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CYP81E1, a Cytochrome P450 cDNA of Licorice (*Glycyrrhiza echinata* L.), Encodes Isoflavone 2′-Hydroxylase

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The microsome of yeast cells overexpressing CYP81E1, a cytochrome P450 cDNA recently cloned from licorice (*Glycyrrhiza echinata* L., Fabaceae), catalyzed the hydroxylation of isoflavones, daidzein and formononetin, to yield 2′-hydroxyisoflavones, 2′-hydroxydaidzein, and 2′-hydroxyformononetin, respectively. The chemical structures of the reaction products were confirmed by mass spectrometric analysis. Genistein also yielded a putative 2′-hydroxylated product, but flavanones and cinnamic acid derivatives were not used as substrates for the reaction with the recombinant yeast microsome. CYP81E1 protein was thus demonstrated for the first time to be isoflavone 2′-hydroxylase involved in the biosynthesis of isoflavonoid-derived antimicrobial compounds of legumes.

Isoflavonoids constitute a large group of naturally occurring flavonoid subclasses. They are distributed almost exclusively in the leguminous plants (Fabaceae) and participate in the defense responses of the legumes against phytopathogenic microorganisms (1, 2). Major antimicrobial isoflavonoid derivatives have characteristic oxygen functionalities at C-2′ (Fig. 1) (1, 3). For instance, most of isoflavanone and isoflavan phytoalexins are hydroxylated at C-2′. The dihydrofuran ring of pterocarpans, and presumably of coumesterols, is formed through the dehydration from hydroxyls at C-2′ and C-4 on isoflavanol skeletons (4, 5). Furthermore, rotenoids should be biosynthesized from isoflavonoids with 2′-methoxy groups. 2′-Hydroxylation of isoflavones is thus an essential part of the biosynthesis of these biologically-active compounds. Isoflavone 2′-hydroxylase (I2′H) activities have been identified in the microsomal fractions of elicitor-treated soybean (6), chickpea (7, 8) and alfalfa (9) cells, and the enzyme has been considered to be a cytochrome P450 (P450) monooxygenase. Neither enzyme purification nor molecular cloning of its gene, however, has been reported.

Isoflavonoid-derived compounds are also important in the symbiotic relationship between the soybean root and rhizobial bacteria eventually resulting in the organogenesis of nitrogen-fixing root nodules (10, 11). Thus, the structural genes of isoflavonoid branch pathway and their expression are of certain interest from the viewpoint of adaptation of leguminous plants to environmental microorganisms which are either pathogenic or symbiotic (12). However, while cDNAs or genomic clones encoding isoflavone reductase of alfalfa (13), chickpea (14) and pea (15), and vestitone reductase of alfalfa (16), have been cloned, the majority of the reactions within this biosynthetic branch remains to be elucidated at the molecular level. Many reactions among these are presumed to be catalyzed by P450s; e. g., isoflavone synthase (6, 17), isoflavone 2′- and 3′-hydroxylases (6–9), 3,9-dihydroxypterocarpan 6a-hydroxylase (18), 2- and 4-dimethylallylglycinol cyclase (19), and methylenedioxy bridge forming enzyme (20).

Recent progress in the understanding of plant P450 genes has made possible the cloning of new P450 DNAs by the strategy based on sequence homology and functional analysis of overexpressed gene products in heterologous systems without prior purification of the protein (21, 22). We have cloned eight P450 cDNAs, CYP Ge-1 to CYP Ge-8, from elicitor-induced licorice (*Glycyrrhiza echinata* L., Fabaceae) cells by PCR-based method, and identified the catalytic functions of some of the proteins expressed in insect or yeast cells (23-25). In this report, we demonstrate that heterologously expressed CYP Ge-3 (CYP81E1) catalyzes the hydroxylation of isoflavones at C-2′.

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The sequence reported in this paper has been deposited in the DDBJ, GenBank, and EMBL databases (CYP81E1, Accession No. AB001379).

Abbreviations used: I2′H, isoflavone 2′-hydroxylase; P450, cytochrome P450.

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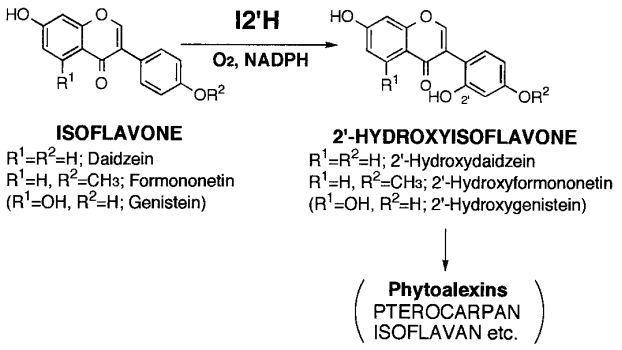

FIG. 1. The reaction catalyzed by isoflavone 2′-hydroxylase.

MATERIALS AND METHODS

Construction of expression vectors, expression in yeast cells and preparation of microsomes. *Kpn*I and *Sac*I sites were introduced by PCR with KOD polymerase (Toyobo, Tokyo, Japan) just upstream of the initiation codon and downstream of the stop codon of CYP81E1 sequence (24), using the Ge-3S1 primer (5′-CCAAGGTACCATGGAGATTCTATCCCTCTT-3′) and Ge-3A1 primer (5′-GGTGAGCTCTTACTGCTTGAAAACCTTGTTGATG-3′), with CYP Ge-3 cDNA in pBluescript SK(−) as the template. The *Kpn*I–*Sac*I fragment of the PCR product was cloned into corresponding sites of pYES2 expression vector (Invitrogen, California, USA). Introduction of the plasmid into *Saccharomyces cerevisiae*, selection of the transformant, induction of the P450 proteins, and preparation of spheroplasts were performed as already described (25). The yeast microsomes were also prepared as before (25) from the spheroplasts in 0.1 M K-Pi (pH 7.5) supplemented with 0.4 M sucrose and 3.5 mM 2-mercaptoethanol, by ultracentrifugation procedures. The final microsomal precipitates were homogeneously suspended in 0.1 M K-Pi (pH 7.0) containing 0.4 M sucrose, 0.35 mM 2-mercaptoethanol, and 5% glycerol (2.8 mg/ml protein).

Enzyme assay. The reaction mixture contained phenolic substrates (1 μg in 10 μl 2-methoxyethanol), 1 mM NADPH, and 1 ml yeast microsomal fraction. After incubation at 25°C, the reaction was terminated by the addition of 10 μl acetic acid. The mixture was then extracted with ethyl acetate, and an aliquots of organic layer were concentrated and subjected to HPLC on Shim-pack CLC-ODS column (6.0 × 150 mm; Shimadzu, Kyoto, Japan) with 40% methanol and 3% acetic acid in H_2O (for daidzein/2′-hydroxydaidzein and genistein) or 50% methanol and 3% acetic acid in H_2O (for formononetin/2′-hydroxyformononetin) at the flow rate of 1 ml/min at 40°C. The eluent was monitored at 248 nm for daidzein/2′-hydroxydaidzein and formononetin/2′-hydroxyformononetin or at 262 nm for genistein. The separation of 2′-hydroxydaidzein and 3′-hydroxydaidzein was performed on HPLC with the same column using a linear gradient elution of 20% to 35% acetonitrile during 35 min in 3% acetic acid/H_2O (flow rate, 0.8 ml/min). For mass spectrometric analysis, the incubations with formononetin and daidzein as the substrates were carried out in a large scale (×10 of the scale described above), and the ethyl acetate extracts of the reaction mixtures were applied to a preparative TLC either on Polyamid $11F_{254}$ [Merck (Darmstadt, Germany); solvent, toluene/ethyl acetate/methanol/light petroleum = 6:4:1:3; for formononetin metabolite] or on Kieselgel F_{254} (Merck; the same solvent as above; for daidzein metabolite). The materials at the putative 2′-hydroxyisoflavone spots were collected and further purified by HPLC. The spectra were recorded on a JEOL JMS-AX505H mass spectrometer under the electron impact mode with the ionization voltage of 70 eV.

Isoflavonoid materials. The samples were obtained from Extrasynthése (Genay, France; daidzein, 3′-hydroxydaidzein and genistein) or Plantech (Reading, UK; 2′-hydroxydaidzein and 2′-hydroxyformononetin). Formononetin was from our laboratory stock originally isolated from cultured licorice cells.

RESULTS AND DISCUSSION

CYP81E1 (CYP Ge-3) has been isolated from the cDNA library of elicitor-induced *G. echinata* cells (23). Transient transcriptional activation of CYP81E1 in elicited cells, and also of a gene hybridizable with CYP81E1 in elicited alfalfa cells, have been demonstrated (23). These cells are known to be stimulated to produce phenylpropanoid/flavonoid/isoflavonoid compounds by elicitor (26–28). In fact, other P450 clones isolated from the same source have been assigned to *trans*-cinnamate 4-hydroxylase (CYP Ge-1, 2) (23), (2*S*)-flavanone 2-hydroxylase (CYP Ge-5) (25) and ferulate 5-hydroxylase (CYP Ge-6, 7) (23), all of which are involved in phenylpropanoid (lignin) and flavonoid biosynthesis. Therefore we examined the function of CYP81E1 protein focusing attention on phenolic metabolism.

The microsome of galactose-treated yeast cells transformed with CYP81E1 was incubated with NADPH and candidate phenolics, and the reaction mixture was analyzed with reversed-phase HPLC. When an isoflavone formononetin (7-hydroxy-4′-methoxyisoflavone) was the substrate, a new peak of the reaction product appeared and increased constantly between 1 and 3 h of the reaction on HPLC chromatogram, and after 12 h the original peak disappeared almost completely (Fig. 2a). The retention time (Rt; 10.6 min) was the same to that of 2′-hydroxyformononetin (formononetin, Rt 16.6 min). The Rf value (0.15) of the product spot and 2′-hydroxyformononetin on polyamide TLC also coincided

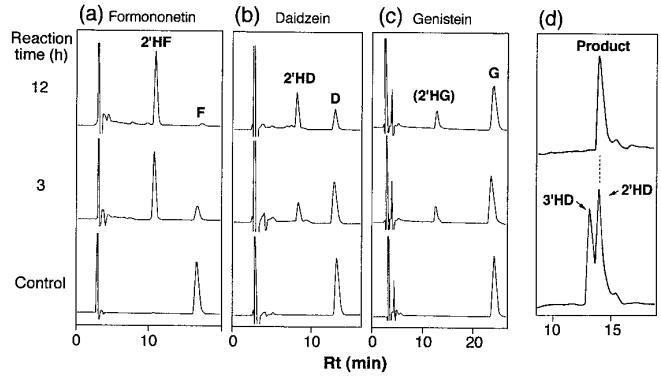

FIG. 2. HPLC profiles of the products from the reactions with microsomes of yeast cells expressing CYP81E1. The products from formononetin (F, in a), daidzein (D, in b) and genistein (G, in c) in 3 and 12 h reactions were analyzed with reversed-phase HPLC. 2′HF, 2′-hydroxyformononetin; 2′HD, 2′-hydroxydaidzein; 2′HG, putative 2′-hydroxygenistein. For the control, a microsome of yeast cells transformed with pYES2 was reacted with each substrate for 12 h. 2′HD peak in b was also collected and reanalyzed with a gradient elution to distinguish from 3′-hydroxydaidzein (3′HD, in d).

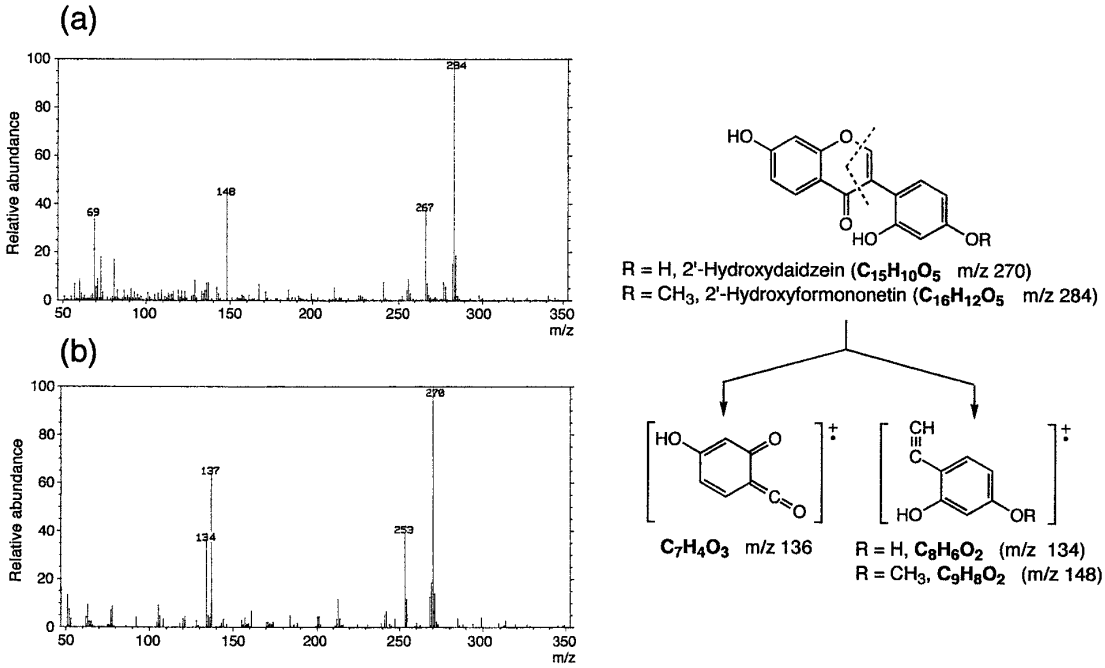

FIG. 3. Electron-impact mass spectra of the CYP81E1-catalyzed reaction products from formononetin (a) and daidzein (b). The scheme of retro-Diels-Alder fragmentation is also shown.

(formononetin, Rf 0.26). Daidzein (7,4′-dihydroxyisoflavone; Rt 13.9 min, Rf 0.24 on silica gel TLC) was also converted to a product which is chromatographically identical with 2′-hydroxydaidzein [Rt 9.0 min (Fig. 2b); Rf 0.22]. Genistein (5,7,4′-trihydroxyisoflavone; Rt 24.6 min) yielded an unknown product (Rt 13.0 min), which is supposed to be 2′-hydroxygenistein judged from the retention time on HPLC (Fig. 2c). Control microsome prepared from the yeast transformed with the vector without inserts did not react with these isoflavones (Figs. 2a–2c). When the 2′-hydroxydaidzein peak in Fig. 2b was recovered and reanalyzed with HPLC by a gradient elution, the reaction product was clearly distinguished from 3′-hydroxydaidzein (Fig. 2d).

The chemical structures of the products from formononetin and daidzein were confirmed by mass spectrometric analysis (Fig. 3). The products purified by TLC and HPLC from the reaction mixtures with formononetin and daidzein exhibited molecular ion peaks at *m/z* 284 and 270, respectively. The retro-Diels-Alder fragment peaks, which are in good agreement with the hydroxylated B-rings, were also observed at *m/z* 148 and 134. The spectra were identical with those of authentic samples of 2′-hydroxyformononetin and 2′-hydroxydaidzein.

The concentrations of CYP81E1 protein and total P450 proteins in the induced yeast cells were under the detection levels by SDS/PAGE with silver staining and CO difference spectrum (data not shown). However, the specific enzyme activity shown in Fig. 2 was certainly due to the plant P450 expressed in the yeast cells since the microsomes of yeast harboring vector plasmid pYES2 without CYP81E1 had no activity. Our observation is consistent with a previous report on the heterologous expression of alfalfa CYP73A3 (29). Also licorice CYP93B1 protein [(2*S*)-flavanone 2-hydroxylase (25)] was only detectable by SDS/PAGE in the recombinant insect cells, while the microsome of the recombinant yeast cells where the protein was invisible in SDS/PAGE was fully catalytically active.

The following phenolic substances were tested for the reaction, but not accepted as substrates (data not shown); naringenin (5,7,4′-trihydroxyflavanone), liquiritigenin (7,4′-dihydroxyflavanone), *trans*-cinnamic and 4-coumaric acids. Thus, the heterologously expressed CYP81E1 was demonstrated to have I2′H activity *in vitro*.

Chickpea and alfalfa are known to produce pterocarpan phytoalexins derived from formononetin, e.g., medicarpin and maackiain. Chickpea I2′H has been reported to be specific to 4′-methoxyisoflavones (formononetin and biochanin A), while the 4′-hydroxy analogues (daidzein and genistein) were scarcely hydroxylated (8). On the other hand, glyceollins, the representative soybean phytoalexins, should be biosynthesized from daidzein. The reaction rates with licorice CYP81E1 protein expressed in yeast were 340 nkat kg^{-1} protein or higher for formononetin, ca. 40 nkat kg^{-1} protein for daidzein and ca. 9 nkat kg^{-1} protein for genistein. At present, it is not known in what biosynthetic pathway the I2′H of licorice is involved, but

vestitol, a methylated isoflavan presumably derived from formononetin, has been isolated from the roots of the original *G. echinata* plant (30). The examination of the distribution and expression of genes homologous to CYP81E1 among the plants producing 2′-hydroxylated isoflavonoids with 5-hydroxy/deoxy and 4′-methoxy/hydroxy substitutions would provide a clearer view about the nature of defense related biosynthesis of isoflavonoids.

The amino acid sequence of CYP81E1 has 47.6% identity with CYP81B1, which encodes in-chain fatty acid hydroxylase and induced by treatment with xenobiotics in *Helianthus tuberosus* (31), and 50.7% identity with wound-inducible CYP91A1 (recently reassigned to CYP81D1) of *Arabidopsis thaliana* (32). The genes of CYP81 and CYP91 families including other functionally-uncharacterized members are possibly stress-inducible in plant cells and have the physiological roles in defense against microbial and chemical challenges.

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REFERENCES

1. Dewick, P. M. (1993) *in* The Flavonoids. Advances in Research since 1986 (Harborne, J. B., Ed.), pp. 117–238, Chapman and Hall, London.
2. Barz, W., and Welle, R. (1992) *in* Recent Advances in Phytochemistry. Vol. 26. Phenolic Metabolism in Plants (Stafford, H. A., and Ibrahim, R. K., Eds.), pp. 139–164, Plenum Press, New York.
3. Ingham, J. L. (1982) *in* Phytoalexins (Bailey, J. A., and Mansfield, J. W., Eds.), pp. 21–80, Blackie and Sons, Glasgow.
4. Guo, L., Dixon, R. A., and Paiva, N. L. (1994) *J. Biol. Chem.* **269,** 22372–22378.
5. Guo, L., Dixon, R. A., and Paiva, N. L. (1994) *FEBS Lett.* **356,** 221–225.
6. Kochs, G., and Grisebach, H. (1986) *Eur. J. Biochem.* **155,** 311–318.
7. Clemens, S., Hinderer, W., Wittkampf, U., and Barz, W. (1993) *Phytochemistry* **32,** 653–657.
8. Hinderer, W., Flentje, U., and Barz, W. (1987) *FEBS Lett.* **214,** 101–106.
9. Kessmann, H., Choudhary, A. D., and Dixon, R. A. (1990) *Plant Cell Rep.* **9,** 38–41.
10. Phillips, D. A. (1992) *in* Recent Advances in Phytochemistry. Vol. 26. Phenolic Metabolism in Plants (Stafford, H. A., and Ibrahim, R. K., Eds.), pp. 201–231, Plenum Press, New York.
11. Loh, J., Garcia, M., and Stacey, G. (1997) *J. Bacteriol.* **179,** 3013–3020.
12. Stafford, H. A. (1997) *Botanical Rev.* **63,** 27–39.
13. Paiva, N. L., Edwards, R., Sun, Y. J., Hrazdina, G., and Dixon, R. A. (1991) *Plant Mol. Biol.* **17,** 653–667.
14. Tiemann, K., Inze, D., Van Montagu, M., and Barz, W. (1991) *Eur. J. Biochem.* **200,** 751–757.
15. Paiva, N. L., Sun, Y., Dixon, R. A., VanEtten, H. D., and Hrazdina, G. (1994) *Arch. Biochem. Biophys.* **312,** 501–510.
16. Guo, L., and Paiva, N. L. (1995) *Arch. Biochem. Biophys.* **320,** 353–360.
17. Hashim, M. F., Hakamatsuka, T., Ebizuka, Y., and Sankawa, U. (1990) *FEBS Lett.* **271,** 219–222.
18. Kochs, G., and Grisebach, H. (1989) *Arch. Biochem. Biophys.* **273,** 543–553.
19. Welle, R., and Grisebach, H. (1988) *Arch. Biochem. Biophys.* **263,** 191–198.
20. Clemens, S., and Barz, W. (1996) *Phytochemistry* **41,** 457–460.
21. Bak, S., Kahn, R. A., Nielsen, H. L., Møller, B. L., and Halkier, B. A. (1998) *Plant Mol. Biol.* **36,** 393–405.
22. Pauli, H. H., and Kutchan, T. M. (1998) *Plant J.* **13,** 793–801.
23. Akashi, T., Aoki, T., Takahashi, T., Kameya, N., Nakamura, I., and Ayabe, S. (1997) *Plant Sci.* **126,** 39–47.
24. Akashi, T., Aoki, T., Kameya, N., Nakamura, I., and Ayabe, S. (1997) *Plant Physiol.* **115,** 1288.
25. Akashi, T., Aoki, T., and Ayabe, S. (1998) *FEBS Lett.* **431,** 287–290.
26. Dixon, R. A., and Paiva, N. L. (1995) *Plant Cell* **7,** 1085–1097.
27. Ayabe, S., Iida, K., and Furuya, T. (1986) *Phytochemistry* **25,** 2803–2806.
28. Kirikae, Y., Sakurai, M., Furuno, T., Takahashi, T., and Ayabe, S. (1993) *Biosci. Biotechnol. Biochem.* **57,** 1353–1354.
29. Fahrendorf, T., and Dixon, R. A. (1993) *Arch. Biochem. Biophys.* **305,** 509–515.
30. Nomura, T., and Fukai, T. (1998) *in* Progress in the Chemistry of Organic Natural Products (Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., and Tamm, Ch., Eds.), Vol. 73, pp. 1–140, Springer, Wien.
31. Cabello-Hurtado, F., Batard, Y., Salaun, J. P., Durst, F., Pinot, F., and Werck-Reichhart, D. (1998) *J. Biol. Chem.* **273,** 7260–7267.
32. Mizutani, M., Ward, E., and Ohta, D. (1998) *Plant Mol. Biol.* **37,** 39–52.