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Review

Impact of biochemical pre-studies on specific metabolic engineering strategies of flavonoid biosynthesis in plant tissues

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Abstract

The role of flavonoids including anthocyanins in providing brilliant and coloured pigments in different plant tissues, their contribution to plant protection against UV-B radiation, microbial and herbivory pathogens and their medicinal and nutritional value is well documented. Furthermore, general and manifold studies led to the accumulation of a vast amount of knowledge on genetics, chemistry, biochemistry and molecular biology of this pigment group. Several expensive and time consuming experiments were performed to introduce or suppress specific flavonoid genes in ornamental plants. For an improvement of such metabolic engineering strategies a careful characterisation of the target plants and the genes of interest is highly recommended. From simple chemical and biochemical studies with well-established methods valuable information on the gene pool, the biosynthetic pathway, the substrate specificity of relevant enzymes of concerned steps can be easily obtained, allowing a prediction of the putative resulting phenotype of the planned experiment. Actual studies were performed with *Zantedeschia*, *Lilium* and *Osteospermum* using supplementation experiments, inhibitor application, heterologous gene expression and enzymological assays with plant protein extracts and recombinant proteins to design new powerful strategies to modify the flavonoid pattern in these plants, leading to new flower colours.

Keywords: Flavonoids; Plant tissue; Pigment

1. Introduction

Until now, the large number of more than 6400 different flavonoid compounds were described in plants, where they were characterised by their wide occurrence and complex diversity. Based on an imposing number of genetic, chemical, biochemical and molecular biological studies the flavonoid biosynthesis became one of the best understood and characterised pathway in plant secondary metabolism (Fig. 1) [1]. In plant tissues flavonoids have many functions, as for, e.g. colour and UV-B screening pigments, pollen fertility factors, activators of bacterial nodulation genes, free-radical scavengers, anti-feedants and phytoalexins. Besides these biochemical, physiological and ecological functions many flavonoids plays a health-protecting role in human-diet and by this gave useful applications in the manufacture of foods, industrial products, and biopharmaceuticals [2].

The diverse functions of flavonoid compounds in plants and in humans offers many potential and attractive targets for metabolic engineering approaches. Structural and also several regulatory genes have been cloned, characterised and used in gene transformation experiments to modify flavonoid synthesis in specific plant tissues [1,3–5]. One of the most important function of flavonoids is their contribution to the colouration of flowers and other tissues. In the first successful metabolic engineering experiment of the flavonoid pathway in plants, the maize *A1* gene encoding dihydroflavonol 4-reductase (DFR) was introduced in a chemicogenetically characterized mutant line of *Petunia hybrida* accumulating kaempferol (Km) and dihydrokaempferol (DHK) in flowers. The native *Petunia* DFR enzyme is unable to convert DHK to leucopelargonidin (LPg), the precursors of pelargonidin (Pg) and an orange pigment not found in this species. The described strategy overcame the natural lack of Pg-derivatives and gave a new orange *Petunia* phenotype [6,7]. This experiment initiated much work in this field in the following years.

In 1996, the first transgenic floricultural crop—the “blue” carnation [3,4] followed by in Japan and USA (F. Brugliera,

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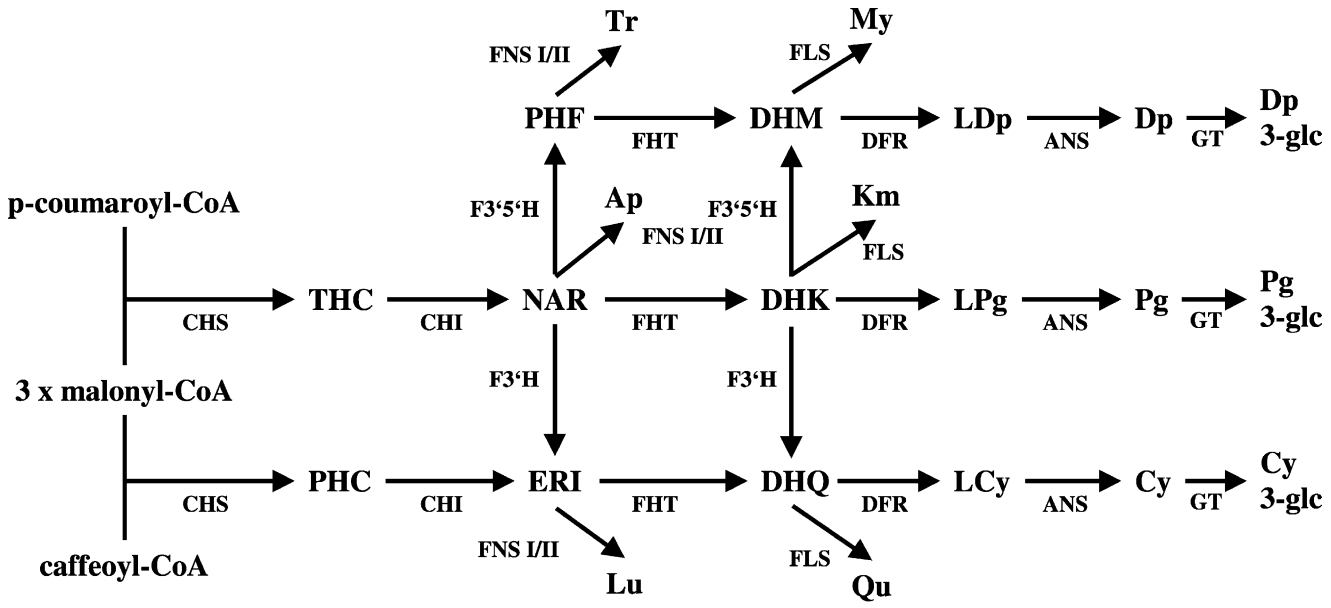

Fig. 1. Scheme of the main flavonoid pathway leading to the most common flavone, flavonol and anthocyanin pigments. Flavonoids—THC: tetrahydroxychalcone; PHC: pentahydroxychalcone; NAR: naringenin; ERI: eriodictyol; PHF: pentahydroxyflavanone; Ap: apigenin; Lu: luteolin; Tr: tricetin; DHK: dihydrokaempferol; DHQ: dihydroquercetin; DHM: dihydromyricetin; Km: kaempferol; Qu: quercetin; My: Myricetin; LPg: leucopelargonidin; LCy: leucocyanidin; LDp: leucodelphinidin; Pg: pelargonidin; Cy: cyanidin; Dp: delphinidin; Pg 3-glc: pelargonidin 3-glycosid; Cy 3-glc: cyanidin 3-glycosid; Dp 3-glc: delphinidin 3-glycosid; Enzymes—CHS: chalcone synthase; CHI: chalcone isomerase; FHT: flavanone 3ß-hydroxylase; FNS I/II: flavone synthase I and II; F3′H: flavonoid 3′-hydroxylase; F3′5′H: flavonoid 3′5′-hydroxylase; DFR: dihydroflavonol 4-reductase; FLS: flavonol synthase; ANS: anthocyanindin synthase; GT: glycosyl transferases.

pers. comm.). The aim to create a real blue colour in petals of carnation by introducing the "blue gene" (flavonoid 3′5′-hydroxylase; F3′5′H) [8] failed due to inappropriate vacuolar pH, copigmentation and/or anthocyanin modification (e.g. acylation). In this context flavones may play an important role as copigments. There are various examples of delphinidin (Dp) containing flowers expressing no blue colouration possibly due to a lack of these potential copigments (e.g. in *Pelargonium*, *Cyclamen*, *Osteospermum*). The blueing effect of specific flavones was described in 1991 by [9].

Other successful examples of single-enzyme manipulations for increasing or decreasing accumulation of selected flavonoid metabolites were reported in the last years not only concerning modulation of flower colouration, but also, e.g. regarding pollen fertility and nutraceutical potential of flavonoids in crop plants (see [5] and reference therein).

Besides the flavonoid pathway, metabolic engineering in plants discover many other pathways, e.g. lipids in soybean seeds [10], vitamin E in *Arabidopsis* [11], terpenoids in mint [12] and carotenoids in tomato [13]. Most of these experiments were also single-enzyme manipulation strategies. But nowadays also the control of multiple steps to achieve net gains product accumulation is reported. On basis of chemical, biochemical and molecular pre-studies [14,15], a double transformation of *Forsythia* × *intermedia* was performed to overcome the lack of anthocyanins in carotenoid accumulating petals by introducing subsequently DFR (*Antirrhinum majus*) and anthocyanidin synthase (ANS) (*Matthiola incana*), finally resulting in orange flowering plants (C. Rosati, pers comm.). Furthermore, Ye et al. [16] were able to increase the carotenoid accumulation in rice, the so-called golden rice, by overexpressing three defined genes.

Generally, three basic strategies could be used for flavonoid pathway modulation in specific plant tissues:

(1) Expression of novel sense derivatives of genes not present in the gene pool of the target plant to open the pathway to new metabolites.
(2) Expression of sense derivative of a suitable gene to open the pathway to new metabolites by overcoming genetic blocks or ratelimiting steps.
(3) Gene silencing to down regulate synthesis of undesired flavonoids. This may include opening of new pathway branches upstream.

Here we will present biochemical methods and their use in respective pre-studies to develop metabolic engineering strategies for the flavonoid pathway in ornamental plants.

2. Biochemical methods

2.1. Supplementing plant tissues with flavonoid precursors

Feeding plant tissues with potential flavonoid precursors has widely been used in the elucidation of the flavonoid pathway. In case of anthocyanin synthesis the phenotypical

results can easily be obtained by visual inspection and additionally proved by chemical characterisation of the pigments formed, using photometric and chromatographic methods [17–19]. Successful applications have been reported for different plant species [20–26]. On the one hand, with defined flavonoid precursors, you can easily localise genetic blocks in the pathway of acyanic mutants. Moreover, the chemical structure of the resulting anthocyanin will reveal the presence or absence of modifying enzymes (hydroxylation, methylation, glycosylation, acylation, etc.). On the other hand, using a defined mutant, you can get information on the question of whether or not a potential flavonoid intermediate is really involved in the formation of a definite flavonoid end product.

With regard to metabolic engineering, supplementation studies are excellent tools to simulate *in planta* the possible results of the introduction of a gene coding for a naturally not present enzyme activity or overcoming a rate limiting step. It can easily be tested, whether the internal enzyme set can convert the new intermediate to the desired end product, and, in addition, whether this end product meets the desired quality, such as flower coloration. For example, feeding of acyanic flowers of *A. majus*, blocked in the flavanone 3-hydroxylase (FHT) step [27], with the Dp precursor dihydromyricetin (DHM), which is naturally not present in the flowers because of the lack of the F3′5′H gene in *A. majus*, led, on the one hand, to the desired formation of Dp-derivatives. On the other hand, however, the Dp-derivatives gave a purple rather than the desired blue coloration may due to inefficient pH-value, copigmentation and/or anthocyanidin copigmentation (Fig. 2). Feeding of DHM to petals of a suitable mutant of carnation led to similar results [25].

Furthermore, the use of flavones and flavonols in such experiments will give results regarding the co-pigmentation effect of these compounds together with naturally occurring anthocyanins and the use of new metabolic engineering strategies concerning these side branches.

2.2. *In vivo* application of specific inhibitors of flavonoid pathway enzymes

In vivo applications of specific enzyme inhibitors might mimic the results of gene silencing experiments allowing a prediction on the outcome of respective metabolic engineering experiments by direct inspection and subsequent chemical analysis of the altered pigment pattern as mentioned above. This strategy may also reveal side effects, such as the synthesis of novel compounds from accumulated intermediates upstream of the inhibited enzyme in the flavonoid pathway. Specific inhibitors are available for 2-oxoglutarate-dependent dioxygenases (e.g. FHT, flavone synthase I (FNS I), flavonol synthase (FLS)), ANS and for cytochrome P450-dependent enzymes (e.g. flavone synthase II (FNS II), flavonoid 3′-hydroxylase (F3′H) and F3′5′H). Unfortunately, there are no specific inhibitors available for chalcone synthase (CHS), chalcone isomerase (CHI) and DFR, respectively.

FHT is the first 2-oxoglutarate-dependent dioxygenase in the flavonoid pathway. Treatment of flower buds with the specific inhibitor prohexadion-Ca strongly inhibits FHT activity leading to an drastic reduction in anthocyanin formation (Fig. 3). As expected, in *Gerbera*, only an increase of the amount of flavones is observed as side effect. Surprisingly, however, after treatment of rose flowers or leaves with prohexadion-Ca, a novel flavonoid pathway was found to be opened leading to synthesis of the rare 3-deoxyflavonoids (Knott, unpublished results). An unexpected side effect was also observed in transgenic carnations harbouring a FHT antisense construct. FHT suppression resulted in the accumulation of yellow chalcone pigments and additionally the metabolic flow was diverted towards an other biosynthetic branch also originating from the phenylpropanoid pathway. In this case, benzoic acid derivatives, which gave a significantly higher fragrance to the transgenic flowers were enhanced in their accumulation [28]. Prohexadion-Ca may also

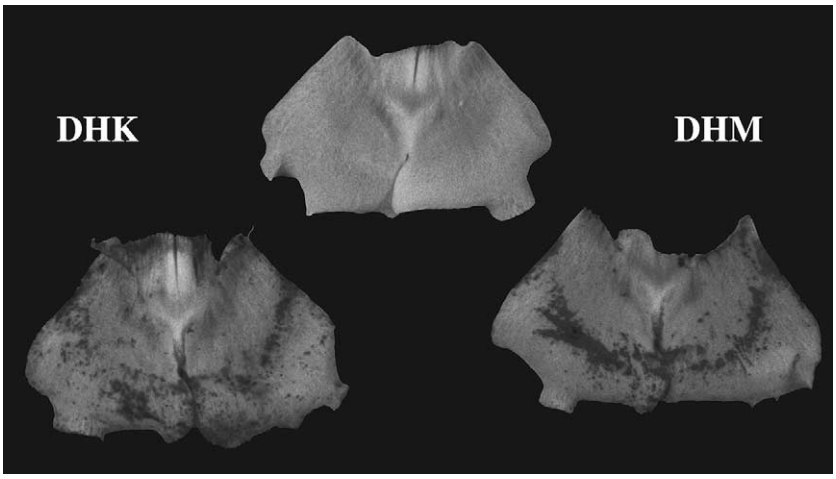

Fig. 2. Supplementation of an FHT mutant of *A. majus* with dihydroflavonols (1 mg/ml sterile water) to overcome the FHT block and to simulate the outcome of a F3′5′H introduction by metabolic engineering.

Fig. 3. Inhibition of anthocyanin formation in *Gerbera hybrida* "Regina" (left: control, right: after application of prohexadion-Ca (1 mg/ml sterile water)).

inhibit FNS I, FLS and ANS. On the one hand, the possibility of a simultaneous inhibition of all 2-oxoglutarate-dependent enzymes in the flavonoid pathway may be a disadvantage. On the other hand, with a suitable experimental design, a separate study of the influence of the inhibitor should be possible.

Several inhibitors for the group of cytochrome P450-dependent enzymes are available, which shows in different plant species a high specificity towards a single enzyme [29]. For example, by using tetcyclacis in vivo a direct simulation of suppression experiments concerning the hydroxylating enzymes F3′H and F3′5′H could be performed and the potential outcome could easily be obtained by visual inspection followed by detailed chemical analysis as mentioned above. Furthermore, inhibition of these enzymes in vivo normally led to an enhanced accumulation of 4′-hydroxylated flavonoids such as apigenin (Ap), Km and Pg. In case of *Anagallis monelli* white flowering petals were found after application of the inhibitor, which accumulate Km in a higher extend as the control (Fig. 4). From this, a strong substrate specificity of DFR enzyme in this plant could be parallel to the results of inhibition of the hydroxylases concluded. Using modified application protocols, e.g. changing time of application in regard to flower development, concentration of inhibitor and number of applications, may also the modelling of the effects of different Pg/cyanidin (Cy) or Pg/Cy/Dp ratio in different plant tissues could be examined.

For all *in vivo* inhibitor experiments it is recommended to test the specificity of the compound *in vitro* enzyme assays (see Section 2.3) with protein extracts from the chosen plant species. It is important to correlate the later results regarding the concerned and other steps of the flavonoid pathway and to establish a possible strategy for metabolic engineering.

2.3. Enzymatic studies of flavonoid pathway steps

Enzymatic characterisation was often the basic for metabolic engineering projects (e.g. *Petunia*, *Dianthus*, *Forsythia*). Several ornamental plant species were included in such studies, but still many important crops are missing.

With enzymatic methods the biosynthesis of flavonoids can be followed step by step starting from *p*-coumaric acid up to the resulting anthocyanin. Except ANS all enzymes involved in the main pathway to the different flavonoid classes have been determined and characterized in plant extracts ([1] and references therein). Additionally, the structural genes off

Fig. 4. Inhibition of anthocyanin formation in *A. monelli* "Blue Sky" (left: after application of tetcyclacis (1 mg/ml sterile water), right: control).

all of the enzymes shown in Fig. 1 have been isolated and functionally expressed in bacteria, yeasts, insects or plant cells. Subsequently the respective gene products were characterized regarding its biochemical properties [1,30–32].

3. Results from biochemical pre-studies

3.1. Lilium sp.

The ornamental cut flower market offers asiatic and oriental *Lilium hybrids*. Besides this two large groups some minor species, e.g. *Lilium longiflorum* (Easter Lilly), became more and more interesting for growers and consumers because of certain positive characteristics as beautiful flowers, early spring bloomer, easy to grow and propagate and vase life quality [33]. *Lilium* sp. are known to contain anthocyanins and carotenoids as flower pigments [34]. We analysed flavonoid and anthocyanin extracts of 11 different hybrids and *L. longiflorum* by UV-Vis spectroscopy and TLC. In six reddish varieties, besides carotenoids, Cy-derivatives are present. All lines including *L. longiflorum* accumulate flavonol derivatives of mainly Km and quercetin (Qu), additionally isorhamnetin was found in four lines.

From these results we postulate blocks of the DFR and/or the ANS step in all acyanic lines of *Lilium* species. The exclusive accumulation of Cy-derivatives could be the result of a distinct DFR substrate specificity as known for the *Petunia* enzyme [6] and/or additionally to a competition of DFR, F3′H and FLS for DHK as common substrate. The observation that extracts of *L. longiflorum* changed from colourless to reddish during acid hydrolysis, indicates the presence of leucoanthocyanidins or related compounds. Therefore DFR activity should be present in this species.

Enzymatic studies revealed a high preference of CHS for *p*-coumaroyl-CoA as substrate even at low pH-values in the enzyme assays. Activity of CHS was found in all investigated lines. FHT converts both flavanones NAR and ERI with protein extracts of acyanic and cyanic lines to the respective dihydroflavonols DHK and DHQ. In enzyme preparations of acyanic lines of *Lilium hybrids* DFR activity was not detectable indicating a genetic block in this step. As postulated from the chemical analysis, in preparations of *L. longiflorum* enzyme activity of DFR was clearly detectable in the same manner as in cyanic lines with DHQ but not with DHK as substrate (Table 1). Although different enzyme preparation methods were used activity of F3′H was not detected until now. A possible explanation could be a large amount of secondary products as phenolic acids making isolation of membrane bound enzymes nearly impossible with standard methods. All results led to the postulated pathway scheme shown in Fig. 5.

3.2. Zantedeschia sp.

Zantedeschia with the commercial available species *Z. rehmanii*, *Z. jucunda*, *Z. elliotiana*, *Z. albomaculata* and *Z. aethiopica* is a new and growing ornamental pot and cut-flower plant. In *Zantedeschia* sp. anthocyanins as well as carotenoids are the major colour giving pigments. The analysis of different extractions of 15 selected *Zantedeschia* species and cultivars including *Z. aethiopica* “Nili” (Bock, Bremen, Germany) by UV-Vis spectroscopy and TLC identified besides carotenoids and chlorophyll also Cy and Paeonidin (Pn) derivatives in cyanic spathas and dark eyes. All acyanic lines including *Z. aethiopica* accumulate derivatives of flavones, C-glycosyl flavones and flavonols mainly Ap, luteolin (Lu) their C-glycosylated derivatives vitexin (Vit), orientin (Ori), isovitexin (Isovit), isoorientin (Isoori), Km, Qu and also procyanidin (ProCy).

From these results we postulate a block of ANS in acyanic lines. Furthermore, DFR enzyme should be active in all lines, but showing distinct substrate specificity for DHQ. An additional high F3′H activity would led to accumulation of Cy/Pn derivatives only. This is further supported by the observation, that extracts of *Z. aethiopica* changed from colourless to reddish during acid hydrolysis, indicating the presence of leucoanthocyanidins or related compounds as found in *L. longiflorum*.

Similar to *Lilium* in enzymatic studies a high preference of CHS for *p*-coumaroyl-CoA even at low pH-values in the enzyme assays was found. Both early enzyme activities, CHS and FHT, were detected in all investigated lines. In enzyme preparations of cyanic and acyanic lines of *Zantedeschia* sp. DFR activity was clearly observed indicating a genetic block in a later step. Surprisingly, besides DHQ DHK serves as a good substrate for DFR reaction. F3′H activity was very weak with naringenin (NAR) but clearly higher with DHK

Table 1
Activity of DFR in *Lilium* sp.

Lilium sp.	Flower colour and identified flavonoid aglyca	Colour of extraction after hydrolysis	Substrate for DFR enzyme assay	Products formed in assay
L. hybrids	Red: Cy, Km, Qu, Isorha	Red	DHK DHQ	None Lcy
L. hybrids	White: Km, Qu, Isorha	Colourless	DHK DHQ	None None
L. longiflorum	White: Km, Qu, Isorha, Lcy	Red	DHK DHQ	None LCy

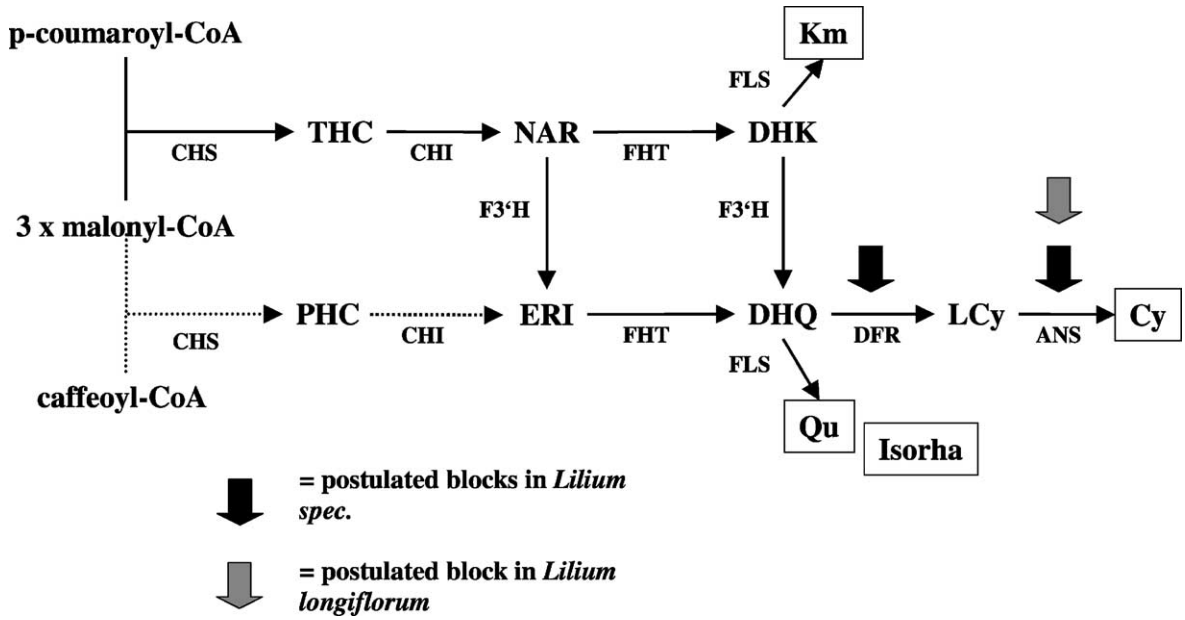

Fig. 5. Postulated flavonoid pathway in *Lilium* sp. based on chemical and biochemical pre-studies (Isorha: isorhamnetin). Solid arrows indicating major pathway; dotted arrows minor pathway (see caption of Fig. 1).

as substrate. This led to the hypothesis that F3′H may also convert LPg to leucocyanidin (LCy). This possibility was discussed by [26] and has to be proven for *Zantedeschia*. The accumulation of only Cy-derivatives must be explained by either high F3′H activity and late expression of ANS or by a yet not described high substrate specificity of ANS for LCy. Both possibilities has to be investigated in detail now. The obtained results from this investigations are summarised in a postulated pathway scheme in Fig. 6.

3.3. *Osteospermum Hybriden*

Osteospermum is a relatively new ornamental mainly used as a summer pot plant with still growing importance on the market. The flowers are white, creamy white, yellow, mauve and purple to deep lilac. The impressing colour effect of the flower heads is contrasted by the differences of discs and petals. True orange, red and blue colours of the petals are still lacking.

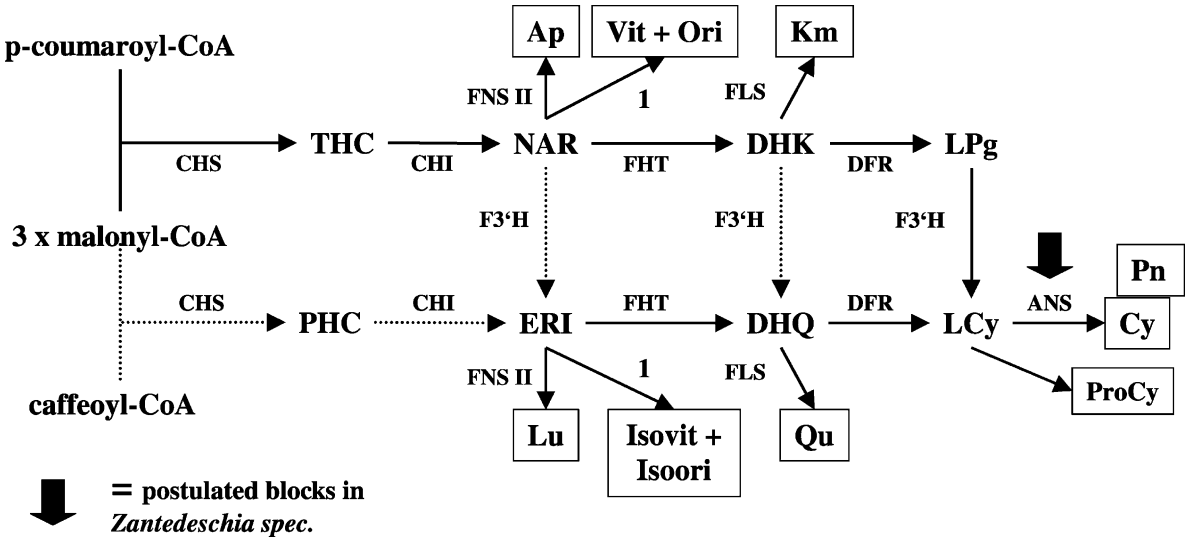

Fig. 6. Postulated flavonoid pathway in *Zantedeschia sp.* based on chemical and biochemical pre-studies. Solid arrows indicating major pathway; dotted arrows minor pathway. 1: flavanone 2-hydroxylase (F2H) and flavonoid 6/8 C-glycosyltransferase; Vit: vitexin; Ori: orientin; Isovit: isovitexin; Isoori: isoorientin (see caption of Fig. 1).

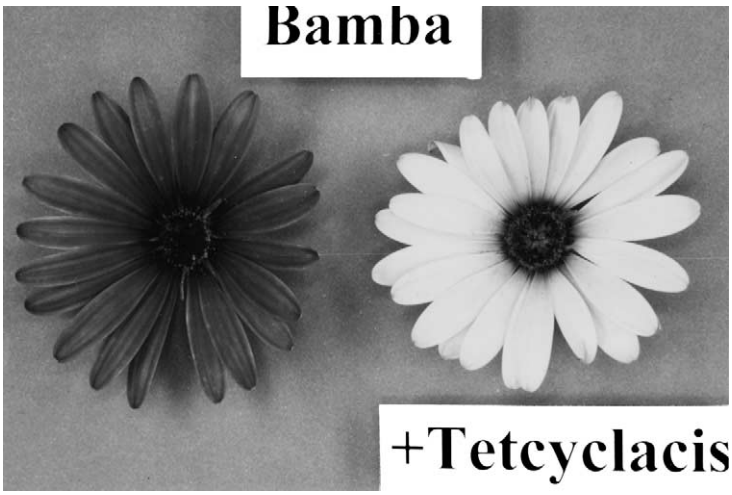

Fig. 7. Inhibition of anthocyanin formation in *Osteospermum hybrid* "Bamba" (left: control, right: after application of tetcyclacis (1 mg/ml sterile water)).

Chromatographic and spectralphotometric investigations of 29 different varieties and genotypes (Cape Daisy, Aarhus, Danmark; Schmülling, Billerbeck, Germany) revealed a comparatively simple flavonoid pattern: the flavonols Km and Qu, and anthocyanins based on Dp were found as major pigments. As minor pigments derivatives of myricetin (My), Cy and petunidin (Pt) were present. In contrast, flavones often reported for members of the *Asteraceae* family were not detected in *Osteospermum*. The yellow coloured lines are based on carotenoids.

For further elucidation of the flavonoid pathway biochemical methods were used. With regard to *Osteospermum*, especially the question of the strong hydroxylation leading to Dp derivatives is of interest. CHS enzyme activity was clearly detectable with *p*-coumaroyl-CoA, whereas caffeoyl-CoA was only a poor substrate. High activity of FHT was shown in enzyme extracts of all *Osteospermum* with NAR as substrate leading to DHK. Only a slight conversion of ERI and pentahydroxyflavanone (PHF) to DHQ and DHM, respectively, was detected. Therefore, it is assumed that *Osteospermum*

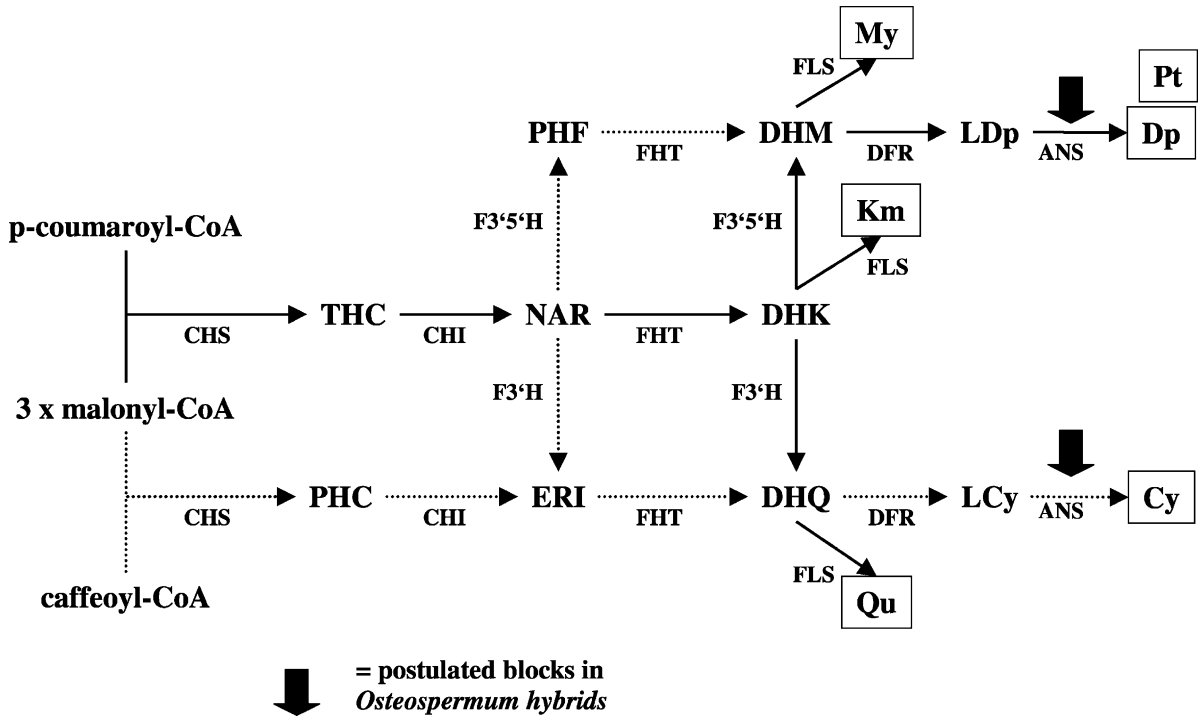

Fig. 8. Postulated flavonoid pathway in *Osteospermum hybrids* based on chemical and biochemical pre-studies. Solid arrows indicating major pathway; dotted arrows minor pathway (see caption of Fig. 1).

Table 2
Developed metabolic engineering strategies based on biochemical pre-studies to achieve modification of the flower colour

Plant species	Strategy	Expected outcome
L. longiflorum	Introducing ANS	Accumulation of red Cy-derivatives
	Introducing DFR accepting DHK as substrate and ANS	Accumulation of naturally not occurring orange-red Pg- together with Cy-derivatives
Zantedeschia aethiopica	Introducing ANS	Accumulation of orange-red Pg- and Cy-derivatives
Osteospermum hybrids	Introducing DFR accepting DHK as substrate and ANS in white flowering lines	Accumulation of orange-red Pg-derivatives
	Suppression of F3′5′H	Increasing red pigmentation based on Cy
	Introducing FNS I or FNS II	Copigmentation and modulation of anthocyanin amount

FHT prefers in vivo NAR as substrate but accepts to a minor extend ERI and PHF too. From these results regarding the substrate specificity of CHS and FHT, it could be postulated that F3′H and/or F3′5′H are responsible for the addition of the two further OH-groups at the B-ring and that hydroxylation happens at the level of the dihydroflavonols rather than at the level of the flavanones. In fact, a high activity of F3′5′H was shown in *Osteospermum* with both NAR and DHK leading to the formation of PHF and DHM, respectively. Furthermore, DHK is not accepted as a substrate by *Osteospermum* DFR. Therefore, Pg is not synthesised in the flowers. In respective enzyme assays DHK is strongly 3′5′-hydroxylated to DHM which is further converted to leucodelphinidin (LDp) by DFR. Additionally results from *in vivo* application of tetcyclacis strongly supports the fact that DHK is not converted to LPg by *Osteospermum* DFR. The inhibition of F3′5′H resulted in white petals accumulating mainly Km (Fig. 7). Surprisingly, also in enzyme preparations of white flowering lines a clear DFR activity was detectable, but in contrast to *Lilium* and *Zantedeschia* the presence of leucoanthocyanidins or related compounds could not be shown. These result led to the hypothesis that anthocyanin synthesis is blocked at the ANS level in *Osteospermum*. Furthermore, FLS activity was detectable. In this case, the enzyme accepted DHK, DHQ and DHM as substrates for the formation of the respective flavonols. A postulated pathway scheme based on these results is shown in Fig. 8.

3.4. Developed metabolic engineering strategies

In case of *Lilium*, *Zantedeschia* and *Osteospermum* molecular techniques of breeding are obvious because of the restricted initial colour palette and the restricted success of interspecific hybridisation techniques. Furthermore, the described investigations demonstrated fundamental lacks on the biochemical and probably also on the molecular level of the flavonoid pathway making dramatic colour modifications with classical breeding methods impossible. Nevertheless, these results gave clear hints for suitable metabolic engineering strategies. Therefore, the detailed knowledge of the flavonoid pathway obtained here is the precondition for well-aimed molecular breeding programmes to broaden the range of flower colours by specific modification of the anthocyanin pattern. The details at the respective developed strategies are summarised in Table 2.

4. Discussion

The increasing knowledge of the flavonoid biosynthesis have made this biosynthetic pathway an excellent target for metabolic engineering. Because barely no species generate the full spectrum of colours, novelty of flower colour is one of the major topic in ornamental plant breeding. Moreover, flower colour is often based on flavonoids compounds, such as anthocyanins. Therefore, the development of strategies to create missing hues in a selected plant species or sortiment is of special interest. Today, this aim could be more promising be reached by a suitable combination of classical and molecular breeding methods. Herein, metabolic engineering strategies provide a powerful tool to overcome natural barriers and have the advantage, that these strategies can be used for creation of novel plant phenotypes without changing other desirable characteristics of a pre-existing cultivar [4, 35 and references therein]. Many transformation experiments were performed to change flavonoid pattern and by this the colouration of specific tissues in selected plant species. In detail, the described engineering strategies led to the establishment of several ornamental plants with novel and/or modified flavonoid pattern, e.g. the generation of new colour pigments, mainly yellow and blue. The most recent results are summarised and discussed in [5].

Well known and even simple chemical and biochemical methods including TLC, HPLC, UV-Vis spectroscopy, supplementation experiments, inhibitor application and enzymatic studies proved to be very helpful to support the development of novel metabolic engineering strategies of the flavonoid pathway and to minimise the risk of such experiments regarding the expected and wanted outcome. This is more and more important as metabolic engineering is a time and money consuming technique, where the outcoming flavonoids and their specific functions is often determined by several factors. A careful characterisation of the target plant on the chemical and biochemical level is recommended to obtain valuable information on the gene

pool, on the flavonoid enzymes involved in the pathway and their biochemical properties (e.g. substrate specificity, expression, etc.) and on the naturally occurring flavonoid compounds in specific tissue. The described results highlight the importance of understanding the adjoining aspects of metabolism when manipulating a metabolic pathway.

The described biochemical pre-studies could simulate transformation experiments using sense or gene silencing strategies to introduce and overcome missing or to suppress undesired steps. Moreover it gave an insight to the properties of the flavonoid pathway enzymes in three specific plant species. By this, not only phenotypical changes could be visually detected, but also effects at the chemical level, not directly obvious, could be screened by analytical methods before establishment of transgenic plants. Modifications could concern the flavonoid biosynthesis and maybe much more important steps or branches upstream the flavonoid pathway. This includes the opening of new pathway branches, the synthesis of new compounds and their properties especially the characterisation of potential risk factors, e.g. new allergic or toxic compound accumulation.

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