Biosynthesis and genetic engineering of proanthocyanidins and (iso)flavonoids

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Received: 13 September 2007 / Accepted: 12 October 2007 / Published online: 31 October 2007
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Abstract Plant natural products have been used since ancient times as medicines and herbal remedies. Over the past two decades, the results of population and intervention studies, or assays in animal or cell model systems, have revealed positive health beneficial effects for various classes of phytochemicals, particularly polyphenols. The results of such studies have ignited an interest in being able to manipulate the levels of such bioactive compounds in plants using biotechnological approaches. Although still in its infancy, this technology promises to deliver health benefits to humans and animals through direct consumption of genetically-modified or -enhanced dietary plant materials. We here review the strategies currently being used for engineering two classes of nutraceuticals, the proanthocyanidins and the isoflavones, in transgenic plants. We also provide an overview of recent advances in our understanding of the biosynthesis of these classes of compounds.

Keywords Isoflavone · Proanthocyanidin · Genetic engineering · Nutraceutical

Abbreviations

AHA	Autoinhibited H ⁺ -ATPase isoform
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
DFR	Dihydroflavonol reductase
DMAPP	Dimethylallyl pyrophosphate
ER	Estrogen receptor
F3H	Flavanone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
FLS	Flavonol synthase
FNS	Flavone synthase
GPP	Geranyl pyrophosphate
GSPE	Grape seed proanthocyanidin extract
GST	Glutathione S-transferase
2HID	2-Hydroxyisoflavanone dehydratase
2HIS	2-Hydroxyisoflavanone synthase
IFS	Isoflavone synthase
LAR	Leucoanthocyanidin reductase
LDL	Low density lipoprotein
MATE	Multidrug and toxic efflux transporter
2-OG	2-Oxoglutarate
OMT	O-methyltransferase
PA	Proanthocyanidin
PAL	Phenylalanine ammonia-lyase
PAP	Production of anthocyanin pigmentation
TT	Transparent testa
TTG	Transparent Testa Glabra
UGT	UDP glycosyltransferase

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Potential health promoting activities of bioactive flavonoids and isoflavonoids

Over the past two decades, a large literature has accumulated describing the effects of various classes of phytochemicals, particularly polyphenolic compounds of the (iso)flavonoid, anthocyanin and proanthocyanidin classes, on human and animal health, assessed either through population and intervention studies, or by assays in animal or cell model systems (see Adlercreutz 2002; Cornwell et al. 2004 for reviews).

Polyphenolic natural products of the (iso)flavonoid, anthocyanin, and proanthocyanidin classes exhibit a number of health promoting activities associated with their antioxidant properties. For example, the anthocyanin glycosides (“anthocyanosides”) present in bilberry extracts have attracted attention because of their potential for preventing or delaying macular degeneration in the retina, a process associated with oxidative damage (Martin-Aragon et al. 1999; Lee et al. 2005). Chocolate contains (–)-epicatechin, and it has been suggested that consumption of dark chocolate is potentially cardioprotective due to subsequent increases in blood plasma antioxidant potential associated with epicatechin (Serafini et al. 2003). The antioxidant activity of grape seed proanthocyanidin extract (GSPE) is well known (Bagchi et al. 2000), and clinical trials with human subjects have suggested that consumption of GSPE reduces oxidized low density lipoprotein, a marker for cardiovascular disease (Vinson et al. 2002). In red wines, oxygen radical absorbance capacity correlates with the presence of anthocyanin (malvidin compounds) and proanthocyanidins (PAs). In white wines, levels of trimeric PAs were correlated with peroxyl radical scavenging activity (Sanchez et al. 2003).

Monomeric flavan-3-ol derivatives are important determinants of the documented health effects of green tea (Dufresne and Farnworth 2001), grapes (wines, juices and GSE) and cranberry juice. Green tea is a rich source of catechin derivatives, particularly (–)-epigallocatechin gallate, and both this compound and (–)-epicatechin protect mammalian cells from free radical-mediated oxidative stress and cause apoptosis of cancer cells (Zhan et al. 1997; Ahmad et al. 2000; Spencer et al. 2001).

Cranberry juice is well known for its ability to protect the urinary tract from adherence of

urinopathogenic bacteria; drinking cranberry juice is a recommended treatment for various urinary tract infections and prostatitis. Cranberry juice contains high concentrations of anthocyanins, flavonol glycosides, phenolic acids, and PAs. The active fraction for prevention of binding of uropathogenic *Escherichia coli* cells to surfaces containing the urinary tract α -Gal(1→4) β -Gal receptor motifs contain PAs consisting primarily of epicatechin tetramers and pentamers (Foo et al. 2000).

Compounds within the flavonoid, isoflavonoid, stilbene and lignan classes are also known to function as phytoestrogens. Isoflavones are the most studied group of phytoestrogens and their estrogenic activities stem from the common structural features that they share with human estrogen 17 β -estradiol (Dixon 2004). The isoflavones daidzein and genistein can bind to human estrogen receptors (ER) and exert estrogenic or anti-estrogenic activities upon their binding to ER. Inverse correlations have been reported between the intake of isoflavones and the incidence of breast and prostate cancers (Lee et al. 1991; Denis et al. 1999). Isoflavones can also relieve the symptoms of other hormone-related illnesses, such as osteoporosis and post-menopausal ailments (Baird and Umbach 1995; Potter et al. 1998). Although flavonoid phytoestrogens have been less studied than the isoflavonoid phytoestrogens, 8-prenylnaringenin isolated from hop extract bound to ER more tightly and was shown to have higher estrogenic activity than the isoflavonoids genistein or coumestrol (Kitaoka et al. 1998; Milligan et al. 1999, 2000, 2002).

In addition to their effects on hormone-related illnesses, flavonoids and isoflavonoids have been shown to impede the growth of cancer cells by inhibiting tyrosine kinase and DNA topoisomerase activities and by modulation of apoptosis (Akiyama et al. 1987; Okura et al. 1988; Boersma et al. 2001). Their potential antioxidant activities are exemplified by the inhibition of 5-lipoxygenase and cyclo-oxygenase activities and low density lipoprotein (LDL) oxidation, which may contribute to lowering the risk of cardiovascular diseases and certain type of cancers (Laughton et al. 1991; Tikkanen and Adlercreutz 2000; Wiseman et al. 2000; Rufer and Kulling 2006). Anti-inflammatory and anti-viral activities have also been reported for some flavonoid and isoflavonoid molecules (Kaul

et al. 1985; Middleton and Kandaswami 1992; Formica and Regelson 1995). Although most studies support the beneficial effects of flavonoids/isoflavonoids on human disorders, potential adverse effects have also been observed and further research is still required to demonstrate the chemopreventive and therapeutic activities of these compounds (Strauss et al. 1998).

In addition to their documented effects on human health, PAs are also important for the well-being of ruminant animals. Consumption of protein-rich forages such as alfalfa results in rapid microbial digestion in the rumen, associated with release of excess methane and trapping of the methane in the protein foam, resulting in the potentially lethal condition of pasture bloat. The presence of PAs in the forage results in a lower rate of protein degradation due to binding of PAs to dietary protein; not only does this prevent pasture bloat (Lees 1992), but it also results in increased protein exiting the rumen to benefit the nutrition of the animal (Barry and McNabb 1999). There is therefore considerable interest in introducing PAs into forage crops such as alfalfa whose aerial portions lack these compounds (Dixon et al. 2005).

Although certain plants provide ample levels of dietary natural products for potential health benefits (e.g. soy seed isoflavones), many compounds are only produced in small amounts, or are found in species that are not suitable for the human or ruminant diet. Development of a robust technology for introducing such compounds into common dietary plant sources is therefore an important goal. Such development requires knowledge of the biosynthetic pathways leading to the compounds, the regulatory networks underlying their biosynthesis and accumulation, and the effects of the introduced compounds on the physiology of the host plant. The remainder of this article briefly summarizes recent advances in our understanding of the biosynthesis of various classes of flavonoid-derived nutraceutical compounds, and gives examples of attempts to either manipulate their levels or introduce their biosynthesis *de novo* through transgenic technology. We also discuss the types of biochemical modifications that alter or enhance the bioactivity of plant natural products, with implications for the engineered synthesis of a range of novel natural products in plant or microbial systems.

Biosynthesis of proanthocyanidins

PAs, also called condensed tannins, are monomeric or oligomeric end products of the flavonoid pathway. The composition of the PAs in different plant species varies considerably. In leaves of the tannin-rich legume *Desmodium uncinatum*, the starter unit of PAs is exclusively catechin, and catechin also accounts for around 10–30% of the extension units, with the remainder being epicatechin, gallocatechin and epigallocatechin (Peel GJ, personal communication). Similarly, in the legume *Lotus corniculatus*, the extension units mainly consist of epicatechin, with the starter units being predominantly catechin (Foo et al. 1996). However, the PAs found in the seed coat of *Arabidopsis thaliana* consist entirely of epicatechin units (Routaboul et al. 2006), and the PAs from the model legume *Medicago truncatula* appear similar (Pang et al. 2007). In grape seed PAs, catechin is the dominant starter unit, with around 70% epicatechin extension units, although the PA composition changes during fruit ripening (Kennedy et al. 2000).

The biosynthesis of PA precursors shares the same upstream pathway as that of anthocyanins. The two precursor groups, 2,3-*trans*-flavan-ols and 2,3-*cis*-flavan-ols, are synthesized by two distinct pathway branches from two different substrates by distinct enzymes, although the only difference between these compounds is the *cis*- or *trans*- stereochemistry at the C2 and the C3 positions of the C-ring. Leucoanthocyanidin can be converted to 2,3-*trans*-flavan-ol (catechin) by leucoanthocyanidin reductase (LAR, EC.1.1.7.1.3) (Fig. 1). Leucoanthocyanidin is also the substrate for anthocyanidin synthase (ANS, EC 1.14.11.19) to produce colored anthocyanidin, which is converted to the other PA precursor group, 2,3-*cis*-flavan-ols (epicatechins), by the action of anthocyanidin reductase (ANR, EC 1.3.1.77) (Fig. 1). Anthocyanidins can be also glycosylated to form anthocyanins.

To date, several *LAR* and/or *ANR* genes have been cloned and characterized from different plant species with different PA accumulation patterns and compositions (Tanner et al. 2003; Xie et al. 2003; Bogs et al. 2005; Pang et al. 2007; Paolocci et al. 2007). The first reported purification of a native LAR enzyme, and the cloning of the corresponding gene, was from leaves of *D. uncinatum* (Tanner et al. 2003). Mutation of the *ANR* gene in *Arabidopsis*

Fig. 1 Pathways for the biosynthesis of PAs. DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. R1=H, R2=H, (epi)afzelechin; R1=H, R2=OH, (epi)catechin; R1=OH, R2=OH, (epi)gallocatechin

results in a PA-deficient phenotype with accumulation of anthocyanin in the seed coat (the *BANYULS* mutation), whereas over-expression of *AtANR* in *Arabidopsis* led to reduced anthocyanin levels and production of epicatechins in floral tissue (Xie et al. 2003), consistent with a role for ANR in the PA biosynthetic pathway. No close *LAR* ortholog has been identified in *Arabidopsis*, consistent with the lack of (+)-catechin in the PAs from this species (Tanner et al. 2003; Routaboul et al. 2006).

The single copy *ANR* and *LAR* genes of *M. truncatula* have been characterized in vitro, and the consequences of their ectopic expression in transgenic plants in vivo have been determined (Xie et al. 2003, 2004; Pang et al. 2007). *MtANR* converted the three different anthocyanidin substrates (cyanidin, pelargonidin and delphinidin) to their corresponding flavan-3-ols in vitro (Xie et al. 2004), and its expression in tobacco significantly reduced anthocyanin levels but increased PA levels in flowers (Xie et al. 2003). Similarly, when the *MtANR* gene was over-expressed in *M. truncatula*, accumulation of PAs was observed at the same time as the loss of the typical anthocyanin-accumulating spots on the leaves (Xie et al. 2006). Further investigation showed that levels of *MtANR* transcripts paralleled the accumulation of PAs in developing seeds, whereas *MtLAR* transcripts appeared to be more transiently expressed (Pang et al. 2007). Although a single *LAR* gene encoding a protein with weak LAR activity was cloned and characterized from *M. truncatula*, the apparent

lack of catechin in the PAs of this species, and the lack of catechin or PA production in transgenic tobacco expressing *MtLAR*, raises questions as to the actual role of LAR in *M. truncatula* (Pang et al. 2007). Two *LAR* genes were reported in *Lotus corniculatus*, but only one of them, *LcLAR1*, was shown to encode a protein with demonstrable LAR activity (a weak activity detected by TLC separation of products from a coupled enzyme assay with recombinant dihydroflavonol reductase (DFR)) (Paolucci et al. 2007). *LcANR1* was more highly expressed than the two other *ANR* genes from this source in all tissues in which expression was detected, and levels of both *LcANR* and *LcLAR1* transcripts correlated with the levels of PAs in the leaves of *L. corniculatus* (Paolucci et al. 2007).

The fruits of the grape (*Vitis vinifera*) are rich in PAs, and two grape *LAR* orthologs were reported. One of them, *VvLAR1*, was functionally characterized by enzymatic assay of the recombinant protein, and possessed enzyme activity comparable to that of DuLAR (Bogs et al. 2005). Expression of *VvANR* in tobacco led to an increase in the levels of PAs with a corresponding reduction of anthocyanin levels (Bogs et al. 2005). The tissue- and temporal-specific expression of the *VvLAR1* and *VvANR* genes determines both PA content and composition during grape berry development (Bogs et al. 2005).

PA transport, oxidation and polymerization

PA precursors are believed to be first synthesized in the cytosol and then transported into the vacuoles. So far, three different types of transporters have been implicated in this process (Debeaujon et al. 2001; Kitamura et al. 2004; Baxter et al. 2005; Marinova et al. 2007). A glutathione *S*-transferase (GST)-like protein encoded by the *TT19* gene was found to participate in the anthocyanin pathway as well as the PA pathway, as a putative transporter of PA precursors (Kitamura et al. 2004), although the exact biochemical and biological functions of TT19 remain unclear. In addition to TT19, a P-type ATPase superfamily member, autoinhibited H^+ -ATPase isoform 10 (AHA10), was also shown to be necessary for the accumulation of PAs in *Arabidopsis* seeds, and is possibly involved in vesicle-mediated trafficking (Baxter et al. 2005), although the protein is predicted to be localized to the plasma membrane.

TT12 encodes a membrane protein of the multidrug and toxic efflux transporter (MATE) family, which was suggested to be involved in the vacuolar accumulation of PA precursors in the Arabidopsis seed coat endothelium (Debeaujon et al. 2001). Further investigation suggested that *TT12* is a tonoplasmic membrane protein acting as a proton antiporter, which mediates the transport of glucosides of monomeric flavan-3-ols and anthocyanidins (Marinova et al. 2007). Taken together, current data suggest that PA precursors (and other flavonoids) are either directly loaded into or guided toward vesicles through the cytosol by binding to a glutathione *S*-transferase such as *TT19*, and these vesicles are destined to fuse with the vacuole. At the same time, *TT12*-mediated proton-dependent transport of glucosylated PA monomers may take place at the vacuole level, and could be energized by the proton gradient established by *AHA10* (Marinova et al. 2007). Although the recent discovery of *TT12* function extends our understanding of PA precursor transport, it is not yet clear whether a PA-monomer-specific glucosyltransferase (cytosolic?) and corresponding glucosyl hydrolase (vacuolar?) exist. It is also not clear whether increasing the transport of PA monomers will increase PA accumulation.

During seed development, the colorless PA precursors first accumulate in vacuoles, and then migrate to the cell wall as insoluble PAs in the senescing testa, until they are no longer visible in the vacuole (Kitamura et al. 2004). The PAs are then oxidized into brown complexes that are cross-linked with the cell wall. The Arabidopsis *TT10* gene encodes a laccase-like protein that may act to oxidize epicatechin units in PAs into the corresponding quinones, thereby initiating additional polymerization and cross-linking of brown PA polymers in the seed coat (Pourcel et al. 2005). Although more than one model has been proposed for the initial oligomerization of PAs, it is still not clear whether this process is enzymatic or non-enzymatic; direct experimental verification of any model is still lacking (Dixon et al. 2005; Pourcel et al. 2005).

PA pathway transcription factors

Like other secondary metabolism pathways, the PA biosynthetic pathway is under complex

transcriptional control by multiple regulatory genes. These regulatory genes in Arabidopsis fall into three different functional categories (Dixon et al. 2005). We here discuss the functions and potential applications of the *production of anthocyanin pigmentation (PAP1)* gene, and a key ternary complex consisting of MYB, HLH and WD family transcription factors from Arabidopsis.

PAP1 is an R2R3 MYB family transcription factor, the ectopic activation of which induces massive anthocyanin accumulation in Arabidopsis associated with the transcriptional up-regulation of the complete anthocyanin pathway (Borevitz et al. 2000; Tohge et al. 2005). Likewise, constitutive expression of *AtPAP1* in tobacco leads to the accumulation of anthocyanin and a deep red-purple phenotype throughout the plant (Borevitz et al. 2000; Xie et al. 2006). An anthocyanin/PA pathway transcriptional complex comprising Transparent Testa 2 (*TT2*, a R2R3 MYB-class protein), *TT8* (a basic helix-loop-helix-class protein), together with Transparent Testa Glabra1 (*TTG1*, a WD40-repeat protein) has been found to regulate the accumulation of PAs in a tissue-specific manner in the seed coat of Arabidopsis (Walker et al. 1999; Nesi et al. 2000, 2001; Zhang et al. 2003; Baudry et al. 2004). This complex, in which *TT2* is regarded as the master regulator, controls PA accumulation by regulating the expression of *DFR*, *ANS* and *ANR* (Baudry et al. 2004).

Genetic engineering of PAs

A number of investigations have focused on engineering PAs in model plants (Arabidopsis, tobacco and *M. truncatula*), as well the forage legumes *L. corniculatus* and alfalfa (*Medicago sativa*). Work on the forage legumes is targeted primarily towards improving dietary nitrogen nutritional efficiency and preventing pasture bloat in ruminant animals. These studies also serve as models for the introduction of PAs into plants as nutraceuticals for human health enhancement. Since transcription factors are capable of regulating the expression of whole pathways, the different transcription factors described above have been ectopically expressed, either alone, in groups, or in combination with the structural genes for PA monomer biosynthesis, in attempts to increase PA

levels or introduce PAs de novo into tissues that do not naturally produce them (Table 1).

In Arabidopsis, ectopic expression of the maize *Lc* gene, a MYC/HLH transcription factor, alone did not induce *ANR* gene expression or PA accumulation, even though it weakly induced the expression of *AHA10* and anthocyanin accumulation (Sharma and Dixon 2006). When the same gene was introduced into alfalfa, both anthocyanin and PA accumulation was observed in leaf tissue, but only under stress conditions such as cold or high light intensity (Ray et al. 2003). In contrast, expression of *Lc* in apple (*Malus domestica*) led to induction of not only the anthocyanin pathway genes *PAL*, *CHS*, *F3H*, *DFR* and *ANS*, but also the PA-specific pathway genes *LAR* and *ANR*. This resulted in increased levels of both anthocyanins and PAs (catechin and epicatechin monomers and dimeric PAs) (Li et al. 2007a).

In *L. corniculatus*, ectopic expression of the maize bHLH transcription factor *Sn* enhanced PA polymer accumulation associated with an increase in the number of PA-containing leaf cells, and also affected anthocyanin accumulation in a tissue-specific manner (Robbins et al. 2003). Further study showed that light and *Sn* have a synergistic effect on PA accumulation, and expression of *LAR*, *ANR*, *DFR* and *ANS* correlated with PA levels in the leaves of *L. corniculatus* (Paolucci et al. 2005, 2007). In rice, the *Rc* locus, encoding a basic helix-loop-helix protein, is involved in the biosynthesis of PAs in the seed pericarp (Sweeney et al. 2006; Furukawa et al. 2007). Taken together, the above examples indicate that ectopic expression of the same transcription factor can lead to different results in different plant systems.

MYB transcription factors are central to the control of PA biosynthesis. When *AtTT2* was constitutively over-expressed in Arabidopsis, *ANR*, *AHA10* and *TT12* were all up-regulated, although no significant accumulation of PAs was observed (Sharma and Dixon 2006). In contrast, the ectopic expression of a MYB transcription factor from grape, *VvMYB5a*, affected expression of a number of endogenous flavonoid pathway genes in tobacco, leading to increased levels of anthocyanins and PAs (epicatechin-derivatives) in the flowers (Deluc et al. 2006). Another *MYB* gene from grapevine, *VvMYBPA1*, regulates PA synthesis during grape berry development and in seed, and can

complement the Arabidopsis *tt2* seed PA-deficient mutant phenotype. However, whereas *TT2* appears specific for activation of downstream PA-pathway genes such as *ANR*, *VvMYBPA1* also activates the general flavonoid pathway (Bogs et al. 2007).

In addition to expression of a single heterologous or native structural gene or transcription factor, two or more transcription factors, or transcription factors in combination with structural genes, have also been employed to increase the accumulation of PAs in transgenic plants. When *AtTT2* was co-expressed with *PAP1* in Arabidopsis, PA levels were increased, but only in a subset of cells where the *ANR* promoter was expressed (Sharma and Dixon 2006), suggesting that such cells may be pre-programmed for PA accumulation. When two MYB transcription factors, *AtTT2* and *PAP1*, together with one HLH (*Lc*) transcription factor, were introduced into Arabidopsis, the *ANR* and *AHA10* genes were both induced and anthocyanins and PAs accumulated (Sharma and Dixon 2006). However, these transformed plants exhibited growth abnormalities followed by death due to possible pleiotropic effects; it is also possible that the levels of anthocyanidin and/or PA accumulation exceed the tolerance limit of the target plant. In tobacco leaves co-expressing *PAP1* and *MtANR*, the high anthocyanin levels typical of the *PAP1* transformants were significantly reduced, with corresponding production of PA monomers and oligomers in which epicatechin units predominated (Xie et al. 2006), suggesting that the combination of a transcription factor to increase the level of anthocyanidin substrate and a PA pathway gene to divert that substrate into the PA pathway is an applicable strategy for metabolic engineering of PAs in plants.

To date, both MYB and HLH transcription factors, alone or in combination, have been used for the metabolic engineering of PAs. The other member of the Arabidopsis ternary transcriptional complex, the WD repeat domain-like protein *TTG1*, has not yet been reported as having been tested for this purpose. Whether or not *TTG1* will be able to induce the ectopic expression of endogenous PA pathway genes and subsequent PA accumulation, or whether the introduction of the whole ternary transcriptional complex will be more efficient than that of a single transcription factor, remains to be investigated.

Table 1 Transcription factors involved in anthocyanin/PA biosynthesis as applied to PA bioengineering

Type	Gene name	Gene source	Target plant	Regulate anthocyanin or PA	Regulated pathway genes	References
MYB	<i>VvMYBPAL</i>	<i>Vitis vinifera</i>	Arabidopsis <i>tt2</i> mutant	PA	<i>CHI, F3'H, ANS, LAR, ANR</i>	Bogs et al. (2007)
	<i>VvMYB5a</i>	<i>Vitis vinifera</i>	Tobacco	Both	<i>CHS, CHI, F3'H, DFR</i>	Deluc et al. (2006)
	<i>TT2</i>	Arabidopsis	Arabidopsis	PA	<i>ANR, AHA10, TT12</i>	Sharma and Dixon (2006)
	<i>TT2+PAP1</i>	Arabidopsis	Arabidopsis	Both	<i>ANR, AHA10, TT12</i>	Sharma and Dixon (2006)
	<i>Sn</i>	Maize	<i>Lotus corniculatus</i>	Both	<i>DFR, ANS, LAR, ANR</i>	Robbins et al. (2003), Paolucci et al. (2005) and Paolucci et al. (2007)
Myc/HLH	<i>Rc</i>	Rice	Rice <i>rc</i> mutant	Both		Sweeney et al. (2006) and Furukawa et al. (2007)
	<i>Lc</i>	Maize	Arabidopsis	Anthocyanin	<i>AHA10</i>	Sharma and Dixon (2006)
	<i>Lc</i>	Maize	Alfalfa	Both	<i>CHS, F3'H</i>	Ray et al. (2003)
	<i>Lc</i>	Maize	Apple	Both	<i>PAL, CHS, F3'H, DFR, ANS, LAR, ANR</i>	Li et al. (2007)
	<i>TT2+PAP1+Lc</i>	<i>TT2</i> and <i>PAP1</i> from Arabidopsis, <i>Lc</i> from maize	Arabidopsis	Both	<i>ANR, AHA10</i>	Sharma and Dixon (2006)
Myb+HLH						
MYB+ANR	<i>PAP1+ANR</i>	<i>PAP1</i> from Arabidopsis, <i>ANR</i> from <i>M. truncatula</i>	Tobacco	Both		Xie et al. (2006)

Biosynthesis of isoflavones

In contrast to the ubiquitous distribution of flavonoids in the plant kingdom, isoflavonoids are present mostly in legumes, presumably due to the lack of isoflavone synthase (IFS), the enzyme catalyzing the committed step for isoflavone synthesis, in most other plant species (Fig. 2). The term IFS has been commonly used to describe the combined aryl migration reaction, catalyzed by 2-hydroxyisoflavanone synthase (2HIS), and the subsequent dehydration reaction, catalyzed by the separate enzyme 2-hydroxyisoflavanone dehydratase (2HID), which also occurs spontaneously under acidic conditions (Akashi et al. 2005).

cDNAs encoding 2HIS have been cloned from sugarbeet, licorice, soybean, red clover, *Lotus japonicus*, *Medicago truncatula* and alfalfa (Akashi et al. 1999a; Steele et al. 1999; Jung et al. 2000; Kim et al. 2003; Deavours and Dixon 2005). These 2HISs belong to the CYP93C subfamily of cytochrome P450 enzymes, share high level of similarity at the amino acid sequence level, and require both NADPH and O₂ as co-factors for the formation of 2-hydroxyisoflavanone. The 2HIS-catalyzed reaction is stereoselective in that only 2*S*-flavanone, not 2*R*-flavanone, can be used as substrate. The aryl migration proceeds from abstraction of a hydrogen at C-3 followed by B-ring migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical. To investigate the key amino acid residues that are required for this unique aryl migration reaction, a three dimensional model of CYP93C2 (2HIS from licorice) was constructed using the P450BM3 structure as template and several amino acids predicted to be located in the substrate binding site were modified by site-directed mutagenesis (Sawada et al. 2002). The wild-type and mutant CYP93C2 proteins were functionally expressed in yeast and the 2HIS enzymatic activities were tested using liquiritigenin (7,4'-dihydroxyflavanone) as substrate. Wild type CYP93C2 converted most of the liquiritigenin to 2-hydroxyisoflavanone with 3-hydroxyflavanone as a by-product (~8%). Several point mutations at the substrate binding site led to altered activities in the mutant enzymes, the most significant being a single amino acid change from Lys375 to Thr (K375T), which totally abolished the 2-hydroxylation and aryl migration activities of CYP93C2 and led to 3-hydroxyflavanone as the sole

product, establishing the essential role of Lys375 in the aryl migration reactions (Sawada et al. 2002). Further multiple point mutations of CYP93C2 revealed that Leu371 was essential for correct folding of the protein. Interestingly, the misfolding caused by the L371V mutation could be reversed by introducing a second K375T mutation to the protein. However, a triple mutant of S310T-L371V-K375T catalyzed the formation of flavone as the only product (Sawada and Ayabe 2005). Taken together, the site-directed mutagenesis studies indicated that Lys375 is essential for the aryl migration reaction and causes the instability in the protein conformation, whereas Ser310 and Leu371 are important for the hydrogen abstraction at C-3 and correct folding of the P450 protein, respectively (Sawada et al. 2002; Sawada and Ayabe 2005).

Genetic engineering of isoflavones

The numerous roles that isoflavonoids play in promoting plant and human health have stimulated intense interest in genetic engineering of isoflavonoid accumulation in leguminous and non-leguminous plants. Three different strategies have been employed for enhanced isoflavone accumulation: ectopic expression of 2HIS singly, expression of 2HIS together with chalcone isomerase (CHI), and a combined over-expression of 2HIS, early pathway genes or transcription factors and suppression of endogenous competitive pathway genes (Jung et al. 2000; Yu et al. 2000, 2003; Liu et al. 2002, 2007; Deavours and Dixon 2005; Sreevidya et al. 2006; Tian and Dixon 2006). These approaches take advantage of the ubiquitous presence of the common biosynthetic precursor, 2*S*-naringenin, for flavonoid and isoflavonoid biosynthesis and all resulted in (enhanced) accumulation of isoflavone conjugates, not aglycones, in different tissues of the transgenic plants. The nature of the genistein glucose conjugates formed in transgenic *Arabidopsis* and tobacco plants was consistent with the sugar donors used for endogenous flavonol glycosylation in this species (Jung et al. 2000; Yu et al. 2000, 2003). However, when a *Medicago truncatula* 2HIS gene was expressed in alfalfa, glucosylated genistein, biochanin A, and pratensein were detected, although the endogenous flavones in this species are glucuronidated (Deavours and Dixon 2005).

Fig. 2 Biosynthesis of flavonoids and isoflavonoids in plants. Enzymes, structures of certain intermediates, and the steps blocked by the *tt3*, *tt5* and *tt6* mutations are shown. Legume-specific isoflavonoid synthesis is highlighted in gray. PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase.

thase; CHR, chalcone reductase; CHI, chalcone isomerase; 2HIS, 2-hydroxyisoflavanone synthase (also commonly, and incorrectly, known as isoflavone synthase); 2HID, 2-hydroxyisoflavanone dehydratase; F3H, flavanone 3-hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase

Increase in overall carbon flow to the isoflavonoid pathway in tobacco was accomplished by introducing an artificial bi-functional isoflavone synthase/

chalcone isomerase (IFS/CHI) enzyme constructed by in-frame gene fusion (Tian and Dixon 2006). The fusion protein was localized to the endoplasmic

reticulum membranes, consistent with the intracellular localization of IFS. Both IFS and CHI activities were retained in the IFS/CHI fusion protein when assayed in yeast. A twofold increase in genistein accumulation was observed in petals of IFS/CHI transgenic tobacco compared to petals expressing the *IFS* transgene alone (Tian and Dixon 2006). A similar increase in genistein glucoside accumulation was observed when *IFS* was co-expressed in tobacco petals with a phenylalanine ammonia-lyase (*PAL*) gene from *Astragalus membranaceus* (Liu et al. 2007).

The outcome of the genetic engineering efforts for isoflavone accumulation was not only determined by the introduced 2HIS activity, but also by endogenous pathway competition. When 2HIS was transformed into the *Arabidopsis tt6/tt3* (flavanone 3-hydroxylase (*F3H*)/dihydroflavonol reductase (*DFR*)) double null mutant background, genistein accumulation was 10–50 fold higher compared to that observed when 2HIS was expressed in wild type *Arabidopsis*, indicating that competition from endogenous flavonol biosynthesis was limiting for enhanced isoflavone production (Liu et al. 2002). Similar results were also reported in transgenic tobacco plants, where the average genistein accumulation in 2HIS-overexpressing and *F3H*-antisense suppressed plants was 11-fold higher than in those with 2HIS over-expression alone (Liu et al. 2007). However, co-suppression of *F3H* did not lead to a significantly increased isoflavone level in seeds of transgenic soybean plants (Yu et al. 2003). When the maize transcription factors C1 and R were co-expressed in soybean seeds in conjunction with the suppression of *F3H*, the total isoflavone levels in the transgenic plants reached up to 4-fold higher than in the control plants (Yu et al. 2003).

Overall, a thorough understanding of plant metabolism and a concerted strategy are required for manipulation of flavonoid and isoflavonoid biosynthesis/accumulation. In addition to endogenous competing pathways, the “true” substrate for *in vivo* biosynthesis, subcellular localization and availability of substrates and biosynthetic enzymes, metabolon formation among the biosynthetic enzymes, product stability/turnover, and expression of modification enzymes (e.g. glycosyltransferases that catalyze the formation of isoflavone conjugates) are some of the factors that should be taken into account when planning a genetic engineering experiment.

Nevertheless, in certain cases, intended biological activities might not be achieved. For example, in an attempt to produce isoflavones as nodulation signals in non-nodule forming plants, the soybean *IFS* gene was expressed in rice plants under the control of a 35S promoter. Although production of genistein glycoside was detected in root tissue of the transgenic rice plants and both leaf and root extracts were able to stimulate nod gene expression in some of the rhizobia tested, root nodule formation was, perhaps predictably, not reported in these plants (Sreevidya et al. 2006).

Engineering of novel natural products by enzymatic modifications of core skeletons

More than 8,000 flavonoid and isoflavonoid compounds have been identified in nature (Harborne and Williams 2000). 2S-Naringenin, daidzein and genistein are central intermediates in the biosynthesis of more complex flavonoids and isoflavonoids. Modifications to the ring structure and/or acyclic side structure of these aglycones, such as oxidation, C- or O-methylation, C- or O-glycosylation and C- or O-prenylation, give rise to many (iso)flavonoid derivatives. Representative biosynthetic relationships of potential products from daidzein after hydroxylation, O-methylation, C- and O-glycosylation, and C-prenylation and cyclization are shown in Fig. 3.

Many of the substituted (iso)flavonoids occur naturally, but their distribution is restricted to certain plant species or organs. In addition, they often accumulate at low levels, insufficient for large scale extraction. Chemical synthesis of the modified (iso)flavonoids is difficult due to the demand for high yield and the regio- and stereo-specificity that are essential for biological activities of these compounds. Therefore, it is appealing to use enzyme catalysis as a tool for biosynthesis of bioactive (iso)flavonoid compounds. Such enzymatic modifications could lead to a range of novel products with enhanced/modified bioactivity. Genes encoding some of the modification enzymes have been cloned and characterized in recent years using genetic, genomic and biochemical approaches. We here review enzymatic modifications of isoflavonoid aglycones, enzyme functionalities and structure-function relationships, and potential applications of (iso)flavonoid modification by genetic engineering.

Fig. 3 Biosynthetic relationships of daidzein and its derivatives via various enzymatic modifications

Oxidation

Hydroxylation is the most common substitution of the (iso)flavonoid ring structures. The hydroxyl groups are often used to form ethers with alcohols (e.g. methanol by *O*-methylation), sugars (e.g. glucose by

O-glucosylation) and terpenoids (e.g. isoprenoid by *O*-prenylation). (Iso)flavonoid hydroxylations/oxidations are catalyzed by two families of enzymes: the NADPH-dependent cytochrome P450s and the non-heme ferrous, 2-oxoglutarate (2-OG) dependent dioxygenases. The four dioxygenases that modify the C-ring of flavonoids, flavanone 3-hydroxylase (F3H), anthocyanidin synthase (ANS), flavone synthase I (FNS I) and flavonol synthase (FLS), all belong to the 2-OG dependent oxygenase family (Turnbull et al. 2004). F3H catalyzes the hydroxylation of 2*S*-naringenin, while ANS, FNS I and FLS catalyze desaturation reactions. Two distinct groups of 2-OG dependent dioxygenases for flavonoid biosynthesis have been proposed based on their sequence similarities and substrate- and stereo-selectivities: F3H/FNS I and ANS/FLS (Turnbull et al. 2004). Flavonol 6-hydroxylase from *Chrysosplenium americanum*, that catalyzes 6-hydroxylation on the A-ring, is also a 2-OG dependent dioxygenase (Anzellotti and Ibrahim 2000).

Many steps of (iso)flavonoid oxidation are catalyzed by the cytochrome P450 enzymes. All of the functionally characterized CYP93 family members catalyze oxidation reactions in the (iso)flavonoid pathway: CYP93A is a pterocarpan 6a-hydroxylase that hydroxylates on C-6a of the pterocarpan skeleton; CYP93B is a 2*S*-flavanone 2-hydroxylase that adds a hydroxyl group to the C-2 position of flavanone, which dehydrates to flavone upon acid treatment, and a flavone synthase II (FNS II) that converts flavanone directly to flavone; CYP93C is the 2-hydroxyisoflavanone synthase that catalyzes the aryl migration from C-2 to C-3 followed by 2-hydroxylation (with 3-hydroxyflavanone as a by-product) (Akashi et al. 1998, 1999a, b; Schopfer et al. 1998; Martens and Forkmann 1999). Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) hydroxylate many flavonoid substrates at the 3' position or both the 3' and 5' positions of the B-ring. F3'H and F3'5'H belong to the CYP75 family, and were first characterized at the molecular level in petunia and subsequently isolated from a number of other plant species (Bogs et al. 2006). CYP81E subfamily enzymes catalyze regiospecific 2'- and 3'-hydroxylation of isoflavones, which are essential for methylenedioxy bridge formation in maackiain and pisatin biosynthesis (Clemens and Barz 1996; Liu et al. 2003). The pterocarpan 2- and 4-dimethylallylglycinol cyclases are also cytochrome

P450 family members (Welle and Grisebach 1988; Clemens and Barz 1996).

O-methylation

O-methylation occurs on the hydroxyl groups of oxygenated (iso)flavonoid derivatives. An O-methyltransferase (OMT) transfers a methyl group from S-adenosyl-L-methionine (SAM) to an alcohol, forming a methyl ether and S-adenosyl-L-homocysteine. About 50% of isoflavonoids are 4'-methoxylated (e.g. formononetin and biochanin A) or 3',4'-methylenedioxyated and many function as phytoalexins (Dewick 1993). An isoflavone OMT activity, isoflavone 7-O-methyltransferase (I7OMT), was isolated from alfalfa and proposed to be involved in the phytoalexin response, as indicated by induction of its transcript accumulation and enzymatic activity after elicitation or fungal infection (He et al. 1998; He and Dixon 2000). The recombinant protein can catalyze the conversion of daidzein to isoformononetin (7-O-methoxy daidzein) in vitro (He et al. 1998). Since isoformononetin does not normally accumulate in plants, the in vivo function of I7OMT remains unclear. However, transgenic alfalfa over-expressing I7OMT showed increased accumulation of 4'-O-methylated isoflavonoids upon elicitation and the GFP-fused I7OMT protein was localized to the endoplasmic reticulum, the subcellular compartment for synthesis of hydroxyisoflavanone, the precursor of 4'-methoxylated isoflavonoid (He and Dixon 2000; Liu and Dixon 2001). It was therefore hypothesized that formononetin (4'-O-methoxy daidzein) was synthesized from daidzein by 4'-O-methylation and the regiospecificity of I7OMT changed from 7-hydroxyl to 4'-hydroxyl when targeted to a different subcellular compartment (Liu and Dixon 2001). Interestingly, in the crystal structure of alfalfa I7OMT, 2,7,4'-trihydroxyisoflavanone can bind to the active site when modeled by superimposing the A-ring of isoformononetin and the B-ring of 2,7,4'-trihydroxyisoflavanone (Zubieta et al. 2001).

Licorice (*Glycyrrhiza echinata*) cell cultures accumulate formononetin and medicarpin when treated with yeast elicitor (Nakamura et al. 1999). The biosynthetic route for formononetin was elucidated when 2-hydroxyisoflavanone 4'-O-methyltransferase (HI4'OMT) was cloned by screening a cDNA

expression library prepared from a yeast elicited licorice cell culture. HI4'OMT acted on 2,7,4'-trihydroxyisoflavanone to produce 2,7-dihydroxy-4'-methoxyisoflavanone, which is then dehydrated to form formononetin (Akashi et al. 2003). The licorice HI4'OMT did not display OMT activity toward daidzein and D7OMT (the alfalfa I7OMT homolog in licorice) converted daidzein to isoformononetin (7-O-methoxy daidzein) when assayed in vitro with recombinant proteins, indicating that D7OMT and HI4'OMT have different substrate regiospecificity in vitro (Akashi et al. 2003).

6a-Hydroxymaackian 3-O-methyltransferase (HM3 OMT) converts the relatively non-toxic compound 6a-hydroxymaackian to the potent antimicrobial pisatin in pea (*Pisum sativum*) (Fig. 4). HM3OMT and HI4'OMT share high similarity at the amino acid sequence level (Akashi et al. 2003). The two enzymes have overlapping substrate specificities, both acting on 2,7,4'-trihydroxyisoflavanone, (+)-medicarpin and (+)-6a-hydroxymaackian, but not (–)-6a-hydroxymaackian (Wu et al. 1997; Akashi et al. 2003, 2006). Crystal structures of *Medicago truncatula* HI4'OMT-substrate complexes revealed identical bound conformations of (2S,3R) 2,7,4'-trihydroxyisoflavanone (substrate for HI4'OMT) and (6aR,11aR) 6a-hydroxymaackian (substrate for HM3OMT), suggesting the evolutionary relatedness of the two enzymes (Liu et al. 2006). Furthermore, catalytic specificity and molecular modeling of two pea OMT isozymes suggested that pea HM3OMT evolved from HI4'OMT through an earlier gene duplication event (Akashi et al. 2006).

A genomics approach was taken to evaluate isoflavonoid O-methylation specificity in the model legume *Medicago truncatula* (Deavours et al. 2006). A number of genes related to the above-described OMTs clustered on separate branches of the type I plant small molecule OMT family. Seven of the OMTs were expressed as recombinant enzymes in *E. coli*, purified, and shown to display promiscuous activities. Interestingly, some exhibited dual regiospecificity for the 4' and 7-hydroxyl moieties of the isoflavonoid nucleus, and this observation was rationalized by protein structure homology modeling (Deavours et al. 2006). Although such an approach can reveal interesting details of in vitro catalysis, the true in vivo functions of several of these enzymes still remain obscure.

Fig. 4 Methylation reactions catalyzed by selected flavonoid *O*-methyltransferases. (a) I7OMT, isoflavone 7-*O*-methyltransferase; (b) HI4'OMT, 2-hydroxyisoflavanone 4'-*O*-methyltransferase; (c) HM3OMT, hydroxymaackiain 3-*O*-methyltransferase

C- and O-glycosylation

Glycosylation is one of the most common modifications of (iso)flavonoids. Glycosylated (iso)flavonoids become more soluble and are transported to the vacuole for storage, from where they can potentially be mobilized upon pathogen elicitation. Glycosylation also improves the bioavailability of the aglycones in humans. The sugar moieties on the flavonoid glycosides also participate in the molecular recognition of targets and are essential for activity.

C- and *O*-glycosylflavonoids are widely distributed in the plant kingdom. *O*-glycosylflavonoids can be readily hydrolyzed to their aglycone forms by either acid or enzyme treatment. In contrast, the *C*-glycosyl bond is stable and *C*-glycosylflavonoids, in contrast to *O*-glycosylflavonoids, can not be hydrolyzed to aglycones by acid or enzyme treatment. A well known class of flavonoid *O*-glycosylation products are the anthocyanins, the principle pigments in many flowers and fruits. As already described above, unstable anthocyanidin precursors are glycosylated by 3-*O*-UDP glycosyltransferase to derive the stable anthocyanins, which are then transported to the vacuole for storage. The color of the anthocyanin pigments varies depending on the structure of the glycoside (including subsequent acylation), the presence/absence of co-pigments, and the pH of the environment.

The structures of several representative flavone and isoflavone *C*-glycosides are shown in Fig. 5. Vitexin

(apigenin 8-*C*-glucoside) was first isolated from *Vitex lucens* and has been shown to inhibit thyroid peroxidase in vitro and to have antithyroid activities in vivo (Gaitan et al. 1995). Maysin (*C*-glycosyl flavone) retards growth of corn earworm larvae that feed on fresh silks and developing kernels of maize (Byrne et al. 1996). Puerarin (daidzein 8-*C*-glucoside) was isolated from the root of *Pueraria lobata* (Kudzu), a

Fig. 5 Flavonoid glycosylation. (a) Structures of representative flavone and isoflavone *C*-glycosides. (b) Glycosylation reaction catalyzed by UGT73F1. (c) Structure of quercetin

perennial legume native to East Asia, and has been reported to be efficacious in treating alcohol dependency, coronary heart disease and cardiac infarction (Lin and Li 1998; Zhang et al. 2006).

Glycosyltransferases transfer a nucleotide diphosphate activated sugar moiety to an acceptor molecule. The advancement of genomic tools has enabled the identification of a vast array of glycosyltransferases in plants. In the fully sequenced Arabidopsis genome, there are 117 putative UDP glycosyltransferase (UGT) genes identified (Ross et al. 2001) and 164 putative UGTs have been identified so far in the model legume *M. truncatula* (Achnine et al. 2005). Some of the plant natural product UGTs have been functionally characterized, and shown to catalyze *O*-glycosyl (iso)flavonoid formation and exhibit promiscuous activity toward sugar acceptors (Bowles et al. 2006). An exception to the broad substrate recognition of phenylpropanoid glycosyltransferases, a UGT specific for isoflavonoid substrates, UGT73F1, was identified from *Glycyrrhiza echinata* cell suspension culture treated with yeast elicitor (Nagashima et al. 2004). UGT73F1 converts formononetin and daidzein to their corresponding 7-*O*-glucoside derivatives efficiently, but does not use flavones or flavonols as substrate (Fig. 5). A gene encoding a *C*-glycosyltransferase has not yet been characterized from any plant. However, early labeling studies suggested that flavone *O*- and *C*-glycosides were synthesized via different pathways (Wallace et al. 1969; Wallace and Grisebach 1973). *C*-glycosylation occurs at the flavanone level and the *C*-glycosyl flavanone is oxidized to form *C*-glycosyl flavone, whereas flavanone is oxidized then glycosylated to form *O*-glycosyl flavone (Wallace et al. 1969; Wallace and Grisebach 1973).

Recently, the three dimensional structures of several plant natural product UGTs have been solved, and this has greatly facilitated the understanding of structure-function relationships in these enzymes (Shao et al. 2005; Offen et al. 2006; Li et al. 2007b). In general, plant natural product UGTs are more regio- than substrate-specific. The regiospecificity of these enzymes can further be altered by generating mutants of UGTs. For example, UGT71G1 catalyzes glycosylation on every hydroxyl group of the quercetin molecule, with 3'-*O*-glycosylated quercetin being the major product (Shao et al. 2005). The amino acids forming the sugar acceptor binding pocket in the crystal structure of UGT71G1

were mutated and the glycosyltransferase activity and substrate specificities were examined with the recombinant mutant proteins (Shao et al. 2005; He et al. 2006). The single mutation of Phe148 to Val resulted in quercetin 3-*O*-glucoside as the only product. Tyr202 is close to Phe148 in the three dimensional structure of UGT71G1, and mutation of Tyr202 to Ala also dramatically enhanced the regiospecificity of the enzyme toward the hydroxyl group at the C-3 position (He et al. 2006).

A genomics approach has been applied to identify the glycosyltransferases that are active toward quercetin in Arabidopsis (Lim et al. 2004). Out of 91 recombinant UGTs analyzed, 29 were able to glucosylate quercetin at one to four sites. Regiospecific mono- and di-glucosides were produced with purified recombinant proteins and the regioselectivity of Arabidopsis UGTs was retained when they were used as whole-cell biocatalysts in *E. coli*, a step forward for large scale regiospecific synthesis of small molecule glycosides (Lim et al. 2004).

A similar approach was recently taken to identify (iso)flavonoid *O*-glycosyltransferases from *M. truncatula*, although in this case multiple potential acceptor substrates were screened (Modolo et al. 2007). Eight glucosyltransferases were identified with activity against isoflavones, flavones, flavonols or anthocyanidins, and several showed specificity for more than one class of substrate. Phylogenetic analysis indicated that the gene sequences fell into a number of different clades, and several clustered with UGTs annotated as glycosylating non-flavonoid substrates, indicating the difficulties in ascribing functional annotations based on DNA sequence information alone. Unique tissue-specific transcript expression patterns were observed for each of the enzymes, and comparison with phytochemical profiles suggested *in vivo* functions for several of the enzymes. However, because of the promiscuous and overlapping substrate specificities recorded *in vitro*, it will be necessary to develop genetic tests before true *in vivo* functionalities can be ascribed.

C- and O-Prenylation

Prenylation, also termed isoprenylation, of (iso)flavonoids involves the addition of an isoprenyl group, e.g. from dimethylallyl pyrophosphate (DMAPP) or

geranyl pyrophosphate (GPP), to an aromatic ring. Prenylation enhances the biological activities of the prenyl acceptor molecules, presumably due to increased lipophilicity and stronger affinity or permeability to biological membranes. Comprehensive surveys on the occurrence of prenylated isoflavonoids and flavonoids in various plant species have been summarized in several excellent reviews (Tahara and Ibrahim 1995; Barron and Ibrahim 1996; Epifano et al. 2007).

Prenylation can occur on either the carbon skeleton or hydroxyl group of the (iso)flavonoid ring structures, distinguished as either *C*- or *O*-prenylation. *O*-prenylation has only been studied in the last decade. It can occur on a variety of phenolic compounds. A few *O*-prenylated chalcones, coumarins and flavonoids have been identified from plants (Epifano et al. 2007). Both DMAPP and GPP can be used as prenyl donors for *O*-prenylations. The pharmacological activities of *O*-prenylated (iso)flavonoids have not been extensively studied due to their low level of accumulation in plants, though they have been used for medicinal purposes (Epifano et al. 2007).

The *C*-prenylated (iso)flavonoids have diverse structures and are broadly distributed. The structural variation of *C*-prenylated derivatives lies in: (1) the carbon skeleton of the substrates for prenylation, which include isoflavonoid, flavonoid, coumarin and chalcone; (2) The C5 (DMAPP) or C10 (GPP) side chain; (3) the substitution position on the ring structure and the level of substitution (mono-, di- or tri- prenylated); and (4) the cyclization of the prenyl groups to produce pyrano- or furano-derivatives. For example, mono-prenylated genistein and 2'-hydroxygenistein (luteone and wightone respectively) accumulate in white lupin, and di-prenylated (with one of the DMAPP groups cyclized) genistein and 3'-hydroxy genistein (osajin and pomiferin respectively) accumulate in fruits of the osage orange (*Maclura pomifera*) (Fig. 6). Structures of representative prenylated pterocarpan, chalcones and flavanones are shown in Fig. 6.

Prenyltransferases catalyze the transfer of isoprenyl groups to a wide variety of receptors. Despite the abundance of prenylated (iso)flavonoids in plants, prenyltransferase activities have only been reported from crude plant or cell culture extracts and, in some cases, were partially purified. The plant prenyltransferases have not been characterized at the

molecular level. Biochemical characterizations of the soybean and lupin prenyltransferases have shown that they are membrane-associated enzymes (Welle and Grisebach 1991). The 3,9-dihydroxypterocarpan prenyltransferase from soybean was localized to chloroplast inner envelope membranes and could be solubilized with various detergents (Welle and Grisebach 1991). Prenylated isoflavones are produced constitutively in lupin roots from days 7–12 post-germination (Gagnon et al. 1995). Microsomal preparations of white lupin root and cell culture could catalyze the prenylation at the C-6, C-8 and C-3' positions of genistein and 2'-hydroxygenistein (LaFlamme et al. 1993). The prenyltransferase activity required Mn^{2+} and was most active at pH 7.5. Distinct enzymes were proposed to catalyze the prenylation reactions based on the correlation between positional specific activity and the quantity of detergent applied (LaFlamme et al. 1993).

Two classes of prenylated compounds exist in hop glandular trichomes: bitter acids and prenylated flavonoids. The two related prenylated chalcones desmethylxanthohumol and xanthohumol are stable in the trichomes but unstable in hop extract and isomerize to 8-prenyl naringenin, the most potent phytoestrogen, and isoxanthohumol, respectively (Fig. 6). Using protein extracts from hop flowers and cones, it was shown that the prenyltransferase activity for bitter acid synthesis is soluble, in contrast to the membrane-bound lupin and soybean prenyltransferases (Zuurbier et al. 1998). However, it is unclear whether the same prenyltransferase(s) is involved in both xanthohumol and bitter acid biosynthesis.

The soil bacterium *Streptomyces* produces potent antibiotics that are prenylated derivatives of small aromatic molecules, such as aminocoumarin and naphterpin (Fig. 6). A class of bacterial prenyltransferases has been characterized in recent years, including CloQ and NovQ that attach a DMAPP group to C-3 of 4-hydroxyphenylpyruvate, Orf2 for naphterpin biosynthesis, and Fnq26 for furanonaphthoquinone I biosynthesis (Pojer et al. 2003; Kuzuyama et al. 2005; Haagen et al. 2007). These bacterial prenyltransferases are monomeric, soluble, do not contain the prenyl diphosphate binding motif (N/D)DXXD common to UbiA family aromatic prenyltransferases, and do not depend on Mg^{2+} for activity except for Orf2. CloQ and NovQ

Fig. 6 Structures of prenylated phenylpropanoids from (a) lupin, (b) Osage orange, (c) hop and (d) bean and soybean; (e) *O*-prenylated chalcones and flavonoids; (f) Reaction products catalyzed by bacterial prenyltransferases

use DMAPP as the prenyl donor; whereas Orf2 and Fnq26 use GPP as the prenyl donor and catalyze both *C*- and *O*-geranylation of different aromatic substrates. Fnq26 is a unique member of the bacterial

prenyltransferase family in that it attaches C-3 rather than C-1 of the prenyl group to the aromatic substrate, which is termed a “reverse” prenylation (Haagen et al. 2007). The three dimensional structure

of Orf2 has been solved and represents a new type of antiparallel β , α barrel fold. The ten antiparallel β strands form a barrel that contains the spacious binding sites for aromatic substrates and prenyldiphosphate. Consistent with the structure prediction, Orf2 shows relaxed specificity toward aromatic substrates, including isoflavones, flavonoids and stilbenes (Kuzuyama et al. 2005).

Conclusions and perspectives

With the exception of plant derived prenyltransferases and C-glycosyltransferases, genes encoding oxygenases, methyltransferases and O-glycosyltransferases have been cloned and characterized from various plant species and are available for in vitro and in planta manipulation of bioactive (iso)flavonoid accumulation. Several strategies focusing on different properties of the modification enzymes can be applied for enzyme and/or genetic engineering.

- (1) Regiospecificity of the enzyme. Regiospecific chemical synthesis is often complicated due to the demand for adding and removing protecting groups. Regiospecific (iso)flavonoid hydroxylases, methyltransferases and glycosyltransferases have been either isolated in their native forms or constructed through mutations of amino acid(s) at the active sites of the wild type enzymes. These enzymes will facilitate regio-specific introduction of hydroxyl, methyl and glycosyl groups to (iso)flavonoid substrates.
- (2) Substrate specificity of the enzyme. Site-directed mutagenesis, domain swapping and directed evolution can be applied to alter/improve the substrate specificity of an enzyme. The modified enzymes can be used as biocatalysts in vitro or can be introduced into plant systems.
- (3) Promiscuity of the enzyme. Bacterial enzymes with relaxed substrate specificity can be used as an alternative for enzyme engineering when the plant derived enzymes are not isolated. For example, bacterial prenyltransferases (e.g. Orf2) are soluble and recognize (iso)flavonoids as substrate, making them potentially more suitable for engineered biocatalysis than the membrane bound plant prenyltransferases yet to be characterized at the molecular level.

- (4) A combination of different modification enzymes. Manipulation of F3H, F3'5'H and FLS expression levels, together with ectopic expression of DFR and glycosyltransferases, has generated flowers with colors that are not produced by the native plants (Tanaka et al. 1998; Zuker et al. 2002; Fukui et al. 2003; Mizutani et al. 2003; Ono et al. 2006). Similar strategies can be applied to alter the oxidation, methylation and glycosylation levels of (iso)flavonoid compounds for beneficial anti-microbial and health promoting activities.

Engineered isoflavones accumulate as glycosides, presumably in the cellular vacuole. It appears that endogenous glycosylation and transport systems can “handle” some novel compounds (eg. genistein in tobacco) and “process” them correctly. PA engineering may be more challenging, since less is understood of the mechanisms whereby the monomeric units (catechins and epicatechins) are transported, polymerized, and delivered to their final destination (vacuole or cell wall). Success in PA engineering may ultimately involve manipulations to cell biological as well as biochemical processes.

In vitro enzyme engineering does not require knowledge of an enzyme's substrate specificity in planta and is relatively straightforward. High throughput approaches are available to screen a broad range of potential substrates, which provides a new frontier for developing novel anticancer and antimicrobial compounds. A post-engineering evaluation pipeline should be constructed where the structure of the novel compounds will be determined and their efficacy tested through in vitro bioassays and evaluated using animal models, cancer and tumor cell lines, and eventually through clinical trials. Engineering bioactive (iso)flavonoid accumulation in plants has the advantage of readily available substrate pools. Furthermore, the overall substrate level can be enhanced by ectopic expression of transcriptional regulators. However, besides the above mentioned bottlenecks for in planta genetic engineering, other complications include potential pleiotropic effects on plant growth, development and reproduction caused by metabolic perturbations. The physiological status of the transgenic plants should be carefully and thoroughly examined and the added antimicrobial and antifungal activities should also be assessed.

Acknowledgements We thank Dr. Gregory Peel for critical reading of the manuscript.

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