Plant Physiology, November 1999, Vol. 121, pp. 821–828, www.plantphysiol.org © 1999 American Society of Plant Physiologists

Cloning and Functional Expression of a Cytochrome P450 cDNA Encoding 2-Hydroxyisoflavanone Synthase Involved in Biosynthesis of the Isoflavonoid Skeleton in Licorice[1]

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Isoflavonoids are distributed predominantly in leguminous plants and play critical roles in plant physiology. A cytochrome P450 (P450), 2-hydroxyisoflavanone synthase, is the key enzyme in their biosynthesis. In cultured licorice (*Glycyrrhiza echinata* L., Fabaceae) cells, the production of both an isoflavonoid-derived phytoalexin (medicarpin) and a retrochalcone (echinatin) is rapidly induced upon elicitation. In this study, we obtained a full-length P450 cDNA, CYP Ge-8 (CYP93C2), from the cDNA library of elicited *G. echinata* cells. When the flavanones liquiritigenin and naringenin were incubated with the recombinant yeast microsome expressing CYP93C2, major products emerged and were readily converted to the isoflavones daidzein and genistein by acid treatment. The chemical structures of the products from liquiritigenin (2-hydroxyisoflavanone and isoflavone) were confirmed by mass spectrometry. CYP93C2 was thus shown to encode 2-hydroxyisoflavanone synthase, which catalyzes the hydroxylation associated with 1,2-aryl migration of flavanones. Northern-blot analysis revealed that transcripts of CYP93C2, in addition to those of other P450s involved in phenylpropanoid/flavonoid pathways, transiently accumulate upon elicitation.

Isoflavonoids form a distinct class among flavonoids and have a characteristic structure. Flavonoids in general have linear C_6-C_3-C_6 skeletons derived from a phenylpropanoid (C_6-C_3) starter and three C_2 elongation units, whereas the C_3 part is rearranged in the isoflavonoids (Fig. 1). Isoflavonoids are distributed almost exclusively in leguminous plants (Fabaceae) and play essential roles in interactions with other organisms in the environment (Dewick, 1993). Compounds possessing pterocarpan and isoflavan skeletons are the most abundant antimicrobial phytoalexins and participate in the defense responses of legumes against phytopathogenic microorganisms (Smith and Banks, 1986; Barz and Welle, 1992; Dixon et al., 1995). Isoflavones also act as signals in the early steps of symbiosis between the soybean root and soil bacteria (*Rhizobium* spp.) to form nitrogen-fixing root nodules (Phillips, 1992; Spaink, 1995). In addition, several isoflavonoids (e.g. rotenoids and coumestans) deter insect feeding (Dewick, 1986).

All isoflavonoids are derived from isoflavones, and formation of isoflavone skeletons is the critical process of isoflavonoid biosynthesis. This reaction has been detected in the microsomes of elicitor-treated soybean (Kochs and Grisebach, 1986), *Pueraria lobata* (Hashim et al., 1990; Hakamatsuka et al., 1991), and alfalfa (Kessmann, 1990) cells, and proven to consist of two steps (Kochs and Grisebach, 1986; Hashim et al., 1990). The first step is a P450-dependent oxidative aryl migration of flavanones to yield 2-hydroxyisoflavanones. The enzyme catalyzing this unique reaction is called 2-hydroxyisoflavanone synthase (IFS) (Heller and Forkmann, 1994). The second step is catalyzed by a dehydratase to introduce a double bond between C-2 and C-3 (Hakamatsuka et al., 1998). Solubilization and partial purification of IFS in *P. lobata* has been reported (Hakamatsuka et al., 1991; Hakamatsuka and Sankawa, 1993), but no complete purification of IFS has been achieved. This is not unusual in spite of the intense interest in the enzyme as the first step of isoflavonoid biosynthesis, because plant P450s are generally extremely difficult to purify due to their scarcity, the large number of homologous proteins in the cells, and their instability.

Since 1990 (Bozak et al., 1990), molecular cloning of plant P450 genes based on nucleotide sequence information without prior purification of the protein has revealed more than 200 sequences divided into more than 40 gene families. Some of the catalytic activities of plant P450s have been identified in heterologous expression systems, but many are functionally uncharacterized (Bolwell et al., 1994; Schuler, 1996; Chapple, 1998). In cultured cells of a leguminous plant, licorice (*Glycyrrhiza echinata* L.), a retrochalcone, echinatin, and its biosynthetic intermediate, licodione, are synthesized by treatment with elicitors, and an isoflavone, formononetin, is also constitutively produced (Fig. 1) (Ayabe et al., 1986). Two P450s, IFS and (2*S*)-flavanone 2-hydroxylase (F2H), participate in divergent pathways to isoflavone and retrochalcone (Otani et al., 1994), and several other P450s are also involved in the phenolic metabolism in *G. echinata*. These cells were thus expected to be a good source of P450 genes encoding enzymes of the phenylpropanoid and flavonoid/isoflavonoid pathways, especially IFS and F2H. We isolated eight P450 fragments (Ge-1 to Ge-8) from the cDNA library

[1] This work was supported by a Grant-in-Aid (no. 09640782) from the Ministry of Education, Sports, Science and Culture of Japan. T. Akashi was also supported by a research fellowship (no. 3,883) from the Japan Society for the Promotion of Science for Young Scientists.

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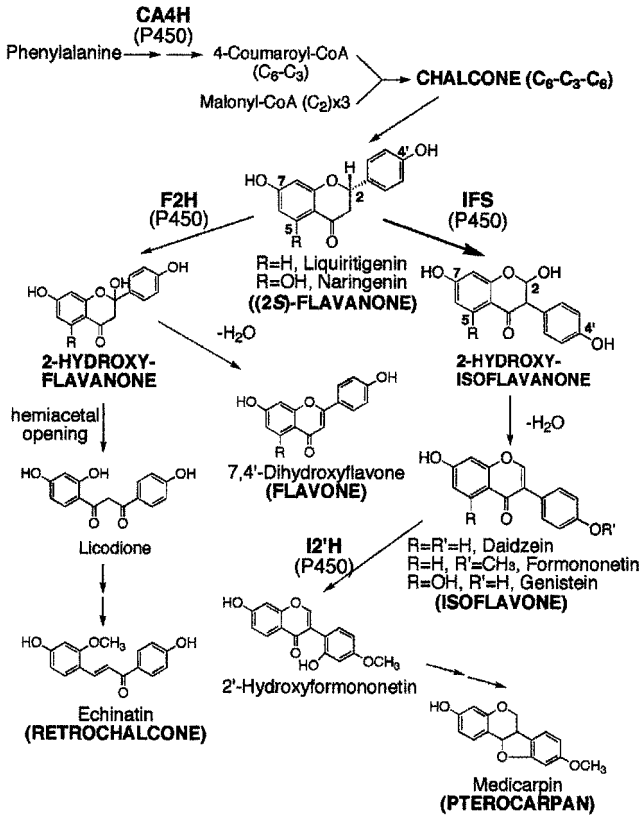

Figure 1. Biosynthesis of isoflavonoids, retrochalcone, and flavones.

prepared from elicitor-treated *G. echinata* cells using a PCR-based method (Akashi et al., 1997b). Full-length P450 cDNAs, CYP93B1 and CYP81E1, corresponding to the fragments Ge-5 and Ge-3, were then cloned (Akashi et al., 1997a). CYP93B1 and CYP81E1 proteins have been identified as F2H (Akashi et al., 1998b) and isoflavone 2′-hydroxylase (I2′H) (Akashi et al., 1998a), respectively (Fig. 1); however, IFS cDNA has not yet been identified.

Recently, we established a new cultured cell line of *G. echinata* that is induced to produce an isoflavonoid-derived phytoalexin (medicarpin) in addition to a retrochalcone by elicitor treatment (Nakamura et al., 1999). In this study, we cloned a full-length P450 cDNA, CYP Ge-8 (CYP93C2), from this cell line and demonstrated that it was IFS. The sequence reported in this paper has been deposited in the DDBJ, GenBank, and EMBL databases (CYP93C2 [CYP Ge-8], accession no. AB023636).

MATERIALS AND METHODS

Plant Materials and Culture Methods

Callus cultures were established from young leaves and petioles of licorice (*Glycyrrhiza echinata* L.). Callus grown on half-strength Murashige and Skoog's medium (solidified with 0.3% [w/v] gellan gum) containing naphthylacetic acid (1 μg mL^{-1}) and benzyladenine (1 μg mL^{-1}) under a 12-h light (6,000 lux)/12-h dark cycle was used to construct the cDNA library. Suspension cultures were maintained in Murashige and Skoog's medium supplemented with 2,4-dichlorophenoxyacetic acid (0.1 μg mL^{-1}) and kinetin (1 μg mL^{-1}) in the dark. Elicitation was performed with 0.2% (w/v per medium) yeast extract (Difco Laboratories, Detroit) (Ayabe et al., 1986). The elicited cells were collected and stored as described previously (Akashi et al., 1997b).

cDNA Library and Screening

Poly(A$^+$) RNAs were isolated from the callus culture 6 and 12 h after elicitation using an mRNA isolation system (Straight A's, Novagen, Madison, WI). These RNAs (2.5 μg each) were mixed and a cDNA library was constructed using the ZAP cDNA Synthesis Kit (Stratagene, La Jolla, CA). Plaques (2×10^5) of the cDNA library were screened with horseradish peroxidase-labeled Ge-8 (Akashi et al., 1997b) using an enhanced chemiluminescence direct nucleic acid labeling system (Amersham-Pharmacia Biotech, Uppsala, Sweden). Hybridization and washing conditions were the same as previously described (Akashi et al., 1997b). Positive clones were converted to pBluescript SK(−) phagemids by in vivo excision according to the manufacturer's protocol (Stratagene). The length of the insert cDNA was determined by PCR using plasmid-derived T3 and T7 primers (Akashi et al., 1997b), and the complete nucleotide sequence of the clone with a length of approximately 2,000 bp was determined by the dideoxynucleotide method.

Construction of Expression Vectors, Expression in Yeast Cells, and Preparation of Microsomes

The coding region of CYP93C2 with modified end sequences was amplified by PCR with KOD polymerase (Toyobo, Tokyo) and a CYP Ge-8 cDNA clone as the template. The sequences of the specific primer were as follows: Ge-8S1 (5′-AAACAGGTACC*ATG*TTGGTGGAACTTGC-3′) in which the original six nucleotides (AACACC) upstream of the initiation codon (italicized) are converted to a *Kpn*I site and Ge-8A1 (5′-CGCGCGAATTCT*TTA*CGAC-GAAAAGAGTT-3′) in which the original six nucleotides (ACATAC) downstream of the termination codon (italicized) are converted to an *Eco*RI site. The *Kpn*I-*Eco*RI fragment of the PCR product was cloned into corresponding sites of a pYES2 expression vector (Invitrogen, Carlsbad, CA). Introduction of the plasmid into *Saccharomyces cerevisiae* BJ2168, selection of the transformant, and induction of the P450 protein were performed as described previously (Akashi et al., 1998b). Yeast cells were suspended in 0.1 M potassium phosphate (pH 7.5) containing 10% (w/v) Suc and 14 mM 2-mercaptoethanol. The cells were disrupted by vigorous shaking with glass beads (0.35–0.6 mm in diameter, Sigma, St. Louis), and the microsomes were prepared by ultracentrifugation (Akashi et al., 1998b). The final microsomal precipitates were homogeneously suspended in the same buffer as above (approximately 1.0 mg mL^{-1} protein). The yeast cells transformed with pYES2 were used as controls in the enzyme assay and for immunoblotting.

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  1'  MLVELAITLLVIAL--FIHLRPTLSAKSKSLRHLPNPPSPKPRLPFVGHLHLLDKPLLHYSLIDLSKRYGPLYSLYFGS
        .*...** **  .. .** .  .*  .*  ****  ***..**.*.*. **** *. .**.*****.** ***
  1" MEPQLVAVSVLVSALICYFFFRPYFHRYGK---NL--PPSPFFRLPIIGHMHMLG-PLLHQSFHNLSHRYGPLFSLNFGS

 78' MPTVVASTPELFKLFLQTHEASSFNTRFQTSAIRRLTYDNSVAMVPFGPYWKFIRKLIMNDLLNATTVNKLRPLRSQEIR
     .  ******.. * .***.*  .** *....*...***..*.*..*.* **.**.** **.**.. ..*....**.**..
 75" VLCVVASTPHFAKQLLQTNEL-AFNCRIESTAVKKLTYESSLAFAPYGDYWRFIKKLSMNELLGSRSINNFQHLRAQETH

158' KVLRVMAQSAESQVPLNVTEELLKWTNSTISRMMLGEAEEIRDIARDVLKIFGEYSLTDFIWPLKKLKVGQYEKRIDDIF
     ..**.....* .  ..*.****** **..** **.***** **..*** .****....**** .**...  ..***.*.*
154" QLLRLLSNRARAFEAVNITEELLKLTNNVISIMMVGEAEEARDVVRDVTEIFGEFNVSDFIWLFKKMDLQGFGKRIEDLF

238' NRFDPVIERVIKKRQEIRK--KRKERNGEIEEGEQSVVFLDTLLDFAEDETMEIKITKEQIKGLVVDFFSAGTDSTAVAT
     .***...**.*.**...**  .*....** ..*.    ***.*** .***. **** . .**.*..***.****.**..*
234" QRFDTLVERIISKREQTRKDRRRNGKKGEQGSGDGIRDFLDILLDCTEDENSEIKIQRVHIKALIMDFFTAGTDTTAIST

316' DWALSELINNPRVFQKAREEIDAVVGKDRLVDEADVQNLPYIRSIVKETFRMHPPLPVVKRKCVQECEVDGYVIPEGALI
     .*** **...*.*.**.*****.********.*.* .****...*.*****.***.*.*.*.**.**.*..*****..*.
314" EWALVELVKKPSVLQKVREEIDNVVGKDRLVEESDCPNLPYLQAILKETFRLHPPVPMVTRRCVAECTVENYVIPEDSLL

396' LFNVWAVGRDPKYWDRPTEFRPERFLENVGEGDQAVDLRGQHFQLLPFGSGRRMCPGVNLATAGMATLLASVIQCFDLSV
     . ***..**.**.**.* ********.  *... .**.** ****************.** .....**...*****. *
394" FVNVWSIGRNPKFWDNPLEFRPERFLKLEGDSSGVVDVRGSHFQLLPFGSGRRMCPGVSLAMQEVPALLGAIIQCFDFHV

476' VGPQGKILKGNDAKVSMEERAGLTVPRAHNLICVPVARSSAVPKLFSS
     ***.*.****.*  ....**.***.******.****.*.*.   *
474" VGPKGEILKGDDIVINVDERPGLTAPRAHNLVCVPVDRTSGGGPLKIIEC
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Figure 2. Amino acid sequence of CYP93C2 (CYP Ge-8; upper row) aligned with CYP93B1 (lower row). Gaps (−) are inserted to optimize alignment. Positions with identical and similar amino acid residues in both sequences are marked by asterisks and dots, respectively.

Enzyme Assay

(2*S*)-[^{14}C]Liquiritigenin and (2*S*)-[^{14}C]naringenin were prepared by the incubation of 4-coumaroyl-CoA and [^{14}C]malonyl-CoA with the cell-free extract of cultured *G. echinata* cells as described previously (Otani et al., 1994). Either radiolabeled substrate (6.4 kBq nmol^{-1} each, 0.08 nmol) in 30 μL of 2-methoxyethanol was incubated with approximately 1 mg of microsomes in the presence of 1 mM NADPH (total volume, 1.05 mL) at 30°C for 2 h. After termination of the reaction with 30 μL of acetic acid, the ethyl acetate extract of the mixture was subjected to cellulose thin-layer chromatography (TLC) (Funacel SF, Funakoshi, Tokyo; solvent, 15% [v/v] acetic acid), and analyzed with a radiochromatoscanner (TLC linear analyzer, model no. LB2820–1, Berthold, Wildbad, Germany) and/or autoradiography. Acid-catalyzed conversion of 2-hydroxyisoflavanones into isoflavones was performed by stirring the concentrated ethyl acetate extracts in 500 μL of 10% (v/v) HCl in methanol at room temperature for 1 h and 50°C for 10 min. The mixture was extracted with ethyl acetate, and the product was analyzed by TLC.

For the assay with non-labeled substrates, the reaction mixture contained 10 μg of cold (*RS*)-liquiritigenin. High-performance liquid chromatography (HPLC) was performed using a CLC-ODS column (6.0 × 150 mm; Shim-Pack, Shimadzu, Kyoto) with 40% (v/v) methanol in water at a flow rate of 1 mL min^{-1} at 40°C. The eluant was monitored at 285 nm. To determine the stereoselectivity of the reaction, the remaining substrate (R_F 0.39) recovered from the TLC plate (Kieselgel F_{254}, Merck, Darmstadt, Germany; solvent, toluene:ethyl acetate:methanol:light petroleum [6:4:1:3, v/v]) was analyzed by HPLC on an OD-RH column (4.6 × 150 mm; Chiralcel, Daicel, Tokyo) with 30% (v/v) acetonitrile at a flow rate of 0.4 mL min^{-1} at 30°C. For MS, the incubation with (*RS*)-liquiritigenin was carried out on a large scale (×200 that described above). The ethyl acetate extract of the reaction mixture was applied to a preparative silica gel TLC as above, and the products at the P1 (R_F 0.20) and P3 (R_F 0.30) spots were collected and further purified by HPLC. The spectra were recorded on a mass spectrometer (JMS-AX505H, JEOL, Tokyo) in the electron impact mode with an ionization voltage of 70 eV.

Northern-Blot Analysis

Suspension-cultured cells were harvested 3, 6, 12, 24, and 48 h post elicitation. mRNAs extracted were subjected to electrophoresis on a 1% (w/v) agarose-formaldehyde gel (900 ng per lane) and transferred by capillary onto a Hybond-N$^+$ membrane (Amersham-Pharmacia Biotech). Probes for the hybridization were labeled by alkaline phosphatase using a chemiluminescence system (AlkPhos Direct, Amersham-Pharmacia Biotech). The blot was hybridized with the probes in a hybridization buffer containing 500 mM NaCl and 4% (w/v) blocking reagent for 12 h at 55°C. The membranes were washed twice with the primary buffer at 55°C for 10 min and twice with the secondary buffer for 5 min at room temperature according to the manufacturer's protocol.

RESULTS

Isolation of CYP Ge-8 (CYP93C2) from a New Cultured Cell Line of *G. echinata*

IFS and F2H (CYP93B1) act on the same substrates (flavanones) and perform the same regiospecific hydroxylation at C-2. Therefore, P450 proteins of the CYP93 family were plausible candidates for IFS. Among the fragments of eight P450 cDNAs isolated from *G. echinata* (Akashi et al., 1997b), Ge-8 was presumed to be a member of this family. Further, we predicted that the new *G. echinata* cell line, in which an isoflavonoid-derived phytoalexin (medicarpin) is induced on elicitation (Nakamura et al., 1999), would be appropriate material for the cloning of IFS. Thus, we prepared a cDNA library of cells that begin to accumulate medicarpin and a retrochalcone, echinatin, 6 and 12 h after

elicitation. A full-length cDNA CYP Ge-8 was isolated from the library using Ge-8 as a probe. This cDNA contained 1,895-bp nucleotides and encoded a polypeptide of 523 amino acids (Fig. 2). The deduced amino acid sequence of CYP Ge-8 shared the highest identity (82.8%) with that of CYP93C1 of soybean (accession no. AF022462). CYP Ge-8 was also 54.4% identical with CYP93B1 (Fig. 2) and 42.4% identical with CYP93A1 (Suzuki et al., 1996), but only 29.7% identical with CYP81E1 (Akashi et al., 1998a). This sequence of CYP Ge-8 has been designated CYP93C2 by the Committee on Cytochrome P450 Nomenclature.

Catalytic Function of CYP93C2 Protein

CYP93C2 protein was heterologously expressed in yeast cells to explore the catalytic function of the gene product. The existence of a new P450 protein in the yeast cells transformed with CYP93C2 cDNA was visualized after SDS/PAGE separation with both Coomassie Brilliant Blue staining and immunoblotting with rabbit antisera raised against multiple-antigen peptides based on conserved plant P450 sequences (data not shown). The estimated molecular mass of the specific band (59 kD) was consistent with that of the deduced amino acid sequence of CYP93C2 (59,428 D).

(2*S*)-[^{14}C]Liquiritigenin (7,4′-dihydroxyflavanone) and NADPH were aerobically incubated with the microsomal fraction of the recombinant yeast, and the ethyl acetate extract of the reaction mixture was analyzed by TLC autoradiography. As shown in Figure 3A, three radioactive

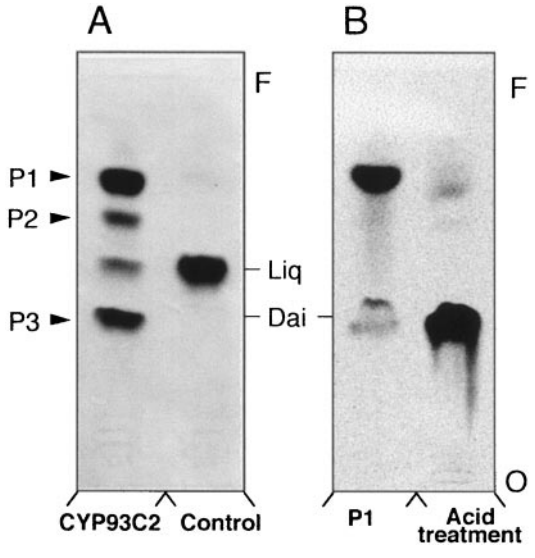

Figure 3. TLC autoradiograms of products from aerobic incubation of (2*S*)-[^{14}C]liquiritigenin and NADPH with microsomal fractions of yeast cells expressing CYP93C2. A, Ethyl acetate extract of products from the incubation with recombinant yeast microsome (left lane) and with control yeast (transformed with the vector pYES2 without insert) microsome (right lane). B, Product after acid treatment of P1 scraped from the TLC plate: left lane, the material isolated from spot P1 in (A); right lane, acid treated sample. TLC, Cellulose; solvent, 15% (v/v) acetic acid. Liq, Liquiritigenin; Dai, daidzein; O, origin; F, solvent front.

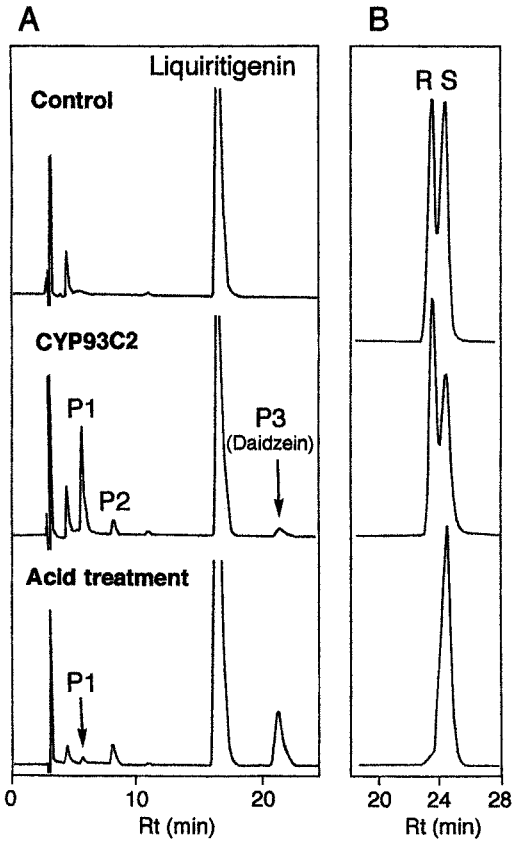

Figure 4. HPLC analysis of products from (*RS*)-liquiritigenin in the IFS assay. A, Direct reaction products with control yeast microsome (top), microsome of the yeast transformed with CYP93C2 (middle), and products after acid treatment of the reaction mixture from IFS reaction (bottom). B, Substrate [(*RS*)-liquiritigenin] before the reaction (top) and recovered after 120 min of incubation (middle) on a chiral separation column. For reference, the chromatogram of (*S*)-liquiritigenin produced by the chalcone isomerase reaction is shown (bottom).

compounds (P1, P2, and P3) were produced in addition to the unreacted substrate. The R_F value of P3 was identical to that of the isoflavone daidzein (7,4′-dihydroxyisoflavone). When the ethyl acetate extract was treated with HCl, the relative radioactivity at the R_F of P1 greatly decreased and that of P3 increased (data not shown). Furthermore, when radioactive P1 was isolated from the TLC plate and reacted with HCl, pure radioactive P3 was produced (Fig. 3B). These results could easily be explained if P1 is acid-labile 2,7,4′-trihydroxyisoflavanone, which should be readily dehydrable to daidzein (Hashim et al., 1990).

Chemically synthesized (*RS*)-liquiritigenin without radiolabel was also incubated with the recombinant microsome, and the reaction mixture was analyzed by reverse-phase HPLC. As shown in Figure 4A, a small peak of daidzein (retention time [Rt], 20.7 min) and two additional peaks (Rt, 5.6 and 8.0 min) were observed. The product giving the Rt 5.6-min peak was proven to be P1 by TLC analysis. Acid treatment of the ethyl acetate extract increased the relative intensity of the daidzein peak, whereas it greatly decreased that of P1 (Fig. 4A), corroborating that

P1 is 2,7,4′-trihydroxyisoflavanone. The substrate stereoselectivity of the IFS reaction was determined by HPLC analysis using a chiral separation column of the substrate recovered after a 120-min incubation with (*RS*)-liquiritigenin (Fig. 4B). (2*S*)-Liquiritigenin, the enantiomer produced by the chalcone isomerase reaction, was reduced, while the (2*R*)-form remained unconsumed. Therefore, CYP93C2 protein acts only on natural substrates with (2*S*)-chirality.

The chemical structures of P1 and P3 were confirmed by electron-impact MS and UV spectroscopy of the TLC- and HPLC-purified samples. As shown in Figure 5A, the mass spectrum of P1 displayed a molecular ion peak at *m/z* 272 ($C_{15}H_{12}O_5$) and $[M\text{-}H_2O]^+$ peaks at *m/z* 254. The UV spectrum of P1 showed a maximum at 276 nm and a shoulder at 309 nm. This coincided well with the reported spectrum of 2,7,4′-trihydroxyisoflavanone (Hashim et al., 1990; Hakamatsuka and Sankawa, 1993). P3 exhibited a molecular ion peak at *m/z* 254 ($C_{15}H_{10}O_4$) and fragment ion peaks at *m/z* 137 and 118 that resulted from retro-Diels-Alder fragmentation in the mass spectrum (Fig. 5B) and absorptions at 248 and 303 (shoulder) nm in its UV spectrum. These spectra were identical to those of the standard sample of daidzein.

When (2*S*)-[^{14}C]naringenin (5,7,4′-trihydroxyflavanone) was used as the substrate for the reaction with the recombinant yeast microsome, major radioactive spots of the products (P4 and P5) were observed on TLC (Fig. 6A). P4 was then scraped from the plate and treated with HCl to produce a new radioactive spot that co-migrated with the carrier sample genistein (5,7,4′-trihydroxyisoflavone) (Fig. 6B). Therefore, P4 is likely to be 2,5,7,4′-tetrahydroxyisoflavanone, indicating that IFS of *G. echinata* employs both 5-deoxyflavanone (liquiritigenin) and 5-hydroxyflavanone (naringenin) as substrates.

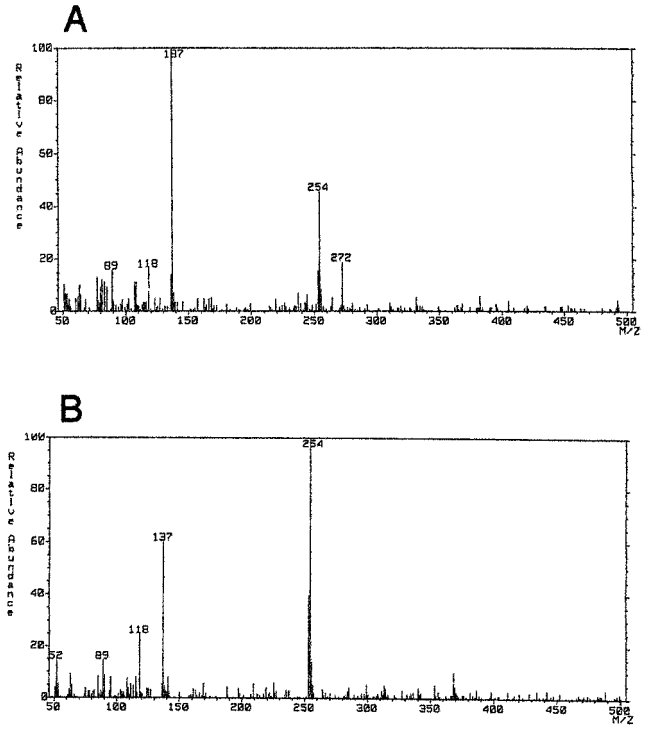

Figure 5. Electron impact mass spectra of P1 (2,7,4′-trihydroxyisoflavanone) (A) and P3 (daidzein) (B).

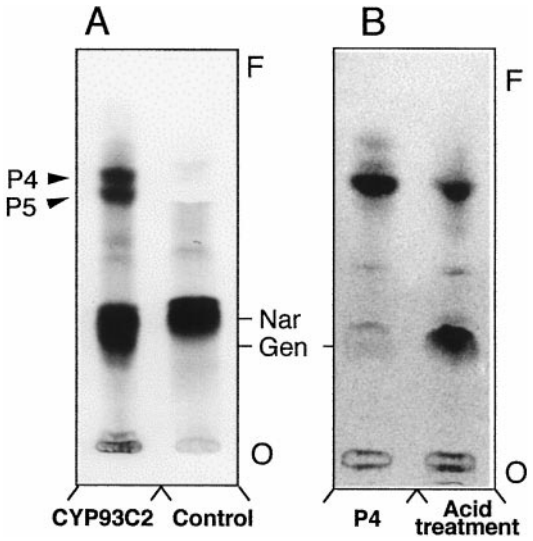

Figure 6. TLC autoradiograms of the reaction products from (2*S*)-[^{14}C]naringenin with microsome of yeast expressing CYP93C2 (A, left lane) or yeast harboring vector pYES2 without insert (A, right lane), and product (B, right lane) after HCl treatment of P4 (B, left lane) separated from the TLC plate (A). TLC, Cellulose; solvent, 15% (v/v) acetic acid. Nar, Naringenin; Gen, genistein.

IFS Activity in *G. echinata* Microsomes

To compare the reactivities of the recombinant yeast and plant cells with the flavanone substrate, the catalytic function of the microsome of elicited *G. echinata* cells toward (2*S*)-[^{14}C]liquiritigenin was examined. Figure 7A shows the TLC scan data of the products formed by incubation with microsomes prepared from the cells 12 h after elicitation. In addition to the radioactive spots of P1 (2,7,4′-trihydroxyisoflavanone) and P3 (daidzein), a strong radioactive spot of licodione was observed. The mixture of radioactive compounds was converted to major products, i.e. daidzein (from P1) and 7,4′-dihydroxyflavone (from licodione) (Akashi et al., 1998b), by acid treatment (Fig. 7B).

Northern-Blot Analysis of CYP93C2 and Other P450s of the Phenylpropanoid/Flavonoid/Isoflavonoid Pathways

As depicted in Figure 1, four P450s are involved in the biosynthesis of flavonoids of *G. echinata* cells. Cinnamic acid 4-hydroxylase (CA4H) is a P450 of the general phenylpropanoid pathway. IFS and F2H hydroxylate flavanones (with or without aryl migration) and lead the phenolic metabolism into divergent pathways. I2′H is specifically involved in pterocarpan biosynthesis. Northern-blot analysis using the P450 probes including IFS (CYP93C2) revealed that these P450 mRNAs all accumulated transiently upon elicitation of the cells (Fig. 8). Intense signals were observed in preparations from the cells 3 to 6 h post elicitation, prior to the time of maximum

accumulation of the direct or end products of each enzyme, which is 24 h for medicarpin (a product through IFS and I2′H reactions), 48 h for echinatin (an end product of F2H), and 12 h for licodione (the direct product of F2H) (Nakamura et al., 1999).

DISCUSSION

In this study, we cloned a full-length P450 cDNA, CYP93C2 (CYP Ge-8), from elicited *G. echinata* cells and identified the catalytic function of the gene product to be IFS. CYP93B1 protein, another P450 of the same source, has been shown to be F2H (Akashi et al., 1998b). IFS and F2H apparently compete for the same substrates ((2*S*)-flavanones; Fig. 1). In fact, microsomes of the original plant cells display combined IFS and F2H reactions (Fig. 7A). Acid treatment of both reaction products produced isoflavones and flavones (Fig. 7B). In living cells, substituting dehydratases like the one from *P. lobata* (Hakamatsuka et al., 1998) for acid would efficiently produce isoflavones and flavones (Otani et al., 1994), and a methyltransferase methylates licodione (a chain tautomer of the hemiacetal, 2-hydroxyflavanone) (Haga et al., 1997) to lead to the retrochalcone pathway (Fig. 1). The production of small quantities of daidzein (P3) from liquiritigenin in reactions with the microsomes of both recombinant yeast (Figs. 3A and 4A) and *G. echinata* (Fig. 7A) should be the result of spontaneous dehydration of 2-hydroxyisoflavanone (P1) during the assay and isolation procedures.

In previous reports (Kochs and Grisebach, 1986; Hashim et al., 1990), the production of both 2′-hydroxyisoflavanone and isoflavone in assays with microsomes prepared from plant cells was described. Furthermore, byproducts of the IFS reactions (P2 in Figs. 3A and 4A; P5 in Fig. 6A) are likely to be 3-hydroxyflavanones (P2, 3,7,4′-trihydroxyflavanone; P5, 3,5,7,4′-tetrahydroxyflavanone) (Hakamatsuka and Sankawa, 1993; Hakamatsuka et al., 1998). The UV spectrum (maximum at 231 nm and shoulders at 275 and 312 nm) of HPLC-purified P2 agreed well with that of 3,7,4′-trihydroxyflavanone (Oyamada and Baba, 1966). The chemical identification of these compounds is now in progress.

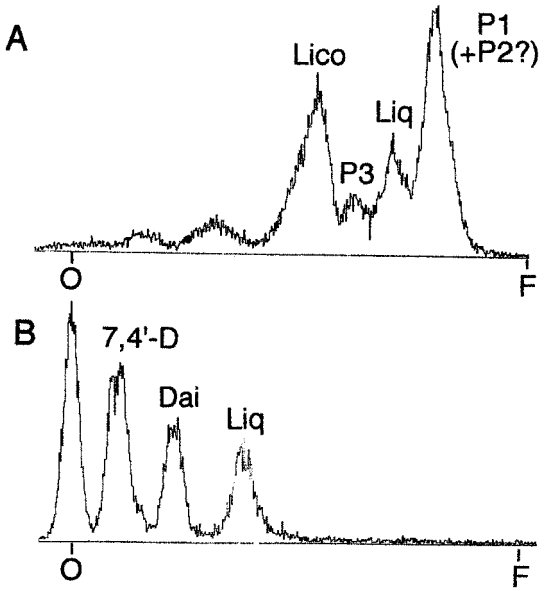

Figure 7. TLC-chromatoscan data of reaction products from (2*S*)-[^{14}C]liquiritigenin with microsomal fraction of elicitor-treated *G. echinata* cells (A) and products after HCl treatment (B). TLC conditions in A are the same as Figure 3; in B, TLC was performed on silica gel with the solvent toluene:ethyl acetate:methanol:light petroleum (6:4:1:3, v/v). Lico, Licodione; Liq, liquiritigenin; Dai, daidzein; 7,4′-D, 7,4′-dihydroxyflavone.

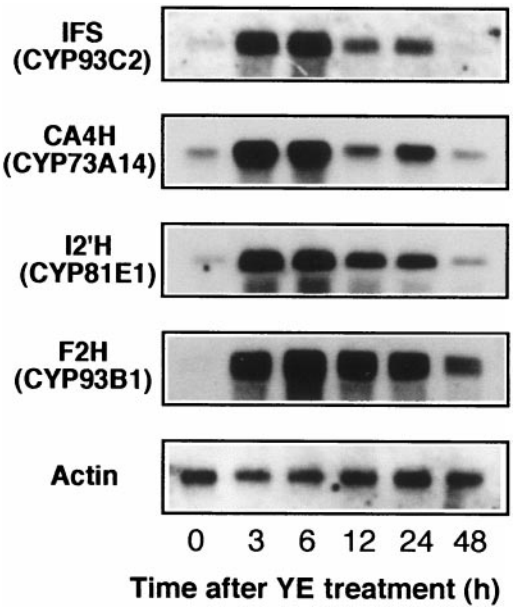

Figure 8. Northern-blot analysis of RNAs from cultured *G. echinata* cells after treatment with yeast extract (YE). For probes, P450 (IFS, CA4H, I2′H, and F2H) coding regions and the PCR fragment of the *G. echinata* actin gene (accession no. AB023637) labeled with alkaline phosphatase were used.

In our continuing study, P450 PCR fragments and full-length cDNAs have been isolated from the cDNA library of elicited *G. echinata* cells (Akashi et al., 1997a; 1997b). Among them, CYP Ge-5 (CYP93B1) and CYP Ge-8 (CYP93C2) were found to be within the CYP93 family, possessing >40% sequence homology (Nelson et al., 1993). The full-length CYP93C2 cDNA was isolated in this study by screening the library with the PCR fragment Ge-8, but was also obtained by screening using CYP93B1 as a probe under low stringency (data not shown). Both CYP93B1 and

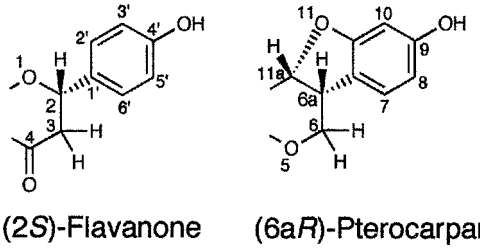

Figure 9. Comparison of partial stereostructures of (2*S*)-flavanone and (6a*R*)-pterocarpan.

CYP93C2 have now been assigned to the class of enzymes employing (2*S*)-flavanones as substrates, while CYP93A1 has been identified as dihydroxypterocarpan 6a-hydroxylase (Schopfer et al., 1998). The sp^3 carbons that are hydroxylated in these reactions, C-2 of (2*S*)-flavanone and C-6a of (6a*R*)-pterocarpan (natural substrate), share identical configurations (Fig. 9) with the three ligands (hydrogen, *p*-hydroxyphenyl, and methylene) they have in common (the fourth is an ether oxygen in flavanone and a methine in pterocarpan). Thus, the CYP93 family proteins are considered to have common substrate recognition functions.

The mechanism for aryl migration in the IFS reaction has been a matter of debate, but no conclusive scheme has been delineated. In the mechanism proposed by Hashim et al. (1990), the hydrogen at C-3 is first removed by the P450 and, after the 1,2-aryl shift, recombination of hydroxy and carbon radicals takes place at C-2. In this mechanism, however, the stereochemistry of the hydroxylation/migration has yet to be clarified (Hakamatsuka et al., 1998). The ionic mechanism (Crombie and Whiting, 1992) assumed a reaction via an intermediate with spirodienone-type B-ring involving deprotonation from the 4′-hydroxyl of flavanone, followed by the reprotonation or methylation to the 4′-carbonyl of the intermediate. On the other hand, the exact nature of 4′-*O*-methylation in the production of formononetin (7-hydroxy-4′-methoxyisoflavone), an intermediate of medicarpin biosynthesis, is unknown; only isoflavone 7-*O*-methyltransferase has been characterized (He and Dixon, 1996) and the cDNA encoding it cloned (He et al., 1998) from alfalfa. The transmethylation to 4′-hydroxyl by isoflavone 7-*O*-methyltransferase during the aryl migration has been supposed to be one of the possible mechanisms for formononetin biosynthesis (He et al., 1998). Now that cDNAs for P450s (IFS and I2′H [Akashi et al., 1998a]) and isoflavone 7-*O*-methyltransferase (He et al., 1998) have been cloned, the molecular mechanism of aryl migration and possible metabolic channeling in medicarpin biosynthesis will be explored in the near future.

Seven of the eight P450 cDNAs cloned from elicited *G. echinata* (including two CA4H and two ferulate 5-hydroxylase isozymes [Akashi et al., 1997b]) have been designated as enzymes of the phenylpropanoid pathway (Akashi et al., 1998a, 1998b). Furthermore, all of the P450s involved in the biosynthetic pathways to medicarpin and echinatin (CA4H, F2H, IFS, and I2′H; see Fig. 1) have been identified among these clones. Thus, the advantage of using cultured cells capable of simplified phenolic biosynthesis, combined with the elicitation technique in the cloning of specific P450s of secondary metabolism, has been demonstrated. Furthermore, Northern-blot analysis with these P450 cDNAs as probes (Fig. 8) showed that the induction of both retrochalcone and isoflavonoid biosynthesis in elicited *G. echinata* cells can be at least partly attributed to transcriptional activation of P450 genes of specific phenolic metabolism.

Isoflavonoids are the characteristic metabolites of legumes (Dewick, 1993): 95% of all natural isoflavonoid aglycons are produced by legumes (Hegnauer and Grayer-Barkmeijer, 1993; Harborne, 1994). Indeed, the nucleotide sequences belonging to the CYP93C family have been found only in the Fabaceae. We believe that soybean CYP93C1 encodes IFS. In addition, we have identified a CYP93C-like sequence from another leguminous plant, *Lotus corniculatus* var *japonicus* (N. Shimada, T. Aoki, T. Akashi, and S. Ayabe, unpublished data). The relationship between the distribution of IFS genes and plant taxonomy is of great interest, and genomic Southern-blot analysis of several leguminous and non-leguminous plants is in progress in this laboratory.

Finally, isoflavonoids are known to possess several biological and physiological activities, both in the producing plants and in organisms ranging from invading microbial pathogens to higher animals, including humans (Middleton and Kandaswami, 1993). The cloning of IFS will enable the transgenic production of either isoflavonoid (phytoestrogen)-enriched plants, which are expected to be highly resistant to phytopathogenic microorganisms (Dixon et al., 1996) and beneficial to human health (Stavric, 1997), or isoflavonoid-reduced forage that will not cause infertility in livestock (Middleton and Kandaswami, 1993).

ACKNOWLEDGMENTS

The authors wish to thank Dr. David R. Nelson (University of Tennessee, Memphis) for the assignment of the P450 (CYP Ge-8) sequence to CYP93C2 and Yuji Sawada (Nihon University) for technical assistance.

Received April 15, 1999; accepted July 14, 1999.

LITERATURE CITED

Akashi T, Aoki T, Ayabe S (1998a) CYP81E1, a cytochrome P450 cDNA of licorice (*Glycyrrhiza echinata* L.), encodes isoflavone 2′-hydroxylase. Biochem Biophys Res Commun **251:** 67–70

Akashi T, Aoki T, Ayabe S (1998b) Identification of a cytochrome P450 cDNA encoding (2*S*)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.; Fabaceae) which represents licodione synthase and flavone synthase II. FEBS Lett **431:** 287–290

Akashi T, Aoki T, Kameya N, Nakamura I, Ayabe S (1997a) Two new cytochrome P450 cDNAs (accession nos. AB001379 and AB001380) from elicitor-induced licorice (*Glycyrrhiza echinata* L.) cells (PGR 97–167). Plant Physiol **115:** 1288

Akashi T, Aoki T, Takahashi T, Kameya N, Nakamura I, Ayabe S (1997b) Cloning of cytochrome P450 cDNAs from cultured *Glycyrrhiza echinata* L. cells and their transcriptional activation by elicitor-treatment. Plant Sci **126:** 39–47

Ayabe S, Iida K, Furuya T (1986) Stress-induced formation of echinatin and a metabolite, prenyl-licodione, in cultured *Glycyrrhiza echinata* cells. Phytochemistry **25:** 2803–2806

Barz W, Welle R (1992) Biosynthesis and metabolism of isoflavones and pterocarpan phytoalexins in chickpea, soybean and phytopathogenic fungi. *In* HA Stafford, RK Ibrahim, eds, Recent Advances in Phytochemistry, Vol 26. Phenolic Metabolism in Plants. Plenum Press, New York, pp 139–164

Bolwell GP, Bozak K, Zimmerlin A (1994) Plant cytochrome P450. Phytochemistry **37:** 1491- 1506

Bozak KR, Yu H, Sirevåg R, Christoffersen RE (1990) Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc Natl Acad Sci USA **87:** 3904–3908

Chapple C (1998) Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu Rev Plant Physiol Plant Mol Biol **49:** 311–343

Crombie L, Whiting DA (1992) The mechanism of the enzymic induced flavanone-isoflavone change. Tetrahedron Lett **33:** 3663–3666

Dewick PM (1986) Isoflavonoids. *In* JB Harborne, ed, The Flavonoids. Advances in Research since 1980. Chapman and Hall, London, pp 125–209

Dewick PM (1993) Isoflavonoids. *In* JB Harborne, ed, The Flavonoids. Advances in Research since 1986. Chapman and Hall, London, pp 117–238

Dixon RA, Harrison MJ, Paiva NL (1995) The isoflavonoid phytoalexin pathway: from enzymes to genes to transcription factors. Physiol Plant **93:** 385–392

Dixon RA, Lamb CJ, Masoud S, Sewalt VJ, Paiva NL (1996) Metabolic engineering: prospects for crop improvement through the genetic manipulation of phenylpropanoid biosynthesis and defense responses–a review. Gene **179:** 61–71

Haga M, Akashi T, Aoki T, Ayabe S (1997) A cDNA for *S*-adenosyl-L-methionine: isoliquiritigenin/licodione 2′-*O*-methyltransferase (accession no. D88742) from cultured licorice (*Glycyrrhiza echinata* L.) cells (PGR 97–014). Plant Physiol **113:** 663

Hakamatsuka T, Hashim MF, Ebizuka Y, Sankawa U (1991) P-450-dependent oxidative rearrangement in isoflavone biosynthesis: reconstitution of P-450 and NADPH:P-450 reductase. Tetrahedron **47:** 5969–5978

Hakamatsuka T, Mori K, Ishida S, Ebizuka Y, Sankawa U (1998) Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*. Phytochemistry **49:** 497–505

Hakamatsuka T, Sankawa U (1993) Recent progress in studies of the biosynthesis of isoflavonoids: oxidative aryl migration during the formation of the isoflavone skeleton. J Plant Res Special Issue **3:** 129–144

Harborne JB (1994) Phytochemistry of the Leguminosae. *In* FA Bisby, J Buckingham, JB Harborne, eds, Phytochemical Dictionary of the Leguminosae. Chapman and Hall, London, pp xx-xxiii

Hashim MF, Hakamatsuka T, Ebizuka Y, Sankawa U (1990) Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis. FEBS Lett **271:** 219–222

He XZ, Dixon RA (1996) Affinity chromatography, substrate/product specificity, and amino acid sequence analysis of an isoflavone *O*-methyltransferase from alfalfa (*Medicago sativa* L.). Arch Biochem Biophys **336:** 121–129

He XZ, Reddy JT, Dixon RA (1998) Stress responses in alfalfa (*Medicago sativa* L.). XXII. cDNA cloning and characterization of an elicitor-inducible isoflavone 7-*O*-methyltransferase. Plant Mol Biol **36:** 43–54

Hegnauer R, Grayer-Barkmeijer RJ (1994) Relevance of seed polysaccharides and flavonoids for the classification of the Leguminosae: a chemotaxonomic approach. Phytochemistry **34:** 3–16

Heller W, Forkmann G (1994) Biosynthesis of Flavonoids. *In* JB Harborne, ed, The Flavonoids. Advances in Research since 1986. Chapman and Hall, London, pp 499–535

Kessmann H, Choudhary AD, Dixon RA (1990) Stress responses in alfalfa (*Medicago sativa* L.). III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts. Plant Cell Rep **9:** 38–41

Kochs G, Grisebach H (1986) Enzymic synthesis of isoflavones. Eur J Biochem **155:** 311–318

Middleton E Jr, Kandaswami C (1993) The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. *In* JB Harborne, ed, The Flavonoids. Advances in Research since 1986. Chapman and Hall, London, pp 619–652

Nakamura K, Akashi T, Aoki T, Kawaguchi K, Ayabe S (1999) Induction of isoflavonoid and retrochalcone branches of the flavonoid pathway in cultured *Glycyrrhiza echinata* cells treated with yeast extract. Biosci Biotechnol Biochem **63:** 1618–1620

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K, Nebert DW (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol **12:** 1–51

Otani K, Takahashi T, Furuya T, Ayabe S (1994) Licodione synthase, a cytochrome P450 monooxygenase catalyzing 2-hydroxylation of 5-deoxyflavanone, in cultured *Glycyrrhiza echinata* L. cells. Plant Physiol **105:** 1427–1432

Oyamada T, Baba H (1966) A new synthesis of polyhydroxydihydroflavonols. Bull Chem Soc Jpn **39:** 507–511

Phillips DA (1992) Flavonoids: plant signals to soil microbes. *In* HA Stafford, RK Ibrahim, eds, Recent Advances in Phytochemistry, Vol 26. Phenolic Metabolism in Plants. Plenum Press, New York, 201–231

Schopfer CR, Kochs G, Lottspeich F, Ebel J (1998) Molecular characterization and functional expression of dihydroxypterocarpan 6a-hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (*Glycine max* L.). FEBS Lett **432:** 182–186

Schuler MA (1996) Plant cytochrome P450 monooxygenases. Crit Rev Plant Sci **15:** 235- 284

Smith DA, Banks SW (1986) Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochemistry **25:** 979–995

Spaink HP (1995) The molecular basis of infection and nodulation by rhizobia: the ins and outs of sympathogenesis. Annu Rev Phytopathol **33:** 345–368

Stavric B (1997) Chemopreventive agents in foods. *In* T Johns, JT Romeo, eds, Recent Advances in Phytochemistry, Vol 31. Functionality of Food Phytochemicals. Plenum Press, New York, pp 53–87

Suzuki G, Ohta H, Kato T, Igarashi T, Sakai F, Shibata D, Takano A, Masuda T, Shioi Y, Takamiya K (1996) Induction of a novel cytochrome P450 (CYP93 family) by methyl jasmonate in soybean suspension-cultured cells. FEBS Lett **383:** 83–86