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Cloning and expression of flavone synthase II from *Gerbera* hybrids

Stefan Martens* **and Gert Forkmann**
Lehrstuhl für Zierpflanzenbau, Technische Universität München, Am Hochanger 4, 85350 Freising, Germany

Summary

In *Gerbera* hybrids, flavone synthesis is controlled by the locus *Fns*. The responsible enzyme, flavone synthase II, belongs to the NADPH-dependent cytochrome P450 monooxygenases. From two different chemogenetic defined *Gerbera* lines with the dominant (*fns*$^+$.) or recessive (*fns fns*) alleles at the locus *Fns*, a cytochrome P450 fragment (CypDDd7a) was isolated using a differential display technique with upstream primers based on the conserved heme-binding region of cytochrome P450 proteins. The full-length cDNA (CYP93B2) which contained the open-reading frame and part of the CypDDd7a sequence was isolated via 5′-RACE and end-to-end PCR with gene specific primers. Northern blot analysis of total RNA of *Gerbera* hybrids indicated that the CYP93B2 gene was only transcribed in lines with the dominant allele *fns*$^+$ and that the transcript levels during flower development are in agreement with the measured enzyme activity of FNS II and flavone accumulation. Microsomes from yeast cells expressing CYP93B2 catalysed the direct formation of [^{14}C]-flavones from the respective [^{14}C]-flavanones. Thus, CYP93B2 was shown to encode flavone synthase II. This is the first report of the isolation and expression of a functional FNS II cDNA clone from any species. The comparison of amino acid sequences revealed that CYP93B2 had 54% identity with the sequence of CYP93B1, which has recently been reported as a (2S)-flavanone 2-hydroxylase of *Glycyrrhiza echinata* L.

Introduction

Within the naturally occurring flavonoid compounds, flavones represent one of the most abundant and important classes. A soluble flavone-forming enzyme (flavone synthase I; FNS I) has been described using parsley (*Petroselinum hortense*) cell suspension cultures. This enzyme was classified as a 2-oxoglutarate-dependent dioxygenase (Britsch *et al.*, 1981). In contrast, in a wide range of plant species the flavone synthase II (FNS II) was found to be the enzyme responsible for flavone formation. Using flavanones as substrate, flavones were formed by the introduction of a double bond between C-2 and C-3 (Figure 1) (Heller and Forkmann, 1994). Recently, the genetic control of FNS II activity by the gene *Fns* was described for the first time (Martens and Forkmann, 1998). Biochemical studies (Kochs and Grisebach, 1987; Stich *et al.*, 1988) identified FNS II as a microsomal cytochrome P450 (P450) monooxygenase. This group of membrane-bound heme plant proteins, which interact with NADPH-cytochrome P450-reductase (CPR), form a superfamily which is involved in a range of oxidative reactions in the metabolism of secondary compounds. The respective pathways lead, for example, to important molecules such as lignins, coumarins, isoflavonoids and flavonoids. Apart from this, the role of P450 reactions in detoxification of xenobiotic compounds has often been reported (Bolwell *et al.*, 1994; Durst and Nelson, 1995; Schuler, 1996). At present, more than 300 plant P450 sequences are registered to different databases and are classified in approximately 46 gene families (D.R. Nelson, personal communication). In contrast to many other isolated clones coding for P450s with still unknown functions, the enzymatic reaction of several gene products belonging to the phenylpropanoid and flavonoid pathways has been described. Cinnamate 4-hydroxylase (C4H) belongs to the CYP73A and catalyses the hydroxylation of cinnamic acid (Hotze *et al.*, 1995). The resulting 4-coumarate is the origin of the B-ring structure of flavonoids. Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′, 5′-hydroxylase (F3′,5′H) catalyze the B-ring hydroxylation of flavonoids (Heller and Forkmann, 1994). They are of enormous commercial importance to the horticultural industry and more recently with respect to the dietary intake of efficient antioxidants such as hydroxylated flavonoids (Bolwell *et al.*, 1994). An F3′H was cloned from Petunia (CYP75B2) and the function of the gene product was identified (Brugliera *et al.*, 1999). F3′,5′H was cloned from several sources and belongs to the CYP75A family (Holton *et al.*, 1993). The gene of FNS II, which is also an important P450 enzyme of the flavonoid pathway, has, however, not been cloned until now.

Based on the domain that contains the heme-binding cysteine, which is regarded as a fingerprint for P450 proteins (Gonzalez, 1989), and the chemogenetic and enzymatic data (Martens and Forkmann, 1998; Tyrach and Horn, 1997), PCR methods, which have been reported

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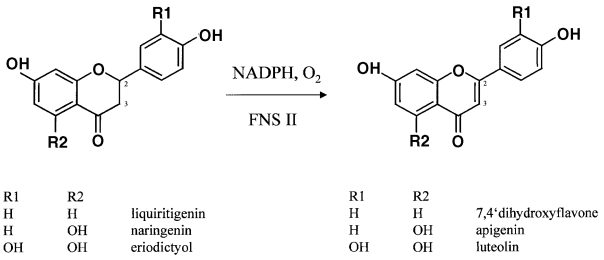
*For correspondence (fax +49 8161 713886;
e-mail martens@lzw.agrar.tu-muenchen.de).



R1	R2	
H	H	liquiritigenin
H	OH	naringenin
OH	OH	eriodictyol

R1	R2	
H	H	7,4'dihydroxyflavone
H	OH	apigenin
OH	OH	luteolin

Figure 1. Enzymatic formation of flavones from flavanones.

for several approaches to isolate P450 genes (Holton *et al.*, 1993; Schopfer and Ebel, 1998), were now found to be a useful tool for the direct cloning of the FNS II P450 gene from *Gerbera* hybrids.

We report here on the first successful molecular cloning of a cDNA encoding FNS II which catalyses flavone synthesis in the flavonoid pathway and on its functional expression in yeast.

Results

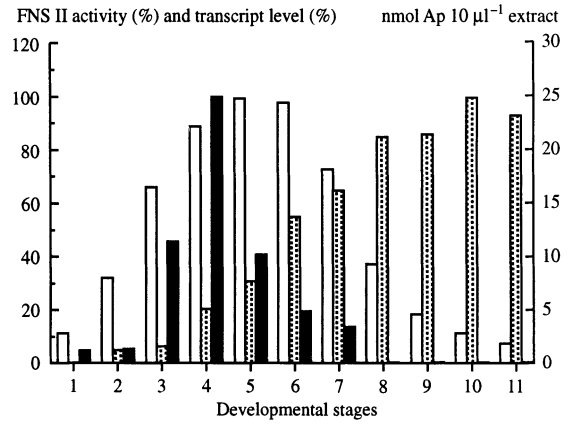
Isolation of a Gerbera *cDNA fragment encoding differentially expressed P450 protein*

Previous biochemical studies with different *Gerbera* lines showed that the formation of flavones is controlled by the gene *Fns*. FNS II activity was only present in lines with the dominant allele (*fns*$^+$.) and not in lines with recessive alleles (*fns fns*). Furthermore, in petals from *Gerbera* 'Regina' the enzyme activity for FNS II was found to increase until developmental stage five (see Figure 2), indicating that corresponding high transcript levels should be present in stages three to four (Martens and Forkmann, 1998). Thus, we compared mRNA populations isolated from respective stages of *Gerbera* lines with the dominant allele *fns*$^+$ and mutants with recessive alleles by means of differential display (DD-RT-PCR).

The DD-RT-PCR based screening revealed five clearly differentially expressed bands. All of them were reamplified and analysed. One PCR product contained high probability amino acid matches to known eukaryotic P450 sequences, an open reading frame with the conserved P450 fingerprint and intact 5′ and 3′ PCR primers. The clone designated as CypDDd7a (Figure 3), represented the 3′-terminal coding region of a P450 cDNA starting from the heme-binding region to first stopcodon (74 amino acids) and a 186 bp untranslated region. The length was in accordance with the estimated size in the differential display gel.

Temporal and spatial expression of Gerbera *CypDDd7a*

Northern blot analysis confirmed the differential expression of the obtained P450 fragment. Transcript levels were



Length of ray floret corolla (mm)	<5	5–10	10–15	15–23	23–26	26–35	35–40	40–50	50–55	55–60	55–60
Description	closed buds	ray floret visible		start of pigmentation	pigmented ray floret ligule		inflorescence half-opened	fully opened inflorescence			senescent ray florets

Figure 2. Transcript levels, FNS II activity and flavone accumulation in *Gerbera* cultivar 'Regina'.
Values of transcript levels (black), determined by Northern Blot analysis, enzyme activity (white) and flavone content (grey) determined as described by Martens and Forkmann (1998).

investigated in different genotypes, in 11 stages of flower development and in leaves of *Gerbera*. Hybridisation under high stringency conditions of total RNA using CypDDd7a DNA probe revealed a 1.7 kb transcript present in genetically defined *Gerbera* lines and cultivars with the dominant allele (*fns*$^+$.) but not in lines and cultivars with recessive alleles at the locus *Fns*. In leaves, which do not naturally form flavones, no hybridisation signal could be detected (Figure 4). Concomitantly, with FNS II activity and flavone accumulation, the maximum rate of the CypDDd7a transcript peaked at stage 4 and then declined (Figure 4).

Isolation of the 5′-end cDNA clone of the Gerbera *P450 cDNA*

For the isolation of the 5′-end cDNA clone, the 5′-RACE technique was used, i.e. three gene specific primers (GSP7–9; see Experimental procedures) from CypDDd7a were deduced. After two PCR rounds a 1.4 kb fragment (CypTÁBATA, Figure 3) was observed on agarose gel stained with ethidum bromide. The obtained fragment again contained high probability amino acid matches to known eukaryotic P450 sequences, an open reading frame with a startcodon, the conserved heme-binding region and

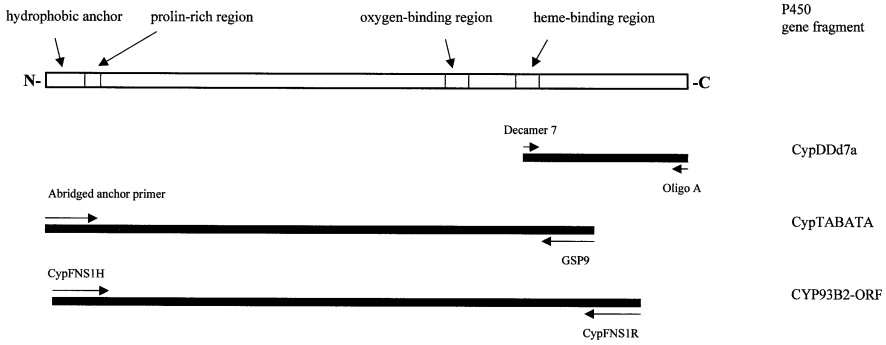


Figure 3. Sequence structure of P450 and isolated fragments from *Gerbera*.
Conserved regions: hydrophobic membrane anchor, prolin-rich region, oxygen binding region and heme-binding region.

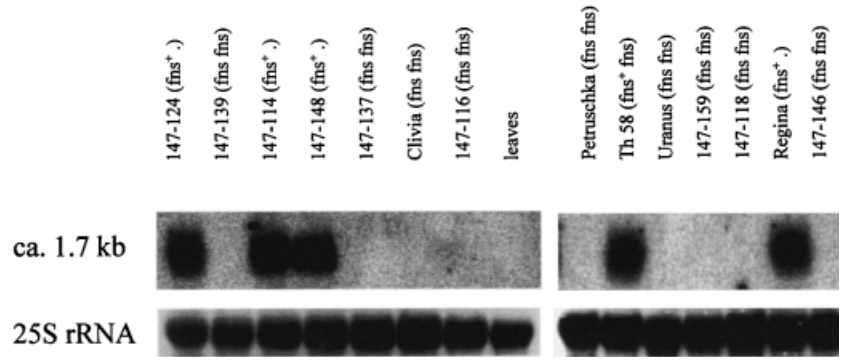


Figure 4. Expression of the respective CypDDd7a gene transcript in different genotypes and in the leaves of *Gerbera* analyzed with phosphorimager.

the intact 3′ gene-specific PCR primer (GSP9) in frame. Therefore, the CypTÁBATA clone represented the 5′-terminal coding region of the *Gerbera* P450 cDNA (460 amino acids) starting with a 23 bp length leader.

Expression of the Gerbera *P450 cDNA in yeast*

To determine which functional protein is encoded by the isolated gene, an additional PCR with gene specific primers (CypFNS1H/R; see Experimental procedures) was performed that enabled the cloning of the full-length P450 coding region (CYP93B2-ORF; Figure 3) into the yeast expression vector (pYES2) without including any of the endogenous 5′ non-translated flanking sequence. This was necessary because in some cases superfluous 5′ untranslated sequences from foreign genes might impede gene expression in yeast (Pompon *et al.*, 1996). FNS II activity was detected in microsomal fractions prepared from yeast strain INV Sc1 transformed with CYP93B2-ORF but not in microsomal preparations from the non-transformed yeast strain INV Sc1 (Figure 5). The product which was formed from naringenin (NAR) as a substrate was identified as apigenin (Ap) in four different solvent systems by co-chromatography with an authentic sample. Under the same conditions, the incubation of eriodictyol (ERI) and liquiritigenin (LIQ) led to the formation of the respective flavones, luteolin (Lu) and 7, 4′-dihydroxyflavone (data not shown). These results demonstrated that the isolated *Gerbera* P450 cDNA codes for functional FNS II.

Sequence of the Gerbera *P450 cDNA clone representing FNS II*

The isolated clone represents 1.7 kb cDNA with a single open reading frame (ORF) of 1536 bp. The ORF codes for a polypetide of 511 amino acid residues to a predicted molecular mass of 58.2 kDa. The deduced amino acid sequence contained not only the conserved amino acids of the heme-binding region but also [(A/G)Gx(D/E)T(T/S)], which is thought to be essential for oxygen binding and activation. Furthermore, an expected hydrophobic membrane anchor and a proline-rich region close to the 5′ N-terminus could be detected. Comparison of the deduced amino acid sequence with known P450 sequences revealed that the isolated cDNA belongs to the CYP93 family. Moreover, the isolated cDNA is highly homologous to the recently published sequence for a (2S)-flavanone 2-hydroxylase (F2H) (CYP93B1; Akashi *et al.*, 1998; GenBank accession no. AB001380) from licorice (*Glycyrrhiza echinata*) with an identity value for the protein of 54% (Figure 6). Consequently this clone was named CYP93B2 (D. Nelson, personal communication). Phylogenetic analysis using the deduced amino acid sequences of a range of

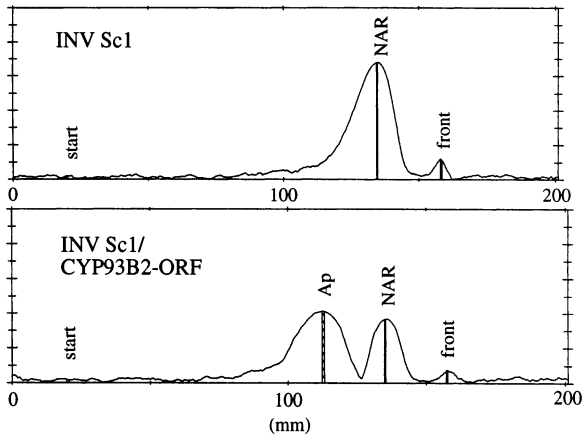


Figure 5. Radiochromatograms of the enzyme assay of FNS II cDNA clone expressed in yeast.
The cDNA insert CYP93B2-ORF was cloned into yeast expression vector pYES2 (INV Sc1-CYP93B2-ORF). Enzyme extracts of non-transformed yeast (INV Sc1) and yeast transformed with CYP93B2 were assayed in double tests for FNS II activity using (^{14}C)-naringenin as substrate and analyzed with phosphorimager.

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CYP93B1         -MEPQLVAVSVLVSALICYFFFRPYFHRYGKNLPPSPF
CYP93B2         MNTLQLIFLLFFFPTLLFLYCLPYKRNQNHRRLPPSPP
                    **. .     .*.  .       ..  . *****

CYP93B1         LSHRYGPLFSLNFGSVLCVVASTPHFAKQLLQTNELAF
CYP93B2         LSTRYGSLIHLRLGSVPCVVVSTPDLAKDFLKTNELAF
                ** *** *  *  *** *** ***  **  *.******

CYP93B1         YGDYWRFIKKLSMNELLGSRSINNFQHLRAQETHQLLR
CYP93B2         YGTYWKFIKKLFTVELLGTQNLSHFLPIRTHEIRELLR
                ** **.*****   ****.. . .*  .*..* ..***

CYP93B1         NNVISIMMVG--------EAEEARDVVRDVTEIFGEFN
CYP93B2         NNVISQMMMSIRCSGTNSEADEAKNLVREVTKIFGQFN
                ***** **.          **.**. .**.** ***.**

CYP93B1         LFQRFDTLVERIISKREQTRKDRRRNGKKGEQESGDGI
CYP93B2         THRRYDALLERIIMGREENR--RRGKIKDGE---G---
                  .*.*.*.****  **..*  **   * **   *

CYP93B1         VHIKALIMDFFTAGTDTTAISTEWALVELVKKPSVLQK
CYP93B2         DHIKALILDFLTAGTDTTAIAIEWALVELINNPNALEK
                 ******.** *********. *******.  *  *.*

CYP93B1         LPYLQAILKETFRLHPPVPMVTRRCVAECTVENYVIPE
CYP93B2         LPYIQAIIKEALRLHPPIPMLIRKSTENVIVQGYDIPA
                ***.***.**. *****.**. *..     *..* **

CYP93B1         EFCPERFLKLEGDSSGVVDVRGSHFQLLPFGSGRRMCP
CYP93B2         EFKPHRFLDG-GDLKSSLDIKGHNFQLLPFGTGRRGCP
                ** * ***   **    .*..* .*******.*** **

CYP93B1         QVVGPKGEILKGDDIVINVDERPGLTAPRAHNLVCVPV
CYP93B2         DVVGER---------LLNTDERAGLTAPRAVDFVCVPI
                 *** .          ..* *** *******   ****.
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Figure 6. Alignment of CYP93B1 and CYP93B2.
Conserved amino acids are indicated by asterisks and conservative substitutions are indicated by dots.

plant P450s involved in the phenylpropanoid and flavonoid pathway is shown in Figure 7. It also indicates that CYP93B2 is more similar to the (2S)-flavanone 2-hydroxylase than to other P450s.

Discussion

It is currently estimated that more than 200 000 secondary metabolites may exist in plants and that at most 10% have been characterised to date (Durst *et al.*, 1994). Many of these have proven to be economically important compounds. One group of important metabolites are the flavones which are widespread, and which occur not only in the flowers of several higher plant species but also in many other plant parts such as leaves, stems and roots (Wollenweber, 1994). They may act as yellow pigments (Harborne, 1978), co-pigments in flower colouration (Goto and Kondo, 1991; Scott-Moncrieff, 1936), inducters of nodulation (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986), signal compounds in different insect–plant interactions (Harborne and Grayer, 1994), bioactive compounds in animal and human diets (Böhm *et al.*, 1998), and as pharmaceuticals (Middleton and Kandawami, 1994). Because of the importance of flavones, it is essential that the processes by which they are formed in response to their different functions are characterised not only at the biochemical level but also at the molecular level. This work describes the first successful cloning and expression of the FNS II gene.

With a DD-RT-PCR strategy we could isolate five differential expressed bands, of which one was identified as a P450 sequence (CypDDd7a). When total RNAs of different genotypes of *Gerbera* hybrids were analyzed by Northern blotting, hybridisation could only be detected in lines with the dominant allele (*fns*$^{+}$.). In recessive lines (*fns fns*), which lack FNS II activity, no hybridisation was found. Moreover, the time course of the transcript levels during flower development corresponded to the measured enzyme activity and flavone accumulation, respectively (Figure 2). Therefore, the gene corresponding to the CypDDd7a cDNA clone had an expression pattern expected for FNS II.

The *Gerbera* P450 complete cDNA was obtained using 5′ RACE technique and end-to-end PCR. As expected, the resulting P450 cDNA sequence has high homology to P450 sequences. Upon comparison with known P450 the isolated clone showed a high homology with CYP93B1, a P450 isolated from elicitor-treated suspension-cultured cells of *Glycyrrhiza echinata*. This clone was identified to encode (2S)-flavanone 2-hydroxylase. The expressed gene product catalyzed the formation of licodione and 2-hydroxynaringenin (2-OH-NAR) from liquiritigenin and NAR, respectively. The flavone Ap was only formed from 2-OH-NAR after acid treatment of the respective enzyme assay (Akashi *et al.*, 1998). *In G. echinata* C-glucosyl-

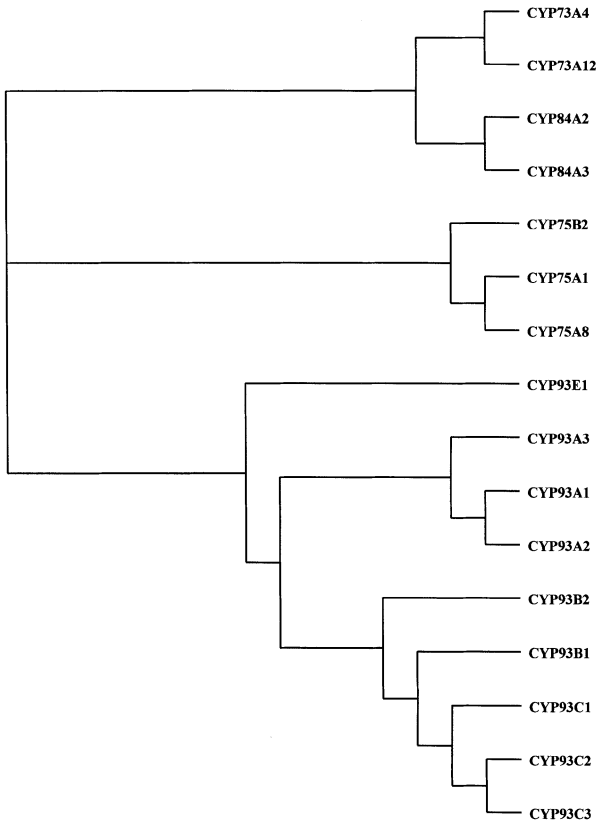

Figure 7. Phylogenetic analysis of plant P450 involved in flavonoid biosynthetic pathway.
The phylogenetic tree was obtained as described in the text. Included in the alignment were sequences of P450 clones involved in the phenylpropanoid and flavonoid pathway. The accession numbers are CYP73A4 *Catharanthus roseus* cinnamate 4-hydroxylase (C4H) (Z32563); CYP73A12 *Zinnia elegans* C4H (U19922); CYP75A1 *Petunia hybrida* flavonoid 3′,5′-hydroxylase (F3′,5′H) (Z22545); CYP75A8 *Catharanthus roseus* F3′,5′H (AJ011862); CYP75B2 *Petunia hybrida* flavonoid 3′-hydroxylase (F3′H) (AF155322); CYP84A2 *Lycopersicon esculentum* ferulate 5-hydroxylase (F5H) (AF150881); CYP84A3 *Liquidambar styraciflua* F5H (AF139532); CYP93A1 to A3 *Glycine max* (D83968, D86351 and Y10492); CYP93B1 *Glycyrrhiza echinata* (2S)-flavanone 2-hydroxylase (F2H) (AB001380); CYP93B2 *Gerbera* hybrids FNS II (AF156976); CYP93C1 *Glycine max* (AF022462); CYP93C2 *Glycyrrhiza echinata* (AB023636); CYP93C3 *Cicer arietinum* AJ243804); CYP93E1 *Glycine max* (AF135485).

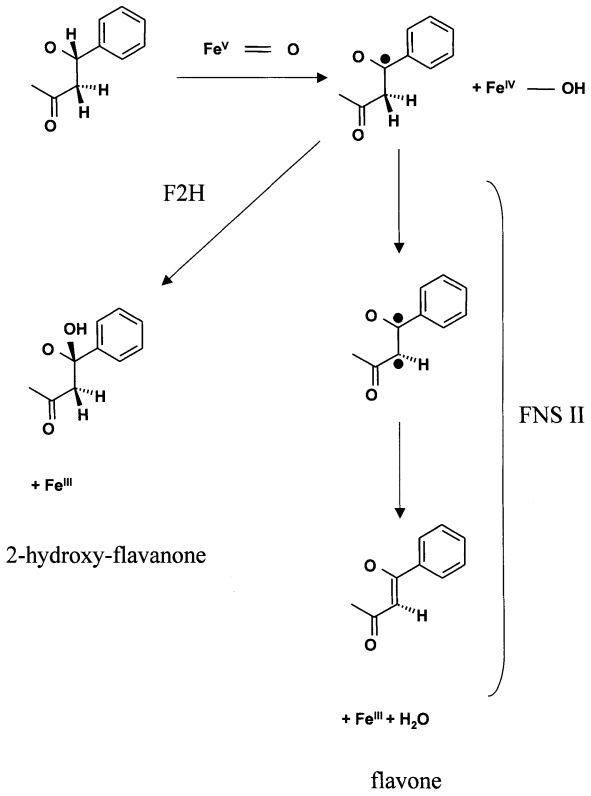

Figure 8. Monooxygenation and dehydrogenation of flavanones catalyzed by (2S)-flavanone 2-hydroxylase (F2H) and flavone synthase II (FNS II), respectively.
The proposed mechanism for FNS II and F2H reaction.

flavones are well documented (Afchar *et al.*, 1984). It is also well known that 2-OH-flavanones (e.g. 2-OH-NAR) were the direct substrates in C-glucosyl-flavone biosynthesis (Kerscher and Franz, 1987). Therefore, we postulate that CYP93B1 codes for the (2S)-flavanone 2-hydroxylase which is involved in this pathway. In contrast, heterologous expression of the clone CYP93B2 described in this paper led, under the mild conditions used in the enzyme assay, to a direct formation of Ap from the NAR without any chemical treatment. In addition, enzyme assays using ERI and LIQ as the substrates led to the formation of the respective flavones. This result and the changing transcript levels which were concomitant with the genotype, enzyme activity and appearance of flavones in *Gerbera* petals proved the first successful cloning of cDNA coding for FNS II.

We postulate a reaction mechanism for FNS II and F2H, respectively, as described for the dehydrogenation of alkane CH-CH bonds resulting in the formation of the corresponding double bond (Mansuy, 1998). It was shown that the double bond is not introduced by a dehydration of the hydroxylated metabolites, but it was shown that during two competing reactions either a transfer of OH from the Fe(IV)-OH species to the substrate (monooxygenation; CYP93B1) or the abstraction of the second hydrogen atom resulting in abstraction of water from the iron species (dehydrogenation; CYP93B2) could occur (Figure 8). A slight shift of the reactive center could be responsible for the different reactions of CYP93B1 and CYP93B2 (W. Heller, personal communication). A similar explanation has been given for the reaction mechanism of FNS I (Britsch, 1990).

Sequence analysis of CYP93B2 with other CYP450s, and especially members of the CYP93 subfamily, provides valuable information concerning the structural aspects of FNS II (see Figure 3). An initial hydrophobic variable

Table 1. Different chemogenetic defined *Gerbera* lines and cultivars

Gerbera linie/cultivar	Genotype	Origin
Th58	fns^+fns	Tyrach and Horn (1997)
Petruschka	fns fns	Tyrach and Horn (1997)
Uranus	fns fns	Tyrach and Horn (1997)
Clivia	fns fns	Tyrach and Horn (1997)
Regina	fns^+.	Terra Nigra
147–116	fns fns	Th 58 × S*
147–118	fns fns	Th 58 × S*
147–124	fns^+.	Th 58 × S*
147–137	fns fns	Th 58 × S*
147–139	fns fns	Th 58 × S*
147–140	fns^+.	Th 58 × S*
147–146	fns fns	Th 58 × S*
147–148	fns^+.	Th 58 × S*
147–159	fns fns	Th 58 × S*

* × S = self crossing.

anchor region of 17 amino acids, which is important for the proper orientation and targeting of the protein to the microsomal membrane (Murakami *et al.*, 1994; Sato *et al.*, 1990), is followed by the high conserved proline-rich hinge region (LPPSPXXXXP) of the enzyme (Yamazaki *et al.*, 1993). Furthermore, the oxygen binding pocket (AGTDT(T/S)) was found to be highly conserved in all CYP93 members. This region, commonly known as [(A/G)Gx(D/E)T(T/S)], is thought to be essential for oxygen binding and activation. This indicates that the corresponding gene product belongs to the so-called group-A P450s (Durst and Nelson, 1995). This group is expected to mediate classic monooxygenations (Schuler, 1996). We also found the P450 fingerprint of the heme binding region (PFGXGRRXCPG), which is highly conserved in all P450 polypeptides. Phylogenetic analysis indicates that FNS II and F2H are closely related (Figure 7). Furthermore, a close relationship could only be described for FNS II and the other members of the CYP93 family. A relationship with the other P450 enzymes from phenylpropanoid and the flavonoid pathway is not as evident.

In summary, we have shown that CYP93B2 cDNA encodes FNS II (a P450 of the flavonoid pathway responsible for flavone formation). This enzyme enables the control of a step in flavonoid biosynthesis that is located at an important junction of this pathway leading to different flavonoids, such as flavones, isoflavones, flavonols, proanthocyanidins and anthocyanidins. Thus, this new P450 gene might be useful in transgenic approaches to control the synthesis of these compounds by changing the FNS II activity. The direct regulation of flavone formation in different plant tissues is a useful tool to create new flower colours and to influence a wide range of economic factors in plant performance, including extending the plant disease resistance, the novel products for mammalian nutrition and the nodulation capacity in legumes.

Experimental procedures

Plant material, yeast strains and plasmids

Genetically defined flavone-producing (genotype fns^+ .) and non-flavone-producing (genotype *fns fns*) lines of *Gerbera* hybrids were obtained from chemogenetic and biochemical investigations (Table 1; Martens and Forkmann, 1998; Tyrach and Horn, 1997). The commercial variety 'Regina' (fns^+ .) was obtained from Terra Nigra (De Kwakel, the Netherlands).

For heterologous gene expression, the yeast strain INVSc1 and the expression vector pYES2 were used (Invitrogen, Groningen, the Netherlands).

Isolation and analysis of RNA

Total RNA from *Gerbera* lines at 11 different stages of floral development (Figure 2) was isolated according to Giuliano *et al.* (1993) using 200 mg of frozen plant tissue. The integrity of RNA and the absence of DNA were checked by agarose gel electrophoresis upon ethidium bromide staining. Northern blot analysis (10 μg total RNA per lane) using formaldehyde agarose gels and Hybond-NX membranes (Amersham Buchler, Braunschweig, Germany) was performed according to standard techniques (Sambrook *et al.*, 1989). ^{32}P-labeling of probes was undertaken using Ready-prime Kit (Amersham Buchler). Pre-hybridisation and hybridisation were performed at 42°C for 10–16 h in 50% (v/v) formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS and 100 μg ml^{-1} denatured salmon sperm DNA. The membranes were washed twice in 2 × SSPE, 0.1% (w/v) SDS at 42°C for 15 min, once in 1 × SSPE, 0.1% SDS at 65°C for 10 min. Quantification of the hybridisation signals was performed using a phosphorimager (BAS1000, Fujifilm, Kanagawa, Nakamura, Japan; software package TINA, Raytest, Straubenhardt, Germany).

Differential display of mRNA

Reverse transcription was performed with Superscript II reverse transcriptase (Gibco BRL, Paisley, UK) in a total volume of 25 μl following the supplier's instructions. Differential display and analysis of differential bands was carried out according to the protocol of Liang *et al.* (1993) with primer sets, containing eight P450-specific upstream primers and three anchored oligo(dT) primers as described by Schopfer and Ebel (1998). Reamplified PCR products were cloned into the pCR2.1 vector using a TA Cloning Kit (Invitrogen). Replitherm polymerase was from Epicentre (Madison, USA).

5′-RACE

Gerbera gene-specific primers (GSP7–9) were deduced from the sequence of CypDDd7a and were used for a 5′-RACE which was carried out essentially according to the manufacturer's instructions using the kit from Gibco-BRL. Total RNA from petals was reverse transcript using GSP7 (5′-ATCTTCAAAGTGTTTCCTCG-TTC-3′) as the primer. After purification and terminal transferase reaction, 5 μl of the resulting TdT product was used as a template

in first-round PCR in the presence of the Abridged Anchor primer (Gibco-BRL) and GSP8 (5′-AATGGAACACACACAAAATCTACC-3′). For the second round PCR, 5 µl of the diluted first-round PCR product was reamplified with AUAP (Gibco-BRL) and GSP9 (5′-TCACCACTGAGAGTTCTCTCATGG-3′).

DNA sequencing and analysis

Both strands of the cDNAs were sequenced using a Sequenase DNA sequencing kit (Amersham Buchler), [^{35}S]dATP (ICN, Eschwege, Germany) and specific oligonucleotide primers (MWG-BIOTECH, Ebersberg, Germany). Computer analysis of the DNA and inferred amino acid sequences was carried out using the OMIGA software package (Oxford Molecular, Oxford, UK). For the alignment and dendrogram of sequences retrieved from the GenBank, CLUSTALW (Thompson *et al.*, 1994) was used and a phylogram was drawn with the TREEVIEW (Page, 1996) program.

Heterologous expression in yeast

The coding region of CYP93B2 was inserted into the yeast vector pYES2 as described by Urban *et al.* (1994). A final PCR was performed to create a full-length version of the P450 ORF that completely lacked endogenous 5′ untranslated sequences. The following PCR primers were used: CypFNS1H (5′-caaaggatcccAACACCATGAATACA CTCC-3′) and CypFNS1R (5′-AGATAGACCGACTGCCATCAAGAAAGC-3′). The forward primer contained a *Bam*H1 restriction site immediately followed by the ATG start codon and the next 10 bases of the reading frame. The downstream primer was specific for the 3′ untranslated region of the gene. To facilitate the subcloning of the P450 into the yeast expression vectors, the internal *Eco*R1 site downstream of the plasmid pCR2.1 (Invitrogen) was used. The PCR amplification was performed with Expand High Fidelity enzyme mix according to the manufacturer's instructions (Boehringer Mannheim).

Yeast strain INV Sc1 was cultured in YPGA, SGI and YPGE mediums as described by Urban *et al.* (1997). Transformation was done by a modified lithium acetate method using 50 µg carrier DNA (Gietz *et al.*, 1992). Microsomes were prepared as described previously (Urban *et al.*, 1994).

Assay of FNS II enzyme activity

The standard FNS II assay, chromatography, identification of products, quantification and protein determination were performed as described by Martens and Forkmann (1998).

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The nucleotide sequence reported in this paper has been deposited in the GenBank, DDBJ and EMBL databases with the accession number AF156976.