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Licodione Synthase, a Cytochrome P450 Monooxygenase Catalyzing 2-Hydroxylation of 5-Deoxyflavanone, in Cultured *Glycyrrhiza echinata* L. Cells

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Cultured *Glycyrrhiza echinata* L. (Leguminosae) cells produce a retrochalcone echinatin (4,4′-dihydroxy-2-methoxychalcone) and its biosynthetic intermediate licodione [1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propanedione, a dibenzoylmethane (keto form) or its enol tautomer (β-hydroxychalcone)], when treated with elicitor-active substances, e.g. yeast extract. A microsomal fraction (160,000*g* pellet) prepared from yeast extract-induced suspension cultures of *G. echinata* catalyzed the formation of licodione from (2*S*)-liquiritigenin (7,4′-dihydroxyflavanone) in the presence of NADPH and air. This licodione synthase activity was shown to be dependent on cytochrome P450 by its microsomal localization, requirement of NAD(P)H and O_2 for activity, and inhibition by typical cytochrome P450 inhibitors. Licodione synthase activity transiently increased in the cells after treatment with yeast extract. When (2*S*)-naringenin (5,7,4′-trihydroxyflavanone) and NADPH were incubated with the same microsomal preparation, a polar compound, which further converted into apigenin (5,7,4′-trihydroxyflavone) when treated with acid, was produced. The reaction mechanism of licodione synthase is likely to be 2-hydroxylation of the flavanone molecule and subsequent hemiacetal opening and is possibly the same as the previously suggested mechanism of flavone synthase II from soybean and, furthermore, closely related to isoflavone synthase from *Pueraria lobata*.

Characteristic phytoalexins of Leguminosae belong to the 5-deoxyisoflavonoid class, and elicitor treatment of leguminous cells often results in a rapid induction of enzymes of both the general phenylpropanoid and flavonoid/isoflavonoid pathways (Ebel, 1986; Smith and Banks, 1986). In *Glycyrrhiza echinata* L. (Leguminosae) cells, however, treatment with YE and other elicitor-active substances causes rapid and transient accumulation of a retrochalcone (echinatin) and its biosynthetic intermediate (licodione; a dibenzoylmethane or a β-hydroxychalcone), whereas an isoflavone (formononetin) is a constitutive component of regular (noninduced) cells (Fig. 1; Ayabe et al., 1986). Induction of the activities of the enzymes involved in retrochalcone synthesis in YE-treated cells has been demonstrated (Ayabe et al., 1987, and refs. cited therein).

The activity of 6′-deoxychalcone synthase, the crucial enzyme of 5-deoxy(iso)flavonoid pathway, was first demonstrated in these induced cells (Ayabe et al., 1988a). Subsequently, this activity was shown to consist of conventional CHS and a new reductase in elicitor-challenged soybean cells (Welle and Grisebach, 1988). Recently, in *G. echinata* cells, too, the involvement of the reductase in deoxychalcone formation was demonstrated (Haranô et al., 1993). In isoflavonoid biosynthesis, the chalcone product isomerizes to a flavanone, which undergoes an oxidative aryl migration to give an isoflavone. IFS has been characterized in soybean (Kochs and Grisebach, 1986) and *Pueraria lobata* (Hakamatsuka et al., 1990) cell cultures. The retrochalcone branch should originate at the same 6′-deoxychalcone/5-deoxyflavanone stage, but the enzymic nature of this transformation has not yet been clarified.

In this paper we describe the activity of LS found in the microsomal fraction of *G. echinata* cells treated with YE. A possible relationship between LS, FS, and IFS is discussed. LS proved to be one of multiple plant Cyt P450s (Donaldson and Luster, 1991) involved in flavonoid biosynthesis, and its role in symbiotic plant-microbe interactions can also be hypothesized.

MATERIALS AND METHODS

Chemicals

Standard flavonoid samples were either extracted from *Glycyrrhiza echinata* L. cell cultures or chemically synthesized (Ayabe et al., 1980; Ayabe and Furuya, 1982). 4-Coumaroyl-CoA was synthesized according to the method of Stöckigt and Zenk (1975), and [2-^{14}C]malonyl-CoA was purchased from DuPont/NEN Research Products. Silica gel TLC plates used were Kieselgel F_{254} (0.25 mm thick; E. Merck, Darmstadt, Germany) and cellulose TLC plates were either Cellulose F (E. Merck) or Funacel SF (Funakoshi Co., Tokyo, Japan). Other chemicals and biochemicals were purchased from Wako Chemicals (Osaka, Japan) and Sigma, unless otherwise stated.

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Abbreviations: CHS, (6′-hydroxy)chalcone synthase; EtOAc, ethyl acetate; FS, flavone synthase; IFS, isoflavone synthase; LS, licodione synthase; YE, yeast extract.

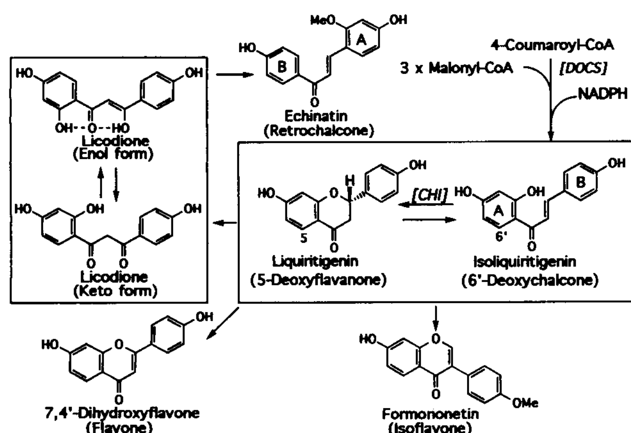

Figure 1. Flavonoid biosynthesis in *G. echinata* L. cells. Licodione exists in an equilibrium mixture of keto and enol forms in solution (Ayabe and Furuya, 1980). DOCS, 6'-Deoxychalcone synthase; CHI, chalcone isomerase.

Cell Cultures and Induction Methods

G. echinata suspension cultures were started by the inoculation of callus cells (3 weeks old) into liquid media, and after 2 weeks a portion of the culture was diluted with 4 volumes of freshly prepared medium. The second passage of the culture was grown for 7 to 10 d and used for the induction experiments. Murashige-Skoog medium was used throughout supplemented with 1 $\mu g\ mL^{-1}$ IAA and 0.1 $\mu g\ mL^{-1}$ kinetin with (for callus) or without (for suspension cultures) 0.9% (w/v) agar, and the cultures were maintained in the dark at 25°C. Treatment with 0.1% (w/v per medium) YE (Difco, Detroit, MI) was as described by Ayabe et al. (1986).

Enzymic Synthesis of (2*S*)-[^{14}C]Liquiritigenin and (2*S*)-[^{14}C]Naringenin

Crude enzyme (10,000*g* supernatant) was prepared from YE-induced (approximately 12 h) *G. echinata*-cultured cells as described by Ayabe et al. (1988a). The 6'-deoxychalcone synthase reaction (coupled with chalcone isomerase reaction) was also carried out essentially by the same method (Ayabe et al., 1988a) but on a larger scale. Normally 120 nmol of 4-coumaroyl-CoA and 23.4 nmol of [2-^{14}C]malonyl-CoA (44 kBq) were incubated with 5 mL of crude enzyme (approximately 5 mg of protein) in the presence of 0.5 mM NADPH. The EtOAc extracts of the reaction mixture were subjected to preparative TLC on silica gel (developed twice with toluene:EtOAc [3:2, v/v]). The radioactive products were visualized by autoradiography, and radioactive liquiritigenin bands (R_F 0.37) were separated and eluted with EtOAc. For the synthesis of (2*S*)-naringenin, the same procedure was used except for the omission of NADPH from the reaction mixture, and the radioactive band at R_F 0.50 on silica gel TLC was collected. The radioactive yield was about 10% for both products, and the specific activity of these products was 5.6 kBq/nmol.

Preparation of Microsomal Fractions

All procedures were carried out at 0 to 4°C. The cells harvested were ground in a mortar with the same volume (per weight) of 0.1 M potassium phosphate buffer (pH 7.5) containing 14 mM 2-mercaptoethanol and 10% (w/v) Suc (grinding buffer), one-tenth weight of Polyclar AT, and one-half weight of sea sand. The homogenate was filtered through eight layers of cheesecloth and centrifuged (10,000*g* for 10 min). The supernatant was treated with Dowex 1-X2 (equilibrated with 0.1 M potassium phosphate buffer [pH 7.5]) for 20 min, followed by filtration through a glass filter. The filtrate was centrifuged at 25,000*g* for 20 min, and the resulting supernatant was finally ultracentrifuged at 160,000*g* for 90 min. The precipitates formed were suspended in appropriate volumes of the grinding buffer and used as the enzyme preparation.

CO-difference spectra were recorded after bubbling a gentle stream of CO (20 s) into a cuvette containing 3 mL of enzyme preparation (300–400 $\mu g\ mL^{-1}$ protein) that had been mixed with approximately 2 mg of solid $Na_2S_2O_4$. For solubilization of the enzyme preparation, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (15 mM) and EDTA (1 mM) were added to the microsomal suspension, and after sonication (three times for 30 s at 5-min intervals), the mixture was ultracentrifuged at 100,000*g* for 60 min. The supernatant solution was used for the measurement of CO-difference spectra as described above.

Enzyme Reactions

The reaction mixture contained (2*S*)-[^{14}C]liquiritigenin (670–1670 Bq) in 2-methoxyethanol (30 μL), 1 mM NADPH (20 μL), and 1 mL of crude enzyme (800–1200 $\mu g\ mL^{-1}$ protein) in a total volume of 1.05 mL. The reaction was started by the addition of NADPH. After the mixture was incubated at 25°C for 1 h, the reaction was terminated by the addition of 20 μL of acetic acid. Standard samples of liquiritigenin, isoliquiritigenin, and licodione were further added as carriers to the mixture, which was subsequently extracted with EtOAc (2 mL). An aliquot of the EtOAc extract was concentrated and analyzed with a TLC-linear analyzer (Berthold, LB2820–1). TLC solvents were toluene: EtOAc:methanol:light petroleum (6:4:1:3, v/v) for silica gel and 30% (v/v) acetic acid for cellulose. For quantitative measurement of enzyme activity (e.g. for inhibitor studies), the radioactive products were separated from the TLC, and the radioactivity was measured with a liquid scintillation counter. Protein content was determined with a protein assay kit (Bio-Rad).

(2*S*)-[^{14}C]Naringenin (600 Bq) and NADPH (1 mM) were also incubated with the enzyme preparation by the same method, and without the addition of carrier flavonoids, the concentrated EtOAc extract was subjected to cellulose TLC (solvent, chloroform:acetic acid:H_2O, 10:9:1). The radioactive spot was scraped from the plate, eluted with methanol, and concentrated. The residual material was dissolved in 0.8 mL of ethanol and stirred at room temperature for 2 h with 2 drops of 12 N HCl, after which the EtOAc layer was prepared and analyzed with a TLC-linear analyzer as described above.

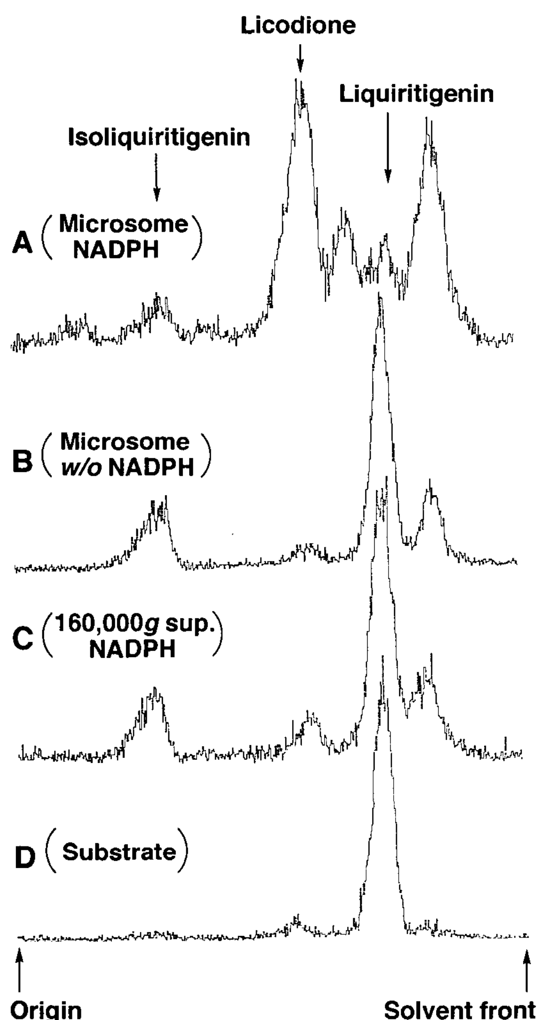

Figure 2. Radiochromatograms of the reaction products from (2*S*)-[^{14}C]liquiritigenin with enzyme preparations from YE-induced (18 h) *G. echinata* cells. The microsomal fraction was prepared by ultracentrifugation at 160,000*g*. The substrate and microsomes were incubated with (A) or without (B, *w/o*) 1 mM NADPH, and the EtOAc extracts of the reaction mixture were analyzed by a TLC-linear analyzer. A reaction with the 160,000*g* supernatant (sup.) plus 1 mM NADPH (C) was also carried out. D, Chromatogram of the substrate. TLC, Cellulose; solvent, 30% acetic acid.

RESULTS

The reaction products formed from (2*S*)-[^{14}C]liquiritigenin when it was incubated with the microsomal fraction of *G. echinata* cells that had been treated with YE were analyzed by cellulose TLC. Although general separation of flavonoids by silica gel TLC was much better than that with cellulose TLC, discrimination of licodione and isoliquiritigenin, which was the expected product from liquiritigenin from the reaction of chalcone isomerase (reverse reaction; Fig. 1), was very difficult with this adsorbent (Kirikae et al., 1993). Reverse-phase HPLC with the gradient solvent system, which is commonly used for flavonoid analysis (Köster et al., 1983), was also inadequate in the analysis of dibenzoylmethane compounds because of extreme broadening of the peak due to the presence of keto-enol tautomeric structures (Ayabe and Furuya, 1980; Fig. 1). Figure 2 shows typical radiochromatograms, in which radioactivity at the R_F of licodione is clearly seen in the lane loaded with the products from the reaction involving NADPH (Fig. 2A). Additional radioactive materials have occasionally been detected, but the identity of these compounds has yet to be determined. The localization of the activity in the 160,000*g* pellet (microsomes) but not in the supernatant and requirement of NADPH for activity were also demonstrated (Fig. 2, B and C).

The material extracted from the licodione spot from cellulose TLC was mixed with a sample of nonradioactive licodione and repeatedly recrystallized. As shown in Table I, the specific radioactivity in samples from successive recrystallizations is constant, confirming the identity of the radioactive product with licodione. Thus, the detected enzyme activity can be designated LS. The apparent mode of action of LS is to introduce one atom of oxygen into the substrate.

Table II shows the effects of substitution of NADPH by NADH and also deletion of O_2 from the reaction by replacement with N_2 and using an O_2-consuming system. The reaction was shown to require molecular O_2 and NADPH, although NADH also served as the hydride donor less effectively. The microsomal localization of the activity and these co-factor requirements strongly suggested that the activity is attributable to a Cyt P450. The effects of known typical inhibitors of Cyt P450s were then tested (Table III). As expected, they inhibited licodione formation substantially. The microsomal suspension and a solubilized microsome actually showed an absorption at 450 nm (Fig. 3) when CO gas was bubbled after reduction with sodium dithionite (Omura and Sato, 1964). This confirmed the existence of Cyt P450 species in the preparations, with about 20 and 60 pmol mg^{-1} protein of Cyt P450s estimated to be in the 160,000*g* pellet and solubilized microsomes, respectively.

The activity change of LS in the microsome preparation of *G. echinata* cells after the addition of YE is shown in Figure 4. There was weak constitutive activity, and on elicitation, the activity rapidly increased to the highest value at 18 h and gradually declined by 24 h. This time course of activity changes was similar to the changes reported for echinatin and licodione levels (maximum, 16–24 h; Ayabe et al., 1986, 1987; A. Udagawa and S. Ayabe, unpublished results) and

Table I. *Dilution analysis of [^{14}C]licodione*

The licodione spot separated from the cellulose TLC plate loaded with the EtOAc extract of a standard LS reaction mixture was eluted with methanol and diluted with 35.4 mg of cold licodione sample. This was repeatedly recrystallized from ethanol/H_2O, and the radioactivity in weighed portions of the crystals at each stage was measured with a liquid scintillation counter.

No. of Recrystallizations	Yield	Specific Activity
	mg	*Bq/mmol*
0	35.4	295
1	22.4	242
2	12.7	235
3	7.2	243

Table II. *Requirements for LS activity*

The microsome was prepared from YE-induced (18 h) *G. echinata* cells. Representative data from two independent experiments are shown. The O_2-consuming system consisted of 50 mM Glc, 5 units of Glc oxidase, and 10 units of catalase. After the microsomal suspension containing (*S*)-[^{14}C]liquiritigenin and the O_2-consuming system were preincubated for the time indicated, NADPH was added to start the reaction. The enzyme activity of the complete system (100%) was 0.53 pmol h^{-1} g^{-1} fresh weight.

Condition	Relative Activity
	%
Complete system (NADPH [1 mM], air)	100
−NADPH	23
−NADPH + 1 mM NADH	89
Air replaced by N_2	87
N_2 + O_2-consuming system (5-min preincubation)	61
N_2 + O_2-consuming system (30-min preincubation)	24

the levels of CHS (maximum, about 10 h; Ayabe et al., 1988b) and *S*-adenosyl-L-Met:licodione 2′-*O*-methyltransferase (maximum, 16–24 h; Ayabe et al., 1987) in the cells treated with YE.

When (2*S*)-[^{14}C]naringenin instead of (2*S*)-[^{14}C]liquiritigenin was incubated with the same microsomal preparation and NADPH (1 mM), a polar radioactive substance emerged as a major product (Fig. 5A), the R_F value of which on cellulose TLC (0.36) was approximately identical with the reported R_F value (0.41) of 2-hydroxynaringenin (Britsch, 1990). This product further converted into a compound comigrating with apigenin when treated with acid (Fig. 5B).

DISCUSSION

The reaction catalyzed by LS is demonstrated to be dependent on a Cyt P450, which is induced in YE-treated cells coordinately with CHS, the reductase yielding 6′-deoxychalcone (Haranô et al., 1993), and a methyltransferase involved in retrochalcone synthesis (Fig. 4). A plausible mechanism of

Table III. *Effects of Cyt P450 inhibitors on LS activity*

The assay was carried out with microsomes prepared as described in Table II. The reaction mixture contained appropriate volumes (30 or 35 μL) of the inhibitor solutions (solvent, 2-methoxyethanol), and the activity was compared to that of the control containing the same volumes of the solvent. Microsomes were separately prepared for each experiment with individual inhibitors, and the enzyme activity of the control was 0.45, 0.53, and 0.37 pmol h^{-1} g^{-1} fresh weight for the ancymidol, ketoconazole, and metyrapone experiments, respectively.

Inhibitor	Concentration	Inhibition
	μM	%
Ancymidol	1	33
	10	68
	100	81
Ketoconazole	1	44
	10	86
	100	72
Metyrapone	1	23
	10	57
	100	74

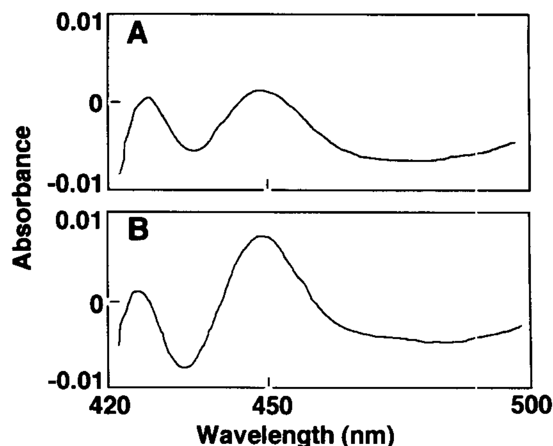

Figure 3. CO-difference spectra of sodium dithionite-reduced microsome (A) and solubilized microsome (B) prepared from YE-induced (18 h) *G. echinata* cells.

LS reaction is the direct hydroxylation of C-2 of liquiritigenin to yield 2-hydroxyliquiritigenin, which undergoes hemiacetal opening to give licodione (keto form, Fig. 6). The tautomeric structures of licodione have been examined by ^{1}H- and ^{13}C-NMR (Ayabe and Furuya, 1980). Licodione exists in major enol (about 70%) and minor keto (about 30%) forms (both chain tautomers) but not in a hemiacetal form (a ring tautomer). Thus, the direct product of the enzymic hydroxylation would spontaneously convert into chain tautomers.

Cyt P450-dependent 2-hydroxylation of flavanone has been postulated in the enzymic conversion of naringenin (5-hydroxyflavanone) into apigenin (5-hydroxyflavone) catalyzed by FS II in soybean cells (Kochs and Grisebach, 1987). Flavone production is the result of 2,3-dehydration of the 2-

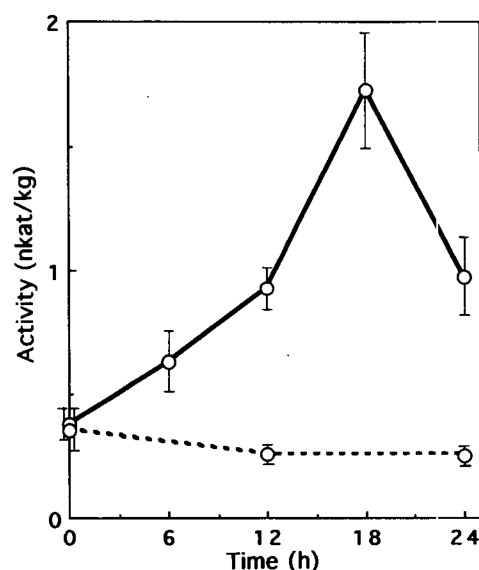

Figure 4. Activity changes of LS in cultured *G. echinata* cells after treatment with YE (0.1% [w/v] of medium; solid line). Control cells received the same volume of water instead of YE at time 0 (dotted line). Data are the average values ± SE from four independent YE-treated or two independent control experiments.

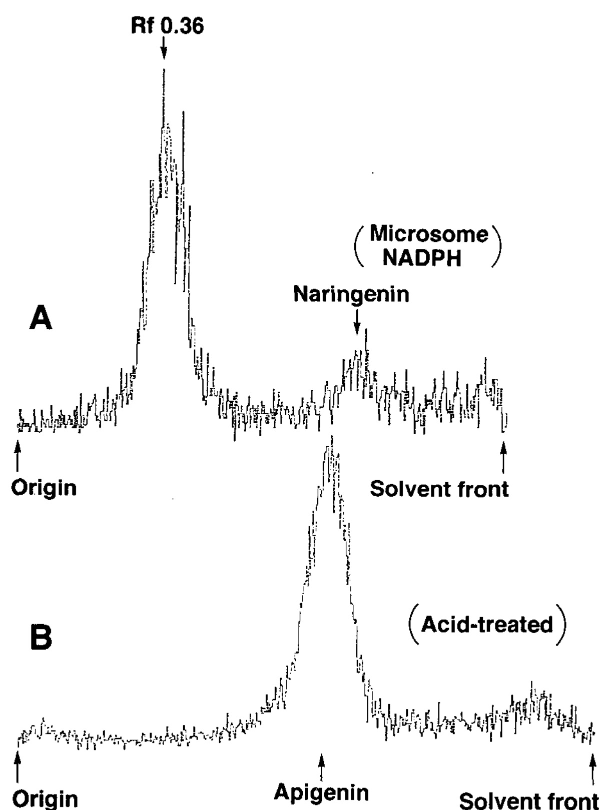

Figure 5. Radiochromatograms of the reaction products from (2S)-[^{14}C]naringenin with the microsomal preparation from YE-induced (18 h) *G. echinata* cells. The substrate and NADPH (1 mM) were reacted with microsomes, and the products were analyzed (A) as in Figure 2. The major radioactive product (R_F 0.36) was then separated from the TLC plate and treated with HCl, and the product was again analyzed (B) in the TLC-linear analyzer. TLC, Cellulose; solvent, chloroform:acetic acid:H_2O, 10:9:1.

hydroxyflavanone. This reaction mechanism is in sharp contrast to that of FS I from parsley in which a 2-oxoglutarate-dependent desaturase reaction has been demonstrated (Britsch, 1990). FS II activity is induced in soybean cells in response to osmotic stress, whereas IFS is induced by elicitor (Kochs et al., 1987). Whether FS II can utilize 5-deoxyflavanone (liquiritigenin) as a substrate (and whether 7,4′-dihydroxyflavone is produced) is not reported, but if it does, the former half reaction is the same as the LS reaction. When *G. echinata* microsome was tested with [^{14}C]naringenin as a substrate, a compound that is most likely to be 2-hydroxynaringenin was formed (Fig. 5), further supporting the identities of LS and FS II.

Another Cyt P450 enzyme closely related to LS in view of the reaction mechanism is IFS (Fig. 6). Among several proposed mechanisms of IFS, the reaction pathway composed of two parts (Hashim et al., 1990) seems to be reasonable. In the first reaction, 2-hydroxyisoflavanone is produced by a Cyt P450-dependent hydroxylation accompanied by aryl migration; then, in the second reaction, a dehydratase catalyzes 2,3-dehydration to yield an isoflavone (daidzein). 2-Hydroxyisoflavanone (5-deoxy type) was detected (by NMR) in the reaction of liquiritigenin with washed microsomes from *P. lobata* cells. Also, the synthesis of genistein (5-hydroxyisoflavone) from naringenin (5-hydroxyflavanone) by the enzyme from soybean cells has been reported to involve 2-hydroxyisoflavanone (5-hydroxy type) as a possible intermediate (Kochs and Grisebach, 1986), although the origin of the oxygen atom of the 2-hydroxyl group is supposed to be water.

Therefore, LS, FS II, and IFS are all likely to be Cyt P450 hydroxylases acting on a flavanone skeleton at C-2, and the former two are possibly the same activity. IFS would have the additional catalytic activity of aryl migration.

Licodione and its prenyl derivatives have been found from *Glycyrrhiza* sp. (Ayabe et al., 1980, 1986; Demizu et al., 1992; Zeng et al., 1992) and from alfalfa (Kobayashi et al., 1988), in which elicitor-induced synthesis of licodione from a remote precursor, Phe, was recently demonstrated (Kirikae et al., 1993). In view of its biosynthetic relationship to commonly found 7,4′-dihydroxyflavone, the distribution of licodione could be even wider than already reported. Recently, several reports have appeared concerning the cloning of Cyt P450 genes involved in phenylpropanoid and flavonoid biosynthesis in higher plants (Fahrendorf and Dixon, 1993; Holton et al., 1993; Mizutani et al., 1993; Teutsch et al., 1993). Among these, a cinnamic acid 4-hydroxylase gene was identified in the cDNA library of alfalfa (Fahrendorf and Dixon, 1993), in which IFS activity has also been demonstrated (Kessmann et al., 1990). Thus, the cloning of IFS and LS constructing a cDNA library from poly(A)$^+$ RNA of YE-induced *G. echinata* cells, and cloning of Cyt P450s is expected in addition to the reductase co-acting with CHS to yield 6′-deoxychalcone (T. Furuno, T. Akashi, T. Takahashi, and S. Ayabe, unpublished data).

Transcriptional activation of *nod* genes in *(Brady)Rhizobium* by leguminous flavonoids is of interest, especially from the viewpoint of symbiotic (establishing N_2-fixing root nodules) and pathogenic (inducing defense responses in host cells) interactions between plants and microorganisms (Kape et al., 1992; Dakora et al., 1993). The LS (or FS II) activity produces potent *nod*-inducer flavones, e.g. 7,4′-dihydroxyflavone and luteolin (Peters et al., 1986; Redmond et al., 1986; Maxwell et al., 1989), whereas IFS is involved in the synthesis of phytoalexins, which play a role in pathogenic interactions (although some 5-deoxyisoflavones have also been found to have *nod*-inducing activity: Kosslak et al., 1987). Biosynthetic branching into the most active *nod*-inducer methoxychalcone of alfalfa (Maxwell et al., 1989) also takes place at the 6′-deoxychalcone stage (Maxwell et al., 1992). Hence, the con-

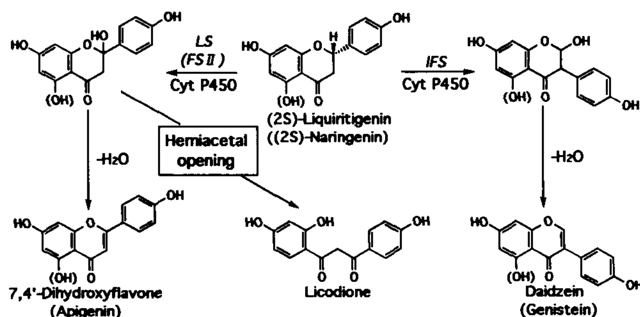

Figure 6. Proposed mechanisms of LS, FS II, and IFS. Compounds of 5-hydroxy series are indicated in parentheses.

trol of activities of these Cyt P450s, as well as of chalcone isomerase and specific methyltransferase, must be very important in the biochemical adaptation of leguminous plants in their environments.

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