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Review

Modification of flavonoid biosynthesis in crop plants

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Abstract

Flavonoids comprise the most common group of polyphenolic plant secondary metabolites. In plants, flavonoids play an important role in biological processes. Beside their function as pigments in flowers and fruits, to attract pollinators and seed dispersers, flavonoids are involved in UV-scavenging, fertility and disease resistance.

Since they are present in a wide range of fruits and vegetables, flavonoids form an integral part of the human diet. Currently there is broad interest in the effects of dietary polyphenols on human health. In addition to the potent antioxidant activity of many of these compounds in vitro, an inverse correlation between the intake of certain polyphenols and the risk of cardiovascular disease, cancer and other age related diseases has been observed in epidemiological studies. The potential nutritional effects of these molecules make them an attractive target for genetic engineering strategies aimed at producing plants with increased nutritional value.

This review describes the current knowledge of the molecular regulation of the flavonoid pathway and the state of the art with respect to metabolic engineering of this pathway in crop plants.
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Contents

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1. Introduction

In order to be able to respond to an ever-changing environment, plants use their enormous metabolic capacity to produce a large variety of secondary metabolites. The basic biosynthetic pathways are conserved over a wide range of plants, resulting in the occurrence of a limited number of basic skeletons. These can then be modified by an array of specific reactions resulting in the generation of the enormous number of different secondary compounds. Flavonoids represent a large family of low molecular weight polyphenolic secondary metabolites that are widespread throughout the plant kingdom, ranging from mosses to angiosperms (Koes et al., 1994). In nature they are involved in a wide range of functions, for example (I) in providing pigmentation for flowers, fruits and seeds to attract pollinators and seed dispersers, (II) in protection against ultraviolet light, (III) in plant defence against pathogenic micro organisms, (IV) in plant fertility and germination of pollen, and (V) in acting as signal molecules in plant-microbe interactions (Koes et al., 1994; Dixon and Paiva, 1995; Dooner et al., 1991).

To date, more than 6000 different flavonoids have been described and the number is still increasing (Harborne and Williams, 2000). By definition, they all share the same basic skeleton, the flavan-nucleus, consisting of two aro-matic rings with six carbon atoms (ring A and B) interconnected by a hetero cycle including three carbon atoms (ring C). According to the modifications of the central C-ring they can be divided in different structural classes like flavanones, isoflavones, flavones, flavonols, flavanols and anthocyanins (Fig. 1). In this review also the closely related chalcones and stilbenes are included. The huge diversity in flavonoid structures is due to modifications of the basic skeleton by enzymes such as glycosyl transferases, methyl transferases and acyl transferases. In a single plant species dozens of different flavonoids may be present and most of these are conjugated to various sugar moieties (Forkmann and Heller, 1999).

Since flavonoids impart much of the colour and flavour of fruits, vegetables, nuts and seeds, they form an integral part of the human diet (Parr and Bowell, 2000). Rich dietary sources of flavonoids are for example soybean (isoflavones), citrus (flavanones), tea, apple and cocoa (flavanols), celery (flavones), onions (flavonols) and berries (anthocyanins) (Rice-Evans et al., 1995; Ross and Kasum, 2002; Le Gall et al., 2003).

Historically, flavonoids have been an attractive research subject mainly because of the coloured anthocyanins. In particular the eye-catching anthocyanin pigments have been very useful to perform genetic experiments, including Gregor Mendel's study of the inheritance of genes responsible for pea seed coat colour, and
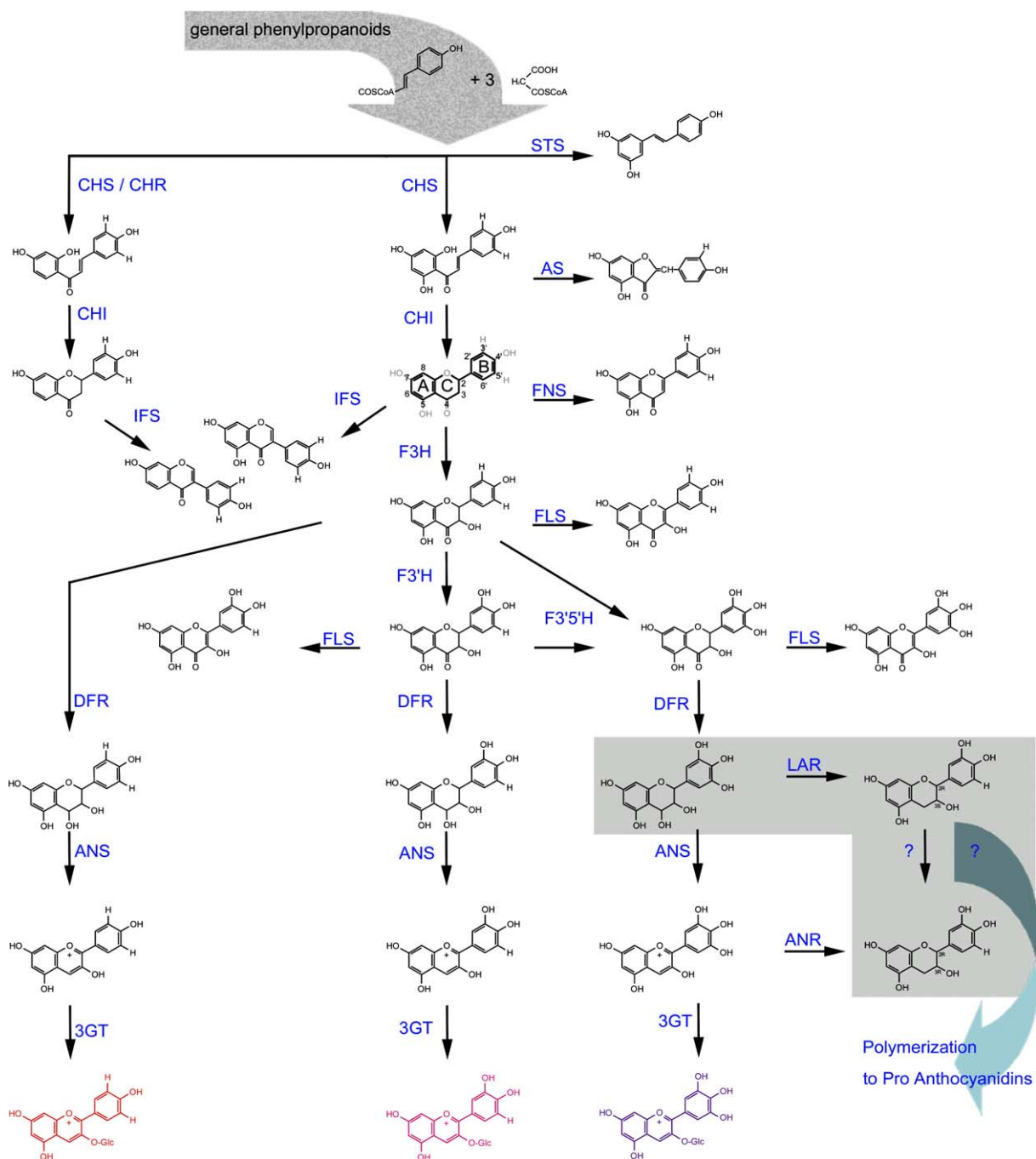
Fig. 1. Schematic overview of the major flavonoid pathway in plants. Enzymes are indicated in blue, abbreviated as follows: CHS, chalcone synthase; CHR, chalcone reductase; STS, stilbene synthase; AS, aureusidin synthase; CHI, chalcone isomerase; F3H, flavanone hydroxylase; FNS, flavone synthase; IFS, isoflavone synthase; FLS, flavonol synthase, F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′,5′-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; 3GT, flavonoid-3-glycosyltransferase.

the discovery of transposable elements interrupting maize pigment biosynthetic genes by McClintock (McClintock, 1967; Lloyd et al., 1992; Koes et al., 1994).

Nowadays, the flavonoid biosynthetic pathway has been almost completely elucidated. Many of the structural and some of the regulatory genes have been cloned from several model plants, including maize, *Antirrhinum*, tobacco, *Petunia* and *Arabidopsis* (Holton and

Cornisch, 1995) and have been expressed in genetically modified model plants and micro-organisms (Dixon and Steele, 1999; Forkmann and Martens, 2001; Winkel-Shirley, 2001; Hwang et al., 2003). Today, standard molecular tools are available to genetically modify plants including several world-wide important crops such as maize, potato, tomato, sugar beet and wheat (Sévenier et al., 2002).

Although some plants contain high levels of certain flavonoids, in other species the composition of these secondary metabolites is 'sub-optimal'. Attempts to modify flavonoid biosynthesis have been made for different reasons. During the last decade, in several ornamental plant species newly pigmented flowers have been developed by genetic modification of the flavonoid pathway (Van der Krol et al., 1988; Courtney-Gutterson et al., 1994; Deroles et al., 1998; Tanaka et al., 1998; Davies et al., 1998; Mol et al., 1999; Aida et al., 2000a,b; Suzuki et al., 2002; Zuker et al., 2002; Fukui et al., 2003). Since defence against pathogens is one of the functions of flavonoids in planta, also for this reason plants with improved flavonoid production have been made (Yu et al., 2003; Fischer et al., 1997; Jeandet et al., 2002). In the past decade it has become more and more clear that the composition of secondary metabolites greatly influences the quality and health potential of food and food products (Stobiecki et al., 2002). In this respect flavonoids are important since they have been suggested to protect against oxidative stress, coronary heart disease, certain cancers, and other age related diseases (Kuo, 1997; Yang et al., 2001; Ross and Kasum, 2002). At least part of these presumed health promoting properties of flavonoids can be attributed to the well-documented antioxidant properties of these compounds. The chemical structure of most polyphenols appears to be ideal for free radical scavenging and in vitro studies have shown that the majority of flavonoids are more effective antioxidants on a mole to mole basis than the antioxidant nutrients vitamins E and C. For example, the flavonol quercetin and the flavan-3-ol epicatechin gallate have a five fold higher total antioxidant activity than the vitamins E and C, when measured as trolox equivalent antioxidant activity (Rice-Evans et al., 1995, 1997). Beside antioxidant activity, the inhibitory effect of flavonoids on enzymatic activities (Castelluccio et al., 1995; Rice-Evans et al., 1997; Pietta, 2000) and their interaction with signal transduction pathways, leading to changes in the expression of genes involved in cell survival, cell proliferation and apoptosis (Yang et al., 2001; Sarkar and Li, 2003; O'Prey et al., 2003; Van Dross et al., 2003) may contribute to their health-promoting properties. For this reason, there is currently a growing interest in the development of agronomically important food crops with optimized levels and composition of flavonoids.

The aim of this review is to present the state of the art of flavonoid pathway engineering in crop plants.

2. The flavonoid biosynthetic pathway

Two classes of genes can be distinguished within the flavonoid pathway: (I) the structural genes encoding enzymes that directly participate in the formation of flav-onoids, and (II) regulatory genes that control the expression of the structural genes. The precursors of the synthesis of most flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derived from carbohydrate metabolism and phenylpropanoid pathway, respectively (Forkmann and Heller, 1999).

The biosynthesis of flavonoids is initiated by the enzymatic step catalysed by chalcone synthase (CHS), resulting in the yellow coloured chalcone. In the majority of plants chalcones are not the end-products, but the pathway proceeds with several enzymatic steps to other classes of flavonoids, such as flavanones, dihydroflavonols and finally to the anthocyanins, the major water-soluble pigments in flowers and fruits. Other flavonoid classes (i.e. isoflavones, aurones, flavones, pro-anthocyanidins (PA) and flavonols) represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation.

3. From the beginning of the pathway towards anthocyanins

3.1. Chalcone synthase

The enzyme CHS catalyzes the stepwise condensation of three acetate units starting from malonyl-CoA with p-coumaroyl-CoA to yield 4,2′,4′,6′-tetrahydroxy-chalcone (Tanaka et al., 1998; Holton and Cornisch, 1995). Genomic and cDNA sequences encoding CHS have been isolated and characterised from many plant species and the expression of endogenous CHS genes has been studied in detail. CHS has been an attractive target for genetic engineering and there are numerous examples of co-suppression or down regulation of this gene in order to modify flower colour towards pure white as a result of a complete absence of flavonoids. However, blocking the flavonoid pathway may also lead to pleiotropic effects such as male sterility (Napoli et al., 1999; Van der Meer et al., 1992; Ylstra et al., 1994; Jorgensen et al., 1996; Deroles et al., 1998).

3.2. Chalcone isomerase

Most plants do not accumulate chalcones. After its formation, naringenin chalcone is rapidly isomerized by the enzyme chalcone isomerase (CHI) to form the flavanone naringenin. Even in the absence of CHI, naringenin chalcone may spontaneously isomer-ise to form naringenin (Holton and Cornisch, 1995). However, in some plants chalcone accumulation does occur. CHI mutants of aster (Callistephus chinensis) and carnation (Dianthus cayophyllus) appeared to have yellowish flower petals; a CHI mutant of Arabidopsis contains a changed seed coat colour (Forkmann and

Heller, 1999), and also the fruit peel of tomato accumulates the yellow coloured naringenin-chalcone (Muir et al., 2001). Two types of CHI are known: one type that can isomerise 6′-hydroxyl- as well as 6′-deoxy chalcones, and another that only converts 6′-hydroxychalcones to flavanones. Although it was thought that the former type of CHI enzyme was restricted to the legumes, it has recently been reported that the tobacco CHI is able to isomerize the 6′-deoxychalcone isoliquiritigenin to the 5′-deoxyflavanone liquiritigenin as well (Joung et al., 2003).

3.3. Flavonoid hydroxylation (F3H/F3′H/F3′5′H)

The subsequent hydroxylation in position C-3 of flavanones to dihydroflavonols has been demonstrated for a wide variety of plant species including *Petunia*, snapdragon, tomato and maize. The reaction is carried out by flavanone-3-hydroxylase (F3H), a member of the 2-oxoglutarate-dependent dioxygenase family which is highly conserved among widely divergent plant species as shown by sequence comparison (Britsch et al., 1993). A mutation resulting in a loss of F3H activity, both in *Petunia* and *Antirrhinum*, prevents the progression along the anthocyanin pathway and white flowers are the consequence (Britsch et al., 1992; Martin et al., 1991).

Dihydrokaempferol (DHK), the product of F3H catalyzed hydroxylation of naringenin, can be further hydroxylated, either at the 3′ position or at both the 3′ and 5′ positions of the B-ring. The former reaction leads to the formation of dihydroquercetin (DHQ) and ultimately to the production of cyanidin based pigments. This is carried out by the P450 hydroxylase flavonoid 3′-hydroxylase (F3′H). The latter hydroxylation steps are catalysed by the P450 enzyme flavonoid 3′,5′-hydroxylase (F3′5′H), responsible for the conversion of DHK into dihydromyricetin (DHM), which is required for the production of delphinidin-based anthocyanins (Forkmann, 1991; Winkel-Shirley, 2001; Toda et al., 2002).

3.4. Dihydroflavonol 4-reductase

The enzyme dihydroflavonol 4-reductase (DFR) catalyzes the stereo specific reduction of dihydroflavonols to leucoanthocyanidins (flavan-3,4-diol) using NADPH as a cofactor (Kristiansen and Rohde, 1991). These leucoanthocyanidins are the immediate precursors for the synthesis of anthocyanins. In addition, they are also precursors for the production of catechins and PA, which are involved in plant resistance and are considered as potential health-protecting compounds in food and feed. Clear evidence for the role of DFR in anthocyanin synthesis came from biochemical supplementation with flavan-3,4-diol as well as from complementation experiments with *DFR* clones in *dDFR* mutants (Goldsbrough et al., 1994; Yoder et al., 1994; Forkmann and Heller, 1999; Martens et al., 2002).

3.5. Anthocyanidin synthase

The leucoanthocyanidins are converted into anthocyanidins by the anthocyanidin synthase (ANS), another member of the 2-oxoglutarate-dependent dioxygenase family. ANS shows large homology to F3H and FLS (Martin et al., 1991; Tanaka et al., 1998). Genomic or cDNA sequences encoding ANS have been obtained from several plant species including *Arabidopsis*, *Antirrhinum*, *Petunia*, *Vitis vinifera* and maize. *ANS* mutants, as well as mutations in regulatory genes affecting *ANS* gene expression have been studied in these plants (Martin et al., 1991; Jackson et al., 1992; Pelletier et al., 1999; Bradley et al., 1998).

3.6. Flavonoid 3-O-glucosyltransferase

In general, flavonoids and anthocyanidins with a free hydroxyl group at the 3 position of the heterocyclic ring are unstable under physiological conditions and are therefore not found in nature (Forkmann and Heller, 1999). The enzyme UDP-glucose:flavonoid 3-O-glucosyltransferase (3GT) is responsible for the transfer of the glucose moiety from UDP-glucose to the hydroxyl group in position 3 of the C ring. Since this is an essential final step required to stabilise anthocyanidins so that they can accumulate as water soluble pigments in the vacuoles, 3GT is regarded as an indispensable enzyme of the main biosynthetic pathway to anthocyanins, rather than simply a modifying enzyme. In this regard it is interesting to note that mutants with decreased DFR and ANS activity also show decreased 3GT activity, suggesting that the late genes of the anthocyanin pathway are co-regulated or may exist as a functional complex (Hrazdina and Wagner, 1985; Hrazdina and Jensen, 1992). Depending on the B-ring hydroxylation pattern, three major types of anthocyanins can finally be distinguished. Each has a characteristic colour since the visible absorption maximum becomes longer as the number of hydroxyl groups in the B-ring increases: pelargonidin-derived pigments are responsible for orange, pink or red colours, cyanidin-derived pigments for red or magenta, and delphinidin-derived pigments for purple or blue (Zuker et al., 2002). Beside the structures of the anthocyanins, differences in vacuolar pH, intermolecular stacking (self-association of anthocyanins and co-pigmentation of anthocyanins with other polyphenols), intramolecular stacking of aromatically modified anthocyanins, glycosylation, metal complexation and cell shape give an almost infinite range of flower colours (Tanaka et al., 1998).

Further flavonoid modification by acylation, additional glycosylation to flavonoid disaccharides or trisaccharides, methylation or hydroxylation may occur within each flavonoid class, whereas modifications like prenylation, sulfation and C-glycosylation are restricted to particular flavonoid groups. Most modifications are performed on the endproducts such as anthocyanin-3-glucosides, flavonols, flavones and (iso)flavones, but also intermediates of the pathway can be used as substrate (Heller and Forkmann, 1994).

4. Formation of other flavonoid classes

Beside going to anthocyanins, the pathway can also branch to other classes of flavonoids, such as stilbenes, aurones, deoxy-(iso)flavonoids, flavones, flavonols, cate-CHINs and PA. The next section gives an overview of how these flavonoids are synthesised.

4.1. Stilbenes

Chalcone synthase, the enzyme responsible for the first step in the flavonoid pathway, is a member of the plant polyketide synthase super family, which catalyses the production of a wide variety of secondary metabolites from a limited set of substrates (malonyl CoA and 4-coumaroyl CoA, in exceptional cases cinnamoyl-CoA), using subtly different reaction mechanisms. For instance, stilbene synthase (STS), the enzyme which catalyses the production of stilbenes, is also a member of this family. Based on the results of mutational and structural analysis and the fact that STS is found in a limited number of unrelated plant species, it has been suggested that STS has evolved from CHS (Tropf et al., 1994; Schröder, 1997). Indeed, CHS and STS share a deduced amino acid sequence similarity of up to 70 percent (Schröder and Schröder, 1990; Eckermann et al., 2003).

Genes encoding STS have been isolated from grape (vitis vinifera sp.) but are also found in peanut and pine. Originally, the stilbene type phyto-alexins such as resveratrol have gained a lot of interest due to their fungicidal properties. In addition, resveratrol and its glycoside piceid are of great interest due to their presumed health effects (Jang et al., 1997; Hall, 2003; Finkel, 2003).

4.2. Aurones

In some popular ornamental plants, such as Antirrhinum, the yellow flower colour is mainly provided by glycosides of flavonoids belonging to the class of aurones (aureusidin and bracteatin). The formation of aureusidin and bacteatin, derived from tetra-hydroxychalcone and penta-hydroxychalcone, respectively, have been demonstrated to be a single enzymatic process catalyzed by the same enzyme, aureusidin synthase (Nakayama et al., 2000). This enzyme and the encoding cDNA sequence (AmAS1) have been purified and isolated from Antirrhinum flower petals. Based on its high deduced amino acid similarity to plant polyphenol oxidases (PPO), aureusidin synthase was classified as a PPO homologue, an enzyme family which occurs ubiquitously in higher plants.

5. DFR

5.1. 5'-Deoxy-(iso)flavonoids

A branch in the first step of the flavonoid pathway, resulting in deoxyflavonoids, has a very limited distribution to just leguminous plants. When CHS alone is present in a plant, 6'-hydroxychalcones are produced. The first deoxy-flavonoid, isoliquiritigenin (2',4',4-trihydroxychalcone or 6'-deoxychalcone), is synthesized by the co-action of CHS and polyketide reductase (PKR), also referred to as chalcone reductase (CHR) (Welle and Grisebach, 1988; Davies et al., 1998; Forkmann and Martens, 2001).

Isoliquiritigenin is the precursor of 5-deoxy-(iso)flavonoids and can, in two subsequent steps, be converted to the 5-deoxy-isoflavonoid daidzein, through the combined action of CHI, leading to the production of the deoxyflavanone liquiritigenin, and the cytochrome P450 enzyme CYP93C1 (2-hydroxy-isoflavanone synthase, commonly termed isoflavone synthase (IFS)). This is the key enzyme for the production of isoflavonoids and carries out the 2,3 migration of the B-ring of liquiritigenin or naringenin, resulting in the production of the isoflavones daidzein and genistein, respectively (Yu et al., 2000).

5.2. Flavones

In several plants (e.g. Petroselinum, Chrysanthenum, Dahlia, Gerbera) naringenin can be used as a precursor for the production of flavones. Two enzymes capable of converting naringenin into the flavone apigenin have been identified from different plant species. In some plant species this conversion is catalysed by a cytochrome P450, whereas in other plants a dioxygenase is responsible for this reaction (Heller and Forkmann, 1994; Forkmann and Martens, 2001). This demonstrates an example of parallel evolution of these flavonoid pathway branches. Flavones play an important role in determining flower colour since they can function as co-pigments by forming complexes with anthocyanins (Aida et al., 2000a,b; Ueyama et al., 2002; Nielsen et al., 2002). In addition, they are proposed to have health beneficial properties (Van Dross et al., 2003).

5.3. 3-Deoxy-anthocyanins and phlobaphenes

In maize and other cereals, two major branches within the flavonoid pathway exist. Beside the common pathway leading to 3-hydroxy-flavonoids such as anthocyanins, a second branch specifically leads to the production of three types of 3-deoxy-flavonoids: C-glycosyl flavones, 3 deoxy-anthocyanins and the pigment phlobaphene. This branch of the pathway is active in maize floral organs and is controlled by the MYB-type transcription factor P (Styles and Cheska, 1975; Grotewold et al., 1998). Flavanones are the common precursors for the production of these 3-deoxyflavonoids. C-glycosyl flavones, a special type of flavones with a C-glycoside attached to the A-ring, are produced from flavanones by an as yet unclear reaction mechanism (Heller and Forkmann, 1988; Grotewold et al., 1998). The 3-deoxy-anthocyanins and the phlobaphene pigments are derived from flavan-4-ols, which are produced from flavanones through the action of DFR (Styles and Cheska, 1975; Grotewold et al., 1998). So, depending on substrate availability, the maize DFR enzyme can either convert flavanones into flavan-4-ols, which are precursors for the formation of 3-deoxy-anthocyanins and phlobaphenes, or convert dihydroflavonols to leucoanthocyanidins, which lead to the production of anthocyanins. This suggests that the branching of the pathway towards either anthocyanins or 3-deoxy anthocyanins and phlobaphenes is, at least in part, determined by the activity of the F3H enzyme, which catalyses the conversion of flavanones into dihydroflavonols.

5.4. Flavonols

One step further downstream in the main pathway towards anthocyanins, the dihydroflavonols (DHK, DHQ, DHM) can be converted into flavonols by the enzyme flavonol synthase (FLS). This enzyme catalyzes the introduction of a double bond between carbon 2 and 3 of the C-ring, producing the flavonols kaempferol, quercetin and myricetin respectively. With regard to the over-all flavonoid biosynthesis leading to anthocyanin formation, there is clearly competition between the enzymes FLS and DFR for the common substrate dihydroflavonols FLSDFR. For instance, transgenic anti-sense FLS flowers of Petunia and tobacco accumulate increased levels of anthocyanins (Holton et al., 1993; Nielsen et al., 2002). It has been reported that FLS activity was high in still uncoloured flowers of Petunia, Dianthus and Matthiola, but that the activity rapidly declined when anthocyanin formation starts (Forkmann and Heller, 1999). This temporal regulation of the activity of enzymes using the same substrate is an attractive way to prevent competition for the dihydroflavonols to be used for either anthocyanin or flavonol biosynthesis.

5.5. Catechins and pro-anthocyanidins

There are two branching points in the anthocyanin pathway which lead to the production of (epi)catechins and PA: one at the level of leucoanthocyanidins and one at the level of anthocyanidin aglycons (Fig. 1). Leucoanthocyanidins can be converted either to anthocyanidins and subsequently anthocyanins through the subsequent action of ANS and 3GT (i.e. the 'normal pathway'), or reduced to catechins through the action of the enzyme leucoanthocyanidin reductase (LAR). The LAR gene has recently been isolated from the legume Desmodium. Expression of this recombinant LAR cDNA in E. coli, transgenic tobacco and white clover resulted in detectable LAR activity and the synthesis of catechin (Tanner et al., 2003; Marles et al., 2003).

Evidence for a second branch point towards catechins at the level of anthocyanidins came from the analysis of a loss-of-function Arabidopsis mutant, called BANYULS, which accumulated high levels of anthocyanins. In first instance it was thought that the BANYULS gene (BAN) encoded a negative regulator of pigmentation. However, ectopic expression of the Arabidopsis as well as the Medicago BAN genes in tobacco showed that both genes encode an anthocyanidin reductase, which converts anthocyanidins to the epicatechin 2,3-cis-flavan-3-ol (Bartel and Matsuda, 2003). Since BANYULS and 3GT compete for the common precursor anthocyanidin, expression of BAN resulted in a loss of anthocyanins, as well as an accumulation of condensed tannins (Xie et al., 2003).

Pro-anthocyanidins, also known as condensed tannins, are polymeric flavonoids that are thought to be synthesised by sequential addition of intermediates derived from flavan-3,4-diol (e.g. leucocyanidin) to a flavan-3-ol initiating unit (e.g. catechin) or a pre-existing chain. In planta, complex PA polymers can be found as the result of a combinatorial incorporation of several isomers, and the degree of polymerization as well as the composition may change during plant development. A condensation reaction resulting in a PA polymer has been demonstrated in vitro (Delcour, 1983), but until now little is known about the genes and enzymes responsible for the polymerisation reactions leading to PA.

Much of our knowledge on PA's to date has been derived from extensive analysis of Arabidopsis seed coat mutants. A number of tannin deficient seed (tds) and transparent testa (tt) genes have been identified in Arabidopsis which, when mutated, cause a disruption in pro-anthocyanin biosynthesis (Abrahams et al., 2002).

6. Regulators controlling the flavonoid pathway

Coordinate transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the

final levels of secondary metabolites in plant cells. This regulation is achieved by specific transcription factors. These DNA binding proteins interact with promoter regions of target genes and modulate the rate of initiation of mRNA synthesis by RNA polymerase II (Ranish and Hahn, 1996). Regulatory genes, in particular those controlling pigmentation intensity and pigmentation pattern through influencing the expression of many different structural genes, have been identified in many plants (Holton and Cornisch, 1995). The regulation of these regulatory genes appeared to be highly dependent on tissue type and/or response to internal signals, such as hormones, and to external signals such as microbial elicitors or UV radiation (Memelink et al., 2000; Martin et al., 2001; Vom Endt et al., 2002).

Transposon tagging has proven to be a very useful tool for isolating flavonoid regulatory genes for which no prior information existed concerning their gene sequence, function or final products (Holton and Cornisch, 1995). In particular three species have been important for elucidating the anthocyanin biosynthetic pathway and its control by regulatory genes: maize, Antirrhinum and Petunia. More recently, regulatory genes have been found in Arabidopsis and tomato. In general the isolated regulatory genes can be divided into two transcription factor families: one with sequence homology to a protein encoded by the vertebrate proto-oncogene c-MYB, and the other with similarity to the vertebrate basic-Helix–Loop–Helix (bHLH) protein encoded by the proto-oncogene c-MYC (Mol et al., 1998). In various plant species the tissue-specific regulation of the structural genes involved in the flavonoid biosynthesis is controlled by the combination of regulators from these two transcription factor families. In different plant species, homologous sets of transcription factors activate different sets of structural genes, thus allowing regulatory diversity in the pathway. Ectopic expression of transcription factor genes in various plant species has confirmed that these regulatory genes are functionally conserved among different plant species. However, the final quantity and class of flavonoids that is produced is determined by a number of parameters: the binding affinity of the transcription factor to the promoter sites of their target structural genes; the ability to cooperate with endogenous transcription factors; the functionality of these endogenous transcription factors (Quattrocchio et al., 1993). There is however a remarkable sequence homology between the flavonoid transcription factors from different plant species, indicating that they are derived from a common ancestor. Some of them have already been successfully expressed ectopically in various transgenic plant species such as tobacco, tomato and Petunia. It appeared therefore that they have been functionally conserved among plant species during evolution (Koes et al., 1994; Quattrocchio et al., 1993).

7. Pathway engineering using the maize R and C1 transcription factors

Several of the anthocyanin myc and myb regulatory genes have been tested for their ability to improve anthocyanin accumulation when expressed in heterologous plants. However, up to now the success of this strategy has been highly variable. Amongst the most well characterized regulatory plant genes are the maize leaf colour (LC) gene belonging to the MYC type R gene family and the MYB type C1 (colourless) gene.

Already more than a decade ago, activation of anthocyanin production was achieved in Arabidopsis and tobacco plants by the introduction of the maize regulators R and C1. In both plant species, expression of the R regulatory gene alone resulted in enhanced anthocyanin pigmentation of tissues that normally produce anthocyanins, whereas in Arabidopsis also an increased trichome production was observed. The C1 gene alone had no visible effect. Accumulation of anthocyanins in tissues that normally do not contain any anthocyanins was observed in hybrid transgenic Arabidopsis plants expressing both C1 and R (Lloyd et al., 1992).

Also in other dicot species, expression of LC resulted in enhanced anthocyanin pigmentation. For example, in LC over-expressing cherry tomato plants, anthocyanins accumulated in leaves, stems, sepals, petal, main vein and, to a lesser extent, in developing green fruits (Goldsbrough et al., 1996). Not only anthocyanins, but also other classes of flavonoids have been reported to accumulate when LC and C1 are ectopically expressed. For example in red ripe tomato fruits, which normally produce only small amounts of the flavonols kaempferol and quercetin in the fruit peel tissue, the introduction and co-ordinate expression of the maize regulatory genes LC and C1 under the control of a combination of general and fruit specific promoters, was sufficient to up-regulate the flavonoid pathway in the fruit flesh, a tissue that normally does not produce flavonoids (Bovy et al., 2002). The main compounds accumulating in the fruits were glycosides of the flavonol kaempferol. A more modest increase in glycosides of the flavanone naringenin was also observed. Total flavonol content of ripe transgenic tomatoes over-expressing LC/C1 was about 20-fold higher than that of the controls (Bovy et al., 2002; Le Gall et al., 2003). Remarkably, no increase of anthocyanins was detected in these fruits. However, RNA expression analysis revealed that all of the structural genes leading to kaempferol-type flavonols and pelargonidin-type anthocyanins were strongly induced by the introduced LC/C1 transcription factors. Biochemical and transcriptional analysis of the transgenic lines indicated that the absence of anthocyanins was primarily due to a low, LC/C1 independent expression of the gene encoding flavanone-3′,5′-hydroxylase, together with a strong preference of the tomato dihydroflavonol

reductase (DFR) enzyme for DHM (Forkmann and Heller, 1999), the precursor for delphinidin-type anthocyanins. In contrast to fruits, old leaves and nodes of some *LC/C1* tomato plants, as well as light-stressed *LC/C1* seedlings, revealed a clearly visible accumulation of delphinidin-type anthocyanins *LC*. In purple-coloured *LC/C1* leaves, *F3'5'H* gene expression was at least 10-fold higher than in fruits (Bovy et al., 2002).

In potato, another globally important crop belonging to the same *Solanaceae* family, expression of *LC* and *C1* resulted in a marked accumulation of kaempferol in the whole tuber and an increased anthocyanin pigmentation of the peel (Fig. 2, De Vos et al., 2000).

In alfalfa, no induction of anthocyanin formation was found in transgenic plants expressing the maize regulatory genes *LC*, *B-Peru*, or *C1* when grown under normal conditions. The *LC* gene, but not *B-Peru* or *C1*, was found to stimulate anthocyanin and pro-anthocyanin biosynthesis in alfalfa, but only in combination with the presence of one or more unknown environmental stress-responsive factors. After cold or light stress the red to deep purple colouration of the leaves and stems of *LC* transgenic alfalfa plants was accompanied by a rapid accumulation of *CHS* and *F3H* transcript levels. Weak expression levels of these two structural genes in non-transgenic plants suggests that the expression of both genes must bypass a certain threshold level before the anthocyanin pathway is induced (Ray et al., 2003).

Although ectopic expression of *LC* alone was sufficient to enhance pigmentation in tobacco, *Arabidopsis*, *Petunia* and to a lesser extent in alfalfa and tomato, it has not been demonstrated to do so in some other plant species, such as Lisianthus and Pelargonium. This apparent inconsistency in response to ectopic *LC* expression is reflected in the remarkable variation between plant species in their transcriptional response to the introduced *LC* genes. For example in *Petunia*, the introduced *LC* gene slightly induced the steady state mRNA levels of *CHS*, *CHI*, and *F3H* and resulted in a stronger activation of *DFR*, *F3'H*, *F3'5'H*, *ANS*, *UFGT*, and *3RT*. In tobacco plants, only the expression levels of *CHS* and *DFR* were enhanced by *LC* (Bradley et al., 1998), and in *Lisianthus* and *Pelargonium*, *LC* over-expression was not sufficient to up-regulate expression levels of flavonoid biosynthesis genes (Bradley et al., 1999).

A deficient transcriptional activation may be due to a lack of appropriate transcription factor binding sites on the promoter sequence of one or more structural genes. Alternatively, it is possible that the introduced *LC* gene product is not able to interact with the endogenous MYB like proteins that may be required to up-regulate flavonoid gene expression or that these MYB like proteins are rate-limiting. Since, in many cases, the induction of flavonoid biosynthesis is enhanced by environmental stress, other endogenous regulators such

Fig. 2. Increased flavonoid biosynthesis in transgenic potato tubers. In addition to kaempferol accumulation in the whole tuber (De Vos et al., 2000), the *Lc/C1* over-expressing tubers (left) clearly show an increased accumulation of anthocyanins in the peel, compared to the control tuber (right).

as stress-induced transcription activators or repressors may also play a role in the final activation of the flavonoid pathway. In this respect, a transgene-dosage effect could be of importance. This dependence on additional transcription factors could, at least in part, be overcome by the coordinate expression of both *LC* and *C1*: whereas LC/C1 together were sufficient to upregulate the flavonoid biosynthesis pathway in tomato fruit, neither LC, nor C1 alone were sufficient to induce the pathway in tomato fruit.

All the above-mentioned examples of introducing the *LC* and/or *C1* transcription factors in heterologous plants clearly show that these genes can be functionally expressed in many, though not all, plant species. Whereas the introduction of *LC* alone may be sufficient to enhance anthocyanins in those tissues normally accumulating flavonoids, in most cases both LC and C1 seem to be necessary to produce significant amounts of anthocyanin pigments or flavonols in plant tissues that normally do not accumulate these compounds.

8. Dicot representatives of Myc/Myb type transcription factors

Homologs of the maize flavonoid transcription factor genes *LC* and *C1* have also been isolated from dicot

species. *Petunia*, *Arabidopsis*, tomato and *Antirrhinum* contain genes with sequence homology to transcription factors that regulate the structural genes of the anthocyanin biosynthetic pathway (Cone et al., 1986; Goodrich et al., 1992; Grotewold et al., 1994; Quattrocchio et al., 1998; Ramsay et al., 2003).

In contrast to maize, where LC and C1 regulate all genes of the of the pathway from *CHS* until *3GT*, it has been shown that in dicots such as *Petunia* and *Antirrhinum* distinct sets of MYB/MYC transcription factors are responsible for regulating the early part (CHS up to F3H) or the late part (DFR to 3GT) of the pathway. For example, in *Antirrhinum*, three anthocyanin regulatory genes – *Delila*, *Eluta* and *Rosea* – have been identified. The *Antirrhinum Delila* gene (*DEL*), a MYC (bHLH) homologue, is required for pigmentation of the flower tube. The first two steps in the flavonoid pathway, *CHS* and *CHI*, show minimal regulation by Delila but subsequent steps (*F3H*, *DFR*, *3GT*) have an absolute requirement for the *Delila* gene product and show quantitative regulation by *Eluta* and *Rosea* (Martin et al., 1991).

Over-expression of *Delila* in tobacco and tomato resulted in enhanced pigmentation of vegetative tissues in tomato whereas only the flowers were affected in tobacco. In both plants this was at least due to increased expression of the *DFR* gene. A 10-fold increase of *DFR* mRNA levels was observed in tomato and a 4-fold increase in tobacco when *DEL* was over-expressed. Transcript levels of *CHS* were only slightly increased, 2 and 3-fold, for tobacco and tomato, respectively (Mooney et al., 1995).

Recently, a Myb transcription factor gene was identified by activation tagging in a tomato line accumulating anthocyanins (Mathews et al., 2003). This gene encoded the ANT1 protein, which shows strongest similarity with the Petunia AN2 (MYB) protein. This gene is responsible for the intense purple colour of vegetative tissue and purple spots in the fruit epidermis of the transposon mutant. Strong constitutive over-expression of a single genomic *ANT1* gene in the tomato cultivar Micro-Tom demonstrated phenotypes ranging from weak to strong anthocyanin accumulation. The same result was obtained with *ANT1* transformed tobacco plants. The anthocyanin levels in 3-week-old in vitro-grown seedlings of transgenic *ANT1* tomato plants were increased up to 3574 μg/g fresh weight, an almost 500-fold increase compared to untransformed seedlings. Beside the 3-rutinoside-5-glucosides of delphinidin-type anthocyanins (delphinidin, petunidin and malvidin), six additional acylated pigments were found. These appeared to be the same three delphinidin-type anthocyanins acylated with either *p*-coumaric acid or caffeic acid. Over-expression of *ANT1* resulted in the up-regulation of early (*CHS*) as well as late (*DFR*) genes of the anthocyanin biosynthesis. Furthermore, genes encoding flavonoid modifying enzymes such as 3-O-glucosyltransferase and 5-O-glucosyltransferase, as well as the flavonoid binding protein GST-I, required for transport, were increased. In addition, three new genes involved in tomato anthocyanin biosynthesis were found in the *ANT1* over-expressing line: a *CHI*-like gene, a gene similar to *Arabidopsis* transcription factor *HD-GL2*, and a gene with strong homology to an *Arabidopsis* permease required for vacuolar transport of PA (Mathews et al., 2003).

Beside transcription factors which increase activity in the flavonoid pathway, also negative regulators have been found. Over-expression of the FaMYB1 transcription factor isolated from red strawberry fruits resulted in suppression of anthocyanin as well as flavonol accumulation in tobacco (Aharoni et al., 2001). Also two MYB transcription factors from *Antirrhinum*, AmMYB308 and AmMYB330 were shown to have repressing effects on genes involved in phenylpropanoid biosynthesis when expressed in tobacco (Tamagnone et al., 1998). Interestingly, all these suppressing MYB factors share a conserved motif in their C-terminal end (Vom Endt et al., 2002), suggesting that additional suppressing transcription factors may more easily be identified in the future. Vom Endt et al. speculated that these MYB suppressor genes encode weak activators/repressors which compete with endogenous MYB-related activators.

9. Modifying the structural genes

Down-regulation or over-expression of structural flavonoid genes in transgenic plants have shown to be useful tools to elucidate the function of flavonoid pathway genes. Furthermore, over-expression of structural genes can be used in metabolic engineering strategies to overcome rate-limiting enzymatic steps in the pathway. In this way, the flux through an already existing pathway of the hostplant can be increased, which may lead to enhanced levels of specific flavonoids or even new flavonoids. As outlined below, this approach has been used extensively to increase the flavonoid content of tomato fruit, in order to improve the food quality of this important crop.

In tomato, naringenin chalcone accumulates almost exclusively in peel tissue and is simultaneously formed with colouring of the fruit, peaking at the turning peel stage. In addition, the flavonols quercetin-rutinoside (rutin) and, to a lesser extent, kaempferol-rutinoside, also accumulate almost exclusively in the peel of ripening tomato fruits. Expression analysis of the endogenous tomato flavonoid genes *CHS*, *CHI*, *F3H* and *FLS* revealed that *CHS*, *F3H* and *FLS* were expressed in peel tissue during all stages of fruit development, peaking in the turning stage. In contrast, the *CHI* transcript levels remained below detection levels (Muir et al., 2001). Based on these biochemical and gene-expression data it was suggested that a block exists

at the level of CHI. Indeed, ectopic expression of a single *CHI* gene from *Petunia* resulted in a tissue specific increase of total flavonols in the fruit peel. This was mainly due to the accumulation of the flavonol rutin (quercetin 3-rutinoside) and quercitrin (quercetin-3-glucoside), and to smaller but still substantial increases in kaempferol glycosides (Fig. 3). In these high-flavonol transformants, naringenin chalcone levels were strongly reduced, suggesting that the *Petunia* CHI enzyme utilizes the natural naringenin chalcone pool as substrate (Muir et al., 2001; Verhoeyen et al., 2002). Although it was not possible to distinguish CHI transformants from the parental variety based on their vegetative phenotype, transgenic lines showed dullness in the tone of their red ripe fruits. The correlation of dullness with the CHI over-expressing phenotype suggests that naringenin-chalcone in some way may be involved in determining the shininess of fruits. In fruit flesh and leaves of CHI-overexpressing plants, the total amount of flavonoids remained unchanged (Muir et al., 2001).

In flesh tissue of tomato fruits, the transcript levels of *CHS*, *CHI*, *F3H* and *FLS* were below detection levels. To enhance the levels of flavonols in the fruit flesh, a four-gene construct has been used and concomitant

ectopic expression of *Petunia CHS*, *CHI*, *F3H* and *FLS* in tomato fruit resulted in increased levels of flavonols in both peel (primarily quercetin glycosides) and flesh (primarily kaempferol glycosides) (Colliver et al., 2002). When expressed separately, none of these four genes was sufficient to produce flavonols in the fruit flesh: *CHS* over-expression resulted in accumulation of naringenin in the flesh, *CHI* only affected flavonol levels in the peel, and *F3H* and *FLS* showed no effects on flavonoid levels, neither in peel, nor in flesh. Crossing experiments with parental single gene transformants revealed that concomitant expression of both *CHS* and *FLS* had a synergistic effect, resulting in accumulation of naringenin- as well as kaempferol-glucosides in tomato flesh. So, the transgenes that appear to be critical to achieving flavonol biosynthesis in tomato flesh (pericarp and columella) tissue are *CHS* and *FLS*, while *CHI* gene activity appears to be the key to flavonol accumulation in the peel tissue (Colliver et al., 2002; Verhoeyen et al., 2002). Furthermore, it can be concluded that, in tomato flesh, ectopic expression of *CHI* and *F3H* are not required for flavonol production. Possibly, this could be due to the presence of sufficient endogenous CHI enzyme or spontaneous conversion of naringenin chalcone to naringenin.
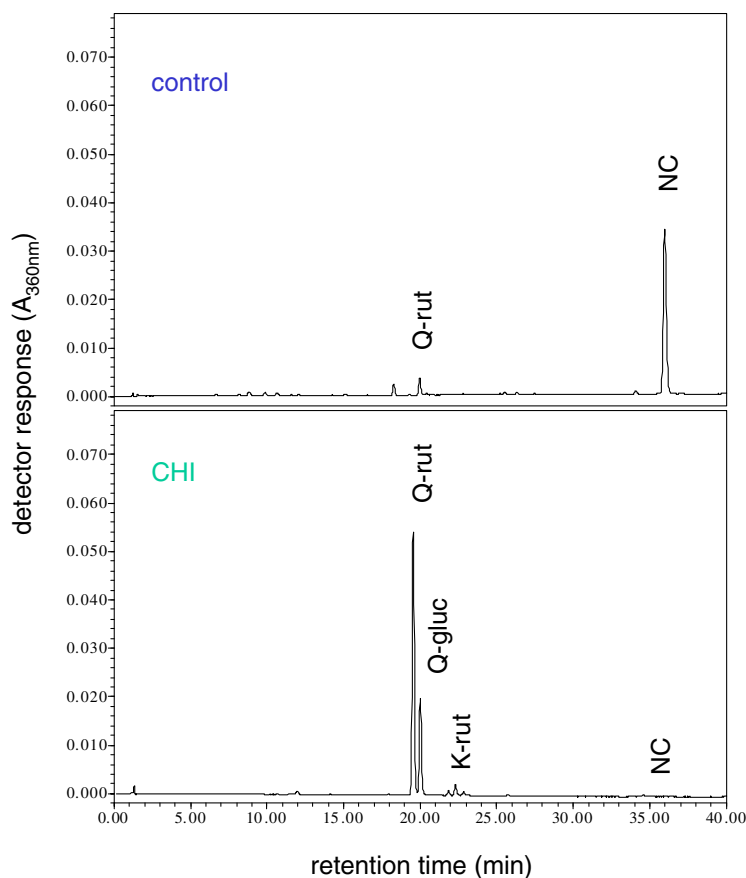

Fig. 3. HPLC chromatogram recorded at 360 nm of non hydrolysed peel of control and *CHI* over-expressing tomato fruit. In the control tomato the major peaks correspond to naringenin chalcone and quercetin rutinoside. In *CHI* over-expressing tomato naringenin chalcone is decreased and flavonol (quercetin and kaempferol) derivatives are the major flavonoids in the fruit peel.

In addition, preliminary results from in vitro experiments show that FLS can use naringenin to form kaempferol, suggesting that the FLS enzyme harbours an intrinsic F3H-like activity as well (Martens et al., 2003; Lukačin et al., 2003).

Also in potato, another *Solanaceous* crop, several attempts have been made to increase the flavonoid production in the tubers by introducing structural genes. Over-expression of a CHS cDNA from *Petunia* resulted in an increase in petunidin and pelargonidin-type anthocyanins in potato tubers. This result could not be obtained, however, with all *CHS* genes. For example the cDNA encoding CHS J from barley was not functional in transgenic potato (Stobiecki et al., 2002; Lukaszewicz et al., 2004). Transgenic potato plants over-expressing a *Petunia* cDNA encoding DFR resulted in an increase of tuber anthocyanins, namely a 3-fold increase in petunidin and a 4-fold increase in pelargonidin derivatives. A significant decrease in anthocyanin levels was observed when the plants were transformed with a corresponding anti-sense construct (Lukaszewicz et al., 2004). In addition, a lower but still significant increase of anthocyanins was observed after over expressing *CHI*. Antisense suppression of the genes encoding CHS and DFR, but not CHI resulted in a significant decrease in anthocyanin content. A likely explanation for the lack of co-suppression with the *Petunia CHI* cDNA may be that this cDNA was not homologous enough to repress the endogenous potato *CHI* gene (Stobiecki et al., 2002). Alternatively, it is possible that the lack of reported *CHI* (co-)suppression could rely on the spontaneous conversion of chalcones to naringenin even in the absence of CHI enzyme activity (Heller and Forkmann, 1994).

10. Introducing new pathway branches to crop plants

The previous section gave several examples of how genetic engineering can be used to enhance or reduce the flux through the endogenous flavonoid pathway in a plant, tissue or organ or even to activate the whole pathway. In addition, one can target flavonoid synthesis towards branches that are normally not present in the host plant by introducing foreign genes that branch off from the existing pathway towards new compounds.

11. From flavonoid precursors to stilbenes

The flavonoid-related stilbenes are well known for their anti-microbial properties and several research groups have expressed *STS* in their favourite host plants to increase disease resistance. To date, *STS* genes have been ectopically expressed in rice, tomato, alfalfa, kiwifruit, barley, wheat and very recently apple (Stark-Lorenzen et al., 1997; Hain et al., 1993; Leckband and Lörz, 1998; Kobayashi et al., 2000; Szankowski et al., 2003).

In all cases, expression of *STS* genes led to a significant increase in STS enzyme activity, with resveratrol accumulating in transformed plants. Independent expression of *STS* genes from three different *Vitis* ssp. in transgenic kiwifruit resulted in the production of piceid, the 3-O-glucoside of resveratrol, rather than resveratrol aglycon. Also in wheat and apple, resveratrol could be detected only after acid hydrolysis, suggesting that the stilbene produced was accumulated in a glycosidic form. Consequently, the resveratrol produced by the action of the introduced *STS* genes seems to be metabolized into piceid by an endogenous glycosyltransferase (Kobayashi et al., 2000). A clear relationship between resveratrol and disease resistance could be demonstrated in all transgenic plants except kiwifruit. In past years, stilbenes have received increasing interest due to their presumed health promoting properties (Jang et al., 1997; Hall, 2003; Finkel, 2003). Over-expression of *STS* in crop plants could therefore be an attractive way to enhance nutritional value. However, very high constitutive *STS* expression also has a dramatic influence on flower colour and pollen development. These undesirable side effects may be due to a competition between CHS and STS for their common substrates, leading to reduced levels of flavonoids, which may give rise to male sterility in some species (Ylstra et al., 1992; Van der Meer et al., 1992). Male sterile phenotypes have been reported for *STS* over-expressing tobacco (Fischer et al., 1997). These plants accumulate relatively high amounts of resveratrol (up to 400 µg/g fresh weight). In contrast, transgenic wheat lines with relatively small amounts of resveratrol (2 µg/g fresh weight) were found to be completely fertile. Therefore it has been proposed that the STS activity in this case may be to weak to give rise to sterility. The reports mentioned here suggest that the reason for diminished flower pigmentation and male sterility is competition for the substrates 4-coumaroyl CoA and malonyl CoA rather than suppression of endogenous *CHS* expression by *STS* sequences (Fischer et al., 1997; Fettig and Hess, 1999; Jeandet et al., 2002). More evidence for the hypothesis that the observed pleiotropic effects in *STS* over-expressing plants are due to reduced flavonoid levels comes from partial chemical complementation of the male sterility phenotype by exogenous addition of flavonols as well as flavonoid precursors from the phenylpropanoid pathway (Fischer et al., 1997).

12. Increasing the production of isoflavonoids

In plants the occurrence of isoflavonoids is limited to the *Leguminoceae*. In members of this family isoflavones have very important functions as inducers of *Rhizobium* nodulation genes and anti-microbial phytoalexins. In addition, isoflavonoids are common constituents of the human diet and are regarded as potentially health-protecting compounds. Some isoflavonoids even exhibit

medicinal properties and there is increasing evidence that these compounds may be protective against certain forms of cancer (Joung et al., 2003; Sarkar and Li, 2003). These properties demonstrate that there is a great potential for isoflavonoid engineering, to enhance the nutritional value of crop plants that normally do not synthesize these compounds (Winkel-Shirley, 2001).

To date, all attempts to produce isoflavonoids in non legumes (Arabidopsis, tobacco and maize), by over-expression of the soybean *IFS* gene, have merely resulted in the production of only small amounts of isoflavones (Liu et al., 2002). These studies have shown that the limited production of isoflavonoids in non-leguminous plants was mainly due to competition for naringenin between F3H and IFS rather than a limiting IFS activity (Yu et al., 2000; Liu et al., 2002). This hypothesis is supported by the recent finding that genistein levels could be increased remarkably by ectopic expression of *IFS* in an *Arabidopsis F3H* mutant line with a reduced flavonol biosynthesis (Liu et al., 2002). In *IFS* over-expressing maize cells, detectable levels of genistein could only be observed when the flux through the pathway, and thereby the availability of substrate, was increased by additional expression of the chimeric transcription factor CRC (a fusion in which the R protein is inserted between the DNA binding and activation domains of C1).

The maize CRC transcription factor has also been used to increase isoflavonoid production in soybean: ectopic CRC expression caused a two-fold increase in isoflavonoid levels of soybean seeds. A substantial increase of daidzein was observed while genistein levels were decreased, resulting in only a slight increase in the total isoflavone levels. The observed decrease of genistein compared to daidzein could be a result of increased transcription levels in the CRC transgenic soybean seeds of (i) *CHR*, thereby channeling the pathway towards liquiritigenin, the precursor of daidzein, and (ii) *F3H*, *DFR* and *FLS* which leads to decreased levels of naringenin, the precursor of genistein, due to competition for the same substrate between F3H and IFS. Co-suppression of *F3H*, to block the anthocyanin and flavonol pathway, together with *CRC* expression, enhanced the isoflavone accumulation in soybean up to 4 times as compared to wild type. Only in the presence of CRC, when the flavonoid pathway is up-regulated, *F3H* co-suppression led to increased accumulation of isoflavonoids in soybean seeds (Yu et al., 2003). Similar results have been described earlier for *Arabidopsis* (Liu et al., 2002).

13. Chalcone reductase; the decision point for deoxy- or hydroxy-flavonoids

In maize cells, expression of the soybean *CHR* cDNA together with the transcription factor CRC and *IFS* led to the synthesis of the deoxy isoflavonoid daidzein in addition to genistein. Although in vitro studies showed that the IFS enzyme was able to convert liquiritigenin to daidzein more efficiently than naringenin to genistein, daidzein was present in these cells at about a 10-fold lower level than genistein. This implies that in the maize cell system the synthesis of the liquiritigenin substrate, rather than substrate specificity, may be limiting. As a possible reason for this substrate limitation in the *CHR* expressing plants it was suggested that the soy CHR was unable to interact properly with the endogenous maize CHS, resulting in a limited liquiritigenin production (Yu et al., 2000).

Beside the enhanced nutritional value obtained by increasing isoflavone levels through *CHR* and *IFS* expression, *CHR* over-expression has also been performed for ornamental purposes. Since in non-legume plants, the isomerization rate of 6′-deoxychalcones by CHI is much lower than that of the comparable 6′-hydroxychalcones, modification of plants to produce 6′-deoxychalcones could be one way to produce stable chalcones, giving more intense yellow flower colours. Expression of CHR not only leads to the production of yellow-coloured 6′-deoxychalcones, but also prevents the formation of 6′-hydroxychalcones, the preferred substrate of endogenous CHI (Welle and Grisebach, 1988). In this way, a phenotype with a 'block' at the CHI step within the hydroxy flavonoid pathway could be obtained. For example the pink flowering Xanthi line of tobacco became white to pink by the introduction and constitutive expression of a *CHR* gene from *Pueraria montana*. This was due to a strongly reduced anthocyanin content in the floral tissues and the production of 5′-deoxyflavonoids (Joung et al., 2003). Also in *Petunia*, the flower colour was changed from white to pale yellow or from deep purple to pale purple by the introduction of *CHR*. As in *Pueraria*, the decreased anthocyanin production and concomitant fading of purple colouration of *Petunia* flowers reflects the competition between the endogenous 5′-hydroxy pathway leading to anthocyanins and the CHR-dependent 5′-deoxy pathway leading to deoxyflavonoids (Davies et al., 1998).

14. Pro-anthocyanins

The presence of PA or condensed tannins in edible plant tissues has gained increasing interest, due to the presumed beneficial health effects of these compounds on animals and human (Dixon et al., 1996; Aerts et al., 1999). The presence of these flavonoid polymers in forage can contribute to: (I) improved efficiency of protein conversion from plant proteins into animal protein, (II) the reduction of greenhouse gasses and (III) increased disease resistance. Furthermore, PA's and their precursors, the catechins, strongly contribute to

the flavour and astringency of two important beverages: wine and tea. The recent cloning of the genes required for the biosynthesis of catechins, *BAN* and *LAR*, will certainly lead to applications in crop plants, aimed at enhancing the levels of catechins and the PA's derived thereof. The isolation of genes encoding the condensing enzymes involved in the synthesis of PA polymers, if any, is one of the major challenges in the years to come.

15. Remarks for genetic engineering

The research described above has led to a huge leap forward in our understanding of the flavonoid biosynthesis. However, despite all the extensive work, there are still challenging tasks ahead.

The isolation and cloning of most of the structural flavonoid genes opens up possibilities to develop plants with tailor-made optimised flavonoid levels and composition. However, it will also be clear that the variation between plant species may lead to complications or unexpected results in pathway engineering. In addition, there are several pathway branches that are still a mystery which remain to be unravelled. The PA branch is as yet largely unknown, and the branches leading to the production of flavones or the relatively unknown aurones have not yet been introduced in transgenic crop plants. Beside this, not much is known about modifying enzymes and the corresponding genes that are responsible for glycosylation, methylation and prenylation reactions that are important for flavonoid stability, cellular distribution, bioactivity and bioavailability. The above mentioned examples are challenging topics to investigate further in the future.

Nevertheless, several considerations have to be kept in mind with regard to genetic engineering. First of all, the final result of the engineering is dependent on the approach used (over-expression or down-regulation). Secondly, it is dependent on the encoded function of the introduced transgene (a transcription factor or an enzyme). Also the activity of the endogenous pathway, as well as its regulation, is of great importance. As illustrated earlier, the stimulation of the flavonoid biosynthesis by the transcription factors *LC* and *C1* in tomato led to the induction of several flavonoid genes, but was not sufficient to induce F3′5′H activity, which appeared to be essential for the production of anthocyanins in tomato fruit. The introduction of a new branch point within the existing pathway could interfere with endogenous flavonoid biosynthesis and/or fail to compete with the endogenous pathway for common substrates. This has been described in detail for the introduction of IFS into non-leguminous plant species, where especially F3H appears to prevent the formation of high levels of isoflavonoids (Yu et al., 2000). Also,

the host plant or tissue may be "incapable" of producing certain compounds due to substrate specificity of endogenous enzymes, as was reported for the tomato DFR that was restricted in its substrate specificity to DHM and thus can only give rise to the production of delphinidin-type anthocyanins (Bovy et al., 2002).

Finally, it has to be taken into account that flavonoids play an essential role in many developmental and physiological processes. Although modification of flavonoid biosynthesis may lead to increased or decreased levels of a desired compound, disruption of the existing pathway can result in pleiotropic effects. Altered growth patterns, enhanced susceptibility to stress and a decreased fertility have been described several times (Van der Meer et al., 1992; Fischer et al., 1997; Ylstra et al., 1992, 1994; Mo et al., 1992; Deboo et al., 1995; Eldik et al., 1997). The use of specific promoters could be a way to circumvent at least part of these pleiotropic effects.

On top of this, controlling the overall metabolic flux within the targeted pathway and endogenous competing pathways is a very important aspect to consider when designing strategies for metabolic engineering.

The concept that the flavonoid pathway may be organized as a multi-enzyme complex was first proposed by Stafford (1974) (Winkel-Shirley, 2001). These multi-enzyme complexes, or metabolons, are organized assemblies that catalyze sequential reactions in a metabolic pathway. They afford important advantages to the cell in terms of metabolic efficiency, providing the means to attain high local substrate concentrations, partition of common metabolites between branch pathways, coordinate the activities of pathways with shared enzymes or intermediates and sequester toxic intermediates (Winkel-Shirley, 1999). Nowadays, a model has been proposed, in which the phenylpropanoid and flavonoid pathways are organized as a linear array of enzymes loosely associated at the cytosolic face of the endoplasmic reticulum and anchored via the cytochrome P450-dependent mono-oxygenases, cinnamate-4-hydroxylase and F3′H (Hrazdina and Wagner, 1985). Co-immunoprecipitation, affinity chromatography and two-hybrid experiments indicate that there are direct associations between CHS, CHI, F3H and DFR in *Arabidopsis* (Burbulis and Winkel-Shirley, 1999; Winkel-Shirley, 2001; Burbulis and Winkel-Shirley, 1999). It has also been suggested that CHS and FLS play a key role in stabilizing a metabolic complex comprising CHS, CHI, F3H and FLS in tomato flesh tissue (Verhoeyen et al., 2002). Moreover, a mutation in the *Arabidopsis* F3′H gene resulted in an altered subcellular localization of CHS and CHI, again indicating that F3′H may function as part of a membrane anchor for other enzymes of the flavonoid pathway. Also findings from the early isoflavonoid pathway, in which a direct interaction between CHS and CHR is involved in pushing the metabolite flow into the iso-flavonoid branch, support the idea of

metabolic channelling (Winkel-Shirley, 1999; Dixon and Steele, 1999). So, in conclusion, it is possible that these metabolic systems, in which catalytic efficiency and control of end-product specificity can be enhanced by macromolecular complexes, could complicate metabolic engineering strategies by limiting the access of substrates to introduced enzymes.

In this paper we have described how different approaches have proven to be more or less powerful in directing the flavonoid biosynthesis in crop plants by genetic engineering. Results have illustrated how modern biotechnology can be used as an invaluable tool to gain insight into the regulation of the flavonoid pathway. In addition to traditional plant breeding, genetic engineering provides great opportunities to develop plants with the desired levels and/or composition of flavonoids.

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Ric de Vos is a specialist in plant physiology and biochemistry working on plant metabolites with potential health-protecting or toxic effects in human. He has 18 years of research experience in extraction, chromatographic separation and identification of plant metabolites, specifically antioxidants such as flavonoids. His current research is mainly focused on setting up and implementing a metabolomics platform at Plant Research International, using high resolution mass spectrometry. He is author of more than 30 peer reviewed papers and 3 filed patent applications.

Arjen J. van Tunen holds a PhD in plant genetics with a specialization in flavonoid biosynthesis. In June 2001, he was appointed Managing Director of the Swammerdam Institute for Life Sciences (SILS) which is a research Institute of the University of Amsterdam that employs around 250 researchers. At SILS, Arjen van Tunen holds the chair Plant Biochemistry and currently he is investigating the molecular control of plant secondary metabolite biosynthesis with a focus on volatiles. The SILS' mission is "Understanding the Fundamentals of Life at the Molecular and Cellular Level for Food and Health". Van Tunen's objective is to further develop SILS into a front runner institute in food sciences and life-science technologies including metabolomics. Arjen van Tunen is chairman of the start up company Renessense and published more than 55 peer reviewed articles and 8 patent applications.

Elio Schijlen obtained his Master of Science in molecular biology at the University of Utrecht. Several years of working experience in the field of metabolic engineering of secondary metabolites in diverse plant species preceded his PhD project at Plant Research International. Currently he is finishing his PhD thesis in plant genetics concerning modification of flavonoid biosynthesis in tomato.

Arnaud Bovy is a trained molecular geneticist with great experience in plant molecular biology and functional genomics (metabolomics and transcriptomics). Within PRI, he is coordinator of the theme "health-related plant metabolic pathways", which focusses on understanding the regulation, biosynthesis and mechanism of action of human-health-protecting plant compounds. Arnaud Bovy has more than eight years of experience in basic flavonoid research and his team has made a significant contribution to the understanding of the regulation and genetic modification of the flavonoid biosynthesis pathway in tomato fruit. His current research is focused on the genetic improvement of food quality traits in crop plants using functional genomics tools. He is author of 17 peer-reviewed scientific papers and 2 filed patent applications.