natural gifts are also beneficial for human health, either as direct medicines or indirectly as nutritional supplements. In nature there is a continuous evolutionary process throughout the millennia to enable plants to survive their microbial and pest enemies. In the last few decades, this has also been the major issue in modern agriculture through chemical tools provided by the Agrochemical Industry. However, this resulted in heavy environmental, and perhaps human health load. Microbes, on the other hand, have proven to be excellent tools and best partners not only for researchers in studying gene biology but also for biotech companies as biofactories whenever either quantity or quality of a native molecule is in demand. By reconstructing multienzyme plant pathways into microbes, metabolic engineering can give solutions wherever plant systems or agricultural techniques have shortcomings. However, the question of economic feasibility of such production schemes is still under evaluation. In this review, we discussed in Part I [1] the diversity and biosynthetic origins of phenylpropanoids, mainly the flavonoid and stilbenoid natural products. We focused in particular on evaluating recent literature data pertaining to the modes of action and biological properties of these compounds, with reference to their effects on human health and physiology, and their roles as plant defense and antimicrobial compounds. The data we collected show that myths (about these natural products) do exist, but so do actual benefits. Recent data, either from original research or from oldstanding knowledge, suggest that the near future of flavonoids and stilbenoids biotechnology will be fruitful. It seems that microbe-aided biosynthesis of phenylpropanoid and flavonoid natural products is feasible, based on current experience. It remains to be seen to what extent transgenic approaches to improve the phenylpropanoid and flavonoid profiles of food crops, with potential benefits to agricultural productivity, food quality, and human health will be adopted for commercial production as this will depend ultimately on public acceptance of this technology.

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