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Flavonoids and isoflavonoids – a gold mine for metabolic engineering

Richard A. Dixon and Christopher L. Steele

Flavonoid-derived plant natural products have long been known to function as floral pigments for the attraction of insect pollinators, as signal molecules for beneficial microorganisms in the rhizosphere, and as antimicrobial defense compounds. New functions for flavonoid compounds continue to be found, particularly in plant–microorganism signaling, and there has been an explosion of interest in flavonoids and isoflavonoids as health-promoting components of the human diet. The flavonoid and isoflavonoid pathways are probably the best characterized natural product pathway in plants, and are therefore excellent targets for metabolic engineering. Manipulation of flavonoid biosynthesis can be approached via several strategies, including sense or antisense manipulation of pathway genes, modification of the expression of regulatory genes, or generation of novel enzymatic specificities by rational approaches based on emerging protein structure data. In addition, activation tagging provides a novel approach for the discovery of uncharacterized structural and regulatory genes of flavonoid biosynthesis.

Flavonoids are a diverse group of plant natural products synthesized from phenylpropanoid and acetate-derived precursors, which play important roles in growth and development, and in defense against microorganisms and pests. These compounds often possess antioxidant activity, and the potential health benefits of fruit, vegetables, green tea and red wine might partly be because of this property of flavonoids and other phytochemicals[1–3]. In addition, the isoflavonoids, which are limited primarily to the Leguminosae, exhibit estrogenic and anti-cancer activity[4,5], and, in common with the flavonoids, are also receiving considerable attention as health-promoting 'nutraceuticals'.

Although the genetic manipulation of plants to improve the composition of health-promoting phytochemicals has been proposed, a major limitation has been the lack of knowledge regarding the complete biosynthetic pathways needed for the synthesis of most biologically active plant natural products. However, because of recent advances, metabolic engineering strategies for quantitatively and qualitatively modifying the plant's ability to synthesize flavonoid and isoflavonoid bioactive natural products for human, animal and plant health can now be considered.

Biological activities of flavonoids that impact on plant and animal health

Because of their *in vitro* antimicrobial activity, specific classes of flavonoid and isoflavonoid compounds have long been thought to play a role in plant–microorganism interactions as part of the host plant's defensive arsenal[5,6]. But, in spite of an extensive literature, direct proof of the 'phytoalexin hypothesis' remains to be found. This could now be obtained by pathway engineering approaches. Genetic manipulation of flavonoid biosynthesis will permit a better appreciation of the roles of flavonoids as inducers of the nodulation genes of symbiotic *Rhizobium* species[7] in natural rhizospheres. It will also allow an assessment to be made concerning the importance of the suppression of host defense responses[8] for the successful establishment of both rhizobial and mycorrhizal symbioses.

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Recent studies have also revealed new roles for isoflavonoids as signal molecules in plant–microorganism interactions. For example, the isoflavonoid pterocarpans, maackiain and pisatin (Fig. 1), act as classical phytoalexins in the interaction between garden pea (*Pisum sativum*) and the fungal pathogen *Nectria haematococca*, and maackiain and pisatin detoxifying enzymes are fungal virulence factors[9,10]. Pisatin itself induces a protein that appears to control the transcription of a fungal pisatin detoxification enzyme[11]. In addition to acting as a cue for phytoalexin detoxification, pisatin, and other flavonoid compounds, might act as stimulants for the germination of spores of *N. haematococca*[12]. Furthermore, the soybean isoflavones, daidzein and genistein (Fig. 1), stimulate chemotropic behavior in hyphal germlings of the oomycete pathogen *Phytophthora sojae*, suggesting that they might help the hyphal tips of zoospores encysted adjacent to roots to locate their host[13]. Because isoflavonoids act as multiple cues for both beneficial and pathogenic microorganisms, metabolic engineering of this pathway might have complex ecological repercussions.

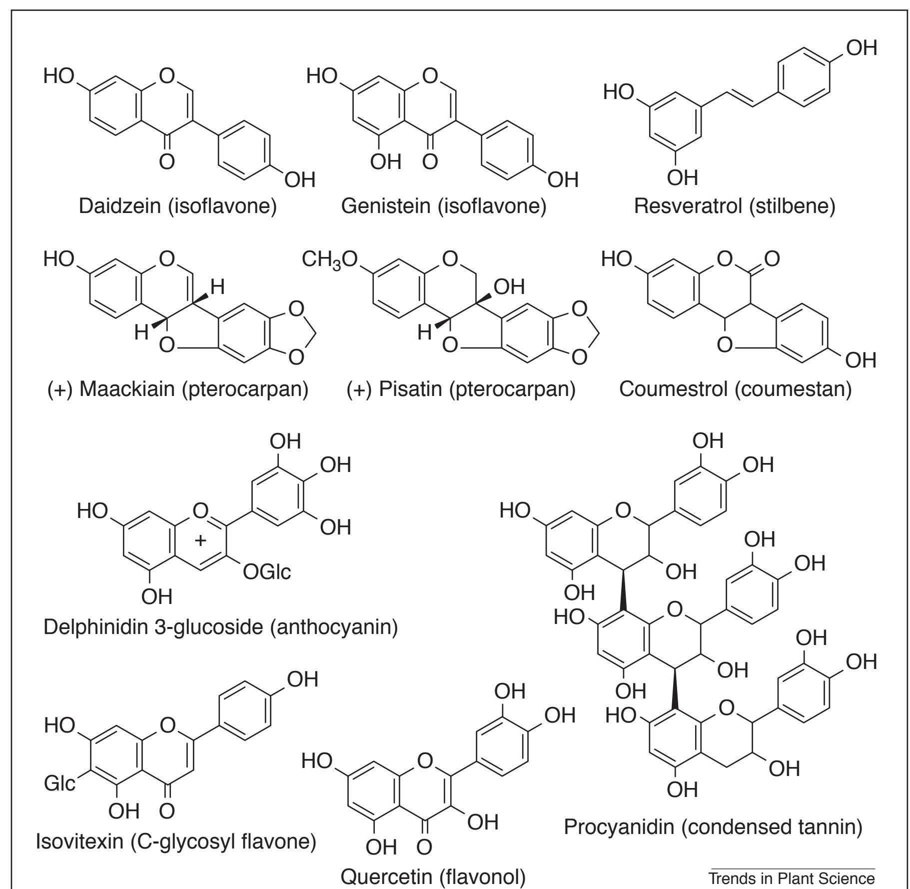

Fig. 1. Chemical structures of selected biologically active flavonoid-derived compounds. The stilbene resveratrol is not strictly a flavonoid, but is synthesized from the same substrates as flavonoids via a chalcone-synthase-type condensation reaction that includes decarboxylation before cyclization.

In addition to playing multiple roles in the interactions between plants and microorganisms, flavonoid and isoflavonoid compounds exhibit a range of mammalian health-promoting activities that are currently the focus of intense study[5,14] (Table 1). Many flavonoids, such as quercetin (Fig. 1) and epicatechin gallate, have high levels of antioxidant activity that might protect against cardiovascular disease and certain cancers[15]. Dietary genistein reduces susceptibility to mammary cancer in rats[16] and helps to prevent bone loss caused by estrogen deficiency in female mice[17]. These effects are mirrored by epidemiological evidence that implicates dietary isoflavones as major factors associated with reduced cancer risk in populations consuming a high soy diet[14]. There is therefore considerable interest in the potential for metabolic engineering to introduce isoflavonoids, the major dietary sources of which are soybean and chickpea, into other food crops.

Recent advances in the biochemistry of the flavonoid and isoflavonoid pathways

The pathways leading to the major classes of flavonoid-derived natural products are summarized in Figure 2. All flavonoids are derived from a chalcone precursor, the product of the condensation of 4-coumaroyl CoA (a product of the central phenylpropanoid pathway) and three molecules of malonyl CoA (formed from acetate via a cytoplasmic form of acetyl CoA carboxylase) by the enzyme chalcone synthase (CHS). It has recently become apparent that CHS is just one member of a family of plant polyketide synthases that together form a variety of natural products. This is achieved through variations in the number of rounds of condensation, differences in starter molecule specificity, and differences in the folding pattern of the intermediate polyketide[18] (Fig. 3). Enzymes previously assumed to be CHS on the basis of sequence identity alone should therefore be re-evaluated by functional expression analysis. For example, in *Gerbera hybrida*, pyrone synthase (previously known as GCHS2) uses acetyl CoA as

Table 1. Selected animal health-promoting effects of isoflavonoid compounds[a]

Compound	Effect	Species
Genistein	Prevention of mammary cancer	Rat
Genistein	Inhibition of prostate tumor growth	Human (cell lines)
Genistein; biochanin A	Inhibition of stomach tumor growth	Human (cell lines)
Genistein, daidzein	Activation of natural killer cells	Human
Genistein	Reduction of serum triglycerides and cholesterol	Rat
Genistein, daidzein	Protection against low density lipoprotein oxidation	Human
Genistein	Prevention of bone loss	Mouse
Genistein	Anti-angiogenesis	Human (cell lines)
Daidzin	Suppression of alcohol consumption	Hamster

[a]See Refs 4,5,14,16,17 for further details on the effects of isoflavonoids, and Refs 1–3,15 for details of the health-promoting effects of flavonoids.

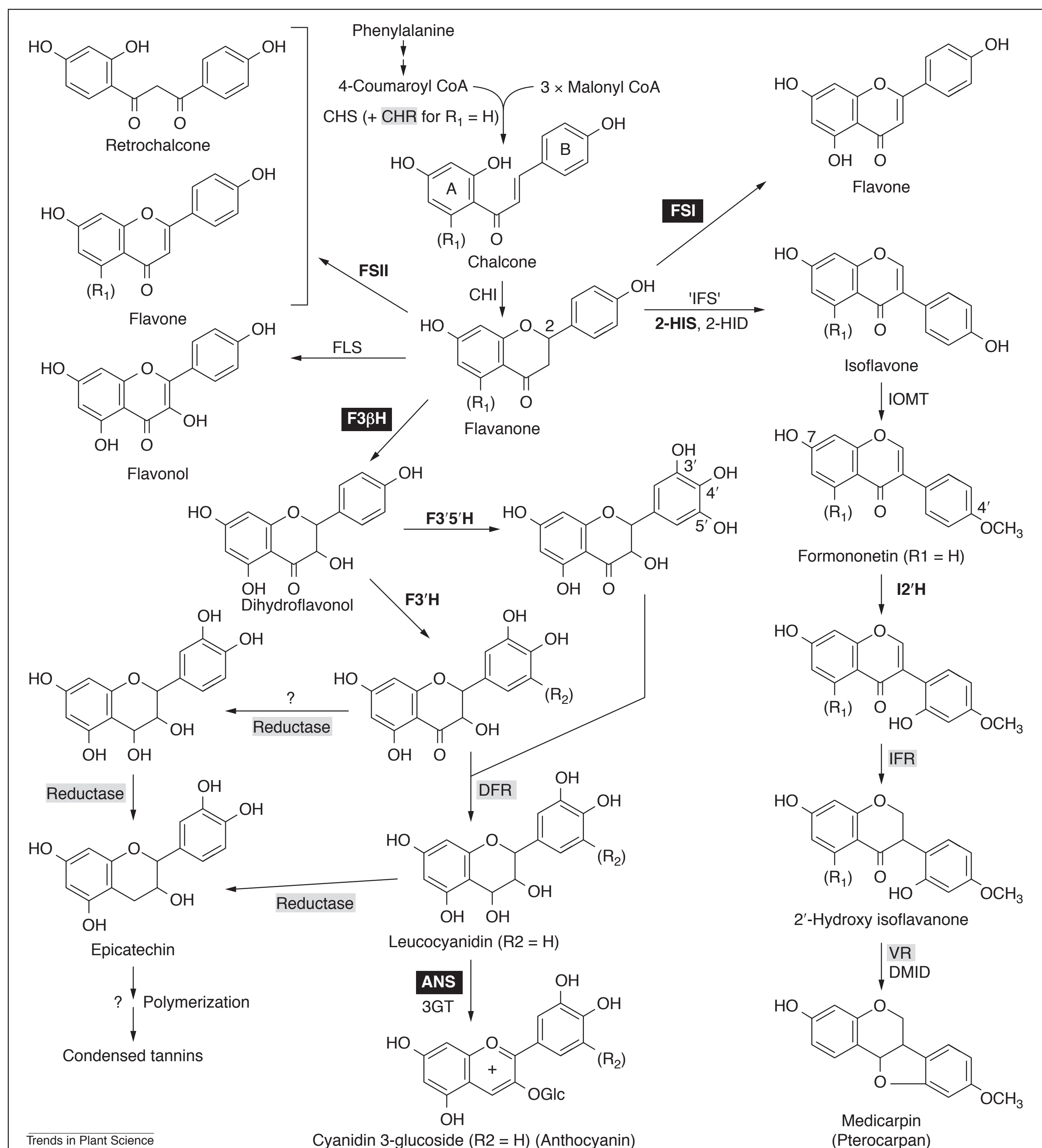

Fig. 2. The biosynthesis of the major classes of flavonoid derivatives. The enzymes are: CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; FSI, flavone synthase I; FSII, flavone synthase II; FLS, flavonol synthase; 'IFS', isoflavone synthase, consisting of 2-hydroxyisoflavanone synthase (2-HIS) and 2-hydroxyisoflavanone dehydratase (2-HID); F3βH, flavanone 3β hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3GT, anthocyanidin 3-glucosyltransferase; IOMT, isoflavone *O*-methyltransferase; IFR, isoflavone reductase; VR, vestitone reductase; DMID, 7,2′-dihydroxy, 4′-methoxyisoflavanol dehydratase. Enzymes in white are 2-oxoglutarate-dependent dioxygenases, in black bold are cytochrome P450s, and highlighted in gray are NADPH-dependent reductases. Simplifications include not discriminating between the 5-hydroxy (R_1 = OH) and 5-deoxy (R_1 = H) flavonoids and isoflavonoids, for which the loss of the 5-hydroxyl occurs because of the co-action of CHR with CHS, and showing only the anthocyanin pathway leading to the compounds with a di-substituted B-ring (cyanidin derivatives). Parallel pathways function in the formation of anthocyanins with mono- and tri-substituted B-rings. In the latter, F3′5′H can act at the level of the dihydroflavonol with a mono- or di-substituted B-ring. The pathway to epicatechin from a dihydroflavonol is shown to follow two routes, both via leucocyanidin. It is unclear whether there is a specific form of DFR that functions only in condensed tannin biosynthesis. The 4′-*O*-methylation of the B-ring of isoflavones occurs in alfalfa, pea and other legumes, but not in bean or soybean.

starter molecule for two rounds of condensation with malonyl CoA to produce 6-methyl-4-hydroxy-2-pyrone (Ref. 19), and acridone synthase shares the same condensation mechanism as CHS but uses *N*-methylanthranilyl CoA as starter molecule[20] (Fig. 3). Stilbene synthases (STSs) from a variety of sources have exactly the same substrate specificity as CHS, but the intermediate polyketide is folded differently, resulting in decarboxylation to yield the stilbene[18] (Fig. 3). The primary amino acid sequences of chalcone and stilbene synthases are sufficiently similar to make it impossible to predict from sequence analysis alone whether an enzyme is a stilbene synthase or a chalcone synthase[18].

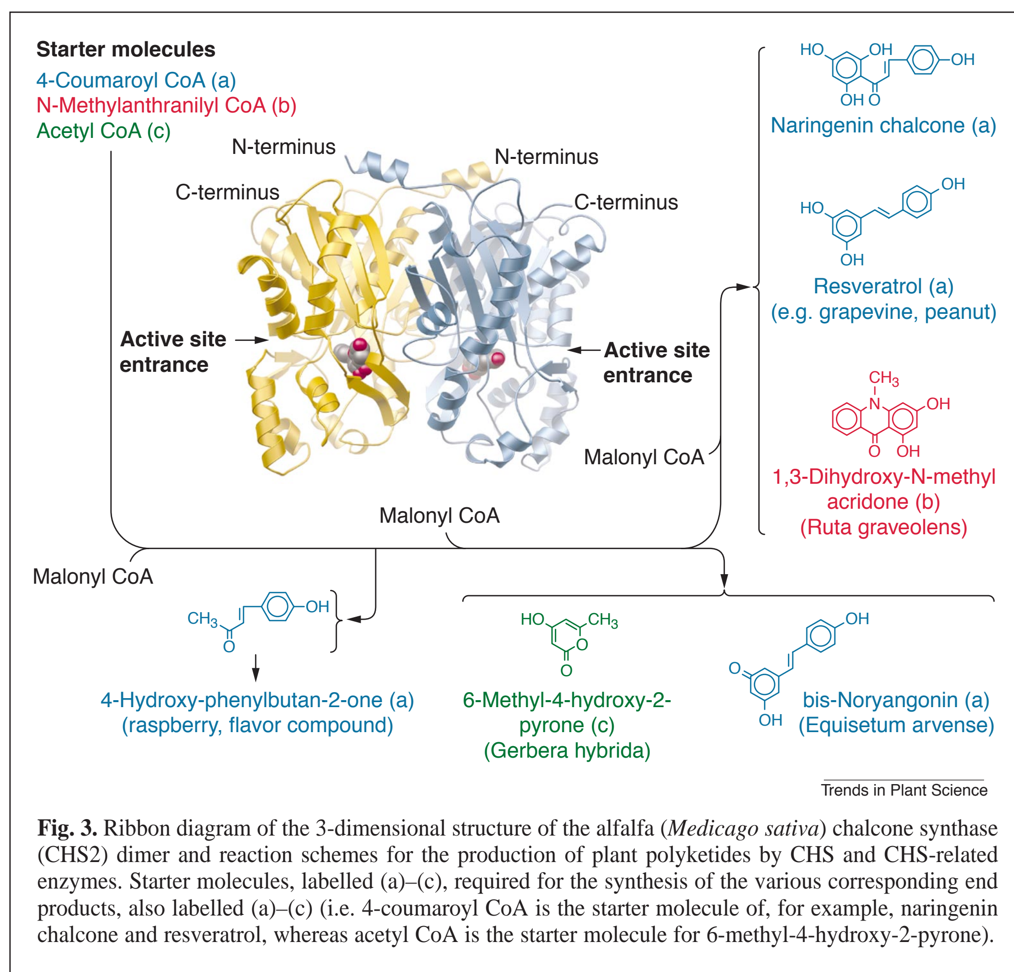

Fig. 3. Ribbon diagram of the 3-dimensional structure of the alfalfa (*Medicago sativa*) chalcone synthase (CHS2) dimer and reaction schemes for the production of plant polyketides by CHS and CHS-related enzymes. Starter molecules, labelled (a)–(c), required for the synthesis of the various corresponding end products, also labelled (a)–(c) (i.e. 4-coumaroyl CoA is the starter molecule of, for example, naringenin chalcone and resveratrol, whereas acetyl CoA is the starter molecule for 6-methyl-4-hydroxy-2-pyrone).

Structural studies

Improved understanding of the molecular basis of catalysis by the CHS-like family of plant polyketide synthases will facilitate the design of novel engineered enzymes with new specificities or improved catalytic properties. To this end, the crystal structure and reaction mechanism of alfalfa (*Medicago sativa*) CHS2 have been reported recently[21]. The CHS homodimer (subunit M_r 42 000) contains two functionally independent active sites (Fig. 3). The architecture of the catalytic pocket that defines the sequence and chemistry of the multiple decarboxylation and condensation reactions, followed by cyclization, is remarkably simple. Four residues are conserved in all CHS-related enzymes. Cys164 serves as the nucleophile and attachment site for polyketide intermediates, His303 might act as a general base during the generation of a thiolate ion from Cys164, and Phe125 and Asn366 might function in the decarboxylation of the first malonyl CoA molecule.

The CHS structural model allows predictions to be made as to the possible catalytic activities of CHS-like enzymes. Thus, modeling the active site of *Gerbera hybrida* GCHS2 indicates that this enzyme cannot catalyze either the CHS or STS reactions because of the reduced volumes of both the coumaroyl-binding and cyclization pockets[21]. This is reflected by the fact that this enzyme uses smaller starter molecules (acetyl or benzoyl CoA) to initiate the reaction, and only catalyzes two acetate additions[19] (Fig. 3). Rational site-directed mutagenesis of the CHS active site, or re-arrangement of the molecule by gene shuffling techniques[22], should generate a range of polyketide synthases for the formation of novel compounds.

Enzymes for the elaboration of flavonoid and isoflavonoid skeletons

Because of its amenability to genetic analysis, the pathway leading to the colored anthocyanin flower and seed-coat pigments is the best understood flavonoid branch pathway[23], particularly in terms of the regulatory genes that control its expression. However, the isoflavonoid branch pathway is also almost completely characterized at the molecular level, and genetic manipulation of flavonoid and isoflavonoid pathways is now feasible at many different biosynthetic stages. The enzymes belonging to these apparently complex pathways for the elaboration of basic flavonoid skeletons, fall into a few major classes (Fig. 2); 2-oxoglutarate-dependent oxygenases, cytochrome P450 hydroxylase enzymes, such as flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H) and the recently characterized isoflavone 2′-hydroxylase (I2′H)[24], NADPH-dependent reductases, such as dihydroflavonol reductase (DFR) and isoflavone reductase (IFR), and glycosyl transferases. The key anthocyanin synthase (ANS) reaction leading to the formation of a colored anthocyanidin (flavylium ion) from a colorless leucoanthocyanidin has been shown recently to be catalyzed by a single 2-oxoglutarate-dependent oxygenase for which the corresponding gene has been cloned[25]. In addition, recent progress has been made in understanding the biochemistry of the substitution of anthocyanins with hydroxycinnamic acids – reactions that contribute to the diversity of flower colors. An acyl-CoA-mediated acyltransferase that can transfer a *p*-coumaroyl or caffeoyl moiety to the glucose bound at the 5-position of anthocyanidin 3,5-diglucoside has now been characterized[26]. Finally, a demonstration of the role of glutathione *S*-transferases for conjugation leading to transfer of glutathionylated anthocyanins[27] and of isoflavonoids[28] to the vacuole via a MgATP-dependent glutathione *S*-conjugate pump, provides a biochemical basis for engineering vacuolar targeting for storage of novel flavonoids in transgenic plants.

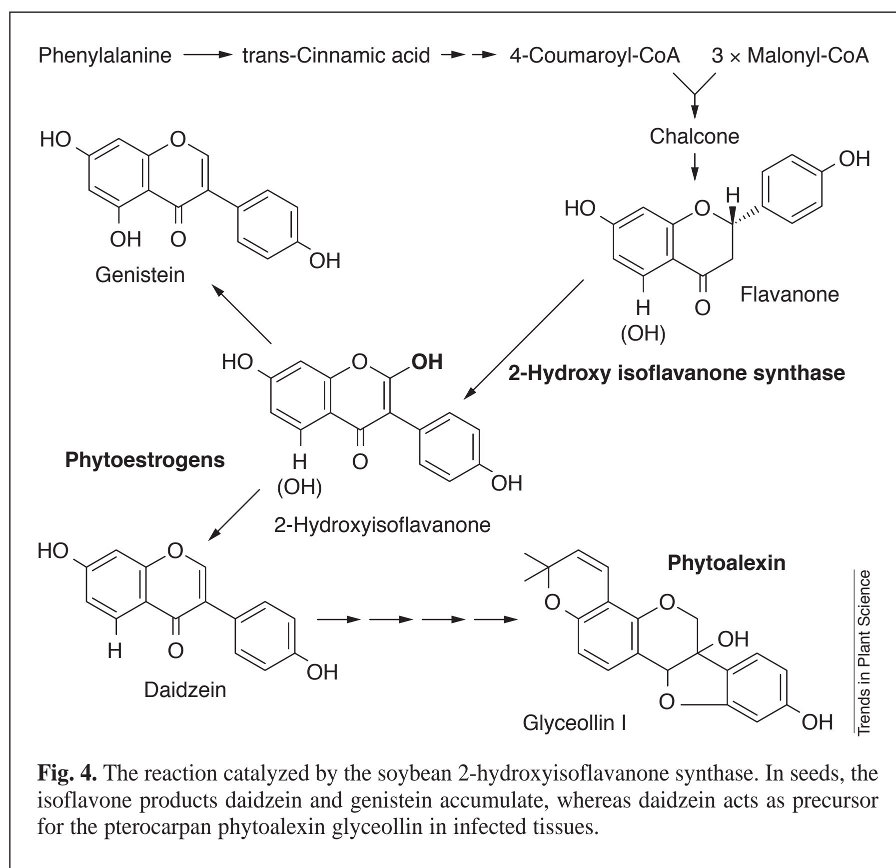

Fig. 4. The reaction catalyzed by the soybean 2-hydroxyisoflavanone synthase. In seeds, the isoflavone products daidzein and genistein accumulate, whereas daidzein acts as precursor for the pterocarpan phytoalexin glyceollin in infected tissues.

The branch pathway for the formation of isoflavonoids shares several mechanistic features with the anthocyanin pathway. However, the first reaction specific for isoflavonoid biosynthesis is unique. It comprises 2-hydroxylation coupled to aryl migration of the B-ring of a flavanone (naringenin or daidzein) to yield, after a dehydration reaction that might be spontaneous or enzyme-catalyzed[29], the corresponding isoflavone (genistein or daidzein, respectively; Figs 2 and 4). The aryl migration enzyme [2-hydroxyisoflavanone synthase (2-HIS)] has been cloned recently from soybean[30], which accumulates isoflavone phytoestrogens in the seed and more complex isoflavonoid-derived phytoalexins, such as glyceollin, in pathogen-infected tissues (Fig. 4). The cloning of 2-HIS opens up the possibility of introducing isoflavone nutraceuticals into a range of food crops.

The 2-HIS is a cytochrome P450 hydroxylase of the CYP93 class[30]. This P450 class also includes flavone synthase II (FSII, CYP93B1)[31], which catalyzes 2-hydroxylation without aryl migration, to form (after dehydration) a flavone from a flavanone (Fig. 2). Interestingly, FSII is also involved in the biosynthesis of retrochalcones in licorice (*Glycyrrhiza echinata*), formed via the hemiacetal opening of the 2-hydroxyflavanone product of FSII (Fig. 2). Thus, three distinct classes of flavonoid compound are produced via the 2-hydroxylation of common flavanone intermediates by related cytochrome P450 enzymes, illustrating the plasticity of plant natural product biosynthesis.

The 4′-*O*-methylation of the B-ring of isoflavones in certain legume species, such as alfalfa and chickpea, is an important reaction for biotechnological exploitation: it is a pre-requisite for further elaboration of the isoflavonoid nucleus to form pterocarpan phytoalexins from isoflavone intermediates with potential anticancer activity. Surprisingly, the isoflavone *O*-methyltransferase (IOMT) responsible for this reaction (Fig. 2) methylates the 7-position of the daidzein A-ring *in vitro*[32], although alfalfa does not accumulate A-ring-methylated isoflavonoids. However, when challenged by abiotic elicitation or fungal infection, transgenic alfalfa over-expressing IOMT produce increased levels of the 4′-*O*-methylated pterocarpan medicarpin and its precursor formononetin (Fig. 2) and, as a result, exhibit reduced susceptibility to the fungal leaf spot pathogen *Phoma medicaginis* (X-Z. He and R.A. Dixon, unpublished). Thus, the regiospecificity of IOMT appears to be different *in vivo* to that *in vitro*. This might result from IOMT associating with the endoplasmic reticulum-associated 2-HIS P450 as part of a complex through which intermediates are channeled. Similar metabolic channeling also occurs between phenylalanine ammonia-lyase and the cinnamate 4-hydroxylase cytochrome P450 at the entry point into the phenylpropanoid pathway[33]. This channeling of intermediates presents a potential complication for pathway engineering by limiting the availability of intermediates for diversion into reactions catalyzed by introduced transgenes.

Metabolic engineering of flavonoid compounds

An early objective for the metabolic engineering of the flavonoid pathway was to introduce blue delphinidin pigments [characterized by the 3′,4′,5′ hydroxylation pattern of the B-ring (Fig. 2)] into ornamentals, such as *Chrysanthemum* and roses, that lack this branch of anthocyanin biosynthesis. However, incorporation of *F3′5′H* genes alone is insufficient to guarantee deep blue coloration because of the need for accessory pigments and correct vacuolar pH, in addition to the recently demonstrated requirement for a flower-specific cytochrome b5 for the full activity of F3′5′H (Ref. 34). However, other changes in flower coloration have been obtained by several strategies including the introduction of dihydroflavonol reductase genes from maize or *Gerbera* into *Petunia*, the co-suppression of flavone synthase in tobacco, and the incorporation of *Antirrhinum* UDP-glucose:flavonoid 3-*O*-glucosyltransferase into Lisianthus (*Eustoma grandiflorum*)[23,35]. A novel approach, introducing alfalfa chalcone reductase (CHR) into *Petunia* has produced yellow flowers in acyanic (white-flowered) varieties, and reduced the degree of purple pigmentation in deep purple-flowered varieties[36]. This is because isoliquiritigenin, the product of the co-action of CHS and CHR, is not a substrate for the *Petunia* chalcone isomerase, and 6′-deoxychalcone derivatives (lacking the R1 hydroxyl in Fig. 2) therefore accumulate.

Condensed tannins (e.g. procyanidin trimer; Fig. 1) are polymerized flavonoids. They are a beneficial agronomic trait in forage legumes because they prevent pasture bloat by collapsing protein foams within the rumen. Much of the condensed tannin pathway is shared with anthocyanin biosynthesis, and attempts have been made to genetically manipulate condensed tannin levels by altering the expression of CHS and DFR genes using antisense strategies. In the latter case, a significant reduction in condensed tannin levels has been achieved in birdsfoot trefoil (*Lotus corniculatus*) without the appearance of obvious default products[37]. The challenge is to characterize the polymerization enzymes of condensed tannin biosynthesis to facilitate the transfer of the pathway to high-bloat forage species that do not contain tannins in their leaf tissue.

The above example serves to highlight a general problem for natural product engineering. The forage legume alfalfa contains condensed tannins in seed coats, but they are not expressed in the tissues in which they would provide agronomic benefit against pasture bloat. The 'classical' strategy for overcoming this problem would be to clone the structural genes for whatever enzymes are lacking in leaf tissue, and to express them under a promoter that directed expression in the leaves. The success of this strategy would depend on the ease with which the genes could be cloned, and the availability of substrates for the introduced downstream enzyme(s). An enzyme system catalyzing the two reductive steps to form a catechin derivative from a dihydroflavonol (Fig. 2) has been partially characterized[38], but no molecular information is available concerning this or the subsequent polymerization reactions leading to condensed tannins (Fig. 2). Genomics-based approaches might facilitate gene discovery in this area if more initial biochemical information were available. An alternative approach would be to up-regulate the expression of the endogenous genes in leaf tissue by the ectopic expression of transcription factors. The principle of this approach has been proven in maize, where dedifferentiated cell lines engineered to express the R and C1 transcription factors accumulate the same cyanidin derivatives (but with different sugars attached) as found in differentiated tissues, such as seed coats. In addition, ectopic expression of the P transcription factor in cell cultures leads to the accumulation of the UV-protective C-glycosyl flavones (e.g. isovitexin; Fig. 1), and other 3-deoxy flavonoids found in maize silks[39]. Cell lines expressing P also accumulate the caffeic acid ester, chlorogenic acid. Interestingly, no chalcone isomerase activity has been detected in the cell lines producing flavonoids in response to expression of R, C1 or P, suggesting that this enzyme is dispensable for flavonoid synthesis in maize, and can be by-passed by spontaneous isomerization of chalcone to flavanone.

Fig. 5. Wild-type (WT, right) and *pap-1D* mutant (left) *Arabidopsis* plants. In *pap1-D*, genomic insertion of an activation tag has led to over-expression of a *myb* family transcriptional regulator of the anthocyanin pathway, leading to intense purple coloration throughout the plant.

Future prospects – panning for gold?

The ability to switch on entire pathways by ectopic expression of transcription factors suggests new approaches for regulatory gene discovery and pathway engineering. Activation tagging was initially developed as a means of obtaining dominant mutations that could be screened for effects on plant growth and development[40]. Given an efficient genetic transformation system, it is possible to generate high numbers of independent transgenic lines harboring T-DNA constructs with multiple transcriptional enhancers at the right border, such that the T-DNA tag can activate transcription of neighboring genes. Confirmation that this approach works in natural product pathways came from the characterization of the *pap-1D* mutant of *Arabidopsis* (Fig. 5). The T-DNA enhancer construct in the *pap-1D* mutant is located proximally to a novel *myb* family transcriptional regulator, the over-expression of which results in the production of a purple anthocyanin pigment throughout the plant (J. Borewitz, Y. Xia, J.W. Blount, R.A. Dixon and C. Lamb, unpublished). Metabolic profiling screens[41] using liquid or gas chromatography coupled to mass spectrometry could, in principle, be used to score for activation-tagged dominant mutations that affect the expression of many secondary metabolic pathways. Ectopic expression of the maize *Lc* regulatory gene up-regulates flavonoid biosynthesis in *Petunia*, but not in Lisianthus or *Pelargonium*[42,43]. Thus, it is not clear whether a regulatory gene characterized by activation tagging in one species could be used to transform other species to uncover the full biosynthetic potential of a particular pathway. Nevertheless, given the necessary high-throughput genetic transformation system, activation tagging is a viable new approach for panning for regulatory or structural genes that control flux through plant natural product pathways. This could lead to altered production of agronomically important flavonoid compounds, such as condensed tannins, or estrogenic and antimicrobial coumestans, such as coumestrol (Fig. 1). Furthermore, because of the dominant nature of activation-tagged mutations, primary transformants can be screened directly for agronomic traits associated with the biochemical phenotype.

Acknowledgements

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