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Differential Expression of Two Cytochrome P450s Involved in the Biosynthesis of Flavones and Anthocyanins in Chemo-Varietal Forms of *Perilla frutescens*

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In *Perilla frutescens*, there are two varietal forms of anthocyanin accumulation, i.e. red and green forms. The cDNA clones encoding flavone synthase II (FSII) and flavonoid 3′-hydroxylase (F3′H), two cytochrome P450s that are involved in the biosynthesis of flavones and anthocyanins, were isolated from *P. frutescens*. The FSII cDNA encoded a 57.1 kDa protein designated as CYP93B6, and the F3′H cDNA encoded 57.5 kDa protein designated as CYP75B4. Recombinant CYP93B6 expressed in yeast converted flavanones to flavones with K_m values of 8.8–11.9 μM. Recombinant CYP75B4 catalyzed 3′-hydroxylation of flavanones to the corresponding compounds with K_m values of 18–20 μM. The CYP93B6 transcript accumulated to an equal level in leaves of both red and green forms of *P. frutescens*, in agreement with the accumulation pattern of flavones in the leaves. However, the CYP75B4 transcript was predominantly expressed in the red form of *P. frutescens*, and its expression was induced by light in conjunction with other transcripts of biosynthetic enzymes of anthocyanin. These results indicate that gene expression of a set of anthocyanin biosynthetic enzymes including F3′H is regulated coordinately only in the red form of *P. frutescens* but not in the green form, whilst FSII gene expression is controlled in a similar manner in red and green forms of *P. frutescens*.

Key words: Anthocyanin — Cytochrome P450 — Flavone — Flavonoid — *Perilla frutescens*.

Abbreviations: F2H, (*2S*)-flavone 2-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FSII, flavone synthase II; HPLC, high performance liquid chromatography; ORF, open reading frame.

The nucleotide sequences reported in this paper have been submitted to DDBJ/GenBank/EMBL under accession numbers AB045592 (CYP93B6) and AB045593 (CYP75B4).

Introduction

Flavonoids constitute a major family of plant secondary metabolites. They play important roles as floral pigments, defense compounds, signal molecules and antioxidants (Holton and Cornish 1995, Mol et al. 1998, Rice-Evans et al. 1997, Shirley 1996). Flavonoids are biosynthesized from phenylpropanoid and acetate-derived precursors. The genes encoding biosynthetic enzymes of the pathway have been cloned from several plant species (Holton and Cornish 1995). Flavones and anthocyanins, two sub-groups of flavonoids, are biosynthesized from a common intermediate, flavanone (naringenin) (Fig. 1). The color of anthocyanin is influenced by the presence of co-pigments such as flavones and flavonols. Co-pigmentation involves the formation of stacked complexes that exclude the anthocyanin pigment from surrounding water, resulting in greater intensity of flower color and a blue shift in the anthocyanin absorption spectrum (Goto and Kondo 1991).

In the biosynthetic pathway of anthocyanins and flavones, two cytochrome P450s, flavonoid 3′-hydroxylase (F3′H) and flavone synthase II (FSII) are involved. The hydroxylation of the B-ring of flavonoids is an important reaction for determining the flower color. F3′H (CYP75B4) and flavonoid 3′,5′-hydroxylase (F3′5′H), which belong to the CYP75 family, are the essential enzymes involved in hydroxylation of the flavonoid B-ring (Heller and Forkmann 1993). F3′H (CYP75B) enzyme activity has been detected in floral extracts from ornamental plant species (Forkmann and Stotz 1981, Stotz et al. 1985). A cDNA encoding F3′H in *Petunia hybrida* was first isolated and functionally confirmed by Brugliera et al. (1999). Recently, a cDNA encoding F3′H has also been isolated from *Arabidopsis thaliana* (Schoenbohm et al. 2000).

FSII, which belongs to CYP93B subfamily, catalyses the reaction that introduces the double bond between C-2 and C-3 of the flavanones to produce flavones (Heller and Forkmann 1993). Recently, a correlation between genotype and enzyme activity for flavone formation in *Gerbera* hybrids was reported (Martens and Forkmann 1998) and a cDNA encoding FSII was isolated (Martens and Forkmann 1999). However, the regulatory mechanism that determines the expression of these two P450s involved in anthocyanin and flavone formation still remains to be clarified.

Recently, we have isolated and characterized a series of structural genes involved in anthocyanin biosynthesis in *Perilla frutescens* (Gong et al. 1997, Saito et al. 1999, Yamazaki et al. 1999). Two forms of *P. frutescens*, red and green, differing

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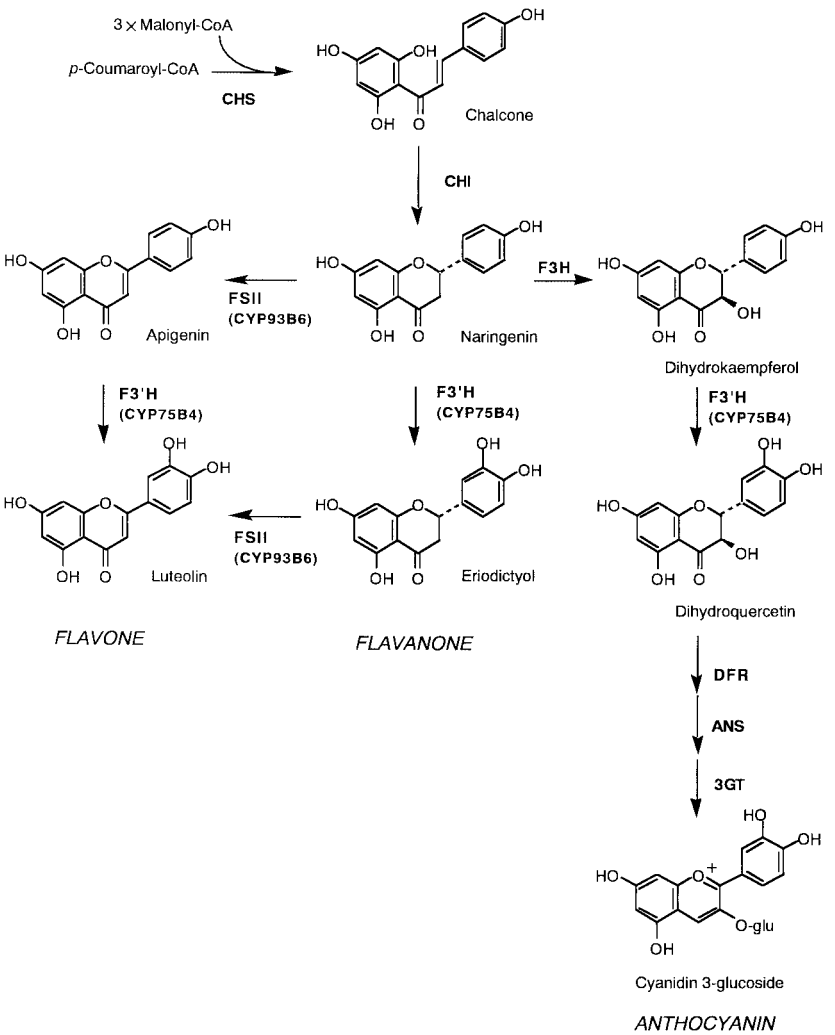

Fig. 1 The biosynthetic pathway of flavone and anthocyanin in *P. frutescens*. The enzymes are: CHS, chalcone synthase; CHI, chalcone isomerase; FSII, flavone synthase II; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose: flavonoid 3-*O*-glucosyl transferase.

in anthocyanin accumulation, are available. Our previous investigations indicated that the structural genes (chalcone synthase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, flavonoid 3-*O*-glucosyltransferase, anthocyanin 5-*O*-glucosyltransferase and acyltransferase) in the biosynthetic pathway of anthocyanin are expressed in the red form but not in the green form. It was also found that the structural genes are coordinately induced by white light in the red form plant. In this study, cDNAs encoding two cytochrome P450s, FSII and F3′H, were isolated from *P. frutescens*. The biochemical properties of the recombinant FSII and F3′H were investigated, and the expression pattern of the corresponding transcripts was analyzed to reveal the regulation mode of flavone and anthocyanin biosynthesis in *P. frutescens*.

Results

Isolation of cDNAs encoding flavone synthase II (FSII) and flavonoid 3′-hydroxylase (F3′H) from red P. frutescens

The FSII-encoding cDNA was isolated from red *P. frutescens* cDNA library using cDNAs from *Antirrhinum majus* (CYP93B3) (Akashi et al. 1999b) and *Glycyrrhiza echinata* (CYP93B1) (Akashi et al. 1998a) as a mixed heterologous probe. The full-length cDNA (*PFSII3*) was 1,770 bp in length and contains an open reading frame (ORF) encoding 506 amino acid residues with a predicted molecular mass of 57,147 Da. The deduced amino acid sequence of *PFSII3*, designated CYP93B6, exhibited 75% and 49% identities with FSIIs from *A. majus* and *G. echinata*, respectively. A multiple sequence alignment of CYP93B6 and other members of the CYP93B sub-family indicates that the oxygen-binding pocket and heme-binding region

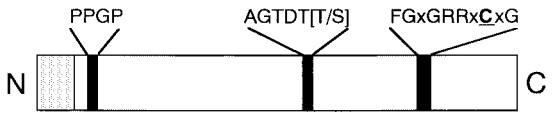

Fig. 2 Schematic presentation of structure of CYP93B6 (FSII) and CYP75B4 (F3′H) proteins. The N-terminal gray part indicates the membrane anchored hydrophobic region. The proline rich region (PPGP), the oxygen-binding pocket (AGTDT[T/S]) and the heme-binding region (FGxGRRxCxG) are indicated. The underlined cysteine (C) is the heme binding site.

of CYP93B6 are highly conserved (Fig. 2). In addition, a hydrophobic helix near the N-terminus and a proline-rich region, which are one of the best conserved areas in P450s (Chapple 1998), could also be found in CYP93B6.

The F3′H-encoding cDNA, *SHT12*, was isolated from a cDNA library of red *P. frutescens* using a F3′H-encoding cDNA from *P. hybrida* (Brugliera et al. 1999) as a heterologous probe. Sequence analysis indicated that *SHT12* is 1,804 bp in length with an ORF of 1,569 bp that encodes a polypeptide of 523 amino acid residues with a predicted molecular mass of 57,520 Da. The deduced amino acid sequence of *Perilla* F3′H, designated CYP75B4, shares 68% and 62% identity with F3′H from *P. hybrida* and *A. thaliana*, respectively. The membrane anchor, proline rich region, the oxygen-binding pocket and the heme-binding cysteine were all conserved between the sequences. *SHT12* also showed a relatively high identity (45–48%) with F3′5′H from *P. hybrida* (Holton et al. 1993), *Solanum melongena* (eggplant) (Toguri et al. 1993), *Gentiana triflora* (Tanaka et al. 1996) and *Catharanthus roseus* (Kaltenbach et al. 1999).

A phylogenic tree based on the deduced amino acid sequences of plant P450 proteins that are involved in flavonoid biosynthesis is shown in Fig. 3. This phylogenic tree suggests that FSIIs from *Perilla* (CYP93B6), *Torenia* (CYP93B4) and *Antirrhinum* (CYP93B3) form a small sub-family in CYP93B. Furthermore, a division of the CYP93B sub-family into two distinct groups is evidently based on whether the B- or C-ring is hydroxylated.

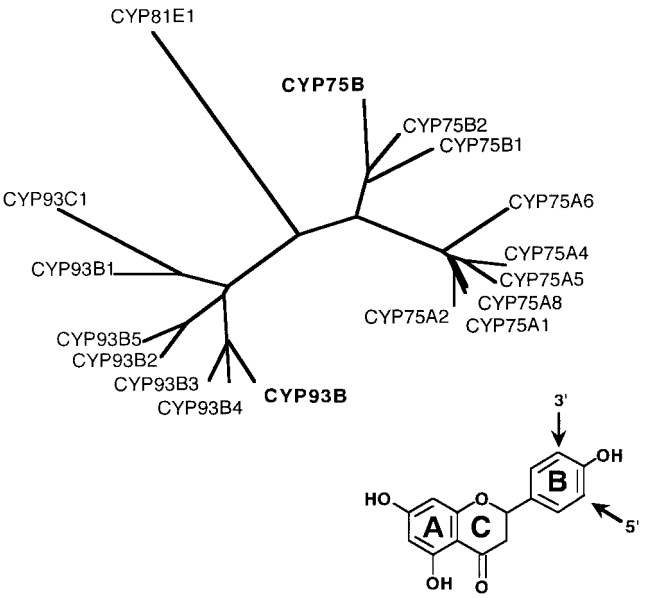

Fig. 3 Molecular phylogenic tree of the amino acid sequences of P450 proteins involved in flavonoid biosynthesis. The tree was produced using the Clustal X program (Jeanmougin et al. 1998). The general structure of flavonoids is included. References and accession numbers are as follows: CYP93B1, F2H from *Glycyrrhiza echinata* (Akashi et al. 1998a) [AB001380]; CYP93B5, FSII from *Callistephus chinensis* [AF188612]; CYP93B2, FSII from *Gerbera* hybrids (Martens and Forkmann 1999) [AF156976]; CYP93B3, FSII from *Antirrhinum majus* (Akashi et al. 1999b) [AB028151]; CYP93B4, FSII from *Torenia hybrida* (Akashi et al. 1999b) [AB028152]; CYP93B6, FSII from *Perilla frutescens* (This study) [AB045592]; CYP93C1, isoflavone synthase from *Glycyrrhiza echinata* (Akashi et al. 1999a) [AB023636]; CYP81E1, isoflavone 2′-hydroxylase from *Glycyrrhiza echinata* (Akashi et al. 1998b) [AB001379]; CYP75B4, F3′H from *Perilla frutescens* (this study) [AB045593]; CYP75B2, F3′H from *Petunia hybrida* (Brugliera et al. 1999) [AF15532]; CYP75B1, F3′H from *Arabidopsis thaliana* (Schoenbohm et al. 2000) [AF271651]; CYP75A6, F3′5′H from *Campanula medium* [D14590]; CYP75A4, F3′5′H from *Gentiana triflora* (Tanaka et al. 1996) [D85184]; CYP75A5, F3′5′H from *Eustoma grandiflorum* (Nielsen and Podivinsky 1997) [U72654]; CYP75A8, F3′5′H from *Catharanthus roseus* (Kaltenbach et al. 1999) [AJ011862]; CYP75A1, F3′5′H from *Petunia hybrida* (Holton et al. 1993) [Z22545]; CYP75A2, F3′5′H from *Solanum melong*ena (eggplant) (Toguri et al. 1993) [X70824].

Functional expression of CYP93B6 (FSII) and CYP75B4 (F3′H) in Saccharomyces cerevisiae

To confirm the function of the proteins encoded by the isolated cDNAs, the coding regions of *PFSII* and *SHT12* were cloned into the yeast expression vector pYES2 to construct pYFSII and pYF3′H, respectively.

FSII activity was detected in microsomal fractions prepared from yeast transformed with pYFSII, but not in that from yeast transformed with the empty pYES2 vector. Transformation of apigenin from naringenin and luteolin from eriodictyol could be detected when appropriate co-factors were incubated with a microsomal preparation of yeast carrying pYFSII. The identity of the products was confirmed by co-chromatography with the authentic standards using high performance liquid chromatography (HPLC) (data not shown). The FSII-mediated reaction required NADPH as a co-factor, like general reaction of P450 proteins. Together, these results demonstrate that *PFSII* cDNA indeed encodes a functional FSII protein.

Similarly, microsomal proteins prepared from yeast transformed with pYF3′H catalyzed the formation of eriodictyol, luteolin and dihydroquercetin from naringenin, apigenin and dihydrokaempferol, respectively, whilst proteins of yeast carrying with the empty pYES2 vector were unable to convert the tested substrates. These results confirmed that *SHT12* cDNA from *P. frutescens* encodes a functional F3′H protein.

Table 1 summarizes the kinetic parameters of the reactions

Table 1 Kinetic parameters of the reaction catalyzed by recombinant FSII and F3′H

Substrate	CYP93B6 (FSII)				CYP75B4 (F3′H)			
	Product	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	V_{max}/K_m	Product	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	V_{max}/K_m
Naringenin	Apigenin	8.8	0.29	0.032	Eriodictyol	19.6	1.32	0.067
Apigenin	–	–	–	–	Luteolin	18.8	1.60	0.085
Eriodictyol	Luteolin	11.9	0.17	0.014	–	–	–	–
Dihydro-kaempferol	–	–	–	–	Dihydroquercetin	19.5	1.01	0.054

The enzymatic assay of CYP93B6 (FSII) and CYP75B4 (F3′H) was carried out by using microsomal proteins from transformed yeast as described in Materials and Methods. The formation of products was determined by HPLC. The reaction mixture contained 200–250 μg microsomal proteins, 2–40 μM substrate and 1 mM NADPH, 100 mM K-P_i (pH 7.5) in 200 μl total volume. The reactions were started by the addition of NADPH, and the reaction mixture was incubated at 25°C for 10 min.

catalyzed by the crude recombinant P450 proteins. Recombinant CYP93B6 exhibited apparent K_m values of 8.8 μM and 11.9 μM with naringenin and eriodictyol, respectively. The apparent K_m values of recombinant CYP75B4, 19.6 μM, 18.8 μM and 19.5 μM, were also similar between the examined substrates, naringenin, apigenin and dihydrokaempferol, respectively. The value obtained for naringenin is comparable to those reported previously for microsomal preparations from *Petroselium hortense* Hoffm. (~0.7 μM) (Hagmann et al. 1983) and *Critrus sinensis* (24 μM) (Doostdar et al. 1995).

The genomic complexity of the genes encoding CYP93B6 (FSII) and CYP75B4 (F3′H) in P. frutescens

Southern blot analysis of the genes encoding CYP93B6 and CYP75B4 was carried out with genomic DNA isolated from red and green forms of *P. frutescens* (data not shown). The results suggested that two copies of both the CYP93B6- and CYP75B4-encoding genes are present in the genome of both forms, consistent with the previous results obtained for other genes encoding anthocyanin biosynthetic enzymes in *P. frutescens* (Gong et al. 1997). This may be explained by the fact that *P. frutescens* is an amphidiploid organism. No restriction fragment length polymorphism was observed for both genes between red and green forms of *P. frutescens*.

For CYP93B6 cDNA cloning, we obtained five clones which exhibited the identical sequences, suggesting that only one of two genes is expressed or the expression level of the second gene is quite low. For CYP75B4 cDNA cloning, we isolated five clones, of which partial sequences were determined. Four clones exhibited an identical sequence with that of *SHT12* and one clone showed small nucleotide substitutions. This might be due to either the expression of two genes or just micro-heterogeneity of individual plants.

Expression of the genes encoding CYP93B6 (FSII) and CYP75B4 (F3′H) in P. frutescens

Northern blot analysis of RNA isolated from red and green forms of *P. frutescens* (Fig. 4A) indicated that mRNA of the expected size of CYP93B6 (1.9 kb) accumulated in both red and green leaves of *P. frutescens*. The CYP93B6 mRNA levels were low in stems compared with leaves. In contrast, the apparent accumulation of a CYP75B4 transcript of the expected size (2.0 kb) was predominantly detected in leaves of red *P. frutescens* but not in green leaves. This expression pattern is similar to the expression of other genes encoding enzymes involved in anthocyanin biosynthesis in *P. frutescens* (Gong et al. 1997). These results indicate that the gene expression of CYP93B6 and CYP75B4 is regulated in a different manner between red and green forms.

Subsequently, the response of the gene expression of CYP93B6 and CYP75B4 to white light was analyzed. The accumulation of both CYP93B6- and CYP75B4-encoding mRNAs increased gradually with increasing exposure time to light (Fig. 4B). In the roots no transcripts of both genes were detected. The accumulation pattern in response to light was consistent with that obtained for other genes involved in anthocyanin biosynthesis in *P. frutescens* (Gong et al. 1997, Saito et al. 1999, Yamazaki et al. 1999).

Accumulation of flavones and anthocyanins in red and green P. frutescens

The contents of flavone and anthocyanin were determined in leaves, stems and roots of red and green *P. frutescens* (Fig. 5). No difference was found in the levels of flavones, e.g. apigenin and luteolin, between red and green plants, consistent with equivalent levels of the flavone-specific CYP93B6 mRNA accumulation in red and green plants (Fig. 4A). However, there was a large difference in the accumulation of anthocyanin between red and green *Perilla* (Fig. 4A). Together with the results of the mRNA expression of CYP75B4, these results confirm the importance of F3′H for anthocyanin biosynthesis in *P. frutescens*, in which the presence of only cyanidin-type of anthocyanins is reported (Kondo et al. 1989).

Discussion

In the present study, cDNA clones encoding CYP93B6 (FSII) and CYP75B4 (F3′H) were isolated from *P. frutescens*

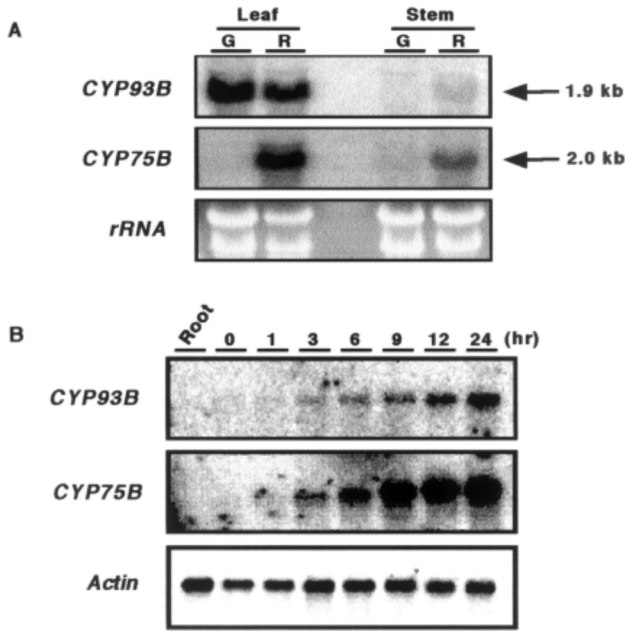

Fig. 4 Northern bolt analysis of RNA isolated from *P. frutescens*. (A) Expression of *CYP93B6* and *CYP75B4* mRNA in red (R) and green (G) forms of *P. frutescens*. Total RNA (10 μg) from leaves and stems was separated by an agarose gel (1.2%) electrophoresis, blotted onto a Hybond-N+ filter (Amersham, Buckinghamshire, U.K.), and then hybridized with ^{32}P-labeled *CYP93B6* and *CYP75B4* cDNA as the probe, respectively. Ethidium bromide staining of the *25S rRNA* is shown as an indication of equal amount of RNA loading. (B) Induction of gene expression of *CYP93B6* and *CYP75B4* by illumination with light. The red-form plants grown in weak light (<480 lux) for 3 weeks were illuminated with strong white light (14,000 lux) for the period indicated. Poly(A)$^+$ RNA was isolated from the leaves and roots and analyzed by Northern blot with *CYP93B6* and *CYP75B4* cDNA as the probes. The cDNA encoding actin from *P. frutescens* (Gong et al. 1997) was used as a control for constitutive expression. Root, poly(A)$^+$ RNA from the roots of red-form plant.

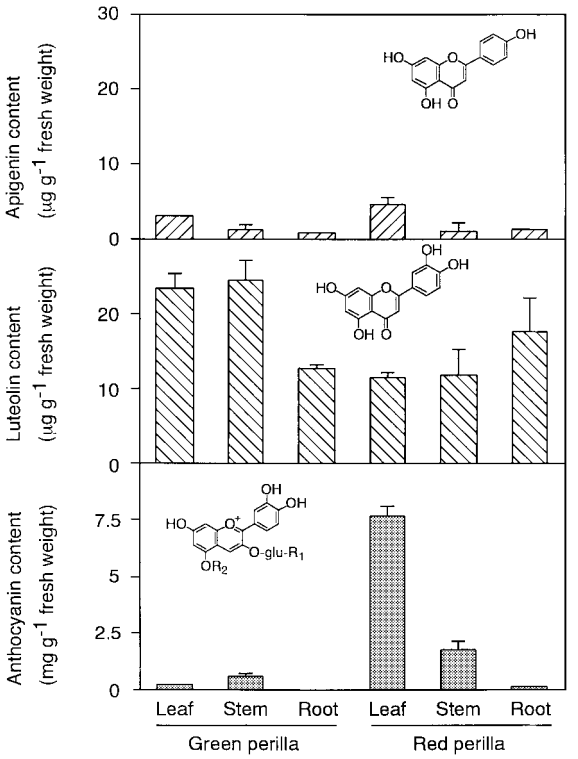

Fig. 5 The contents of flavones and anthocyanin in red and green forms of *P. frutescens*. The contents of flavones and anthocyanin in leaves, stems and roots were measured as described in the Materials and Methods. Data are mean values ±SD of triplicate determinations.

and characterized at a molecular level. Comparison between the deduced amino acid sequences of cytochrome P450 genes have shown that there are several conserved regions (Chapple 1998). The most highly conserved region in all cytochrome P450s is the heme binding domain centered a cysteine (C) residue that binds heme in the active site. The heme binding domain of cytochrome P450 is generally represented by the highly conserved sequence FxxGxRxCxG. This P450 fingerprint motif was found in both of the CYP93B6 and CYP75B4 amino acid sequences (Fig. 2). The characteristic proline rich region (PPxP), which forms a hinge between the membrane anchored N-terminal helix and other parts of the protein, was also found. The (A/G)Gx(D/E)T(T/S) consensus, which is involved in oxygen activation and the transfer of protons to the active site, could also be found. A molecular phylogenic study indicated that within the CYP93B subfamily, *Perilla* FSII (CYP93B6) is most closely related to FSII from *A. majus* (CYP93B3) and *T. hybrida* (CYP93B4) (Fig. 3). The greater phylogenetic distance to FSII from *G. echinata* might be explained by the different mode of reactions catalyzed by these two sub-families of P450. It was reported that the actual function of FSII from *G. echinata* is that of a (*2S*)-flavanone 2-hydroxylase (F2H) (Akashi et al. 1998a). For the formation of apigenin from naringenin, 2-hydroxynaringenin is first formed by F2H, followed by subsequent acid treatment. In contract, FSIIs from other plants directly catalyze the conversion of naringenin to apigenin without any apparent accumulation of 2-hydroxynaringenin (Akashi et al. 1999b, Martens and Forkmann 1999). By the present study, this was also shown for *Perilla* FSII; no intermediary compound such as 2-hydroxynaringenin was detected. Multiple alignment of amino acid sequences of FSII proteins shows that there are several regions between the deduced amino acid sequences of *G. echinata* and those of other plants that differ. These observed differences in the amino acid sequence motifs may be responsible for the observed differences in the reaction mechanism, i.e. C-2 hydroxylation versus direct hydrogen abstraction form C-2 and C-3. Attempts to identify motifs that are responsible for determining the observed differences in reaction mechanisms warrant further investigations.

Heterologous expression in *S. cerevisiae* allowed the biochemical properties of recombinant CYP93B6 and CYP75B4

to be determined (Table 1). Both recombinant CYP93B6 and CYP75B4 exhibited similar K_m and V_{max} values using either of the tested substrates, indicating that there was no particular preference towards a specific substrate. Both CYP93B6 and CYP75B4 can utilize naringenin as a substrate, and the K_m and V_{max} values obtained in vitro are very similar for both reactions.

The interesting findings from the present study concern the expression pattern of the CYP93B6- and CYP75B4-encoding transcripts. The CYP75B4-encoding mRNA specifically accumulated in the leaves of red *P. frutescens*, but not in those of the green form (Fig. 4A). This mode of expression is quite similar to that of other anthocyanin-specific genes as reported previously (Gong et al. 1997), although F3′H is supposed to be involved not only in anthocyanin biosynthesis but also in flavone biosynthesis. Presumably the common regulatory factors that control the expression of genes encoding enzymes of anthocyanin biosynthesis (Gong et al. 1999a, Gong et al. 1999b) may also govern the expression of the CYP75B4-encoding gene. In contrast, the CYP93B6-encoding gene was expressed in both red and green leaves of *P. frutescens* at an equal level (Fig. 4A). This indicates that the expression of the CYP93B6-encoding gene is regulated by a different manner to that of other genes encoding anthocyanin biosynthetic enzymes in red and green forms of *P. frutescens*. These expression patterns of the genes encoding CYP93B6 and CYP75B4 correlate with the accumulation profiles of flavone and anthocyanin in red and green forms of *P. frutescens* (Fig. 5). However, the induction patterns of the transcripts of CYP93B6 and CYP75B4 by light illumination were quite similar to those of enzymes involved in anthocyanin biosynthesis (Gong et al. 1997, Yamazaki et al. 1999). These results suggested that accumulation of transcripts of CYP93B6 and CYP75B4 are regulated by the same manner for the light induction, which is in contrast to the form-specific expression. These may imply that the regulatory elements such as transcription factors and signal transduction systems may be different for form-specific expression and for induction by light illumination. In spite of the low level of CYP75B4 transcript in green plants, luteolin accumulates in both red and green plants. This may be explained by that a weak expression of CYP75B4 would be sufficient to convert apigenin to luteolin and naringenin to eriodictyol. In particular, under the condition that anthocyanin pathway is blocked and thus no dihydrokaempferol is available as a substrate in green plants, a small quantity of CYP75B4 would effectively catalyze the formation of luteolin.

Materials and Methods

Plant materials

The seeds of a reddish-purple form (cv. Chirimenjiso 'Shikun') and a green form (cv. Chirimen-aojiso 'Seikun') of *Perilla frutescens* var. *crispa* (Sakata Seed Company, Kyoto, Japan) were germinated at 25°C under illumination for 16 h d^{-1} in a greenhouse. After 5–7 weeks, when the leaves had grown to a length of approximately 3 cm, the young developed leaves were used for extraction of DNA, total RNA for Northern blotting, and for extraction of flavone and anthocyanin.

Isolation of cDNA and DNA sequencing

A cDNA clone (*PFSII*) encoding FSII was isolated by screening a λgt10 cDNA library, which was constructed from the mRNA of leaves of the red form of *P. frutescens* (Gong et al. 1997), with ^{32}P-labeled mixed probe of *FSII* cDNA from *Antirrhinum majus* (Akashi et al. 1999b) and *Glycyrrhiza echinata* (Akashi et al. 1998a). In addition, the following PCR primers were used to amplify full length cDNA of *PFSII*: 5′-GCT GGG TAG TCC CCA CCT TT-3′ (forward) and 5′-CTT ATG AGT ATT TCT TCC AGG GTA-3′ (reverse). PCR was carried out using Pyrobest DNA polymerase (Takara, Kyoto, Japan). After 30 s heating at 72°C, 30 cycles of amplification were carried out as follows: 1 min denaturation at 94°C, 1 min annealing at 55°C, 3 min extension at 72°C. The reaction was completed by 10 min extension at 72°C. The amplified fragment was cloned into the *Eco*RV site of pBluescriptII SK– (Stratagene, La Jolla, CA, U.S.A.) and fully sequenced on both strands. The F3′H clone from *P. hybrida* (Brugliera et al. 1999) was used to screen a λZAPII cDNA library constructed by Tanaka et al. (1995). Two positive clones were isolated, and their cDNA inserts were excised in vivo into pBluescriptII SK–. A clone showing homology to petunia F3′H was designated *SHT12*.

Heterologous expression in Saccharomyces cerevisiae

The *Bam*HI–*Xho*I fragment of the coding region of *PFSII* cDNA was amplified by PCR using a set of primers, 5′-CAA AGG ATC CAA CAT GGC ACT GTA CGC-3′ and 5′-CCG CTC GAG GGG GTG ATC ACT GGG TGG AAA-3′. *Kpn*I and *Xba*I sites were created at the N- and C-terminals of the coding region of *SHT12* cDNA by PCR-amplification with a primer set, 5′-GGG GTA CCA ATG ATC AGT GCC GCC GTA AG-3′ and 5′-GCT CTA GAG GAT CCT GTT CGC AAT TTG TGC-3′, respectively. The amplified fragments were ligated into the *Bam*HI–*Xho*I site and *Kpn*I–*Xba*I site of pYES2 (Invitrogen, Carlsbad, CA, U.S.A.), respectively. The constructed vectors were transferred into the 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) by the lithium acetate method (Gietz et al. 1992). The transformants were selected on SD minimal medium (Sherman 1991) lacking uracil. The induction of P450-gene expression and preparation of spheroplasts were performed as described by Akashi et al. (1998a). Microsomes were prepared as described previously (Akashi et al. 1998a), except yeast spheroplasts were disrupted with a French press (300 to 400 kg cm^{-2}) instead of glass beads.

Enzyme assay of FSII and F3′H

The incubation mixture (final volume 200 µl) contained 2–40 µM substrate, 200–250 µg microsomal proteins. The reactions were started by the addition of NADPH to a final concentration of 1 mM. The reaction mixture was incubated at 25°C for 10 min and terminated by the addition of 200 µl of cold methanol. After centrifugation, the aqueous supernatants were analyzed by HPLC. HPLC analysis of flavones was carried out on a reverse phase column Mightysil RP-18 GP (4.6 mm ϕ×150 mm; Kanto Chemicals, Tokyo, Japan) using 5% acetic acid in methanol/water (50/50, v/v), or 1% acetic acid in acetonitrile/water (20/80, v/v), at a flow rate of 1.0 ml min^{-1} at 25°C. The detection wavelength was chosen according to the optimal absorption by substances (335 nm for apigenin, 347 nm for luteolin, 290 nm for eriodictyol and dihydroquercetin).

Nucleic acid hybridization analysis

Total RNA was isolated using a RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, U.S.A.). DNA from red and green forms of *P. frutescens* was extracted from young leaves as described previously (Dellaporta et al. 1983). DNA and RNA blot hybridization analysis was performed according to Gong et al. (1997).

Flavone and anthocyanin determination

Flavones were extracted from plant tissues as described previously (Martens and Forkmann 1998). Apigenin and luteolin were quantified by HPLC by their peak area by monitoring the absorbance at 335 nm and 347 nm, respectively. The content of total anthocyanin in plant tissues was determined as described previously (Gong et al. 1997).

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