Metabolic engineering and applications of flavonoids

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During the past decade, the increasing knowledge of flavonoid biosynthesis and the important function of flavonoid compounds in plants and in human and animal nutrition have made the biosynthetic pathways to flavonoids and isoflavonoids excellent targets for metabolic engineering. Recent strategies have included introducing novel structural or regulatory genes, and the antisense or sense suppression of genes in these pathways.

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Abbreviations

ANS	anthocyanidin synthase
CHI	chalcone isomerase
CHKR	chalcone ketide reductase
CHS	chalcone synthase
DFR	dihydroflavonol 4-reductase
F3′H	flavonoid 3′-hydroxylase
F3′5′H	flavonoid 3′,5′-hydroxylase
FGT	flavonoid 3-*O*-glucosyltransferase
FHT	flavanone 3-hydroxylase
FLS	flavonol synthase
FNR	flavanone 4-reductase
FNS	flavone synthase
IFS	isoflavone synthase
LAR	leucoanthocyanidin reductase

Introduction

Their wide occurrence, complex diversity and manifold functions have made flavonoids a very attractive system for chemical, genetic and enzymological studies, and for research on a molecular biological level. A vast amount of knowledge on flavonoids has accumulated; this has provided the tools and the know-how for successful metabolic engineering of the flavonoid pathway. Here we review recent progress in the metabolic engineering of flavonoids and in its applications to industry.

Overview on genetics, biochemistry and molecular biology

We begin by briefly summarising what is known about flavonoids and their functional aspects. First, the coloration of flowers and other plant organs due to flavonoids, in particular the conspicuous colours caused in the presence of anthocyanins, is eye-catching. Thus, alterations in coloration and flavonoid stability can easily be monitored.

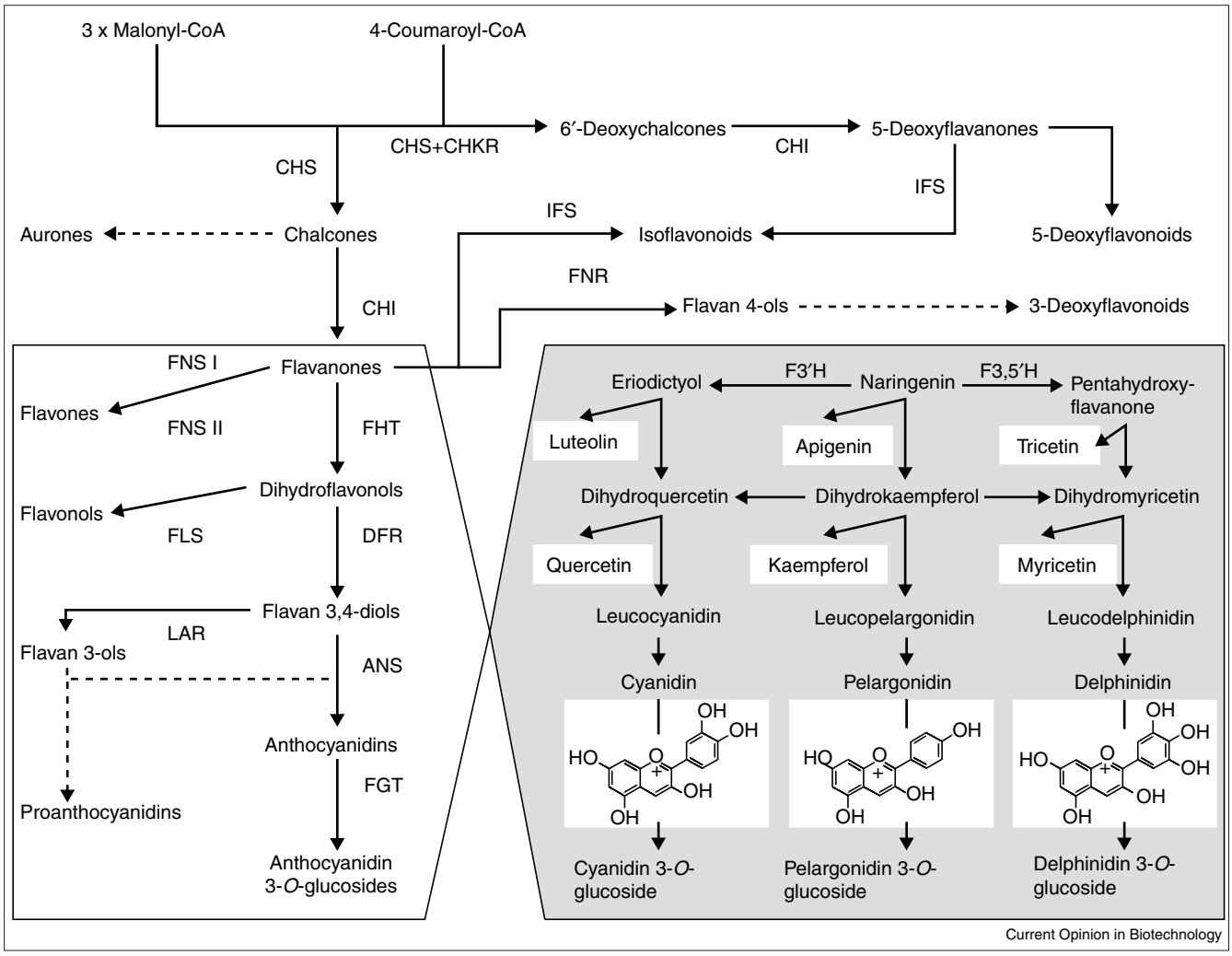
Second, powerful techniques for the chemical characterisation of flavonoids have been developed and used widely. As a result, more than 4000 different flavonoids have been isolated and identified from thousands of plant species.

Third, a wealth of flavonoid genes have been identified by classical experiments. There are structural genes that control single, biosynthetic steps of the various flavonoid classes or steps of flavonoid modification; regulatory genes that switch on or off the whole pathway or parts of it, that influence flavonoid concentration or that lead to pattern formation; and genes that are responsible for vacuolar pH, co-pigmentation and interaction with metal ions.

Fourth, nearly all enzymes involved in the pathways to the different flavonoid classes have been determined (Figure 1). The reactions to the anthocyanins are catalysed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoid 3-*O*-glucosyltransferase (FGT). The other flavonoid classes are derived from intermediates in anthocyanidin formation, and the respective reactions are catalysed by leucoanthocyanidin reductase (LAR), flavone synthase I or II (FNS I, FNS II), flavonol synthase (FLS) and isoflavone synthase (IFS). Proanthocyanidins are most probably synthesised by condensing leucoanthocyanidins (flavan 3,4-diols) with flavan 3-ols. 5-Deoxyflavonoids are synthesised through the combined action of CHS and chalcone ketide reductase (CHKR). The formation of 3-deoxyflavonoids is initiated by flavanone 4-reductase (FNR) through a reaction similar to that of DFR (Figure 1). Flavonoids can be extensively modified by hydroxylation, methylation of hydroxyl groups, glycosylation, acylation, and a number of other reactions. Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) are of particular interest; these enzymes catalyse the introduction of B-ring hydroxyl groups at the appropriate positions to provide the precursors for forming many different flavonoids, including luteolin and tricetin (flavones), quercetin and myricetin (flavonols), and cyanidin and delphinidin (anthocyanidins). The main substrates for B-ring hydroxylation are naringenin and dihydrokaempferol (Figure 1).

Fifth, to date a large number of flavonoid genes have been cloned and characterised. Apart from FNS I, LAR and the ‘condensing enzyme’ responsible for proanthocyanidin formation, the structural genes of all of the enzymes shown in Figure 1 have been isolated. Moreover, genes that modify flavonoids have also been determined, and some regulatory genes encoding transcription factors involved in flavonoid gene expression have also been cloned and characterised. More detailed information on the genetics, biochemistry and molecular biology of flavonoids is presented in a number of reviews [1–5].

Figure 1



Scheme of the individual steps in the flavonoid pathway leading to the most important classes and the hydroxylation of ring B (shaded region shows the respective hydroxylated compounds from some flavonoid classes in the left box). ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHKR, chalcone ketide reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FGT, flavonoid 3-*O*-glucosyltransferase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; FNR, flavanone 4-reductase; FNS, flavone synthase; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase.

Flavonoid functions in plants and humans

Flavonoids have many functions in the biochemistry, physiology and ecology of plants, and they are important in both human and animal nutrition. Flavonoids have functions in protecting against UV light (UV-B screening pigments), in warding off pathogenic microorganisms (phytoalexins) or pests (antifeedants), in the fertility and germination of pollen, in activating bacterial nodulation genes (nitrogen fixation) and in regulating plant growth and enzyme activity. Plant coloration is not only attractive for pollinators and seed distribution, but also provides aesthetically valuable characteristics for humans. The antioxidant activity of flavonoids towards free radicals and reactive oxygen species, and their potential oestrogenic and anticancer activity, such as antiproliferation, promotion of differentiation and apoptosis, draws attention to their health-protecting role in human diet and animal feed (for a review of flavonoid functions see [4,6]).

Putative results of metabolic engineering of flavonoids

Metabolic engineering by introducing or suppressing specific genes is expensive and time consuming. Moreover, a specific flavonoid function is often determined by several factors. Therefore, a careful characterisation of the target plant is necessary to gain valuable information on the gene pool, the biosynthetic pathway, the substrate specificity of the concerned enzymes and the availability of definite substrates. Simple approaches, such as supplementation experiments or the application of specific enzyme inhibitors, can provide additional information. Supplementing plants with flavonoid intermediates, which are not naturally present in the target

plant, is a way to test whether the internal enzyme set can convert these intermediates to the desired flavonoid [7,8]. The *in vivo* application of inhibitors might mirror the results of antisense or sense suppression strategies. Specific inhibitors are available for 2-oxoglutarate-dependent dioxygenases (FHT, FLS, ANS and FNS I) and for cytochrome P450-dependent monooxygenases (F3′H, F3′5′H, FNS II and IFS). These approaches may allow a prediction to be made on the outcome of a planned metabolic engineering experiment either by the chemical analysis of alterations in the metabolite pattern, including the synthesis of novel compounds or, in the case of colour changes, even by visible inspection [9–11].

Examples of metabolic engineering

The metabolic engineering of flavonoid pathways began in 1987 [12], and has rapidly become an accomplished research area in the past decade [13•]. Because of patent applications for property rights, however, very recent results and inventions are often inaccessible. Here we will review the available information with respect to the different flavonoid functions.

Flower and plant coloration

Flower colour novelty, in particular the generation of unknown blue and yellow flowering cultivars, is one of the major driving forces in ornamental plant breeding. In a combination of classical and molecular methods, metabolic engineering provides powerful tools to generate novel flower colours without changing other desirable characteristics of a pre-existing cultivar [14–18,19•].

The successful engineering of pelargonidin synthesis by expressing suitable *DFR* genes from other plants [12,20,21], which led to orange *Petunia* flowers, has initiated much work, including the introduction of novel genes into selected plant species, the complementation of defined mutants, and the suppression of flavonoid synthesis by antisense or sense suppression. The most recent results are summarised in Table 1, and we will discuss some of them here in more detail.

Putting together published information on flavonoid biosynthesis in *Petunia* and *Dianthus* [7,8,22], Florigene Ltd and Suntory Ltd generated transgenic *Dianthus* with violet flowers by introducing delphinidin derivatives, which are not formed naturally. The companies selected a white flowering cultivar that synthesised only dihydrokaempferol owing to a lack of both DFR and F3′H activity. The DNA sequence encoding the *Petunia* DFR enzyme, which accepts dihydromyricetin but not dihydrokaempferol as substrate, was introduced into this cultivar, together with the DNA sequence encoding the *Petunia* F3′5′H enzyme. Expressing both enzymes led to the hydroxylation of dihydrokaempferol to dihydromyricetin (F3′5′H), which is subsequently converted to leucodelphinidin by the *Petunia* DFR and ultimately to delphinidin derivatives by the endogenous enzymes of the plant [P1]. The resulting violet flowering *Dianthus*, named 'Moondust™' and 'Moonshadow™', became the first transgenic floricultural crop on the market. To date, however, truly blue *Dianthus* flowers have not been created, mainly because of inappropriate conditions in vacuolar pH and co-pigmentation. Delphinidin formation by introducing a *F3′5′H* gene has also been achieved in cyanic cultivars of *Dendranthema*, *Dianthus* and *Rosa*, but anthocyanins based on cyanidin and/or pelargonidin are still present in these cultivars [P2].

Table 1

Metabolic engineering of flower or plant colour.

Plant species (Original colour)	Gene construct (Source)	Produced flower colour	Reference
Generation of novel flavonoids			
Dianthus caryophyllus (white)	Sense *DFR* and sense *F3′5′H* (*Petunia*)	Violet	[P1]
Petunia hybrida (pink)	Sense *F3′5′H* (*Eustoma*)	Magenta	[40]
Nicotiana tabacum (pink)	Sense *F3′5′H* (*Eustoma*)	Magenta	[40]
Petunia hybrida (pale lilac)	Sense *F3′H* (*Petunia*)	Dark pink	[41]
Petunia hybrida (white)	Sense *CHKR* (*Medicago*)	Pale yellow	[23]
Eustoma russellianum	Sense *FGT* (*Antirrhinum*)	Altered anthocyanin composition	[42]
Forsythia intermedia (yellow)	Sense *DFR* (*Antirrhinum*) and sense ANS (*Matthiola*)	Brownish	[24]
Petunia hybrida (green tissues)	Sense *Lc* (*Zea mays*)	Anthocyanin in leaves and stems	[43]
Trifolium repens (white leaf tissues)	Sense *B-Peru* (*Zea mays*)	Anthocyanin in leaves	[44]
Dianthus caryophyllus (deep red with pale pink rim)	Sense *difF* and sense *F3′5′H* (*Petunia*)	Deep purple with pale purple rim	[P3]
Suppression of steps in flavonoid biosynthesis			
Torenia hybrida (blue)	Sense *CHS*, *DFR*	White and colour pattern	[45]
	Antisense *CHS*, *DFR*	Pale blue and colour pattern	[46]
Eustoma russellianum (purple)	Antisense *CHS*	White and colour pattern	[47]
Dianthus caryophyllus (orange red)	Antisense *FHT*	Yellow/cream	[P4]
Petunia 'Surfinia' (pink)	Antisense *CHS*	Pale pink, white	[17]
	Sense *FLS*	More anthocyanin and less flavonol	[17]

A novel approach involving manipulation of chalcone synthesis has led to the formation of pale yellow flowers of *Petunia*. Expressing the *CHKR* gene of alfalfa in an acyanic *Petunia* line allowed the synthesis of the 6′-deoxychalcone isoliquiritigenin, through the co-action of endogenous CHS and the introduced CHKR. Because isoliquiritigenin is not a substrate of the *Petunia* CHI, a certain amount of chalcone accumulates in the flowers, leading to the pale yellow coloration [23]. A higher chalcone concentration, and through this the generation of truly yellow flowers, might be achieved by the glycosylation and hydroxylation of isoliquiritigenin. The introduction of CHKR might be successful in all plant species possessing high CHS activity.

The invariably yellow coloration of *Forsythia* petals is caused by accumulation of carotenoids; however, some anthocyanins are formed in the sepals. Studies on this flavonoid pathway revealed that anthocyanin formation in the petals is impaired at the DFR and, in particular, the ANS steps. Introducing the *DFR* gene from *Antirrhinum* and the *ANS* gene from *Matthiola* resulted in transgenic *Forsythia* with slightly brownish flowers, owing to the synthesis of some anthocyanin on the yellow carotenoid background [24].

In 1999, de Vetten *et al.* [25] reported that a flower-specific cytochrome b_5 is required for full activity of F3′5′H in *Petunia*, but they observed no effect on the activity of other cytochrome P450 enzymes. Cytochrome b_5 is encoded by the *difF* gene, which is expressed exclusively in the flower. Introducing and expressing *difF* in *Dianthus* flowers that were also transformed with *Petunia F3′5′H* resulted in the generation of a nearly black flower, owing to a strong accumulation of delphinidin derivatives ([P3]; and JNM Mol, personal communication).

Transforming the orange-red flowering *Dianthus* cultivar ‘Eilat’ with antisense *FHT* caused the production of yellow-cream flowers, owing to the accumulation of naringenin-chalcone 2′-glucoside [P4]. Besides the suppression of anthocyanin formation, however, the overall fragrance intensity of transgenic flowers was significantly higher than in control flowers because of strongly elevated levels of methylbenzoate. Thus, suppressing flavonoid biosynthesis with antisense *FHT* obviously diverts the metabolic flow towards the biosynthesis of benzoic acid derivatives, which also originate from the phenylpropanoid pathway [P4].

Nutriceutical potential of flavonoids

There is increasing evidence for the health-protecting function of flavonoid compounds, such as antioxidative, antitumor, anti-inflammatory and antiatherosclerotic activities. But major crop plants eaten for nutrition often lack the desired flavonoids or contain only small amounts in the relevant tissues. Metabolic engineering has provided a means to improve flavonoid composition as well as content.

In tomato, which produces naturally only low amounts of kaempferol and quercetin in the fruit peel, the introduction and overexpression of the regulatory genes *Lc* and *C1* of maize led to an increase in kaempferol formation of up to 60%, mainly in the flesh of the fruits. Moreover, introducing the *CHI* gene of *Petunia* resulted in an increase of up to 70% in quercetin formation in the peels. Expressing *Lc* and *C1* in potatoes also caused a marked accumulation of kaempferol in the tubers [26]. In the future, the availability of the *FLS* gene from several sources may allow a more directed engineering of flavonol synthesis. Moreover, the recent cloning of the gene encoding FNS II [27•,28•] provides the means for manipulating flavone formation for agronomic and nutritional purposes.

In some forage crops, the accumulation of moderate levels of condensed tannins (proanthocyanidins) confers bloat safety and protein protection in ruminant systems. In *Lotus corniculatus*, both a reduction and an increase in proanthocyanidin levels in leaves and stems has been achieved using antisense strategies for CHS and DFR, respectively. Similar results were observed after introduction of a sense *DFR* gene from *Antirrhinum* [29,30]. In another study, the introduction of the maize transcription factor *Sn* into *L. corniculatus* resulted in the suppression of proanthocyanin synthesis [31]. A more directed approach for improved forage quality will be possible after the biosynthetic pathway for condensed tannins has been finally elucidated and the relevant genes have been isolated.

Isoflavones may act as phytoestrogens, which has generated interest in the use of these compounds for treating and preventing hormone-related disorders in humans [32]. The occurrence of isoflavones is limited primarily to legumes, in which the first committed step in biosynthesis is catalysed by IFS. The recent cloning of the gene encoding IFS has opened the way for engineering isoflavone formation in crop plants that normally lack these compounds. A first successful experiment in *Arabidopsis* has been reported [33•]. Introducing the soybean *IFS* gene driven by the 35S promotor in this non-legume plant resulted in the conversion of naringenin to the isoflavone genistein, which belongs to the phytoestrogens of high medical interest [34].

Pollen fertility

Because male sterility is a prerequisite for the development of hybrid seed systems, the generation of this trait by metabolic engineering is an important goal. In maize, recessive mutations at the two CHS genes *C2* and *Whp* resulted in sterile white pollen lacking flavonoids. In *Petunia*, sense suppression of CHS caused both white flowers and male sterility [35]. Antisense expression of CHS in anthers likewise resulted in male sterility [36]. In tobacco plants, the introduction and overexpression of a *STS* gene encoding stilbene synthase, which competes with the endogenous CHS for common substrates, also caused male sterility in addition to altered flower pigmentation. Moreover, plants with tapetum-specific expression of the *STS* gene were

found to be male sterile [37]. Supplementation studies with flavonoids revealed that the sterile phenotypes can be complemented by the addition of flavonols [38]. Thus, antisense or sense suppression of FLS under the control of a tapetum-specific promotor, as an alternative to suppressing the whole flavonoid pathway, seems to be a very promising approach for the generation of male sterility. Since the demonstration that pollen fertility is unaffected in flavonoid-deficient *Arabidopsis* mutants [39], however, no further efforts have been reported.

Conclusions

Despite the success of recent work, it remains a challenging task to generate desired, or to suppress undesired, flavonoid compounds by highly controlled metabolic engineering of the flavonoid pathway. The isolation of genes encoding, for example, IFS, FLS, FNS II, DFR or FNR, and of promotor sequences directing spatial and temporal gene expression should allow several advances. Not only will it be possible to promote metabolic engineering of health-protecting flavonoids in important crop plants, but it might also be conceivable to engineer improved plant resistance by generating or manipulating phytoalexins (e.g. isoflavonoids, flavanols and proanthocyanidins), enhanced UV protection by a well-aimed synthesis of screening pigments (e.g. flavonols and anthocyanins) and enhanced nodulation efficiency by overproducing *nod* gene inducers (e.g. flavones and isoflavones). In addition, a better understanding of the mechanisms involved in co-pigmentation and vacuolar pH, as well as the availability of the respective genes and improved transformation systems for ornamental plants, will make the modification of flower colour more feasible.

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