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Short Communication

Molecular Cloning and Biochemical Characterization of a Novel Cytochrome P450, Flavone Synthase II, that Catalyzes Direct Conversion of Flavanones to Flavones

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Cytochrome P450 cDNAs, AFNS2 and TFNS5, were isolated from snapdragon and torenia petal cDNA libraries, respectively, based on the sequence homology with licorice CYP93B1 cDNA encoding (2*S*)-flavanone 2-hydroxylase. They were expressed in yeast and identified to encode flavone synthase II catalyzing direct conversion of flavanones to flavones probably via 2-hydroxyflavanones.

Key words: cDNA cloning — Cytochrome P450 — Flavone synthase II — Flavonoid biosynthesis — Snapdragon — Torenia.

Cytochrome P450 (P450) enzymes catalyze various important reactions in flavonoid/isoflavonoid biosynthesis. These include aromatic hydroxylases (flavonoid 3′-hydroxylase (Brugliera et al. 1997), flavonoid 3′,5′-hydroxylase (Holton et al. 1993), and isoflavone 2′-hydroxylase (Akashi et al. 1998a)), and monooxygenases acting on non-aromatic (sp^3) carbons ((2*S*)-flavanone 2-hydroxylase (F2H) (Akashi et al. 1998b), 2-hydroxyisoflavanone synthase (IFS) (Akashi et al. 1999), and pterocarpan 6a-hydroxylase (Schopfer et al. 1998)), which all participate in production of diverse flavonoid/isoflavonoid structures. In spite of their importance, characterization of plant P450 proteins has been extremely difficult due to their scarcity, diversity and the fact that they are membrane proteins bound to endoplasmic reticulum. Thanks to the development of molecular biology, the genes of hundreds of plant P450s have been cloned, and some of them have been functionally identified using heterologously expressed proteins (Bolwell et al. 1994, Chapple 1998, Schuler 1996). P450s are grouped into families based on their sequence homologies and assigned the same CYP numbers when the amino acid sequences are more than 40% identical (Nelson et al. 1993). They are further classified into the same subgroup when they have more than 55% identity. Naturally, the catalytic activities of P450s within the same families/subfamilies are generally the same or similar.

Flavones play important roles in plant-microorganism interaction in legumes (Phillips 1992) and contribute to increasing flower color diversity by forming complexes with anthocyanins (Goto and Kondo 1991). Two independent routes have been suggested in the formation of flavones from flavanones. Flavone synthase I (FNS I) is a soluble 2-oxoglutarate-dependent enzyme found in parsley (Britsch 1990, Britsch et al. 1981), and flavone synthase II (FNS II) is a P450 enzyme demonstrated in crude cell-free extracts or microsomes of snapdragon (Stotz and Forkmann 1981) and soybean (Kochs and Grisebach 1987, Kochs et al. 1987) (Fig. 1). FNS II activity has further been described in widespread plant species (Heller and Forkmann 1994) including a very recent report on the gerbera enzyme (Martens and Forkmann 1998). No enzyme purification work on FNS II, however, has been reported.

F2H of the leguminous plant licorice hydroxylates C-2 of flavanones and has been thought to represent licodione synthase (when the substrate is liquiritigenin) (Otani et al. 1994) and a flavone synthase (when the substrate is naringenin), the latter of which forms 2-hydroxynaringenin and requires a dehydratase catalyzing 1,2-dehydration to form apigenin (Akashi et al. 1998b). A cDNA for F2H has been identified and named CYP93B1 (Akashi et al. 1998b). This has been the only molecular cloning report relevant to flavone biosynthesis, and genes of flavone synthase catalyzing direct conversion of flavanone to flavone have not been reported yet.

To investigate flavone biosynthesis in petals and exa-

Abbreviations: F2H, (2*S*)-flavanone 2-hydroxylase; FNS I, flavone synthase I; FNS II, flavone synthase II; IFS, 2-hydroxyisoflavanone synthase; P450, cytochrome P450.

The nucleotide sequences reported in this paper have been submitted to the DDBJ, GenBank and EMBL under accession numbers: AB028151 (AFNS2), AB028152 (TFNS5).

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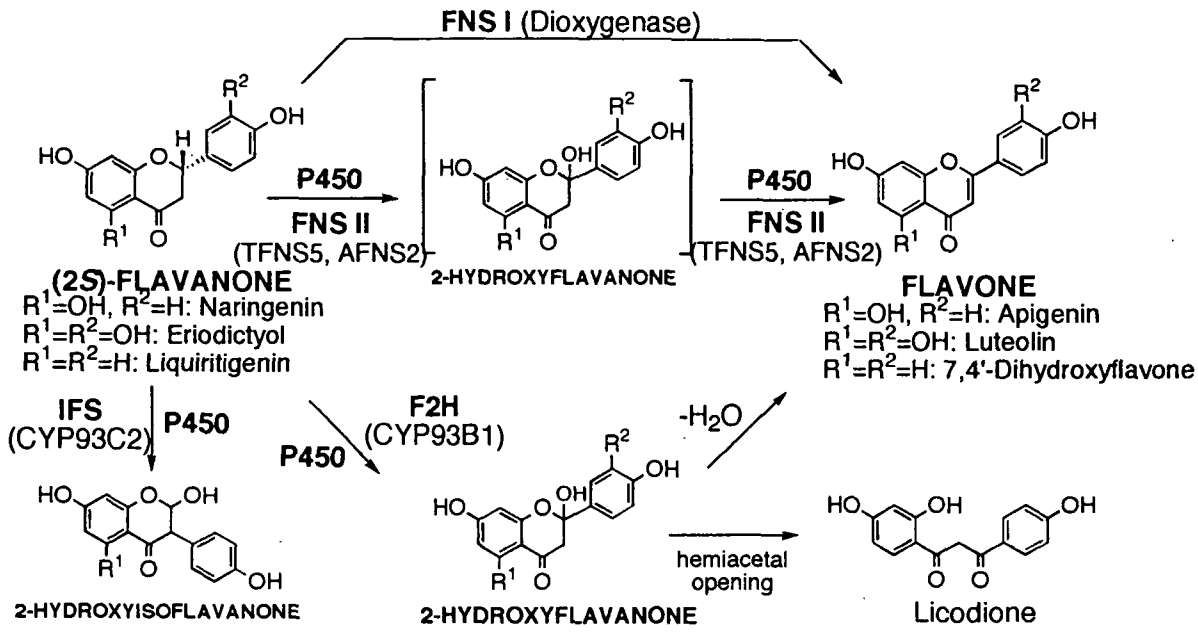

Fig. 1 Involvement of cytochrome P450s and a soluble oxidase in biosynthesis of flavones and related compounds. CYP93B1 catalyzes the first step of flavone and licodione formation. It is assumed that a dehydratase is necessary to complete the flavone synthesis reaction. AFNS2 and TFNS5 catalyze both steps and directly convert flavanones to flavones.

mine the distribution and functions of CYP93B and other related proteins, we screened the petal cDNA libraries of snapdragon and torenia, both belonging to Scrophulariaceae and accumulating a large amount of flavones, using licorice CYP93B1 as a probe. We isolated full-length P450 cDNAs and analyzed the function of snapdragon and torenia enzymes using yeast expression systems. We here report the surprising finding that the new P450s perform apparent direct flavone formation from flavanones (F2H reaction plus dehydration), and thus the protein should be designated a genuine FNS II.

Cloning of AFNS2 and TFNS5—Petal cDNA libraries of a snapdragon cultivar Yellow Butterfly and a torenia cultivar Summerwave Blue were constructed as described previously (Tanaka et al. 1996). The snapdragon cDNA library was screened as reported (Tanaka et al. 1996) with CYP93B1, and a partial cDNA clone encoding a homolog of CYP93B1 (AFNS1) was cloned. Further screening with the partial cDNA afforded a full-length P450 cDNA clone, AFNS2. Screening of the torenia library using the mixture of CYP93B1 and AFNS1 as the probes yielded the full-length clone TFNS5. The amino acid sequences of CYP93B1 and AFNS2, CYP93B1 and TFNS5, and AFNS2 and TFNS5, were 53%, 52% and 77% identical, respectively. The phylogenietic tree (Fig. 2) composed of P450s that are supposed to encode enzymes using flavanones and related compounds as substrates (see below) clearly demonstrates that snapdragon and torenia P450s are the closest relatives, and closely related to CYP93B1. Flavonoid 3′,5′-hydroxylase, an aromatic hydroxylase acting on flavanones, belongs to another P450 family, CYP75.

Spatial and temporal expression of TFNS5 in torenia was examined by Northern analysis as previously reported (Holton et al. 1993). The transcripts were abundant in petals of buds and less in mature petals and leaves (data not shown). This agrees with the reported activity changes of FNS II in gerbera flowers (Martens and Forkmann 1998).

Catalytic function of AFNS2 and TFNS5 proteins—

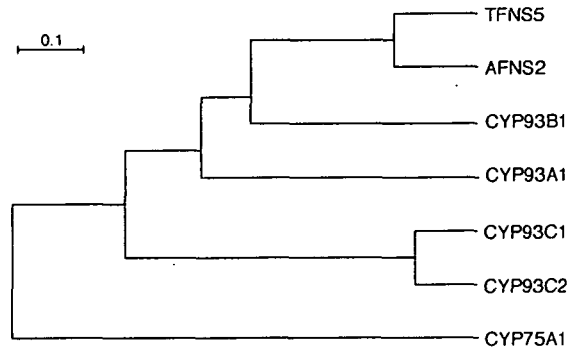

Fig. 2 Phylogenetic relationship of P450s involved in flavonoid biosynthesis. The UPGMA program (Genetyx-Mac Ver 8.0 software; Software Development Co., Tokyo, Japan) was used. CYP93B1 (F2H), CYP93C2 (IFS), TFNS5 and AFNS2 (FNS II), and CYP75A1 (flavonoid 3′,5′-hydroxylase) recognize flavanones as the substrates. The substrate for CYP93A1 (pterocarpan) has a similar stereochemical structure to the flavanone (Akashi et al. 1999). TFNS5 and AFNS2 are the closest.

AFNS2 and TFNS5 cDNAs were integrated into the yeast expression vector pYES2 (Invitrogen) and then used to transform yeast (BJ2168, Nippon Gene) as described before (Akashi et al. 1998b). After induction by galactose (24 h), the microsomes of the recombinant yeast were prepared to perform assay with flavanones (20 μg each for ca. 0.9 mg microsomal protein in 0.5 ml of 0.1 M K-P_i (pH 7.5) containing 10% (w/v) sucrose and 14 mM 2-mercaptoethanol) in the presence of NADPH (1 mM) as the electron donor. The reaction mixture was extracted with ethyl acetate and analyzed by reverse phase HPLC (column, Shim-pack CLC-ODS (6.0 × 150 mm; Shimadzu); solvent, 50% methanol and 3% acetic acid in water; flow rate, 1 ml min^{-1} at 40°C). Acid-catalyzed dehydration of 2-hydroxyflavanones (possible products of the P450 reaction) was performed using 10% (v/v) hydrochloric acid in ethanol as described (Akashi et al. 1998b).

Since TFNS5 was expressed more highly than AFNS2 in yeast, TFNS5 was mainly used for characterization of the catalytic activities. The results shown in Figures 3A and 3B clearly demonstrate the formation of apigenin (Rt 12.4 min; observed at UV 269 nm) from naringenin (Rt 8.9 min) with the yeast microsome expressing TFNS5 protein, regardless of the acid-treatment of the reaction mixture. In contrast, the chromatogram (Fig. 3C) of the reaction mixture from naringenin with CYP93B1 protein displayed the peak of putative 2-hydroxynaringenin (Rt 5.6 min) but not the apigenin peak. Apigenin production was only observed after the dehydration reaction by the acid treatment (Fig. 3D). Based on the P450 content (72 pmol mg^{-1} microsomal protein) calculated from CO-difference spectra of sodium dithionite-reduced microsomes (Fig. 4) (Omura and Sato 1964) and the reaction ratio (28 nkat g^{-1} microsomal protein) measured after the 5 min incubation when the rate of flavone formation is linear, the recombinant yeast microsome was estimated to possess the specific activity of 0.39 kat mol^{-1} P450 to naringenin. This value is comparable to those of heterologously expressed plant P450s in yeast, e.g., *Helianthus tuberosus* cinnamate 4-hydroxylase (0.42 kat mol^{-1} P450) (Urban et al. 1994) and *Persea americana* CYP71A1 (0.092 kat mol^{-1} P450 for *para*-chloro-*N*-methylaniline demethylase activity) (Bozak et al. 1992).

The relative substrate specificity of recombinant TFNS5 was examined using the microsome fraction. Production of luteolin (Rt 8.8 min; observed at UV 354 nm) from eriodictyol (Rt 6.3 min), 7,4′-dihydroxyflavone (Rt 7.6 min; observed at UV 330 nm) from liquiritigenin (Rt 6.2 min), and even a minute amount of quercetin (Rt 7.4 min; observed at UV 374 nm) from dihydroquercetin (Rt 3.9 min), respectively, was observed. The control microsome of the yeast containing pYES2 without P450 insert did not show flavone formation activity with any flavanone substrates. The specificities to naringenin (relative activity, 100%) and eriodictyol (99%), 5-hydroxyflavanones widely distributed in plant kingdom, were almost the same, whereas liquiritigenin, a 5-deoxyflavanone with limited distribution mainly among legumes (Hegnauer and Grayer-Barkmeijer 1993), was the superior substrate (149%) to 5-hydroxyflavanones. Because torenia is a non-legume, the higher reactivity to liquiritigenin was an unexpected observation: this activity, however, may not be significant in vivo where 5-deoxyflavanone would not be produced

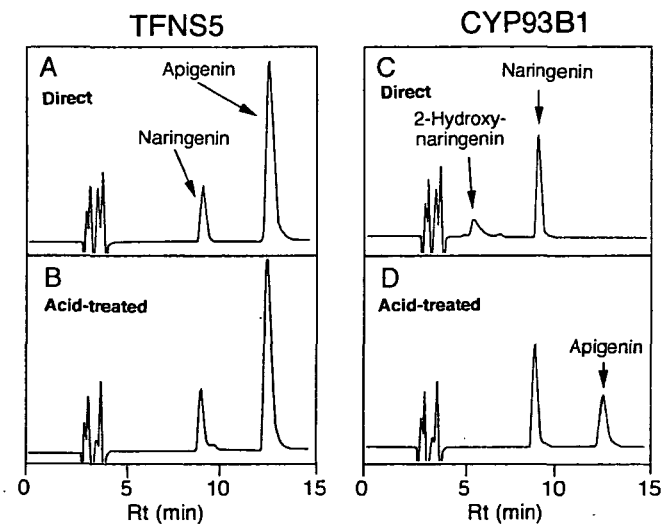

Fig. 3 HPLC analysis of products from naringenin in assays with TFNS5 (A, B) or CYP93B1 (C, D) expressed in yeast. A, C: Direct reaction products. B, D: Products after acid treatment of the direct reaction mixture.

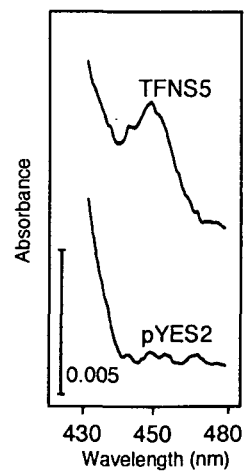

Fig. 4 Carbon monoxide difference spectra of reduced yeast microsomes (0.5 mg protein ml^{-1} each). Base lines were recorded after the addition of excess volumes of sodium dithionite, and the difference spectra were obtained after bubbling carbon monoxide for 30 s.

(Akashi et al. 1997). Another unexpected finding was the low but significant amount of quercetin (flavonol) produced from dihydroquercetin (dihydroflavonol) by TFNS5 reaction (relative activity, 1%). This type of reaction has been attributed to a soluble 2-oxoglutarate-dependent oxidase called flavonol synthase (Heller and Forkmann 1994) and has not been described in flavone synthase reactions (Britsch 1990, Kochs and Grisebach 1987). By analogy with the presence of two types of flavone synthases, i.e., P450 (FNS II) and soluble 2-oxoglutarate-dependent enzyme (FNS I), a P450-type flavonol synthase in addition to the known soluble flavonol synthase may be envisaged.

The direct conversion of naringenin to apigenin without acid treatment was also observed with the yeast microsome expressing AFNS2 protein (data not shown).

FNS II reaction mechanism—The foregoing results suggest that TFNS5 protein, and possibly AFNS2 protein, can be designated FNS II. The HPLC analysis detected no intermediate 2-hydroxyflavanone in the reaction with the recombinant TFNS5 protein, which is consistent with the direct formation of flavones from flavanones reported for FNS II reactions (Kochs and Grisebach 1987, Stotz and Forkmann 1981). However, considering that the amino acid sequence of FNS II is very similar to that of F2H, which produces licodione through a 2-hydroxyflavanone intermediate, it is more likely that flavone formation by FNS II also proceeds through two steps: 2-hydroxylation of flavanone (F2H reaction) and subsequent dehydration. Inspection of the sequence alignments shown in Figure 5 reveals a deletion of 8 amino acids beginning at positions 188 of CYP93B1 (F2H) and 192 of CYP93C2 (IFS) and insertion of several amino acids around position 480 in F2H and IFS, respectively, compared to AFNS2 and TFNS5. Both CYP93B1 and CYP93C2 proteins lack dehydratase activities to produce flavone and isoflavone skeletons. The possible roles of these sequences in the catalytic activities will be examined in the future by producing mutant proteins through domain swapping or insertion/deletion experiments.

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AFNS2     181: MMLGIRCSGTEGEAE
TFNS5     183: MMLSIRCSEDEGDAE
CYP93B1   186: MMV--------GEAE
CYP93C2   190: MML--------GEAE

AFNS2     466: KLPD-------GVKS
TFNS5     471: KLAD-------GSGN
CYP93B1   472: HVVGPKGEILKGDDI
CYP93C2   474: SVVGPQGKILKGNDA
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Fig. 5 Amino acid sequence alignment of snapdragon AFNS2, torenia TFNS5, licorice CYP93B1 and CYP93C2. The alignment was calculated using the MAlign program (Genetyx-Mac Ver 8.0 software). The identical amino acid residues at least three sequences are in reverse type. Gaps (-) are inserted to optimize alignment.

The two-step mechanism for FNS II reaction was supported by the detection of a minor amount of licodione in addition to the major product, 7,4′-dihydroxyflavone, by thin-layer chromatography when liquiritigenin was reacted with the yeast microsome containing the recombinant TFNS5 protein (Fig. 6). Furthermore, when 2-hydroxynaringenin prepared by F2H reaction was incubated with the microsome of yeast expressing TFNS5, a small amount of apigenin formed (relative activity, 1% of that for naringenin; no reaction with the microsome of control yeast). The low yield may be due to the rare exchange between 2-hydroxynaringenin bound to the enzyme and the exogenously added 2-hydroxynaringenin.

Conclusion—The results reported herein suggested that there are two types of P450-dependent flavone formation in plants, a dehydratase-requiring type such as in licorice and a non-requiring type such as in torenia and snapdragon, in addition to P450-independent flavone formation. Such diversity of natural pathways in the production of the flavone skeleton is quite intriguing from the evolutionary viewpoint, and further examination of the distribution of each type of flavone biosynthesis in other plant species is needed to understand the significance of multiple biosynthetic routes. The FNS II genes isolated here provide opportunities to understand structure-func-

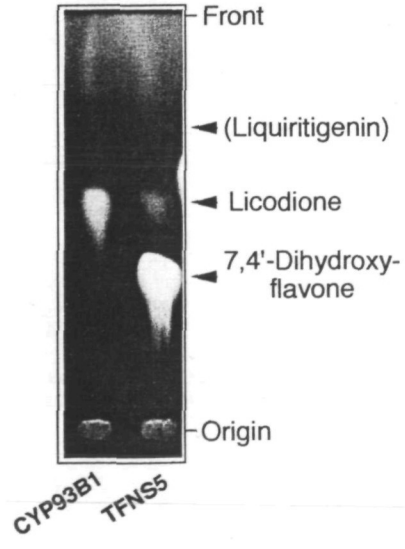

Fig. 6 Thin-layer chromatograms of the products from CYP93B1 (F2H) and TFNS5 reactions. Plate, cellulose (Avicel SF, Funakoshi); solvent, 30% acetic acid; detection, UV 365 nm. Liquiritigenin was converted to 7,4′-dihydroxyflavanone by TFNS5. Licodione was also detected in the same reaction. The result supports the hypothesis that 2-hydroxyflavanone is the intermediate of FNS II reaction.

tion relationships of the related enzymes and will be a new molecular tool to modify plant characteristics by genetic engineering.

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Note added in proof

The sequences of AFNS2 and TFNS5 have been designated CYP93B3 and CYP93B4, respectively, by the Committee on Cytochrome P450 Nomenclature and Dr. David R. Nelson (University of Tennessee), to whom the authors are grateful for the assignment.

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