The Flavonoid Biosynthetic Pathway in Plants: Function and Evolution

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Summary

Flavonoids are a class of low molecular weight phenolic compounds that is widely distributed in the plant kingdom. They exhibit a diverse spectrum of biological functions and play an important role in the interaction between plants and their environment. Flavonoids not only protect the plant from the harmful effects of UV irradiation but also play a crucial role in the sexual reproduction process. A special class of flavonoid polymers, the tannins, plays a structural role in the plant. Yet other classes of flavonoids, flavonols and anthocyanins, have been implicated in the attraction of pollinators. Certain flavonoids participate in the interaction between plants and other organisms such as symbiotic bacteria and parasites. This raises the intriguing question as to how these different compounds arose and evolved. Based on taxonomy and molecular analysis of gene expression patterns it is possible to deduce a putative sequence of acquisition of the different branches of the biosynthetic pathway and their regulators.

Introduction

Plants are well-known producers of a large array of low molecular weight compounds. Most of these compounds were initially classified as secondary metabolites because they seemed to have no clear function for the organism. To man, however, these compounds have had a long history of use as, for instance, precursors of medicines, flavours, fragrances, dyes and various substances for the chemical industry.

Flavonoids are probably the most intensively studied secondary metabolites of plants. Although their widespread presence in the plant kingdom was known for many years, originally they were considered to be of little biological importance. Nevertheless, flavonoids have traditionally been favourite research objects for chemists, enzymologists and geneticists. For example, the laws of Mendel and the discovery of transposable elements by McClintock are based in part on the analyses of anthocyanin pigmentation mutants. Today, flavonoid biosynthesis is one of the best systems available for the study of regulation of plant gene expression. Although this research often addresses issues other than the biological functions of flavonoids, it has helped to make us aware of the wide variety of important biological functions that flavonoids exert in the plant.

Here we will review the current understanding of flavonoid functions and speculate on the evolution of these compounds in the past.

Flavonoid biosynthesis

Flavonoids are a class of secondary metabolites that is common to all higher plants. Over 3000 different flavonoids have been chemically characterised and new structures are still being reported. Flavonoids are phenolic compounds built up of two aromatic rings held together by a C3 unit. Based on the degree of oxidation of the C3 unit, flavonoids are divided into subclasses such as flavonols, flavanones, isoflavonoids and anthocyanins. Each type of flavonoid undergoes further modifications such as hydroxylation, methylation, acylation, glucosylation or rhamnosylation, resulting in the enormous diversity and colours of flavonoids found in nature.

Flavonoid synthesis starts with the condensation of 1 molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA yielding naringenin chalcone (Fig. 1). This reaction is carried out by the enzyme chalcone synthase (CHS). The two immediate precursors of the chalcone originate from two different pathways of primary metabolism. Coumaroyl-CoA is synthesised from the amino acid phenylalanine by three enzymatic steps, collectively called the general phenylpropanoid pathway, because the structures involved are phenylpropane-based and are common to the biosynthesis of a variety of compounds such as lignin, coumarins, stilbenes and flavonoids. Malonyl-CoA is synthesised by carboxylation of acetyl-CoA, a central intermediate in the Krebs tricarboxylic acid cycle. The chalcone is subsequently isomerised by the enzyme chalcone flavanone isomerase (CHI) to yield a flavanone. From these central intermediates the pathway diverges into several side branches, each yielding a different class of flavonoids, as depicted in Fig. 1. A comprehensive review of flavonoid biochemistry can be found in ref. 1.

Functions of flavonoids

Flavonoid pigments as a visual signal for animals

Insect-pollinated plants tend to have large, often brightly coloured petals, whereas wind-pollinated plants generally have small, dull coloured petals or no petals at all (e.g. petunia versus maize). Most of these flower pigments are flavonoids belonging to the class of red or purple coloured anthocyanins or the yellow coloured aurones and chalcones[2]. Besides anthocyanins, many plant species accumulate flavonols or flavanones in the petals. Although these compounds themselves are colourless, they alter the colour of the flower through the formation of complexes with anthocyanins and metal ions, a phenomenon termed co-pigmentation[3]. The strong blue flower colours, in particular, are the result of co-pigmentation.

In most species flower pigments act as a visual signal to attract pollinating animals (insects or birds), telling them that some reward is waiting, e.g. nectar. The spatial and temporal control of anthocyanin biosynthesis is consistent with a role as a visual signal: (i) anthocyanins accumulate mainly in the inner epidermis of the petal, (ii) the transcriptional activity of

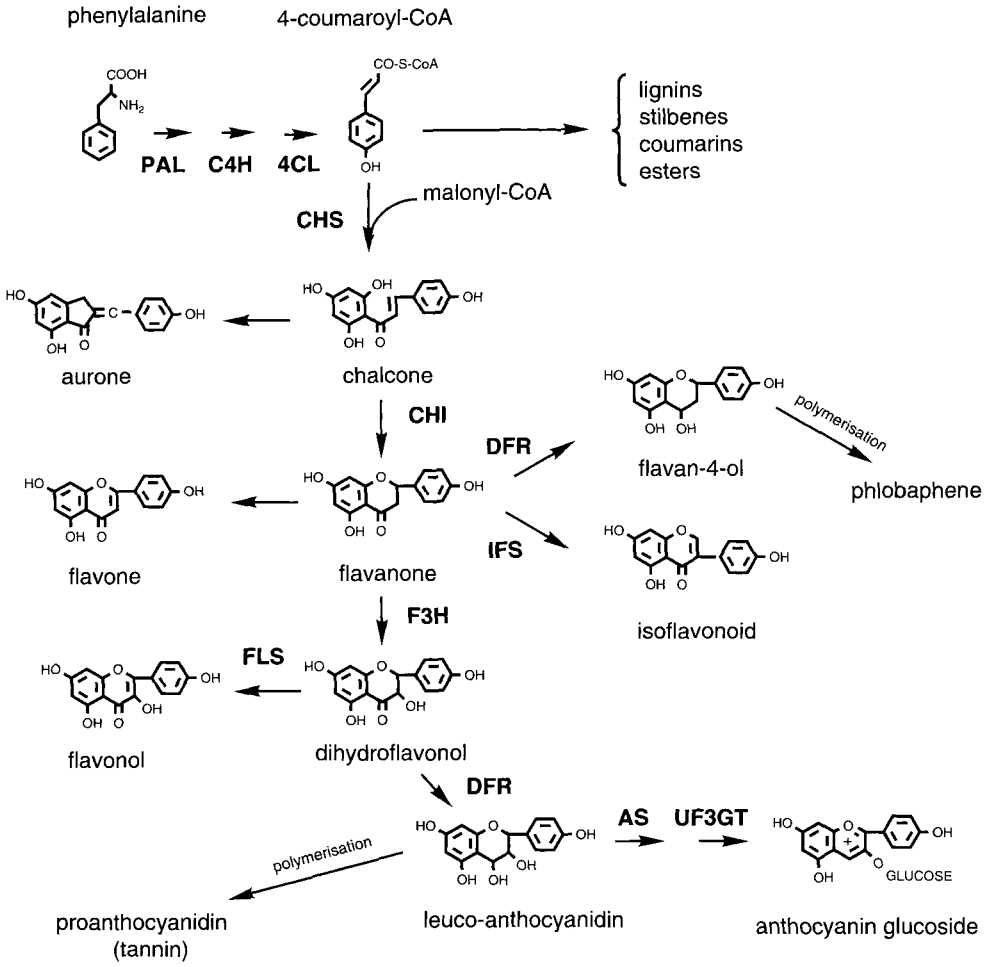

Fig. 1. Simplified diagram of the flavonoid biosynthetic pathway. The main types of flavonoids are represented and the enzymes catalysing some key reactions are indicated by the following abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3β-hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; IFS, isoflavonoid synthase; AS, anthocyanin synthase; UF3GT, UDP-glucose: flavonoid 3-O-glucosyltransferase.

the structural genes, and the rate of anthocyanin biosynthesis, reach a maximum just prior to opening of the flower bud[2,4-7]. Direct evidence for the role of coloured petals in attracting pollinators, comes from field experiments[8,9]. Usually, removal of the petals results in a vast decrease in the number of insect visits to the flower, although it generally does not completely eliminate them. In the field, even flowers from a naturally occurring homeotic mutant (*bicalyx*) of *Clarkia concinna*, in which the bright coloured petals are transformed into green sepal-like structures, are still cross-pollinated, presumably by visiting insects[10]. Clearly, there are additional factors (e.g. fragrance) involved in the attraction of pollinators.

Once pollination has occurred, it is disadvantageous to continue attracting pollinators, as this will lead them away from the other, yet unpollinated, flowers. In most plant species this is prevented by a quick senescence of the petal after pollination. In some plants a remarkable reversal of the signals between plant and animal has been established. *Fuchsia excurata*, a tree, bears flowers with green petals which are visited by birds. Once pollination has occurred, anthocyanin biosynthesis in the petal is quickly induced, and this is a signal to the pollinating birds not to visit the flower[11].

The specific colour shade and the pattern of pigmentation may specifically attract certain species of pollinators only[8,9] and may therefore be of importance for the process of species formation. For instance, the species *Petunia axillaris* and *Petunia integrifolia* are closely related and easily crossed upon manual pollination. *P. integrifolia* flowers are coloured and are pollinated by bees, whereas *P. axillaris* flowers are white, due to a mutation in a regulatory gene (*an2*, see below), and are pollinated by moths[12]. It is assumed that these moths, which visit the flower in the evening, are attracted by the presence of high amounts of (fluorescent) flavonols[12]. Thus, in principle, a single mutation in a regulatory flavonoid gene may contribute to reproductive isolation, ultimately resulting in the formation of separate species.

Wind-pollinated and self-fertile plant species, such as maize, accumulate anthocyanins in several parts of the plant, such as anthers, leaves and the stem. The function of this pigmentation is, however, unclear. The kernels and cob glumen of maize plants are also pigmented and contain besides anthocyanins another class of flavonoid pigments: phlobaphenes. Phlobaphenes are produced by a non-enzymatic polymerisation of flavan-4-ol and have some of the very first biosynthetic steps in common with anthocyanins (cf. Fig. 1), but their synthesis is independently regulated from that of anthocyanins (see below). The function of phlobaphene and anthocyanin pigments in the kernel and surrounding tissues might be to attract fruit-eating animals, thereby contributing to the dispersal of the seed.

Flavonoids protect the plant from UV damage

Plants expose themselves to sunlight in order to drive their photosynthesis. The ultraviolet (UV) component of sunlight, however, is a potential hazard because it can damage DNA and impair several physiological processes[13]. Like all other organisms, plants take counter-measures to protect themselves from UV damage. One of the most general responses of plant seedlings to UV light is the transcriptional activation of flavonoid biosynthetic genes[4,14,15]. Flavonoids are strongly UV-absorbing and accumulate mainly in epidermal cells after UV induction, suggesting that they function as a protective shield[14]. This may explain why UV-induced expression of flavonoid genes is generally transient even under continuous UV light conditions. Once sufficient flavonoids are accumulated most cells will be shielded and biosynthesis ceases. The importance of UV shielding to plant health has been demonstrated by physiological and genetic studies. For instance, rye seedlings grown under flavonoid-inducing conditions (long wave, UV light) are protected against the damaging effects of short wavelength UV light on the photosynthetic apparatus[16]. In the arabidopsis mutants for the genes *tt4* (encoding CHS) and *tt5* (encoding CHI), the synthesis of flavonol derivatives is blocked and consequently these mutants lack flavonols in all tissues. When placed under short wavelength UV light, growth of *tt4* and *tt5* mutants was found to be strongly retarded, directly demonstrating the role of flavonoids in the protection against UV light[17]. Besides flavonoids, other compounds contribute to UV protection of the plant. For instance the *tt5* mutant is more sensitive to UV light than is the *tt4* mutant; this may be related to the reduced levels of sinapic esters, which are derived from 4-coumaroyl-CoA[17]. In *sin1* mutants, synthesis of sinapic esters is blocked[18], resulting in lowered UV absorption and increased UV sensitivity[17,18].

Flavonoids play a role in plant sexual reproduction

Many plant species accumulate flavonoids in the anthers and the pistil, the male and female sex organs respectively. In anthers, the most commonly found flavonoids are anthocyanins, flavonols and chalcones. In young anthers, flavonoid biosynthetic genes and enzymes are active in the tapetum and the connectivum, which are nourishing tissues for the developing pollen grains[6,19-21]. During later stages of anther development, the tapetum and connectivum disintegrate and the cell contents are released into the pollen locule[6,19].

Analyses of spontaneous and engineered mutants showed that flavonoids play an essential role in pollen development. Maize plants with mutations for both *chs* genes, *C2* and *Whp*, produce unpigmented (white) pollen that is sterile in self pollinations[22]. The same phenotype was found in transgenic petunia plants in which *chs* gene expression was blocked via antisense RNA[21] or sense RNA[23,24]. In both cases the white pollen failed to produce a functional pollen tube in an *in vitro* pollen germination assay, as well on stigmas from the same mutant. On wild-type stigmas however, the white pollen was functional and extracts from wild-type stigmas could rescue *in vitro* pollen tube growth, indicating that the stigma contains a factor that can complement the mutation[24]. Using this bioassay the active compound(s) in the stigma were identified as flavonols[24]. The mechanism through which flavonoids promote pollen tube development is at present unknown.

In the pistil of petunia flowers, flavonoid biosynthetic genes like *chs* and *chi* are highly active in the ovary, resulting in the accumulation of flavonols[6,7]. Because the ovules are the primary site for *chs* and *chi* expression, it is tempting to speculate that the flavonols form a gradient along which the growing pollen tubes are guided to their target: the ovule. However, there is, as yet, no experimental evidence available to support this idea.

Flavonoids and symbiotic plant-microbe interactions

Bacteria from the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium,* (further referred to as rhizobia) have the unique capability to live in symbiosis with leguminous plants. These bacteria can infect the roots of a specific host plant and induce the formation of a highly specialised organ, the root nodule, which the bacteria inhabit to fix atmospheric nitrogen. Root nodule formation is highly host specific and each rhizobial species or strain can only infect a limited set of plant hosts. The formation of root nodules is a complex developmental process that requires the action of both bacterial and plant genes. A number of rhizobial nodulation genes required for the early steps of nodule formation (*nod* genes) have been defined by mutations, and several plant genes that are induced during nodule formation (*nodulin* genes) have been identified biochemically[25-27]. As could be expected, there is an extensive cross-talk between the plant and the bacterium during the formation of the root nodule in order to coordinate the genetic programmes on both sides.

In legumes, flavonoid biosynthetic genes are active in young root cap cells and in the zones where root hairs emerge[28]. This results in the synthesis of a mixture of flavonoids, the composition varying between different plant species[29]. By an as yet unknown mechanism the flavonoids are released into the soil. In the rhizobia that live freely in the soil, the *nodD* gene is constitutively expressed and the other *nod* genes are induced by the flavonoids released from the host plant[30-32]. Genetic evidence indicates that the NodD protein functions as the receptor for the flavonoid signal[25-27]. NodD proteins from different bacteria differ in their response to distinct flavonoids and generally respond optimally to the flavonoids excreted by their corresponding host plant[33,34]. Therefore, the spectrum of excreted flavonoids is a first, but certainly not the only, level at which host specificity is determined.

Remarkably, flavonoids can also act as an inhibitor of the *nod* gene expression[29,35,36]. For instance, the isoflavone daidzein induces *nod* gene expression in *Bradyrhizobium japonicum*[37] (nodulates on soybean), but is an inhibitor in *Rhizobium trifolii*[36] (nodulates clovers) and *Rhizobium leguminosarum*[35] (nodulates peas). Possibly, these inhibitors further contribute to the host specificity.

The NodD protein shares sequence homology with the *lysR* type of transcription activators and it can bind to the so-called nod-box, a conserved *cis*-element in the promoters of

the inducible *nod* genes[26]. So far, attempts to show direct binding of flavonoids to the NodD protein have failed. Thus, the precise mechanism by which flavonoids induce *nod* gene transcription remains unclear[26].

Several of the induced *nod* gene products are involved in the synthesis of another signal molecule, the Nod factor. Recently Nod factors from different rhizobia were shown to be substituted (lipo-)oligosaccharides[38,39]. This bacterial signal triggers a number of responses in the plant root which are required for nodulation, such as root hair deformation and initiation of cortical cell divisions[39,40]. Furthermore, newly synthesised flavonoids appear in the plant root as a result of an increase in the levels of flavonoid biosynthetic enzymes and their mRNAs[41-43]. The cells expressing *chs* genes are quite distant from the infecting bacteria. This suggests that some diffusible factor, presumably the Nod factor, is responsible for the induction of flavonoid gene expression[28]. What can be the function of this second burst of flavonoids? It may be that they act as an inducer to maintain bacterial *nod* gene expression in the infection thread. However, they may also be the trigger that induces the subsequent cell divisions in the nodule primordium[28]. Flavonoids have the capacity to inhibit transport of auxin phytohormones[44] and therefore may decrease auxin import into the nodule, resulting in a lowered auxin/cytokinin balance. Artificially applied auxin transport inhibitors and cytokinins can both mimic nodule morphogenesis and induce nodulin gene expression[45,46].

Because flavonoid biosynthetic enzymes in legumes are generally encoded by multigene families, the isolation of flavonoid-deficient mutants is virtually impossible. As summarised by Stacey et al.[47], several laboratories have now constructed flavonoid-deficient legumes via antisense *chs*-constructs, which may be helpful in further delineating the role of flavonoids in root-nodule formation.

Flavonoids and plant-pathogenic microbe interactions

In nature plants are exposed to a large range of potentially pathogenic fungi and bacteria. Microbial plant pathogens do not generally succeed in infecting a plant, usually because the plant is not a host species (non-host resistance) and sometimes because it is a resistant variety of the host species (incompatible interaction). Only interactions between a pathogen and susceptible varieties of the host plant cause disease (compatible interaction). In the majority of cases, plant resistance is correlated with a hypersensitive response, which is characterised by rapid, localised defence responses and death of plant cells surrounding the infection site. These defence responses include: (i) reinforcement of cell walls by increased callose and lignin deposition and the accumulation of hydroxyproline-rich glycoproteins, (ii) induction of lytic enzymes (chitinases, glucanases) and proteinase inhibitors and (iii) synthesis of phytoalexins. The latter have been defined as low molecular weight, anti-microbial compounds that are synthesised by the plant after exposure to microbes[48,49]. Phytoalexins comprise a chemically heterogenous group of compounds which includes terpenoids, stilbenes, polyacetylenes and flavonoids[48,49]. In general, only one or two types of phytoalexin are found within a certain plant family. For instance, in *Leguminoseae* the phytoalexins are isoflavonoids, whereas in *Solaneceae* mainly sesquiterpene phytoalexins are found and no isoflavonoids.

The isoflavonoids of legumes are by far the best studied phytoalexins. However, most research has focussed on the signalling mechanisms that induce isoflavonoid biosynthesis and relatively little attention has been paid to their physiological role. In many plant pathogen systems, the local activation of flavonoid biosynthetic genes and accumulation of isoflavonoids closely correlates with the resistance response, suggesting that isoflavonoids are indeed important for resistance[48,50]. Direct evidence for the role of isoflavonoids in resistance is however scarce. For instance, treatment of soybean seedlings with an inhibitor of phenylalanine ammonia lyase (PAL) causes a strong reduction in isoflavonoid accumulation and loss of the resistance against *Phytophtera megasperma*[51]. However, PAL activity is also required for lignin biosynthesis, hence, lignin biosynthesis was presumably also inhibited in this experiment (*cf.* Fig. 1). In its interaction with pea, the pathogen *Nectria haematococcus* is able to detoxify isoflavonoid phytoalexins via demethylation and loss of this capability results in loss of virulence[52].

The construction of transgenic leguminous plants in which isoflavonoid biosynthesis is blocked via antisense *chs* transgenes may be extremely useful in definitely establishing the role of isoflavonoids in disease resistance[47].

The infection and subsequent transformation of plants by *Agrobacterium tumefaciens* is mediated by host and bacterial genes. In the infection process, part of the bacterial Ti plasmid, the T-DNA, is transferred to the host cell and stably integrated in its genome. The bacterial *vir* genes, responsible for virulence, are induced by a wide range of plant phenolics. The main inducer produced by tobacco cells has been identified as acetosyringone. For a recent review on the plant-*Agrobacterium* interaction, see ref. 27. Flavonols and flavanones are also capable of inducing *vir* gene expression, but 50-100 times higher concentrations are required than for acetosyringone[53]. Therefore, it remains an open question whether the flavonoid compounds are really used in natural habitats.

Flavonoids and parasitic plants

Several plant species are capable of growing as parasites on other plants. Most of these species can also grow and reproduce in the absence of a host (hemiparasites), whereas a few species absolutely require a suitable host plant (obligatory parasites). In *Striga* species, for example, an (obligatory) parasite of grasses, seed germination and subsequent development of the haustorium, a host attachment organ, are dependent on factors released from the host plant[54]. Generally these host-derived factors are phenylpropanoid-derived compounds, such as stilbenes, flavonoids and *p*-hydroxy-acids. However none of these compounds is active as such, but rather their quinone oxidation products, which are synthesised via oxidative enzymes present in both the parasite and the host surface tissue[54].

Flavonoid polymers contribute to the structure of the plant

Most vascular plants accumulate oligomers of leucoantho-

cyanidins called proanthocyanidins or tannins (*cf.* Fig. 1). These compounds are present in large amounts in seed coats of many plant species and in the heartwood and bark of trees, although they can also be found in leaves and roots. There is some limited evidence that proanthocyanidins are important for the reinforcement of plant tissues. For instance, mutant seeds of snapbean which lack proanthocyanidins, are more sensitive to mechanical and water stress than wild-type seeds[55].

A wealth of reports showed that, in solution, proanthocyanidins can precipitate proteins, polysaccharides and RNA or, when applied to seeds, inhibit germination. It is however difficult to evaluate on the basis of such experiments whether these characteristics are of any biological significance. Thus, of all flavonoids, proanthocyanidins remains one of the most mysterious classes.

Different classes of flavonoids appeared sequentially during evolution

The flavonoid pathway appeared in discrete steps during plant evolution

The distribution of different classes of flavonoids throughout the plant kingdom suggests that they have appeared sequentially during evolution[56,57]. This is schematically depicted in Fig. 2. The chalcones, flavanones and flavonols appeared with the ancestors of a class of *Bryophyte* (musci). These compounds are nowadays widely spread in the plant kingdom. With the first vascular plants (ferns) proanthocyanidins appeared, while anthocyanidins did not appear before the emergence of flowering plants (angiosperms). It is therefore tempting to speculate that the different genes and enzymes involved also appeared sequentially (Fig. 2). Most probably, the structural genes encoding the enzymes from secondary metabolism have been derived from genes encoding enzymes of primary metabolism. Many structural genes from the flavonoid pathway have been sequenced and, indeed, their gene products show homologies with enzymes from primary metabolism. For instance, the condensation of *p*-coumaroyl CoA with malonyl-CoA, which is catalysed by CHS, is highly similar to condensation reactions in fatty acid biosynthesis, and based on the sequence homologies between the proteins, the condensing enzymes of fatty acid biosynthetis and CHS might originate from a common ancestor[58]. C4H, flavonoid 3′ hydroxylase and the flavonoid 3′5′ hydroxylase P450 type enzymes are localised in the endoplasmic reticulum, and presumably originate from P450 hydroxylases of primary metabolism[57]. DFR, the first enzyme specific for anthocyanin biosynthesis, has some remarkable homologies with enzymes in steroid metabolism and both are thought to have derived from a common ancestor[59].

If the different flavonoids and their biosynthetic enzymes appeared at different time points during evolution, the same must hold for their functions. Their function as a UV protectant (flavonols and flavanones) is widespread and probably one of the first to have been established. Stafford[57] argued, however, that the first flavonoid biosynthetic enzymes were rather inefficient and could not synthesize sufficient

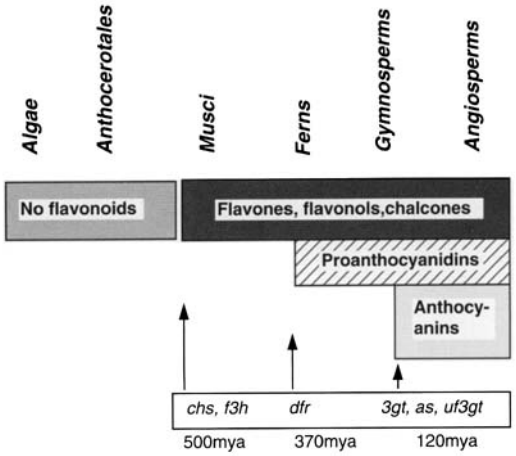

Fig. 2. Appearance of different classes of flavonoids during plant evolution. The enzymes involved in the synthesis of each group of compounds and an estimation of the moment of appearance of such enzymes (mya, millions of years ago) are also indicated[56,57].

flavonoids for effective UV protection. To function as a signal molecule or regulator of hormone action (as in nodulation and pollen development), much lower quantities are required and therefore these may have been the earliest functions of flavonoids[57]. In contrast, pigments involved in the attraction of pollinators (anthocyanin) appeared relatively late, simultaneously with the appearance of flowers as reproductive organs[57]. The acquisition of a function in the interaction with microbes (rhizobia or pathogens) may even be more recent, as this is found nowadays in a single family of plants only (*Leguminoseae*).

Development of regulatory networks controlling flavonoid biosynthesis during evolution

Obviously, the appearance of new biological functions for flavonoids required the establishment of appropriate flavonoid accumulation patterns. For instance, pollinator attractants need to be synthesised in the flower, UV protectants in leaves in response to UV light, and phytoalexins at infection sites in response to a pathogen. The control of flavonoid biosynthesis has been extensively studied in many different plants and plant systems. The results show that flavonoid biosynthesis is controlled primarily at the level of transcription of the structural genes[2,50,60]. Thus, the acquisition of flavonoid functions presumably depended initially on the development of suitable transcriptional control mechanisms for the newly appearing structural genes. Furthermore, the expression of pre-existing genes (e.g. *chs*) needed to be modified accordingly, because their expression is required for the biosynthesis of all flavonoids.

Transcriptional control of structural flavonoid genes has been most intensively studied in relation to the biosynthesis of anthocyanins. Regulatory genes that control transcription of the structural pigmentation genes have been identified in several plant species: maize, snapdragon, petunia and arabidopsis.

In maize, the *C1* and *R* families of transcription activator genes control expression of all the structural genes that are

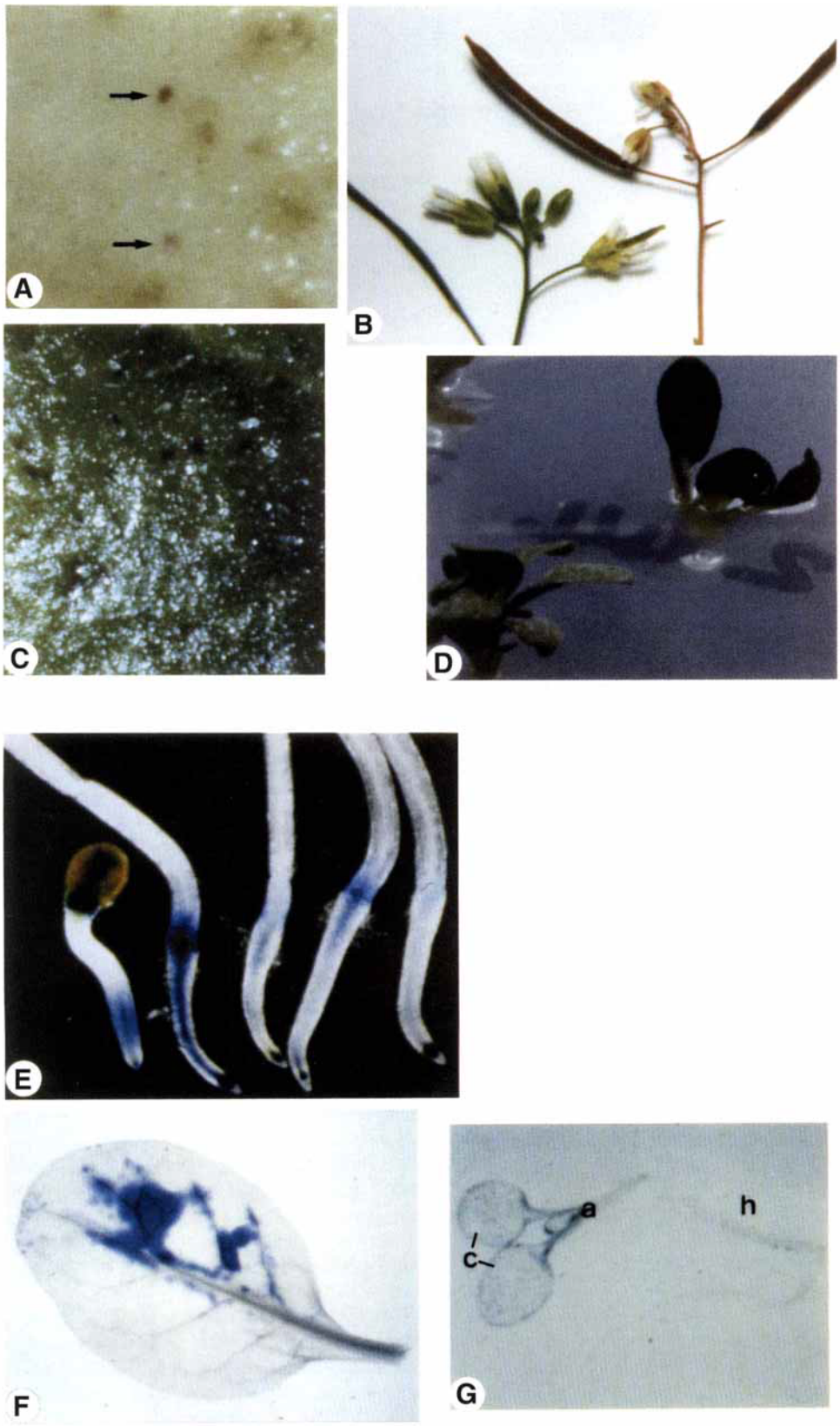

Fig. 3. Exchange of structural and regulatory pigmentation genes between different plant species. (A) Induction of pigmentation (red spots indicated by the arrows) in an *r* maize kernel after delivery via particle bombardment of an *Lc* gene driven by the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter (see also refs 62 and 63). (B) Induction of pigmentation in transgenic arabidopsis plants by expression of *Lc* and *C1* from maize (right) compared with a control untransformed plant (left) (photograph kindly provided by A. Lloyd), (see also ref. 68). (C) Induction of pigmentation in petunia leaves after codelivery via particle gun transformation of an *Lc* and a *C1* gene, both driven by the *CaMV 35S* promoter. (D) Pigmentation in a petunia shoot regenerating from a leaf disk transformed with a *CaMV35S-Lc* gene (top right) in comparison with an untransformed control (bottom left). (E) Expression of a *gus* reporter gene (blue color) driven by a bean *chs8* promoter in roots of transgenic tobacco seedlings (photograph kindly provided by C. Lamb) (see also ref. 75). (F) Fungal elicitor-induced expression of a *gus* gene driven by the *chs8* promoter in leaf tissue from a transgenic tobacco plant (photograph kindly provided by C. Lamb), (see also ref. 75). (G) Expression of a *gus* gene driven by the petunia *chsA* promoter in a transgenic petunia seedling. Note the absence of *chs-gus* expression in the root. a, apical meristem; c, cotyledon; h, hypocotyl.

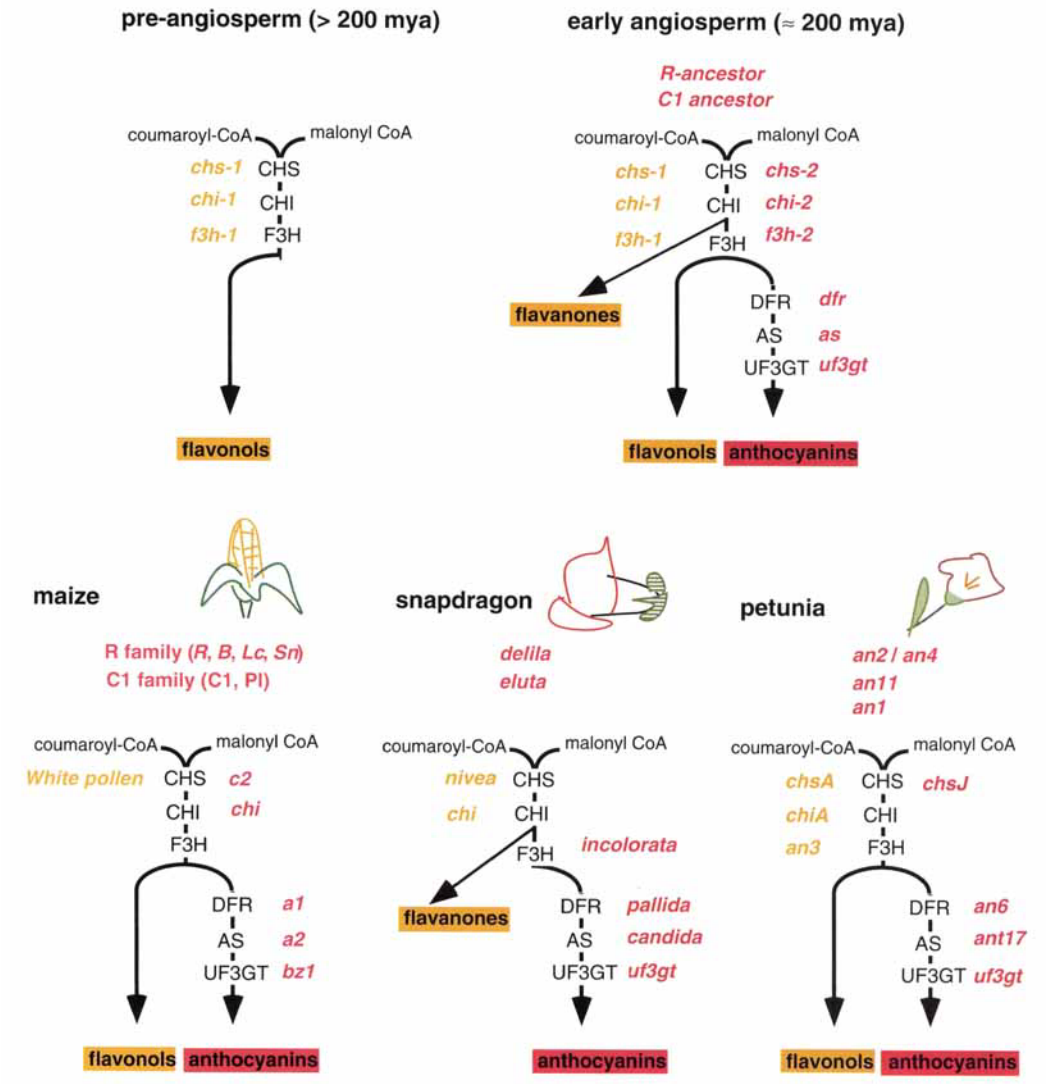

Fig. 4. A model for the evolution of the flavonoid biosynthetic pathway and its regulators. The names of the relevant enzymes are abbreviated as in Fig. 1 and are depicted in black capitals. The names of the genes that encode these enzymes in maize, snapdragon and petunia are shown in red on the right side of the pathway if they are controlled by the regulatory genes shown above the pathway and otherwise in yellow on the left side of the pathway. The organisation of the pathway in early and pre-angiosperms is hypothetical (see text).

required for anthocyanin biosynthesis in the different parts of the plant (*chs*, *chi*, *dfr*, *uf3gt*)[60]. R belongs to the class of helix-loop-helix type transcription factors and C1 bears resemblance to Myb proto-oncogene type factors. Each family of regulators comprises multiple homologous genes that control pigmentation in a specific tissue or organ. For instance, pigmentation of the kernel is controlled by the gene *R*, whereas pigmentation of leaf is controlled by the homologues *Lc* or *B*[60,61]. When *Lc* or *B* gene constructs driven by a constitutive promoter are introduced into unpigmented *r* kernels, anthocyanin biosynthesis is restored[62,63] (see also Fig. 3A). This shows that the regulatory proteins are functionally similar and that the pigmentation patterns are determined by the tissue-specific expression of the regulatory genes[61].

Regulatory genes controlling anthocyanin biosynthesis have also been identified in snapdragon (the genes *delila* and *eluta*)[64,65], petunia (the genes *an1*, *an2*, *an4* and *an11*)[5,66] and arabidopsis (the gene *ttg*) (B. Shirley, W. L. Kubasek, G. Storz, F. M. Ausubel and H. Goodman, personal communication). Cloning and sequencing of *delila* from snapdragon showed that this gene is highly homologous to genes from the R family of maize[67]. Furthermore, functional assays showed that *Lc* can substitute for *ttg* and *an2* in arabidopsis and petunia respectively and that the combination of *C1* and *Lc* can induce expression of structural pigmentation genes in tissues where they are normally silent[66,68] (see Fig. 3B, C and D). This type of swapping experiment has indicated that the regulatory proteins controlling pigmentation in different species are highly similar. These regulators have in common that they control expression of the structural genes acting late in the pathway (from *dfr* on). They differ, however, in their control of the early genes of the pathway. The lower part of Fig. 4 illustrates this. In most maize tissues, the entire pathway from CHS to UF3GT is regulated by *R* and *C*, but in seedlings *chs* expression can occur independently of R[60,69]. In snapdragon the pathway is regulated except for the first two steps, whereas in petunia the regulated part starts with DFR[2,66].

In petunia, the introduction of the maize regulators *Lc* and

C1 only induces the expression of the *an2*-controlled genes *dfr* and *chsJ*, not that of an independently controlled gene (*chsA*)[66]. Taken together, these findings indicate that (i) the regulatory pigmentation genes in different species are functional homologues and presumably derived from common ancestors[67] and (ii) the structural genes differ between species, in that they contain *cis*-elements connecting them to distinct regulatory networks.

In Fig. 4 we present a model showing how the different regulatory mechanisms may have developed from a common origin[66]. In pre-angiosperm plants *chs* expression is needed for the synthesis of chalcones, flavanones and flavonols and is under the control of a yet unknown set of regulatory genes. The structural genes for proanthocyanidin and anthocyanin synthesis (*dfr* and others) should have been linked to another set of regulatory genes, the ancestors of todays *Lc* and *C1*, in order to ensure their co-ordinated expression in the newly evolved flower. In this scenario the early and late genes are still unco-ordinated. Therefore, expression of the early genes (*chs*, *chi* and *f3h*) had to be linked to the same ancestral C1 and R regulatory genes, thereby allowing flavanones, flavonols, proanthocyanidins and anthocyanins to be synthesised independently. This may have been achieved by addition of appropriate modules in the promoters, giving them the desired 'broad' specificity. Alternatively, (some) structural genes may have been duplicated, followed by coupling of of one of the copies to the regulatory anthocyanin genes. This results in multiple 'specialised' structural genes (Fig. 4). During later stages of evolution, either specific *cis*-elements may have been lost from the single multi-purpose gene copy, or single 'specialised' gene copies may have been lost or inactivated (Fig. 4). The pigmentation patterns in distinct (groups of) plants are thought to have been further modified via alterations in the expression patterns of the regulatory genes themselves[67,70].

The biosynthesis of phlobaphenes in the pericarp of maize kernels also seems to have been established via modification of *cis*-elements in the structural genes. Here, the product of the regulatory gene *P* controls expression of *chs* (*c2*)and *dfr* (*a1*)genes[71] by binding to their promoters, whereas the *uf3gt* gene (*bz1*), which is not required for phlobaphene synthesis, lacks a P binding site (E. Grotewold, personal communication).

The acquisition of a role for flavonoids in the interaction of microbes in ancestral leguminous plants, required the establishment of root-specific (for the synthesis of nodulation signals) and pathogen-inducible expression of structural flavonoid genes, features that are nowadays specific for leguminous plants. Several findings suggest that this has again been achieved via a scenario of gene duplications and specific addition of *cis*-elements responding to conserved regulators. For instance, soybean, a legume, harbours 7-8 *chs* genes which are differentially expressed during root nodule formation and the defence response[72]. Pea, another legume, contains three differentially expressed *chs* genes: two are involved in anthocyanin biosynthesis in the flower and are controlled by the regulatory genes *A* and *A2* (*Lc* or *C1* homologs ?), whereas the third *chs* gene is specifically expressed in the root, independently from *A* and *A2*[73]. Furthermore, within a single soybean *chs* promoter, distinct *cis*-elements responding to UV light and pathogens can be discerned[74]. The observation that a leguminous *chs* gene construct retains its typical expression pattern (root expression and elicitor inducibility; Fig. 3E and F) even in a non-leguminous host, suggests that root-specific and pathogen-inducible *chs* expression specific for legumines was established by linking leguminous *chs* genes to universal and well conserved regulatory genes and signal transduction pathways[74,75].

Conclusions and perspectives

Flavonoids are a diverse group of metabolites that is widespread in the plant kingdom. In angiosperm plants they play an essential role in developmental processes and in communication with the outside world. For pre-angiosperm plants information on flavonoid function is lacking. However, one could speculate that the early flavonoids mainly served a role as signal molecules and that during later stages of evolution additional functions were acquired. This demands strict temporal and spatial control of biosynthetic genes specific for each type of flavonoid compound.

The distribution of flavonoids throughout the plant kingdom suggests that they appeared in distinct phases of plant evolution (Fig. 2). Since the most primitive plant species contain chalcones, flavanones and flavonols, the genes *chs, chi* and *f3h* must be older than anthocyanin genes such as *dfr, as* and *rt,* which emerged together with the angiosperms. An intriguing issue is the coordination between early and late genes during evolution. Indications are presented that this may have occurred either by addition of new modules to ancient genes, giving them a broader specificity, or by gene duplication and coupling of one set of genes to newly acquired regulators. Recent studies indicate that these regulators are highly conserved and interchangeable between plant species and that their expression patterns determine the mode of pigmentation in diverse plant species. Thus, plants with novel coloration patterns may be obtained by simultaneous introduction of *C1* and *R*-type genes under the control of cell type-specific promoters. What regulates the regulator genes is unknown at present. Possible candidates are homeotic genes which determine the identity of tissues and organs.

Virtually nothing is known about the regulation of *chs, chi* and *f3h* genes in primitive ferns and mosses. If the evolutionary models presented in this paper are correct, one should expect to see no activation of these genes by the present day regulators *R* and *C1*.

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