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P-450-DEPENDENT OXIDATIVE REARRANGEMENT IN ISOFLAVONE BIOSYNTHESIS: RECONSTITUTION OF P-450 AND NADPH:P-450 REDUCTASE

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ABSTRACT The reaction mechanism of oxidative rearrangement in the conversion of liquiritigenin, a flavanone, into 2,7,4'-trihydroxyisoflavanone was studied in elicitor-challenged *Pueraria lobata* cell cultures. The involvement of cytochrome P-450 in the reaction, hydroxylation associated with 1,2-aryl migration, was proved previously by the inhibition experiments with carbon monoxide and P-450 inhibitors. In order to obtain rigorous evidence proving that the enzyme is a P-450, a reconstitution experiment was performed with solubilized cytochrome P-450 and NADPH:cytochrome P-450 reductase fractions. During these studies we noticed that various biosynthetic reactions can be interpreted as P-450-mediated reactions associated with migration or bond cleavage. Ring contraction of 7-hydroxy-kaurenoic acid in gibberellin biosynthesis, the formation of a furan ring in furanocoumarin biosynthesis and several rearrangement reactions in steroid metabolism are discussed as examples of P-450 reactions associated with migration or bond cleavage.

Phenolic compounds of shikimate origin have important functions in plants. The derivatives of cinnamic acids are not only the precursors of phenylpropanoids, lignans and lignin, but also serve as a building unit of flavonoids. The biosynthetic pathway of flavonoids branches from the pathway of cinnamate metabolism to form a wide variety of compounds having C_6-C_3-C_6 skeleton. Isoflavonoids are a group of phenolic compounds having C_6-C_3-C_6 structures, in which a phenyl group is present at C-3 of the pyrone ring. They are widely distributed among higher plants and exhibit a variety of biological activities.[1] In the defense response of leguminous plants against phytopathogenic microorganisms isoflavonoids are produced as phytoalexins having antimicrobial activity.[2] In *Pueraria lobata* (Leguminosae) for example, tuberosin and glycinol, pterocarpans, have been reported to be phytoalexins of leaves.[3] In stem tissues of *P. lobata*, tuberosin, 8-prenyldaidzein and 8-prenylgenistein were identified as isoflavonoids induced by $CuCl_2$.[4] The pathway of isoflavonoid biosynthesis shares a part of the common pathway of flavonoid biosynthesis. Enzymes relating to flavonoid biosynthesis, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), etc., are essential enzymes in both flavonoid and isoflavonoid biosyntheses. Their ubiquitous distribution and intrinsic functions in plants drew the attention of many researchers. The enzymes were well characterized and some of their genes were cloned and sequenced.[5] Recent developments of molecular biology made it possible to study the regulatory mechanisms of flavonoid biosynthesis at the gene level. Gene expression induced by various stimuli such as elicitors and light as well as that during differentiation and development has been extensively studied.[6] However, information on the isoflavone biosynthesis at the enzyme level became available rather recently.[7]

The rearrangement of flavanone (**1**) into isoflavone (**7**) is the first reaction of isoflavonoid biosynthesis which branches from the general flavonoid pathway.[7a,b,c,e] It has been firmly established by competitive

incubation experiments with [^{14}C]-chalcone and [^{3}H]-flavanone that the substrate of the rearrangement reaction is flavanone.[7e] Since 2-hydroxyisoflavanone was formed as an intermediate,[7b,d] it is evident that the reaction consists of two steps. The first step is the oxidative 1,2-aryl migration of flavanone to yield 2-hydroxyisoflavanone which is catalyzed by membrane bound proteins, whereas the second is the dehydration catalyzed by a soluble enzyme to form a double bond between C-2 and C-3 yielding isoflavone. The above mentioned reaction intermediate, 2-hydroxyisoflavanone, was isolated from the incubation mixture of isoflavone synthesis in soybean cell cultures and was identified to be 2,5,7,4'-tetrahydroxyisoflavanone (**6**, **R**=OH) from the mass and UV spectral data.[7b] In *P. lobata*, we also determined the intermediate as 2,7,4'-trihydroxy-isoflavanone (**6**, **R**=H : **11**) by mass and ^{1}H NMR spectroscopies.[7d]

Several hypotheses were proposed for the reaction mechanism of this oxidative 1,2-aryl migration reaction.[8] Kochs and Grisebach proposed a mechanism which involved an enol epoxide (**3**) of flavanone as an intermediate (Fig. 1).[7a,b] The migration of phenyl group is initiated by the cleavage of the epoxide ring in a fashion that is unfavorable from the view of chemical reaction. Crombie *et al.* suggested another mechanism in which epoxidation occurred on the side phenyl group of flavanone.[9] These mechanisms were ionic ones and the hydroxy group at C-2 of 2-hydroxyisoflavanone (**6**) was expected to be introduced from water. Migration of a 4-hydroxy oxygen of a 4,4-diol (e.g., **5**) to the C-2 position is one of alternative possibilities, but the reaction must proceeds *via* a highly strained compound having a four member ether ring with an acetal and hemiketal structure.

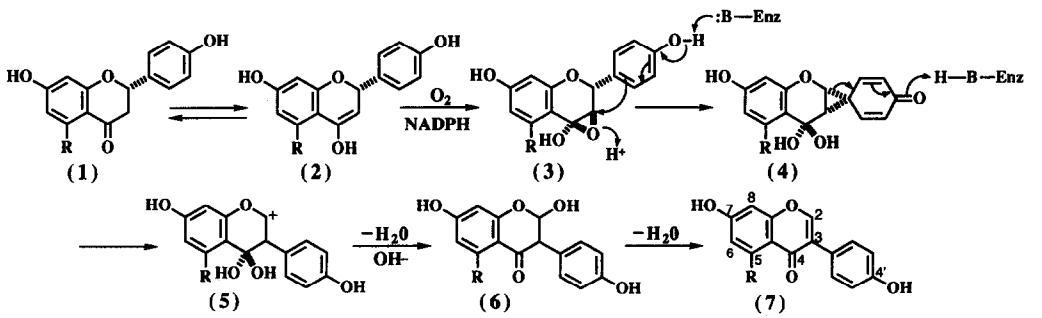


Fig. 1 Proposed Reaction Mechanism for Isoflavone Biosynthesis *via* Enol Epoxide[7a,b]

To clarify the mechanism of the oxidative rearrangement, the origin of two oxygen atoms, the carbonyl oxygen at C-4 in 2-hydroxyisoflavanone (**11**) / isoflavone (**12**) and the hydroxy oxygen at C-2 in **11**, was investigated with the microsomal preparations from *P. lobata* cell cultures.[7d] Almost complete retention of 4-carbonyl oxygen during the conversion was demonstrated by the incubation experiments with [4-^{18}O]flavanone (**8**). This was further supported with an incubation experiment under the atmosphere of $^{18}O_2$ gas which showed no incorporation of ^{18}O into the 4-carbonyl of isoflavone (**12**). On the other hand, an ^{18}O atom from $^{18}O_2$ gas in an incubation mixture was incorporated into the 2-hydroxyl group of 2-hydroxyisoflavanone (**11**). Based on these observations, a new reaction mechanism, hydroxylation associated with 1,2-aryl migration, has been proposed in a previous communication, for the P-450-mediated formation of 2-hydroxyisoflavanone from flavanone (see Fig. 2).[7d] The participation of cytochrome P-450 in the conversion of flavanone to 2-

hydroxyisoflavanone was reported taking account of carbon monoxide inhibition and its partial reversal by light, and inhibition with so called P-450 inhibitors.[7b,d] In addition to a P-450 which contains a protoporphyrin, P-450 reactions require a NADPH:cytochrome P-450 reductase which supplies electrons to P-450 by reducing NADPH. In the studies of P-450 enzymes, reconstitution experiments with solubilized P-450 and reductase are carried out to prove rigorously the P-450 nature of the enzymes under investigation. In contrast to bacterial and mammalian systems, there have been a few reports in higher plants on the resolution of P-450-dependent monooxygenases into individual components and subsequent reconstitution experiments.[10] This paper deals with the results of reconstitution experiments with solubilized P-450 and NADPH:P-450 reductase fractions prepared from the microsomes of elicitor-challenged *P. lobata* cells. During the course of the studies, we have noticed that a considerable number of biosynthetic rearrangement reactions are reasonably explained as P-450-mediated reactions associated with migration. Several biosynthetic reactions which involve rearrangement reactions will be also discussed as examples of oxidative reactions by P-450s.

RESULTS AND DISCUSSION

1. Solubilization of microsomal proteins

Prior to solubilization experiments, several detergents were tested for their influence on enzyme activity in 2,7,4'-trihydroxyisoflavanone synthesis (Table. 2). All the detergents tested decreased enzyme activity around their critical micellar concentration. Though Lubrol and sodium cholate were less inhibitory toward the enzyme, significant activity could not be recovered in a solubilized 105,000 x g supernatant (data not shown). Of the detergent tested, Triton X-100 provided the most consistent results, therefore, this detergent was selected for solubilization experiments.

Table. 2 Effect of Different Detergents on 2,7,4'-Trihydroxyisoflavanone Synthesis Activity

Detergent	CMC* (%)	Concentration (%)	Relative enzyme activity (%)
None	—	—	100
Triton X-100	0.02	0.01	65
		0.02	50
		0.04	34
CHAPS	0.49	0.25	15
		0.49	19
Octyl glycoside	0.73	0.37	62
		0.73	7
Lubrol	0.006	0.003	85
		0.006	75
		0.012	60
Sodium cholate	0.6	0.18	82
		0.36	71
		0.72	44

* Critical micellar concentration

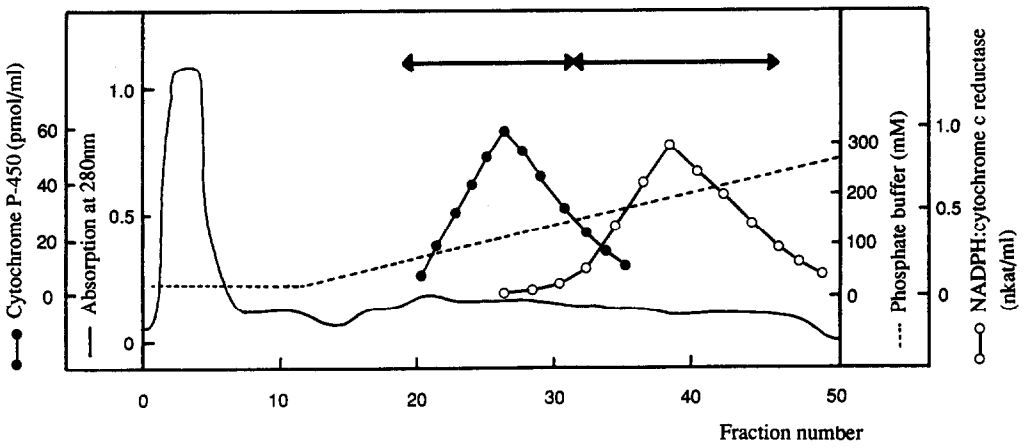


Fig. 2 DEAE-Sepharose Chromatography of *P. lobata* Microsomal Proteins Solubilized with 2% Triton

2. Resolution and reconstitution of cytochrome P-450 enzyme system

DEAE-Sepharose chromatography of the solubilized fraction gave a good separation of cytochrome P-450 from NADPH:cytochrome P-450 (cytochrome c) reductase (Fig. 2). Cytochrome P-450 concentration was determined from the difference spectra of cytochrome-CO adduct reduced with $N_2S_2O_4$. NADPH:cytochrome P-450 reductase activity was assayed by measuring NADPH-dependent reduction of cytochrome c. The fractions containing cytochrome P-450 were pooled as shown in Fig. 2 (specific content: 107 pmol/mg protein) and concentrated. The fractions containing NADPH:P-450 reductase were also pooled (specific activity: 4.17 nkatal/mg protein) and concentrated. With these concentrated fractions, reconstitution experiments were carried out in the presence of *P. lobata* microsomal lipids. As described in Table. 3, enzyme activity of 2,7,4'-trihydroxyisoflavanone synthesis was reconstituted when cytochrome P-450 fractions and reductase fractions were combined. There was some 2,7,4'-trihydroxyisoflavanone synthesis activity with cytochrome P-450 fractions alone. This can be explained by the contamination of the cytochrome P-450 fractions with traces of NADPH:cytochrome P-450 reductase.

Table.3 Reconstitution of 2,7,4'-Trihydroxyisoflavanone Synthesis Activity

Conditions	2,7,4'-trihydroxyisoflavanone synthesis activity (pkat)
Cytochrome P-450 + lipids	0.0026
NADPH:cytochrome P-450 reductase + lipids	0
P-450 + reductase + lipids	0.0104

The assay mixture contained P-450 (9.5 pmol) and reductase (3.7 nkat).
The incubation was carried out at 30° for 45 min.

The results of reconstitution experiments have clearly demonstrated that liquiritigenin (**8**) is converted into 2,7,4'-trihydroxyisoflavanone (**11**) by P-450-mediated reaction. The mechanism proposed in the previous communication (Fig. 3), the hydroxylation associated with rearrangement, is reasonable as P-450 reactions, since the underlying reaction, hydrogen abstraction and subsequent hydroxylation, is typical for P-450. During these studies we noticed that many rearrangement reactions in the biosynthesis of natural products were reasonably interpreted as the hydroxylation reactions associated with rearrangements.

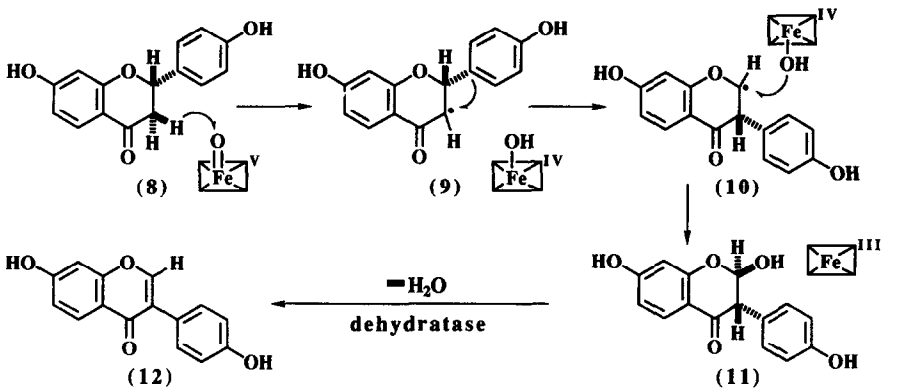
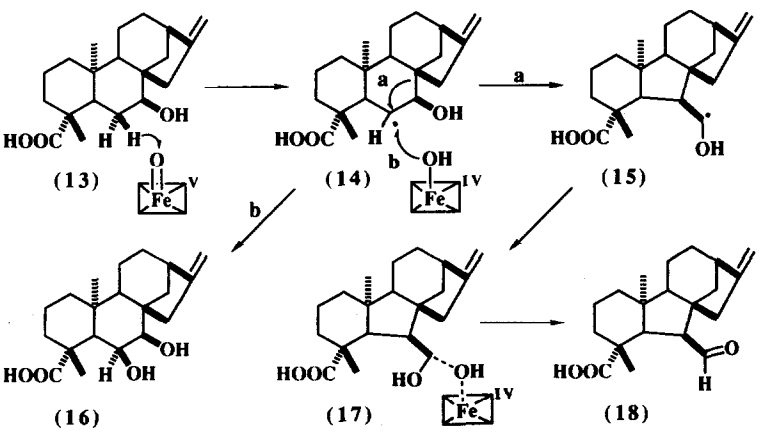


Fig. 3 A New Reaction Mechanism for the Oxidative Aryl Migration in Isoflavone Biosynthesis Catalyzed by Cytochrome P-450 Enzyme System[7d)]

3. Ring contraction of *ent*-7-hydroxykaurenoic acid (13)

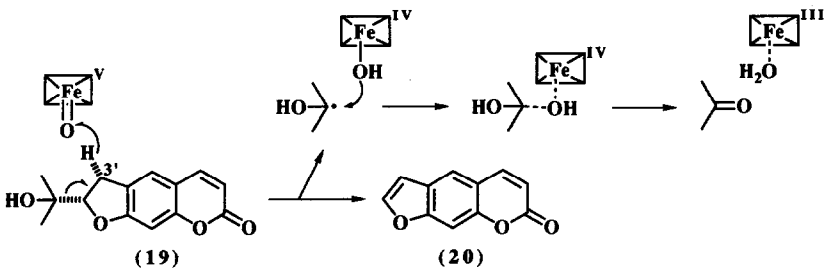
A typical example is the ring contraction of *ent*-7-hydroxykaurenoic acid (**13**) to form a gibberellin (GA_{12} aldehyde (**18**)).



The reaction was reported to be catalyzed by a microsomal preparation in the presence of NADPH and O_2, and the reaction mechanism was explained by the abstraction of hydrogen at C-6 followed by 1,2-migration and hydroxylation with a radical mechanism, though no evidence was presented for the participation of a P-450.[11] *Ent*-6,7-dihydroxykaurenoic acid (**16**) was also formed from **13** along with **18** in the incubation experiment with the same microsomal preparation.[11] Both reactions are explainable by P-450-catalyzed reactions. The mechanism of ring contraction of **13** is strikingly similar to the P-450 reaction of isoflavone biosynthesis. It may be worthwhile to note here that the hydroxylation at the newly generated radical center to give a *gem*-diol is not an absolute requirement in forming the aldehyde group, since desaturation reactions have been recognized in several P-450 reactions.[12] The scheme illustrates a possible structure of a transition state (**17**), in which recombination yields a *gem*-diol and desaturation yields an aldehyde directly. These reactions are schematically regarded as the selective cleavage of the Fe-O or C-O bond in the transition state.

4. Formation of a furan ring of furanocoumarin, psoralen (20)

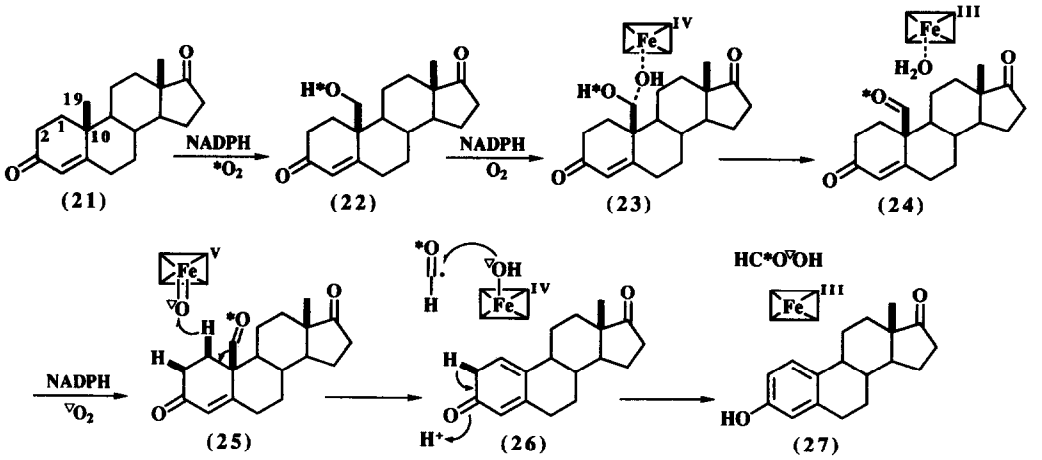
If P-450 catalyzed reaction associated with rearrangement is further extended to those involving 2,3-bond-cleavage, we can find many cases which are explainable by P-450 reactions. Microsomal fractions isolated from parsley cell cultures, which had been challenged with a fungal elicitor, catalyzed the formation of psoralen (**20**) from (+)marmesin (**19**). The reaction requires NADPH, and was inhibited by CO and various P-450 inhibitors, indicating that the responsible enzyme is a P-450.[13] No intermediate was detected in the conversion of **19** into **20** in this reaction system. The reaction is reasonably interpreted as a P-450 reaction associated with 2,3-bond-cleavage. Hydrogen abstraction at C-3' followed by C-C bond cleavage yielded **20** and isopropyl radical, and the latter was further converted into acetone indirectly via *gem*-diol by hydroxylation or directly by desaturation.



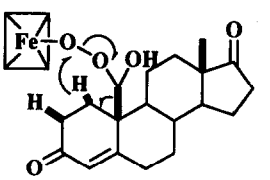
5. Demethylation reaction in steroid metabolism

Several reactions in steroid biosynthesis also fall in this category of P-450 reactions.[14] The best characterized P-450 type enzyme is aromatase which catalyzes the conversion of androstenedione (**21**) into estrone (**27**).

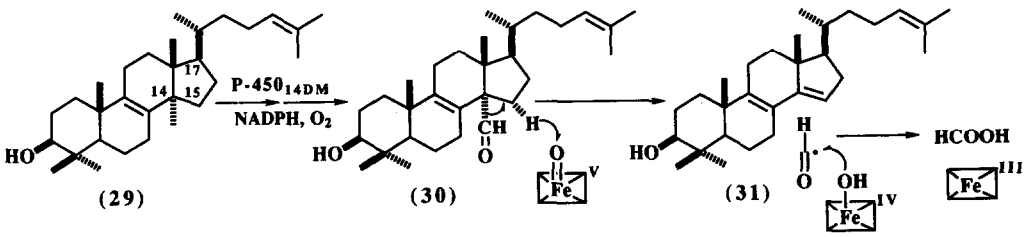
Many experimental evidences have been accumulated in regard to the reaction of aromatase. Aromatase has been completely purified and proved to be a single protein,[15] indicating that it catalyzes three reaction steps;



hydroxylation of the C-19 methyl group, oxidation of the hydroxymethyl group into aldehyde (**24**), and final deformylation and aromatization.[15] Detailed studies on the origin of oxygen atoms of formate formed in the reaction revealed that both oxygen atoms of formate were derived from atmospheric oxygen, but not from water.[16] The two oxygen atoms of formate were introduced in the first oxidation forming the hydroxymethyl group and in the last deformylation step, but not in the second oxidation of the hydroxymethyl group to yield the oxo-group.[16] Stereochemistry in the loss of hydrogen atoms from C-1 and C-2 of A-ring was also investigated with stereospecifically labeled compounds.[17] β-Hydrogen atoms of C-1 and C-2 were lost stereospecifically during the aromatase reaction. To give a reasonable explanation for the third step of the aromatase reaction the participation of a peroxide species of P-450 (**28**) was proposed, [16,18] though the first and second oxidation reactions are typical for P-450 and explainable with feroxene species. The final reaction of aromatase is reasonably explained by a P-450 reaction associated with C-C bond cleavage as it is suggested for psoralen (**20**) biosynthesis. The abstraction of C-1β hydrogen followed by C-C bond cleavage between C-10 and C-19 would yield an unstable dienone(**26**) and formyl radical. The dienone is readily converted into **27** by stereospecific removal of C-2β hydrogen, while formyl radical is hydroxylated by a recombination reaction to give formate. This interpretation is quite reasonable from the capacity a of P-450 reaction.


(28)

The same mechanism is also applicable to the C-14 demethylation reaction of lanosterol (**29**),[19] in which hydrogen abstraction in deformylation reaction occur at C-15α.[20] In addition to the examples discussed above, cholesterol side chain cleavage (SCC) giving pregnenolone, and C-17 side chain cleavage of pregnenolone giving testosterone are also explainable by similar type of P-450 reactions associated with C-C bond cleavage.[21]



The authors wish to emphasize that many reactions in the biosyntheses of natural products are reasonably explained by P-450 reactions associated with migration or bond cleavage. Several reactions are under investigation in our laboratory.

EXPERIMENTAL

General UV spectra were measured with a Hitachi spectrophotometer, model 100-60.
Materials Daidzein (**12**) and racemic-liquiritigenin (**8**) were from our collection. Triton X-100 was from NAKARAI TESQUE(Kyoto, Japan). CHAPS and octyl glycoside were from WAKO(Tokyo). Lubrol and sodium cholate were from SIGMA. DEAE-Sepharose CL-6B was obtained from Pharmacia. Bio-Beads SM-2 was from Bio-Rad.
Cell cultures and elicitor treatment Cell suspension cultures of *Pueraria lobata* were subcultured every three weeks in Murashige and Skoog's medium supplemented with 2 ppm of 2,4-D, 0.1 ppm of kinetin and 3% of sucrose.[7e] Endogenous elicitor was prepared from *P.lobata* cell wall by enzymic digestion with endopolygalacturonase of *Aspergillus niger* (SIGMA).[7e] Seven-day-old cultures were challenged with the endogenous elicitor and incubated for 20-24h. The cells were harvested and immediately frozen in liquid nitrogen for storage at -80°.
Preparation of microsomes The elicitor-challenged cells (100 g fresh weight) were ground in a mortar together with sand (50 g), polyvinylpyrrolidone (10 g) and buffer A (150 ml, pH 7.5) containing potassium phosphate (0.1 M), 2-mercaptoethanol (14 mM) and sucrose (10%). The mixture was filtered through nylon gauze and the filtrate was stirred for 20 min with Dowex I X 2 (20 g) pre-equilibrated with buffer A. After centrifugation at 20,000 x g for 30 min, the supernatant was further ultracentrifuged at 105,000 x g for 60 min. The pellet was suspended in 20 ml of buffer B (pH 7.5) containing potassium phosphate (10mM), 2-mercaptoethanol (14mM) and glycerol (25%, w/v). The slurry was homogenized in a glass homogenizer to give a microsomal suspension with a final protein concentration of 2-2.5 mg/ml.
Solubilization Triton X-100 was slowly added to the microsomal suspension with stirring to give a final concentration of 2% . The mixture was sonicated for three 30-sec bursts in a NISSEI Ultrasonic cleaner (Model NS200 6U) and stirred for an additional 30 min. The material was centrifuged at 105,000 x g for 1 h and the resulting supernatant was used for further reconstitution experiments.
Isolation of microsomal lipids The microsomal lipids were extracted from the microsomal suspension (2 ml) of *P. lobata* by treatment with 40 ml of chloroform/methanol (2:1, v/v). The organic phase was washed with 8 ml of calcium chloride (50 mM) and further with an equal volume of methanol/water (1:1, v/v). The organic phase was evaporated to dryness under nitrogen gas and then suspended in 1 ml of water by sonication. This mixture was used for reconstitution experiments.

Resolution of cytochrome P-450 enzymes The solubilized fraction (20 ml) was applied to a DEAE-Sepharose CL-6B column (1.9 x 7 cm) which had been equilibrated with buffer C (pH 7.5) containing potassium phosphate (10 mM), 2-mercaptoethanol (14 mM), glycerol (25%, w/v) and Triton X-100 (0.02%). The column was washed with 50 ml of buffer C, and then eluted with 200 ml of a linear 10 to 300 mM potassium phosphate gradient in buffer C. Early fractions (fr. 19-31, Fig. 2) which contained cytochrome P-450 were pooled (55 ml, 963 pmol) and concentrated by ultrafiltration with Centriprep-10 (AMICON) to 2.7 ml. Excess detergent was removed by treatment with Bio-Beads SM-2 (1 g/ml). Fractions having NADPH:cytochrome P-450 (cytochrome c) reductase activity (fr. 32-46, Fig. 2) were also pooled (65 ml, 24.6 nkat) and concentrated to 7 ml and treated with Bio-Beads SM-2.

Reconstitution experiments The mixture containing 200 µl of cytochrome P-450 fraction (9.5 pmol), 200 µl of NADPH:P-450 reductase fraction (3.7 nkat), 100 µl of microsomal lipids and 20 nmol of DL-liquilitigenin was kept at 4° for 30 min. NADPH was then added to the assay mixture to give a final concentration of 1 mM and incubated at 30° for 45 min. The reaction products were extracted and analyzed by HPLC as described in standard assay method (see below).

Enzyme assay For standard assay of 2-hydroxyisoflavanone or isoflavone synthesis, microsomal suspension prepared as described above was diluted with buffer B to a final protein concentration of 0.2-0.6 mg/ml. Incubation was carried out for 20 min at 30° with the microsomes (500 µl), racemic-liquiritigenin (**8**) (10 nmol) and NADPH (10 nmol) in a total volume of 520 µl. Reaction was initiated by adding NADPH. The reaction was stopped by rapid chilling on ice and extracted with ethyl acetate (2 ml). 1.5 ml of organic layer was dried under N_2 gas and analyzed by reverse phase HPLC (TSK gel ODS-120A, 4.6 x 250 mm, Tosoh) with a solvent system of acetonitrile-water-acetic acid (19:57:4) at a flow rate of 0.8 ml/min. Retention times (min) of 2,7,4'-trihydroxyisoflavanone (**11**), liquiritigenin (**8**) and daidzein (**12**) were 8.9, 23.0 and 24.5, respectively. NADPH:cytochrome P-450 (cytochrome c) reductase was assayed according to Benveniste et al[22]. The NADPH-dependent reduction of cytochrome c was measured spectrophotometrically at 550 nm in the presence of cytochrome c (0.05 mM) and NADPH (1 mM). The enzyme activity was calculated using a molar absorption coefficient of 21 $mM^{-1}cm^{-1}$.

Analytical methods Determination of cytochrome P-450 was carried out by the procedure of Omura and Sato[23] using a molar absorption coefficient of 91 $mM^{-1}cm^{-1}$. Protein concentration was estimated by the Bio-Rad protein assay with bovine serum albumin as standard.

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