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Flavone synthase II (CYP93B16) from soybean (*Glycine max* L.)

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ABSTRACT

Flavonoids are a very diverse group of plant secondary metabolites with a wide array of activities in plants, as well as in nutrition and health. All flavonoids are derived from a limited number of flavanone intermediates, which serve as substrates for a variety of enzyme activities, enabling the generation of diversity in flavonoid structures. Flavonoids can be characteristic metabolites, like isoflavonoids for legumes. Others, like flavones, occur in nearly all plants. Interestingly, there exist two fundamentally different enzymatic systems able to directly generate flavones from flavanones, flavone synthase (FNS) I and II. We describe an inducible flavone synthase activity from soybean (*Glycine max*) cell cultures, generating 7,4′-dihydroxyflavone (DHF), which we classified as FNS II. The corresponding full-length cDNA (CYP93B16) was isolated using known FNS II sequences from other plants. Functional expression in yeast allowed the detailed biochemical characterization of the catalytic activity of FNS II. A direct conversion of flavanones such as liquiritigenin, naringenin, and eriodictyol into the corresponding flavones DHF, apigenin and luteolin, respectively, was demonstrated. The enzymatic reaction of FNS II was stereoselective, favouring the (*S*)- over the (*R*)-enantiomer. Phylogenetic analyses of the subfamily of plant CYP93B enzymes indicate the evolution of a gene encoding a flavone synthase which originally catalyzed the direct conversion of flavanones into flavones, via early gene duplication into a less efficient enzyme with an altered catalytic mechanism. Ultimately, this allowed the evolution of the legume-specific isoflavonoid synthase activity.

1. Introduction

Flavonoids represent a huge and diverse group of secondary plant metabolites which can be found in all vascular plants and in various tissues (Harborne and Baxter, 1999). In plants, these compounds have various functions in physiology, biochemistry, and ecology. For example, flavonoids are involved in UV-protection, flower coloration, interspecies interaction, and plant defence. In addition, flavonoids are useful tools in phylogenetic studies. Due to the antioxidant or putative anticancer activities of certain flavonoids, they also have nutritional values and medicinal benefits to humans (Dixon and Steele, 1999; Martens and Mithöfer, 2005).

The crucial reaction in the biosynthesis of flavonoids is the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to a chalcone intermediate. Flavonoids are derived from this intermediate after conversion of the chalcone to the corresponding flavanone. Flavanones display the basic structures for all other flavonoid-classes, including flavanols, anthocyanidines, flavonols, flavones, and isoflavones (Harborne and Baxter, 1999; Martens and Mithöfer, 2005). The biosynthesis of flavones from flavanones involves the introduction of a double bond between C2 and C3 in the heterocyclic ring of the flavanone skeleton (Fig. 1). Double bond formation can be catalyzed by two different flavone synthase proteins (FNS I and FNS II), a unique feature within the flavonoid biosynthesis. FNS I, a soluble 2-oxoglutarate- and Fe^{2+}-dependent dioxygenase (Britsch, 1990), is mainly described for Apiaceae, and has only recently been identified from monocotyledonous plants as well (Kim et al., 2008). In contrast, FNS II, a membrane-bound cytochrome P450 monooxygenase, which requires NADPH and molecular oxygen (Heller and Forkmann, 1993), is widespread among higher plants (Martens and Mithöfer, 2005). All FNS II proteins known belong to the plant cytochrome P450 subfamily CYP93B. However, their catalytic mechanisms

Abbreviations: FNS, flavone synthase; 12-OPDA, 12-oxo-phytodienoic acid; DHF, 7,4′-dihydroxyflavone; RACE, rapid amplification of cDNA ends; JA, jasmonic acid; F2H, flavanone 2-hydroxylase; IFS, 2-hydroxyisoflavanone synthase (isoflavonoid synthase).

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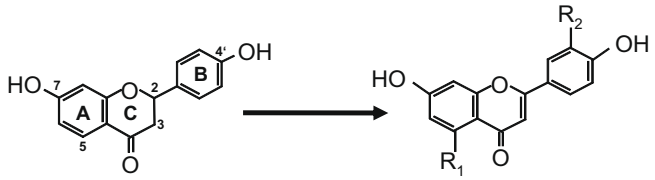

Flavanone	R_1	R_2	Flavone
Liquiritigenin	H	H	7,4'-Dihydroxyflavone
Naringenin	OH	H	Apigenin
Eriodictyol	OH	OH	Luteolin

Fig. 1. Flavone formation catalyzed by flavone synthase II.

might differ from each other. On the one hand, various recombinant proteins heterologously expressed from cloned FNS II-encoding genes showed a direct conversion of flavanones into the corresponding flavones, such as the FNS II from *Gerbera* hybrids (CYP93B2) (Martens and Forkmann, 1999), *Antirrhinum majus* (CYP93B3), *Torenia hybrida* (CYP93B4) (Akashi et al., 1999), and *Perilla frutescens* (CYP93B6) (Kitada et al., 2001). On the other hand, for CYP93B1 from *Glycyrrhiza echinata* (Akashi et al., 1998) and CYP93B10 and 11 from *Medicago truncatula* (Zhang et al., 2007), a 2-OH-flavanone intermediate was suggested that might be further converted, enzymatically or chemically, into flavones. Thus, the latter enzymes can be classified as flavanone 2-hydroxylases.

Here, we show the isolation and heterologous expression of a novel, oxylipin-induced FNS II from soybean (*Glycine max*) as well as the accumulation of its main product, 7,4′-Dihydroxyflavone, in oxylipin-treated soybean tissues. The biochemical characterization indicates a direct and stereoselective formation of the flavone from the flavanone substrate. CYP93B16 from soybean represents therefore the first FNS II known from a leguminous plant which is capable to catalyze the conversion of flavanones directly into flavones.

2. Results and discussion

2.1. Flavone synthesis in soybean cell cultures

Flavones are a large class of plant secondary metabolites which exhibit many different biological functions, such as, for example, antioxidants or UV-protectants, or in plant–microbe interactions (Martens and Mithöfer, 2005). They are synthesized from flavanones, either directly via the action of flavone synthases (either type I or type II), or indirectly via *C*-glycosylated intermediates (Brazier-Hicks et al., 2009; Martens and Mithöfer, 2005). Soybean cell cultures were reported to accumulate the flavone 7,4′-dihydroxyflavone (DHF) after treatment with coronalon, a synthetic jasmonate analogon (Schüler et al., 2004; Fig. S1). For osmotic stress, the induction of a FNS II has already been described (Kochs and Grisebach, 1987; Kochs et al., 1987). In order to identify the underlying enzymatic activity for the generation of DHF by coronalon, induced soybean cell cultures were analyzed for both flavone synthase activities known in plants, FNS I and FNS II. As reported in Table 1, only FNS II activity was detected, which was shown to be enhanced 15-fold by coronalon treatment. FNS I activity could not be found.

As coronalon might mimic both jasmonic acid (JA) as well as its biosynthetic precursor 12-oxo-phytodienoic acid (12-OPDA) (Mithöfer et al., 2005), we compared the efficiency of these three compounds to induce DHF accumulation after 48 h in soybean cell cultures. Whereas JA at a concentration of 100 μM was a comparatively weak inductor, resulting in only about 8 nmol DHF g FW^{-1} (control showed 0.13 nmol DHF g FW^{-1}), 12-OPDA at a concentration of 34 μM induced the production of up to 85 nmol and 10 μM coronalon (Schüler et al., 2004) induced about 40 nmol DHF g FW^{-1}, respectively. Subsequent dose–response analyses revealed EC_{50} values of 7 μM for 12-OPDA and of 0.33 μM for coronalon, respectively.

Table 1
FNS I and FNS II activities in soybean cell cultures treated with coronalon. Soybean cell cultures were treated for 96 h with 2.5 μM coronalon. Soluble and microsomal proteins were extracted and used for FNS I and FNS II specific enzyme assays with liquiritigenin as substrate. DHF as product was determined and quantified by HPLC to calculate the specific enzyme activities. For both, control and induced cell cultures, three independently treated cell samples were pooled for analysis.

Enzyme	Enzyme activity (pkat mg $protein^{-1}$)	
	Control	Induced
FNS I	n.d.	n.d.
FNS II	0.12	1.83

FNS, flavone synthase; n.d., not detected.

2.2. Identification of GmFNS II cDNA from soybean cell cultures

In order to analyse the properties of the enzyme responsible for the inducible accumulation of DHF in soybean cells, a cDNA encoding a putative FNSII was isolated as described in Section 4.4. The cDNA showed an open reading frame of 1581 bp corresponding to 527 amino acid residues (Fig. S2). Northern blotting experiments were used to monitor the time course of transcript accumulation for the putative FNS II in soybean cell cultures in comparison to genes encoding "early" and "late" enzymes of the inducible phenylpropanoid metabolism. Both, the "early" chalcone synthase, as well as the "late" dihydropterocarpan 6a-hydroxylase (CYP93A1), were transiently induced by coronalon at 12 and 24 h, respectively. Transcripts cross-hybridizing with the isolated putative cDNA for FNS II showed to accumulate at even later time points, which coincided with the observed late accumulation of DHF (Fig. S1). The deduced amino acid sequence of the cDNA was 56.6% and 49.7% identical to CYP93B2 from *Gerbera* hybrids and CYP93B1 from *G. echinata*, respectively. The encoded protein was therefore classified as a new member of the CYP93B subfamily, CYP93B16 (D. Nelson, pers. communication).

The recent release of the first chromosome-scale assembly of the soybean genome (http://www.phytozome.net/soybean; Soybean Genome Project, DoE Joint Genome Institute) allowed a first glance on the genomic organization of the gene encoding CYP93B16. A transcript corresponding to the *Gm*FNS II cDNA is predicted on chromosome 12 and was named Glyma12g07190.1 (Fig. S2). A duplication of this gene with 95% identical nucleotide positions in the protein coding sequence is located 6200 base pairs further downstream on chromosome 12 (Glyma12g07200.1). Both genes contain one intron each, highly divergent in sequence and size (Fig. S2). Interestingly, only the gene corresponding to the cloned cDNA encoding CYP93B16 seems to be expressed, since no transcripts corresponding to the second gene can be retrieved from the databases (Fig. S2, and data not shown).

2.3. Heterologous expression and biochemical characterization of CYP93B16

To determine and prove the enzymatic activity of the putative FNS II from soybean, the open reading frame encoding CYP93B16 was inserted into the pYeDP60 vector (Urban et al., 1990). The recombinant protein was expressed in a yeast strain that co-expresses the yeast NADPH-cytochrome P450 reductase gene (*Saccharomyces cerevisiae* W(R)) (Latunde-Dada et al., 2001; Truan

et al., 1993). To characterize the metabolite formed by CYP93B16, microsome suspensions of recombinant yeast cells were incubated with the flavanone liquiritigenin and NADPH. The HOAc/EtOAc extract of the reaction mixture was analyzed by RP-HPLC (Fig. 2) and the only product formed was identified as DHF with authentic samples and by mass spectroscopy. No product was detected from microsomes isolated from yeast cells transformed with the empty vector. The enzymatic reaction was dependent on the presence of NADPH, with a pH optimum at pH 7.9 and a temperature optimum at 25 °C. Using these optimized conditions, the substrate efficiency of *Gm*FNS II for three different flavanones, liquiritigenin, naringenin, and eriodictyol was determined by quantifying the production of their corresponding flavones, DHF, apigenin, and luteolin, respectively. The enzyme was able to accept all substrates with affinities in the low μM range (K_m between 1.8 μM (eriodictyol) and 4.2 μM (liquiritigenin); Fig. S3; Table 2). The efficiency to convert the different flavanones into the corresponding flavones (rel. $V_{max} K_m^{-1}$) was about two times higher for naringenin than for liquiritigenin and eriodictyol, respectively (Table 2). These values in the low μM range are similar to those available for the few recombinant FNS II enzymes, for which quantitative assays have been described up to now. Recombinant FNS II from *Gentiana triflora* (CYP93B13) exhibited K_m values of 8.9 and 19.1 μM with naringenin and eriodictyol, respectively (Nakatsuka et al., 2006). Recombinant CYP93B6 from *P. frutescens* exhibited K_m values of 8.8 μM for naringenin and 11.9 μM for eriodictyol (Kitada et al., 2001). In all cases, the enzymes converted naringenin more efficiently than the other tested flavanones.

Within the CYP93B subfamily, two groups of enzymes have been characterized: one group that directly converts the flavanone substrates into the corresponding flavone products without any free intermediate; and a second group, where the enzymes act as flavanone 2-hydroxylases (CYP93B1 from *G. echinata* and CYP93B10 and 11 from *M. truncatula*). In this latter group, the mechanism of the final conversion of the 2-hydroxylated flavanones into flavones is still unknown. In the case of heterologously expressed CYP93B1 from *G. echinata*, the 2-hydroxyflavanone products were converted to flavones *in vitro* only on acid treatment, suggesting the involvement of an additional plant enzyme, a dehydratase, for the formation of the flavones *in vivo* (Akashi et al., 1998). Zhang et al. (2007) assumed that an endogenous yeast dehydratase was responsible to form flavones from 2-hydroxyflavanones when FNS II-1 (CYP93B10) and FNS II-2 (CYP93B11) from *M. truncatula* were expressed in yeast cells. The novel CYP93B16 isolated from soybean directly converted the substrates into the corresponding flavones. Acid treatment was not necessary for the formation of the flavone products nor could any by-product, representing a 2-hydroxyflavanone, be detected in the experiments performed.

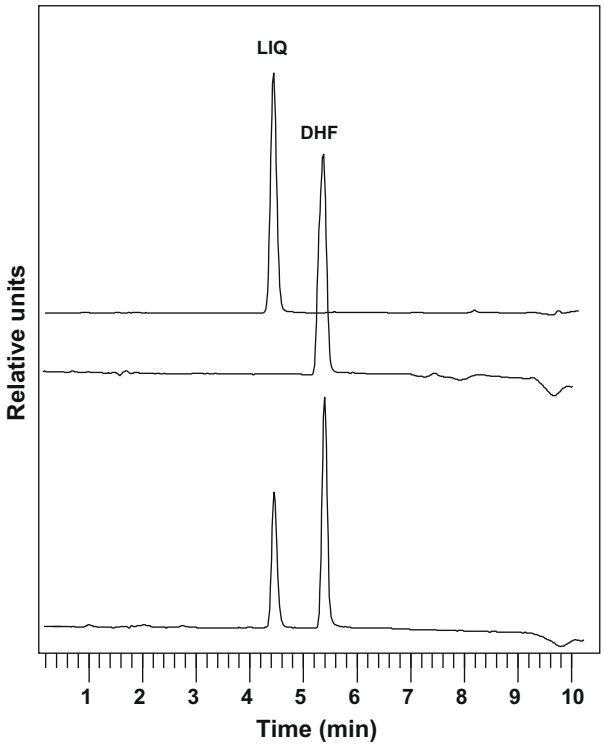

Fig. 2. HPLC analysis of liquiritigenin conversion in the FNS II assay. Upper part: standards of liquiritigenin (LIQ) and 7,4′-dihydroxyflavone (DHF). Lower part: extracts of reaction mixtures after incubation of liquiritigenin and NADPH with a microsomal fraction from yeast cells expressing CYP93B16.

Table 2
Substrate specificity of *Gm*FNS II.

Flavanone	rel. V_{max}	K_m (μM)	rel. $V_{max} K_m^{-1}$ (μM^{-1})
Liquiritigenin	100	4.2	23.8
Naringenin	116	2.5	46.4
Eriodictyol	47	1.8	26.1

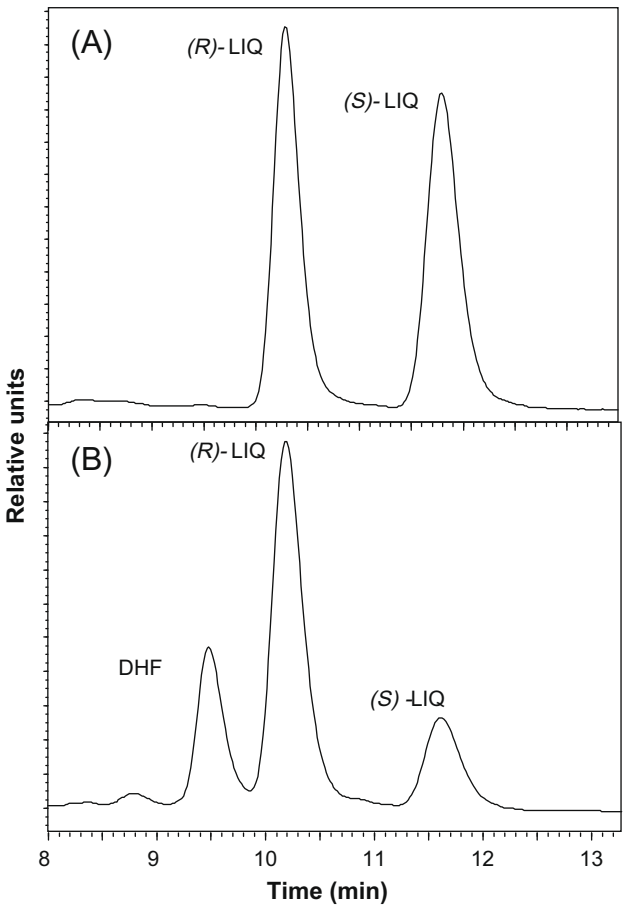

Fig. 3. HPLC analysis of (*R*)- and (*S*)-liquiritigenin enantiomers after conversion in the FNS II assay. (A) Separation of (*R*)- and (*S*)-liquiritigenin (LIQ) on a chiral resolution column. (B) Separation of the reaction mixture after incubation of (*R*)- and (*S*)-liquiritigenin and NADPH with a microsomal fraction from yeast cells expressing CYP93B16. In (A) and (B), the same initial amounts of (*R*)- and (*S*)-liquiritigenin were used.

To investigate the enantiomer selectivity of the *Gm*FNS II-catalyzed reaction, a chiral resolution column, Chiralpak IA, was employed in HPLC analysis. This column was able to separate the (*S*)- and (*R*)-enantiomers of liquiritigenin (Fig. 3A). Only the (*S*)-enantiomer was accepted as substrate (Fig. 3B; Table 3). Interestingly, CYP93B1 from *G. echinata*, which in fact is a flavanone 2-hydroxylase, showed the same selectivity for the (*S*)-enantiomer (Akashi et al., 1998). Increasing concentrations of (*R*)-liquiritigenin, compared to (*S*)-liquiritigenin, in mixtures of both forms inhibited the conversion rate of the soybean enzyme for the (*S*)-form (Table 3). This result suggested the competition of both enantiomers at the same binding site of FNS II. Taken together, these results clearly indicate that the isolated cDNA from soybean encodes a functional FNS II.

Table 3
Enantiomer selectivity of *Gm*FNS II.

Liquiritigenin	FNS II activity (μkat kg^{-1})	Conversion (%)
(*R*)	n.d.	n.d.
(*S*)	14.2 ± 1.1	9.3
(*R*) + (*S*) (1:1)	13.6 ± 0.3	8.9
(*R*) + (*S*) (2:1)	12.8 ± 0.2	8.4
(*R*) + (*S*) (6:1)	7.01 ± 0.8	4.6

FNS, flavone synthase; n.d., not detected.

2.4. Phylogeny of the CYP93B subfamily

CYP93B16 from soybean represents the first FNS II characterized from a leguminous plant which is capable to directly form the flavone from a flavanone precursor. This direct mode of conversion was up to now only reported from Asterales, Lamiales, and Gentianales, whereas the studies on enzymes from the Fabales (*G. echinata* CYP93B1 and *M. truncatula* CYP93B10 and 11) identified them to be flavanone 2-hydroxylases (F2H) rather than flavone synthases. The biochemical diversity is mirrored by the phylogenetic analysis of the subfamily CYP93B (Fig. 4). A clear bifurcation separates the flavanone 2-hydroxylases (CYP93B1, 10, and 11) from all other members of the CYP93B subfamily, for all of which the direct conversion can be predicted, if not experimentally shown already. A CYP93 protein containing flavone synthase activity has been considered to be the ancestor of the present day CYP93 family (Sawada and Ayabe, 2005), which, in addition to FNS and F2H, includes 2-hydroxyisoflavanone synthase (IFS, CYP93C) and pterocarpan 6a-hydroxylase (CYP93A) (Sawada and Ayabe, 2005; Schopfer et al., 1998). Strikingly, a deletion of eight amino acid residues correlates with the disability of the F2H enzymes to introduce the double bond between C2 and C3 of the 2-hydroxylated flavanone to form flavones directly (Fig. S4); however, this finding needs to be confirmed experimentally. A gene duplication event early in the evolution of Leguminosae was postulated (Sawada and Ayabe, 2005), which resulted in the generation of enzymes

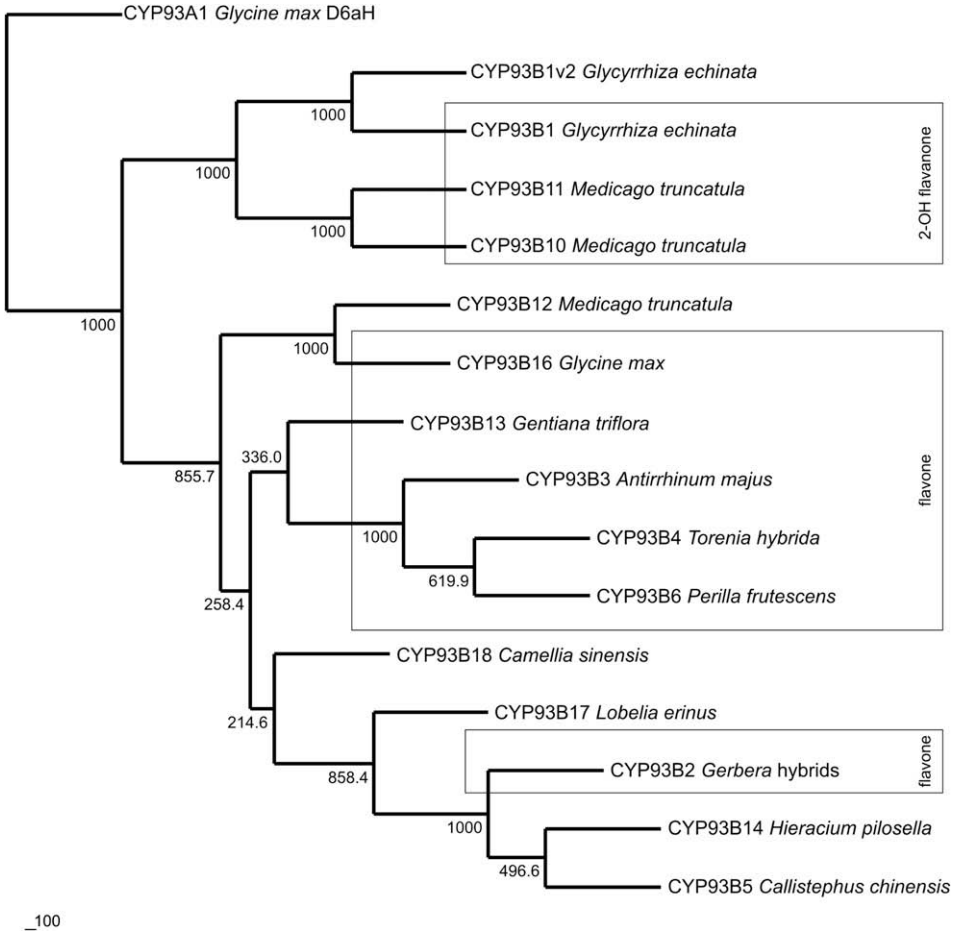

Fig. 4. Phylogenetic analysis of the CYP93B subfamily. The DNA parsimony method was applied on a multiple sequence alignment after exclusion of unaligned overhanging positions (see Fig. S4). Bootstrap values for 1000 replicates are indicated on the consensus tree shown. The boxes group those species for which the enzymatic activity was reported according to the final product of the enzymatic conversion of flavanones to either 2-hydroxyflavanone or flavone. For the description of the species included in the analyses, see Table S2. CYP93A1 (D6aH) from *Glycine max* served as an outgroup.

capable to hydroxylate C2, which is one precondition for the rather unusual aryl migration forming isoflavonoids, a branch of flavonoids present predominantly in leguminous plants, as shown so far. However, the parent was preserved during evolution as well, as shown by the ancient, FNS-type reaction of CYP93B16 in soybean.

Interestingly, *M. truncatula* is up to now the only leguminous plant for which ancient (FNS) as well as modern (F2H) CYP93B activities can be predicted. CYP93B10 and 11 have been shown to be flavanone 2-hydroxylases (Zhang et al., 2007), whereas CYP93B12 clusters unambiguously with FNS in the phylogenetic reconstruction (Fig. 4). Moreover, according to the Medicago Gene Expression Atlas (Benedito et al., 2008), the expression pattern of CYP93B10/11 versus CYP93B12 seems to be mutually exclusive, with the former being expressed in roots and nodules and the latter being expressed mainly in the aerial parts of the plant. These data might indicate that, in a given tissue, always only one type of FNS II reaction will be performed, the reason for which would be interesting to study in the model legume *M. truncatula*.

3. Concluding remarks

Recently, flavone synthases were shown to be involved in mutualistic as well as parasitic interactions of *M. truncatula* (Uppalapati et al., 2009; Zhang et al., 2007, 2009). RNAi-mediated silencing of CYP93B10 and 11 strongly reduced both, the amount of 7,4′-dihydroxyflavone and the level of nodulation in *M. truncatula* roots, which could be restored by exogenous application of DHF. This effect was independent of a pretreatment of the rhizobia with *nod*-gene inducing flavones, which suggested a dual role of flavones in the rhizobial symbiosis of *M. truncatula* (Zhang et al., 2007, 2009). DHF might play a role during the infection of *M. truncatula* roots with the necrotrophic fungus *Phymatotrichopsis omnivora* as well since flavone synthase II (CYP93B10/11) was found to be highly induced early during the interaction (Uppalapati et al., 2009). Interestingly, ethylene and jasmonic acid signalling pathways were activated during the interaction as well, emphasizing our observation that JA-related compounds are potent elicitors of DHF production in soybean cell cultures. Whether, in soybean, flavones are involved in disease and symbiosis, as it is obviously the case in *M. truncatula*, needs to be investigated.

4. Experimental

4.1. General experimental procedures

12-Oxo-phytodienoic acid (12-OPDA) was purchased from Cayman Chemicals, Hornby, Canada. 6-Ethyl-1-oxo-indanoyl isoleucine methyl ester (coronalon) was synthesized as described (Schüler et al., 2001). Naringenin (5,7,4′-trihydroxyflavanone), eriodictyol (5,7,3′4′-tetrahydroxyflavanone), apigenin (5,7,4′-trihydroxyflavone), and luteolin (5,7,3′,4′-tetrahydroxyflavone) were from the lab collection, liquiritigenin (7,4′-dihydroxyflavanone) and 7,4′-dihydroxyflavone (DHF) from Extrasynthèse, Genay, France.

4.2. Biological materials

Soybean (*G. max* L. cv. Harosoy 63) cell suspension cultures were grown as described (Hille et al., 1982). Five-day-old cultures were used for induction studies, extracted and the samples prepared as described (Fliegmann et al., 2003). The plasmid pYeDP60 (provided by D. Pompon, Gif-sur-Yvette, France) (Urban et al., 1990) was used for recombinant protein synthesis in *S. cerevisiae* strain W(R). This strain had previously been engineered to express the yeast CPR (NADPH-cytochrome P450 reductase) upon galactose induction (Truan et al., 1993) and was provided by Rhône-Poulenc Agro (Lyon, France).

4.3. HPLC analysis

Organic residues were dissolved in 200 μl EtOH and analyzed by HPLC (Nova-Pak C_{18}, 4 μm, 4.6 × 150 mm (Waters); flow rate 1 ml min^{-1}; linear gradient either 40–75% (v/v) MeOH in 7 min for the separation of liquiritigenin and DHF, or 25–75% (v/v) MeOH in 18 min) and monitored at 330 nm.

4.4. Cloning of CYP93B16 cDNA, expression in yeast, and phylogenetic analysis

The isolation of a cDNA encoding a putative FNS II was performed in multiple steps. First, a fragment of 352 bp (pos. 411–763, Fig. S2) was amplified from genomic DNA of soybean using degenerated oligonucleotides cons93b-f1/r1 (provided by S. Martens; see supplemental Table S1 for all oligonucleotides used). By comparing the cDNAs encoding CYP93B enzymes from *G. echinata*, *Gerbera* hybrids, *A. majus*, *T. hybrida*, *Callistephus chinensis*, and *P. frutescens*, versus those encoding CYP93A enzymes from soybean (see Table S2 for all accessions used), a degenerated oligonucleotide (cons93b-r2) was designed which enabled the distinction of the closely related gene families in soybean. It was employed, in conjunction with the oligonucleotide gm93b-f2, derived from the first partial soybean DNA sequence, in a RT-PCR reaction using RNA isolated from soybean cell cultures treated for 60 h with 2.5 μM coronalon. The resulting fragment of 919 bp overlapped over 97 bp with the first fragment (Fig. S2). RACE (rapid amplification of cDNA ends; (Frohman et al., 1988)) was used to isolate the missing 5′ and 3′ regions of the cDNA, by using RNA isolated from soybean cell cultures, elicited with coronalon for 96 h. For 5′RACE (Mithöfer et al., 2000) the gene-specific primers GSP1 (for reverse transcription), GSP2, and adaptor primers *bsh–t17* and *bsh* were used (Fliegmann and Sandermann, 1997). 3′RACE was performed accordingly using the gene-specific oligonucleotide GSP3 in combination with *bsh* after reverse-transcription with *bsh-t17*. The open reading frame encoding CYP93B16 was amplified using the oligonucleotide pair gm93b-f3/r3 and proof-reading *Pfu*-polymerase (Promega), subcloned after adenylation into pGEM-T (Promega), and sequenced. Using the restriction sites inserted at the 5′ and the 3′-ends, the open reading frame was released and inserted into appropriately prepared pYeDP60. Yeast was transformed, grown and induced for protein synthesis at 30 °C for 2 days in SC/gal Ura^- medium as described (Fliegmann et al., 2005).

An alignment of all 15 open reading frames encoding CYP93Bs (including the corrected version of CYP93B12 of *M. truncatula*, see supplemental Fig. S5) and one encoding CYP93A1 from soybean was generated by using the Webinterface Multalin (Corpet, 1988). 5′ and 3′-non overlapping regions of the alignment were removed, guided by an amino acid sequence alignment (Fig. S4). The truncated nucleic acid sequence alignment was fed into the phylogeny interference package version 3.66 (Felsenstein, 2004) and was used to estimate the phylogeny by running seqboot (1000 replicates, random number seed 4533), dnapars (random number seed 4533, 33 jumbles, 1818 trees generated), and consense. The consense tree was drawn by using TreeView 1.6.6 (Page, 1996) and the bootstrap values were added manually according to the output file from consense.

4.5. Enzyme assays

For protein extraction, treated and non-treated cells were harvested at the time indicated and separated from the medium.

Soybean cells (8 g), frozen in liquid nitrogen, were homogenized by mortar and pestle and resuspended in 10 ml 0.1 M Tris/HCl, pH 7.5, 0.1% 2-mercaptoethanol in the presence of 2 g Dowex 1 × 2. After centrifugation at 9000*g* at 4 °C for 10 min, the microsomal fraction was collected from the supernatant by adding $MgCl_2$ (final concentration 30 mM) followed by a second centrifugation step at 150,000*g* and 4 °C for 25 min. The pellet obtained was suspended in 500 µl of 0.1 M Tris/HCl, pH 7.5, 0.02% 2-mercaptoethanol and used for enzyme assays as well as the supernatant of the second centrifugation. Microsomes from induced yeast cells were prepared according to Urban et al. (1994) by mechanical disruption using glass beads and recovered by centrifugation for 1 h at 106,000*g*. The pellet was suspended in 50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 20% (v/v) glycerol and stored at −80 °C. Protein was determined as described (Bradford, 1976), using bovine serum albumin as standard.

The enzyme assay for FNS II was performed according to Martens and Forkmann (1998). Briefly, the incubation mixture (final volume 200 µl) contained: 0.1 M Tricine/KOH, pH 7.9, 0.1 mM liquiritigenin, 0.5 mM reduced glutathione, 1 mM NADPH, and 50 µg of crude protein fraction. The enzyme assay for FNS I was carried out according to (Britsch, 1990) in 1 ml final volume. The incubation mixture contained: 0.1 M Tricine/KOH, pH 7.5, 0.1 mM liquiritigenin, 0.5 mM reduced glutathione, 0.25 mM 2-oxo-glutarate, 5 mM ascorbic acid, 15 µM $Fe(II)SO_4$, and 50 µg of crude protein fraction. After 30 min at 25 °C, each reaction was stopped and the mixture extracted twice by an equal volume of EtOAc. For the separation of liquiritigenin and DHF, the pooled organic solvent was evaporated and the remaining compounds used for HPLC analysis as described above. When naringenin and eriodictyol were assayed as substrates, products were analyzed using linear gradients comprising 1% HOAc in MeOH–acetonitrile (1:1, solvent A) and 1% HOAc in H_2O (solvent B; 25–75% of solvent A within 14 min) (Latunde-Dada et al., 2001). For the separation of (*S*)-and (*R*)-forms of liquiritigenin, chiral resolution HPLC with Chiralpak IA (4.6 × 150 mm; Chiral Technologies Europe, Ilkirch Cedex, France) was used. The eluent consisted of 100% MeOH at a flow rate of 0.5 ml min^{-1} at 25 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.01.007.

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