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

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Abstract The microsome of insect cells expressing CYP Ge-5 (CYP93B1), a cytochrome P450 cDNA of licorice (*Glycyrrhiza echinata* L.), catalyzed the formation of [^{14}C]licodione and [^{14}C]-2-hydroxynaringenin from (2*S*)-[^{14}C]liquiritigenin and (2*S*)-[^{14}C]naringenin, respectively. On acid treatment, the products were converted to ^{14}C-labeled 7,4′-dihydroxyflavone and apigenin. Eriodictyol was also converted to luteolin by the reaction with the microsome of yeast expressing CYP93B1 and subsequent acid treatment. CYP93B1 was thus shown to encode (2*S*)-flavanone 2-hydroxylase, which has previously been designated to licodione synthase and flavone synthase II depending on the substrates employed.

Key words: Cytochrome P450; Elicitation; (2*S*)-flavanone 2-hydroxylase; Flavonoid biosynthesis; Heterologous expression; *Glycyrrhiza echinata*

1. Introduction

Flavonoids are widely distributed in vascular plants and play essential roles in the plant physiology [1–3]. They consist of several classes of carbon skeletons which are primarily of tetraketide origin derived from a phenylpropanoid (C_6-C_3) starter and three C_2 elongation units (Fig. 1) [4]. Most of the flavonoid skeletons thus have linear C_6-C_3-C_6 structures, but the isoflavonoids are rearranged in the C_3 part. The enzymes of flavonoid biosynthesis have been extensively studied [4], and recent molecular biological approaches have been impressive in elucidating the mechanism and regulation of flavonoid biosynthesis [5].

However, the enzymes involved in the formation of flavones and isoflavones have not been fully characterized (except for flavone synthase I; see below). These two major flavonoid classes are especially important in the leguminous plants for their interactions with environmental microorganisms [3,6]; e.g. isoflavones and their derivatives are typical phytoalexins [7] and many flavones act as signals in the early steps of symbiosis with soil bacteria (*Rhizobia*) to form nitrogen-fixing root nodules [8,9]. These skeletons are biosynthesized from a flavanone by an oxidative process which introduces a double bond between C-2 and C-3, together with an aryl migration from C-2 to C-3 in the case of isoflavone synthesis (Fig. 1) [4]. Flavone synthesis displays a unique diversity in the reaction mechanism operating in the biosynthesis of the same product from the same substrate. In parsley, 2-oxoglutarate-dependent flavone synthase I abstracts hydrogens from C-2 and C-3 of a flavanone, naringenin, to produce a flavone, apigenin [10,11]. In contrast, in snapdragon [12] and soybean [13,14], a cytochrome P450 (P450) monooxygenase has been presumed to catalyze the formation of a hypothetical 2-hydroxyflavanone from a flavanone, the subsequent dehydration of which yields a flavone. This process is called flavone synthase II (FNSII) reaction. In leguminous cells, a P450 isoflavone synthase also acts on flavanone to produce an isoflavone via the dehydration of 2-hydroxyisoflavanone (Fig. 1) [15,16]. Several P450 monooxygenases including FNSII and isoflavone synthase have been proposed to be involved in flavonoid metabolism (e.g. flavonoid 3′- and/or 5′-hydroxylase of anthocyanin biosynthesis and isoflavone 2′-hydroxylase of the legume phytoalexin pathway) [17], but the structural genes of these P450 proteins have been unknown except for that of flavonoid 3′,5′-hydroxylase [18].

We have characterized a P450 monooxygenase, licodione synthase (LS), from cultured cells of a leguminous plant, licorice (*Glycyrrhiza echinata* L.), which produces a retrochalcone, echinatin, on treatment with elicitor active substances, e.g. yeast extract (Fig. 1) [19]. We have suggested that the mode of action of LS is the hydroxylation of a 5-deoxyflavanone, liquiritigenin, at C-2, and that the formation of licodione is the result of non-enzymatic hemiacetal opening. This proposed mechanism has made LS very likely to be the same enzyme as FNSII. To clone LS cDNA, we have prepared a cDNA library of elicited licorice cells, and isolated four functionally uncharacterized P450 PCR fragments (Ge-3, 4, 5 and 8), in addition to other four fragments highly homologous to known sequences encoding *trans*-cinnamic acid 4-hydroxylase and ferulic acid 5-hydroxylase [20]. The full-length P450 sequences CYP Ge-3 (CYP81E1) and CYP Ge-5 (CYP93B1) containing the sequences of Ge-3 and Ge-5, which are inducible on elicitation of the cells, have recently been cloned [21].

In this report, we demonstrate that heterologously expressed CYP Ge-5 protein displays LS activity and FNSII activities toward different flavanone substrates (after chemical dehydration). We propose that the enzyme responsible for these collective activities may be called (2*S*)-flavanone 2-hydroxylase (F2H).

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Abbreviations: F2H, (2*S*)-flavanone 2-hydroxylase; FNSII, flavone synthase II; LS, licodione synthase; P450, cytochrome P450

The sequence reported in this paper has been deposited in the DDBJ, GenBank and EMBL databases (CYP93B1, accession No. AB001380).

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2. Materials and methods

2.1. Expression of CYP Ge-5 (CYP93B1) in insect cells using baculovirus vector

The coding region of CYP Ge-5 [21] with tailored end-sequences was obtained by PCR with KOD polymerase (Toyobo, Tokyo, Japan) and CYP Ge-5 cDNA clone in pBluescript SK(−) as the template, using specific primers, Ge-5S1 (5′-CTAGGATCC*ATG*GAACCT-CAACTCGTAG-3′) in which the original six nucleotides (AGCGAT) upstream of the initiation codon (italicized) are converted to GGATCC (*Bam*HI site) and Ge-5A1 (5′-*TCA*ACATTCAATGA-TTTTGAGGGG-3′) to generate a blunt-end at the termination codon (italicized). The PCR products were digested with *Bam*HI, and the fragment was subcloned into the *Stu*I and *Bam*HI sites of pFAST-BAC1 donor plasmid (Gibco BRL, Maryland, USA) to produce pFAST Ge-5, which contained the open reading frame in the sense direction under the polyhedrin promoter. The recombinant baculovirus was constructed using the Bac-to-Bac Baculovirus Expression system (Gibco BRL). *Spodoptera frugiperda* (Sf9) cells (Invitrogen, California, USA) were maintained as a monolayer culture in the TMN-FH medium [22] supplemented with 10 μg/ml gentamicin sulfate. For the expression of P450 proteins, Sf9 cells were infected with recombinant virus at a rate of 5 to 10 plaque-forming units per cell. Hemin (2 μg/ml) was added to the culture medium to supplement low endogenous levels of heme in insect cells. Cells were harvested 110 h after infection.

2.2. Expression of CYP93B1 in yeast

The CYP Ge-5 coding region was amplified by PCR as above using Ge-5S1 primer and Ge-5A2 primer (5′-AGACCTCGAG*TCA*A-CATTCAATGATTTTGA-3′) in which six nucleotides (TTATTC) downstream of the termination codon (italicized) of the original cDNA were converted to CTCGAG to generate a *Xho*I site. The *Bam*HI-*Xho*I fragment of the PCR product was cloned into a pYES2 (Invitrogen) expression vector with a URA3 selection marker, and the resultant pYES Ge-5 was transferred into a protease-deficient *Saccharomyces cerevisiae* strain BJ2168 (a; *prc*1-407, *prb*1-1122, *pep*4-3, *leu*2, *trp*1, *ura*3-52; Nippon Gene, Tokyo, Japan). The transformants were selected on a medium containing 6.7 mg/ml yeast nitrogen base without amino acids (Difco, Michigan, USA), 20 mg/ml glucose, 30 μg/ml leucine, 20 μg/ml tryptophan and 5 mg/ml casamino acid. For the induction of P450 proteins, the cells grown in the above medium for 10 h were transferred to 40 volumes of YPGE medium [23] supplemented with 20 mg/ml galactose and 2 μg/ml hemin but without glucose. After 24 h, the cells were harvested, and converted to spheroplasts using Zymolyase 20T (Seikagaku Corporation, Tokyo, Japan).

2.3. Preparation of microsomes and enzyme assay

The insect cells and yeast spheroplasts were disrupted at 4°C with glass beads (0.35–0.60 mm diameter) in buffer A (0.1 M K-Pi (pH 7.5) containing 10% (w/v) sucrose and 14 mM 2-mercaptoethanol). The lysate was successively centrifuged at 10 000×*g* and 15 000×*g* for 10 min each, and the resultant supernatant was ultracentrifuged at 160 000×*g* for 90 min. The precipitates were homogeneously suspended in buffer A (1.5–2.5 mg/ml protein).

(2*S*)-[^{14}C]liquiritigenin or (2*S*)-[^{14}C]naringenin (6.4 kBq/nmol each, 0.08 nmol) [19] in 30 μl 2-methoxyethanol was incubated at 25°C for 2 h with 1.5–2.5 mg microsomes in the presence of 1 mM NADPH in the total volume of 1.05 ml. After the termination of the reaction with 30 μl acetic acid and carrier samples, the ethyl acetate extract of the mixture was analyzed by TLC-radiochromatography/autoradiography. For the assay with non-labeled substrates, the reaction mixture contained 10 μg each of the substrates (liquiritigenin, naringenin (Wako, Osaka, Japan) and eriodictyol (Extrasynthése, Genay, France)) instead of radiolabeled ones. Acid-catalyzed conversion of the reaction products into flavones was carried out by stirring the concentrated ethyl acetate extracts with 500 μl 10% HCl in ethanol at room temperature for 2 h followed by ethyl acetate extraction and HPLC analysis using a Shim-pack CLC-ODS column (6.0×150 mm; Shimadzu, Kyoto, Japan) with a solvent of 50% methanol and 3% acetic acid in water at a flow rate of 1 ml/min at 40°C. The eluent was monitored at 330 nm (7,4′-dihydroxyflavone), 263 nm (apigenin) or 354 nm (luteolin). For the determination of the substrate stereoselectivity of the reaction, (*RS*)-naringenin (10 μg) and the microsome (ca. 2 mg/ml protein) of the yeast expressing CYP Ge-5 were incubated as above, except that incubation times were 20 and 120 min. The remaining substrates recovered from a silica-gel TLC plate (Merck (Darmstadt, Germany); solvent: toluene/ethyl acetate/methanol/light petroleum (6:4:1:3)) were analyzed by HPLC on a Chiralcel OD-RH column (4.6×150 mm; Daicel, Tokyo, Japan) with 35% acetonitrile at a flow rate of 0.5 ml/min at 30°C.

3. Results and discussion

For the radiochemical assay of P450 reactions, (2*S*)-[^{14}C]liquiritigenin (7,4′-dihydroxyflavanone) and (2*S*)-[^{14}C]naringenin (5,7,4′-trihydroxyflavanone) were prepared using a cell-free extract of licorice cells with [2-^{14}C]malonyl-CoA as the isotope source [19]. The assay was first carried out with the insect microsome in which CYP Ge-3 or CYP Ge-5 was expressed. Fig. 2a shows the TLC-autoradiogram of the products of the reaction in the presence of 1 mM NADPH, exhibiting that the preparation containing CYP Ge-5 protein converted (2*S*)-[^{14}C]liquiritigenin into [^{14}C]licodione. CYP Ge-3 protein did not react with liquiritigenin. The identity of [^{14}C]licodione was demonstrated by the formation of [^{14}C]-7,4′-dihydroxyflavone on treatment with HCl to perform dehydrative cyclization (Fig. 2b) [24]. When (2*S*)-[^{14}C]naringenin (5,7,4′-trihydroxyflavanone) was used as the substrate for the reaction with CYP Ge-5 protein, one major radioactive product was observed on TLC-radiochromatogram (Fig. 3a). Although a standard sample was inaccessible to us, this compound is likely to be 2-hydroxynaringenin, as its Rf (0.38), and also the Rf's of naringenin (0.75) and apigenin (0.58), agreed with the reported values (0.41, 0.75 and 0.65, respectively) under the same conditions [11]. Furthermore, it was converted to [^{14}C]apigenin (5,7,4′-trihydroxyflavone) when treated with HCl (Fig. 3b), confirming the 2-hydroxyflavanone structure of the enzyme reaction product.

The finding that a single P450 protein catalyzed the formation of both the theoretical tautomer of 2-hydroxyliquiritigenin (licodione) [25] and acid-labile intermediates of flavone biosynthesis strongly supports the hypothesis that flavone biosynthesis in legumes proceeds via 2-hydroxylation of flava-

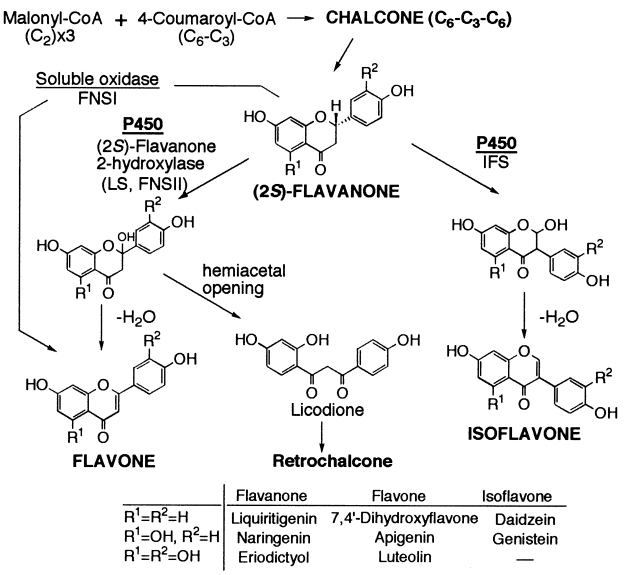

Fig. 1. Biosynthesis of licodione, flavones, and isoflavones. FNSI, flavone synthase I; IFS, isoflavone synthase.

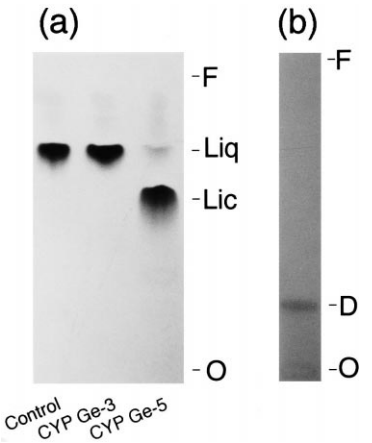

Fig. 2. TLC-autoradiograms of the products from aerobic incubation of (2*S*)-[^{14}C]liquiritigenin and NADPH with the microsomal fractions of Sf9 cells infected with the recombinant baculovirus harboring CYP Ge-3 and CYP Ge-5. a: Direct reaction products on cellulose TLC (solvent, 30% acetic acid). The control lane was loaded with the products from the reaction with uninfected Sf9 cells. b: The product after acid treatment of the spot in CYP Ge-5 lane in a. The radioactive material at the spot (Rf 0.59) was separated from the plate, treated with HCl, and subjected to TLC on silica-gel (solvent: toluene/ethyl acetate/methanol/light petroleum (6:4:1:3)). Liq, liquiritigenin; Lic, licodione; D, 7,4′-dihydroxyflavone; O, origin; F, solvent front.

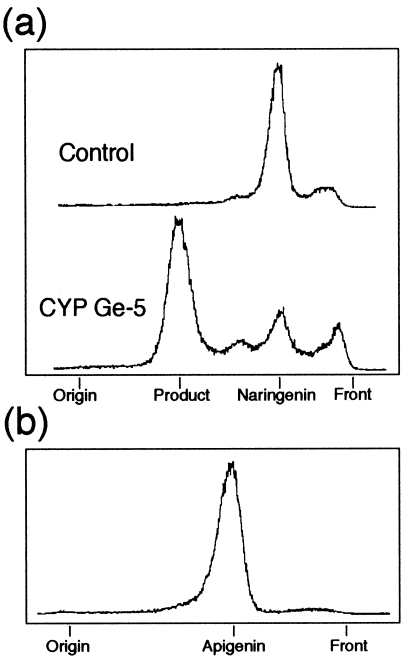

Fig. 3. Radiochromatograms of a: the reaction products from (2*S*)-[^{14}C]naringenin with the microsomal fraction of Sf9 cells expressing CYP Ge-5, and b: the product after HCl treatment of the spot (Rf 0.38) in a. TLC, cellulose; solvent: chloroform/acetic acid/water (10:9:1). For the control, a microsome of uninfected Sf9 cells was reacted with the substrate.

nones (Fig. 1) [13]. The second half of flavone formation should be a dehydratase-catalyzed reaction rather than a spontaneous dehydration, because the intermediate (2-hydroxynarigenin) was found to be stable under neutral conditions (Fig. 3a). In contrast, the hypothetical product from liquiritigenin, 2-hydroxyliquiritigenin, proved to be very unstable; indeed it has not been detected in the tautomeric mixtures of licodione [25]. It should undergo hemiacetal opening to yield licodione as soon as it is formed at the enzyme active site, but when the dehydratase functions quickly, it will be converted to 7,4′-dihydroxyflavone. CYP Ge-5 (CYP93B1) was thus shown to encode (2*S*)-flavanone 2-hydroxylase (F2H) which has previously been designated to LS and FNSII depending on the substrates employed.

The yeast expression system was used to monitor the substrate specificity of the reaction catalyzed by CYP Ge-5 protein (F2H). The unlabeled substrates and NADPH were incubated with the yeast microsomes, and the reaction mixtures were analyzed by HPLC after acid treatment (Fig. 4). As expected, when liquiritigenin and naringenin were the substrates, respective major peaks of 7,4′-dihydroxyflavone and apigenin were observed (Fig. 4a and b). Eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) was converted to luteolin (5,7,3′,4′-tetrahydroxyflavone) through the sequential reactions with CYP Ge-5 protein and acid treatment (Fig. 4c). Thus, licorice

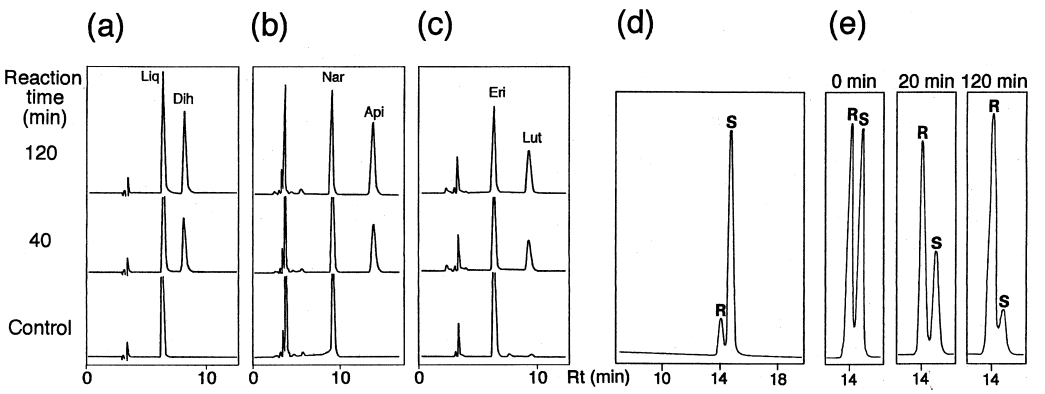

Fig. 4. HPLC analysis of the reaction catalyzed by (2*S*)-flavanone 2-hydroxylase expressed in yeast. a, b and c: Time course of the reaction. The reaction products from non-labeled (*RS*)-liquiritigenin (Liq, in a), (*RS*)-naringenin (Nar, in b) and (*RS*)-eriodictyol (Eri, in c) were acid treated and analyzed with reversed-phase HPLC. Dih, 7,4′-dihydroxyflavone; Api, apigenin; Lut, luteolin. For the control, a microsome of yeast cells transformed with pYES2 was reacted with each substrate for 120 min. d: HPLC of the product of chalcone isomerase reaction in licorice cell-free extract with 4,2′,4′,6′-tetrahydroxychalcone as the substrate on a chiral separation column. The reaction theoretically yields (2*S*)-enantiomer, but a partial racemization must have occurred during workup. e: Time course of the consumption of the substrate, (*RS*)-naringenin, by the F2H reaction displayed by the chiral HPLC.

F2H acts on at least three differently hydroxylated flavanones (liquiritigenin, naringenin and eriodictyol).

The substrate stereoselectivity of the F2H reaction was further explored by HPLC on a chiral separation column, which clearly distinguishes (2*R*)- and (2*S*)-naringenin (Fig. 4d). Racemic naringenin was reacted with yeast microsome expressing Ge-5 protein, and the remaining substrate during the reaction was recovered and analyzed. Results in Fig. 4e show that only (2*S*)-naringenin was consumed and (2*R*)-naringenin remained, confirming the F2H selectivity toward (2*S*)-flavanone.

In this study, heterologously expressed CYP93B1 was demonstrated to have F2H activity toward both 5-deoxy- and 5-hydroxyflavanones in vitro. As the time courses of the induction of the retrochalcone pathway (the highest LS activity and maximum echinatin accumulation at about 18 h and 24 h post-elicitation, respectively; [19,26]) and CYP93B1 mRNA accumulation (about 12 h; [20]) in elicited licorice cells coincide, CYP93B1 is regarded as the actual gene encoding LS. Also, as 7,4′-dihydroxyflavone has been identified from licorice cells [24], CYP93B1 protein is likely to participate in this 5-deoxyflavone biosynthesis in vivo. Although 5-hydroxyflavones, e.g. apigenin and luteolin, have not yet been identified in licorice cells, the F2H activity toward 5-hydroxyflavanone of licorice microsome previously detected in vitro [19] can now be attributed to this protein. It remains to be determined whether CYP93B1 protein catalyzes this reaction on 5-hydroxyflavonoids in licorice cells. The existence of CYP93B1-like sequences and their expression in plants producing 5-hydroxyflavones should be examined to establish that this gene product is the universal component of FNSII.

The substrate specificity of the recombinant licorice F2H casts an interesting problem regarding the biosynthesis of 3′-hydroxyflavones. Luteolin biosynthesis from eriodictyol in the soybean microsome has been reported as one of the FNSII reactions [13], and the present study reproduced this reaction with heterologously expressed F2H. On the other hand, luteolin formation by the 3′-hydroxylation of apigenin by flavonoid 3′,5′-hydroxylase (CYP75) has also been reported [27]. Thus the organization of a 'metabolic grid' [28] in the synthesis of 3′-hydroxylated flavones by two P450 monooxygenases (F2H and flavonoid 3′,5′-hydroxylase) may be envisaged.

Other members (CYP93A1 and CYP93A2) of CYP93 family have been isolated from soybean, another species of Fabaceae [29,30]. CYP93A1 is inducible in the cells by treatment with methyl jasmonate or *Phytophthora* elicitor [29,31]. There is a good possibility that these gene products are involved in stress-induced phenylpropanoid/flavonoid biosynthesis.

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