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Characterization of Flavonoid 3′,5′-Hydroxylase in Microsomal Membrane Fraction of *Petunia hybrida* Flowers[1]

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We have detected a flavonoid 3′,5′-hydroxylase (F3′,5′H) in the microsomal fraction of *Petunia hybrida* flowers. Activity varied with the development of flowers, peaking immediately prior to and during anthesis, but was absent in mature flowers. F3′,5′H activity in flower extracts from genetically defined floral color mutants correlated strictly with the genotypes *Hf1* and *Hf2*. No activity was detected in flowers from mutants homozygous recessive for both alleles. F3′,5′H activity was dependent on NADPH and molecular oxygen; there was only slight activity with NADH. The enzyme catalyzes the hydroxylation of 5,7,4′-trihydroxyflavonone at the 3′ and 5′ positions, and of 5,7,3′,4′-tetrahydroxyflavonone and dihydroquercetin at the 5′ position. Hydroxylase activity was inhibited by plant growth regulators (1-aminobenzotriazole and tetcyclacis) and by CO, *N*-ethylmaleimide, diethyldithiocarbamate, and cytochrome (Cyt) *c*. Activity was not affected by diethylpyrocarbonate or phenylmethylsulfonyl fluoride, but was enhanced by 2-mercaptoethanol. A polyclonal antibody that inhibits higher plant NADPH-Cyt P450 reductase inhibited the F3′,5′H. The data are consistent with the suggestion that the *P. hybrida* F3′,5′H is a monooxygenase consisting of a Cyt P450 and a NADPH-Cyt P-450 reductase. Cyts P450 were detected in microsomal membranes and in solubilized detergent extracts of these membranes. F3′,5′H activity was sensitive to low concentrations of all detergents tested, and therefore solubilization of the active enzyme was not achieved. Reaction products other than flavanones were observed in F3′,5′H assays and these may be formed by enzymic oxidation of flavanones. The possibility of a microsomal flavone synthase of a type that has not been described in *P. hybrida* is discussed.

Important differences in *Petunia hybrida* flower colors result from enzymic modification of anthocyanidin precursor compounds such as the flavanones and 3-hydroxy derivatives, dihydroflavonols (Weiring and de Vlaming, 1984). In particular, hydroxyl group incorporation at the 5′ position of the flavonoid B-ring (Fig. 1) gives rise to flavonoids that, with further enzymic modifications, form purple anthocyanidins. In *P. hybrida* flowers, hydroxylations at the 3′ position are regulated by two separate genetic loci; *Ht1* is responsible for hydroxyl group addition at the 3′ position of precursor flavonoids in the corolla limb and tube, and *Ht2* controls 3′-hydroxylation in the tube only. Similarly, *Hf1* controls hydroxylation at the 3′ and 5′ positions in limb and tube. *Hf2* is responsible for 3′ and 5′ hydroxylation only in the limb (Doodeman et al., 1982; Weiring and de Vlaming, 1984). Enzymic studies of *P. hybrida* mutants have established that the *Ht1* gene corresponds to F3′H activity (Stotz et al., 1985). The enzyme responsible for flavonoid 5′-hydroxylation has been detected but not clearly identified in *P. hybrida* flowers (Stotz et al., 1985; Britsch and Grisebach, 1986).

However, in *Verbena hybrida* flowers a F3′,5′H has been characterized; its expression is controlled by one gene (Stotz and Forkmann, 1982). These flavonoid B-ring hydroxylases have properties in common with Cyt P450-dependent monooxygenases (for review, see Stafford, 1990a). Characteristics include association with microsomal membrane fraction, dependence on NADPH and O_2, and sensitivity to certain inhibitors such as CO. They are distinct from the soluble flavanone 3β-hydroxylase, an enzyme encoded by the *An3* locus in *P. hybrida* flowers (Fig. 1), which does not require NADPH for activity (Britsch and Grisebach, 1986; Britsch, 1990).

Several distinct Cyt P450-dependent monooxygenase systems have been characterized in prokaryotes and eukaryotes (for reviews, see West, 1980; Peterson and Prough, 1986; Rivière and Cabanne, 1987). In eukaryotes NADPH is the source of electrons; in prokaryotes a soluble monooxygenase system may obtain electrons from NADH. Mammalian mitochondria utilize a Cyt P450, a flavin adenine dinucleotide-containing flavoprotein, and an Fe-S protein. Non-mitochondrial eukaryotic monooxygenases consist of a Cyt P450 and a flavin adenine dinucleotide- and flavin mononucleotide-containing NADPH-Cyt P450 reductase (EC 1.6.2.4). In plants only the latter system has been found (Donaldson and Luster, 1991). In some reactions in eukaryotic tissues, Cyt P450-dependent reactions can be partly supported by NADH, a hemoprotein, Cyt b_5, and a flavoprotein, NADH-Cyt b_5-reductase (Møller and Lin, 1986).

This paper is a report on in vitro studies intended to clarify the nature of the enzyme or enzymes responsible for F3′,5′H activity in *P. hybrida* flowers. The correlation between

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; eriodictyol, 5,7,3′,4′-tetrahydroxyflavanone; F3′H, flavonoid 3′-hydroxylase; F3′,5′H, flavonoid 3′,5′-hydroxylase; naringenin, 5,7,4′-trihydroxyflavanone; R_f, fractional retardation.

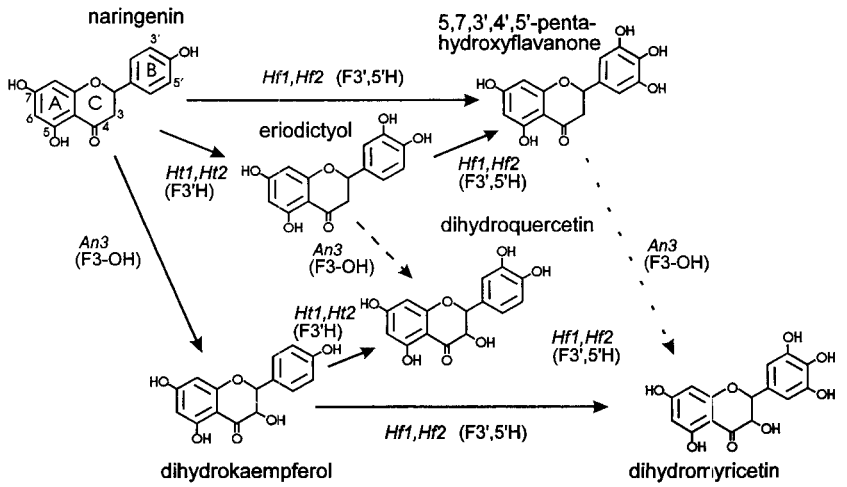

Figure 1. Genetics of the hydroxylation pathway of anthocyanin biosynthesis in *P. hybrida* flowers. Hydroxylations are controlled by the genes *Hf1*, *Hf2*, *Ht1*, *Ht2*, and *An3*. *Hf1* and *Hf2* control hydroxylation at the 3′ and 5′ positions and *Ht1* and *Ht2* control hydroxylation at the 3′ position only. The *An3* gene can convert the flavanone compounds naringenin, eriodictyol, and 5,7,3′,4′,5′-pentahydroxyflavanone (also known as 5′-hydroxyeriodictyol) to the corresponding dihydroflavonols: dihydrokaempferol, dihydroquercetin, and dihydromyricetin by the addition of a hydroxyl group at the 3 position. Three enzyme activities are believed to be responsible for the catalyses: F3′,5′H; F3′H; and flavanone 3-β-hydroxylase (F3-OH). Redrawn from Forkmann et al. (1986).

F3′,5′H activity and dominant and recessive *Hf1* and *Hf2* genotypes is examined, and the expression of F3′,5′H in flowers at various stages of development is also described.

MATERIALS AND METHODS

Source of Plant Material and Growth Conditions

Petunia hybrida plants cv Old Glory Blue (Ball Seed Company, Chicago, IL) and *P. hybrida* mutants (A. Cornu, Institut National de la Recherche Agronomique) were grown at approximately 22°C with light provided by either mercury lamps or fluorescent and tungsten lamps at 17 ± 3 kilolux. Approximately 14 h of light was provided per day. Flowers were harvested daily and stored briefly at 4°C before use.

Preparation of Tissue Extracts

All procedures were carried out on ice or at 4°C and protein was determined by the bicinchoninic acid microassay protocol of Smith et al. (1985). Flower buds were selected on the basis of their morphological similarity to stage 5 flowers, as defined by Stotz et al. (1985).

Cell-free extracts of stage 5 flower buds were used for experimental comparison of F3′,5′H activity in flowers from various mutants. Individual flower buds were separated into limb and tube portions. Limb and tube pieces (0.04–0.1 g) were finely chopped and gently homogenized by 10 passes in a chilled 2-mL glass homogenizer with 1 mL of buffer A (100 mM potassium phosphate, 10^{-7} M pepstatin A, 10^{-7} M leupeptin, 0.1 mg/mL PMSF, 0.25 M Suc, 0.25 M mannitol, 20 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM EGTA, 0.1% BSA, pH 7.5) containing 10 mg Polyclar AT (BDH, Poole, UK). The homogenate was filtered through one layer of Miracloth (Calbiochem, La Jolla, CA), the filtrate was centrifuged at 12,000*g* for 10 min, and samples of supernatant containing 0.5 mg of protein were assayed for F3′,5′H activity.

The microsomal membrane fraction was isolated from the limb portion of stage 5 flower buds. Tissue was homogenized in a mortar and pestle with buffer A (containing 10 mg Polyclar AT/mL and 0.1 g of fine sand) in the ratio of 1 g of tissue (wet weight) to 5 mL of buffer. Homogenization was continued until the homogenate had a smooth consistency. The microsomal fraction was sedimented essentially as described by Diesperger et al. (1974): the homogenate was centrifuged at 5,000*g* and the supernatant was recentrifuged at 12,000*g* for 20 min. The supernatant was filtered through Miracloth, adjusted to 40 mM $MgCl_2$, and incubated for 10 min. A membrane fraction was pelleted by centrifugation at 20,000*g* (20–30 min), and this constituted the microsomal fraction. Membrane pellets were suspended in buffer B (100 mM potassium phosphate, 1 mM EDTA, 20% Suc or glycerol, 0.1% BSA, 20 mM 2-mercaptoethanol [or 1–5 mM DTT], 10^{-7} M leupeptin, 10^{-7} M pepstatin A, pH 7.5). On average the yield of microsomal protein was 86 mg/kg flower (wet weight). The microsomal fraction from young leaves was isolated exactly as for flower buds.

F3′,5′H Assay

F3′,5′H activity was measured by a modification of the method of Stotz and Forkmann (1982). Substrates were [^{3}H]-naringenin (Amersham), [^{3}H]dihydrokaempferol (Amersham), or [^{3}H]eriodictyol. [^{3}H]Eriodictyol was prepared by enzymic modification of [^{3}H]naringenin with *P. hybrida* flower extracts under standard assay conditions and was purified by chromatography on cellulose TLC plates. For the standard F3′,5′H assay, the following were mixed and incubated (2 h, 23°C) in a total of 210 μL: 10 to 100 μL of microsomal suspension or cell-free extract, 1 μM ^{3}H-labeled substrate containing 2 μCi of [^{3}H]naringenin, [^{3}H]dihydrokaempferol, or [^{3}H]eriodictyol in buffer C (100 mM potassium phosphate, 20 mM 2-mercaptoethanol, 1.25 mM NADPH, pH 8.0). The reaction was stopped and flavonoids were extracted by addition of 1 mL of ethylacetate containing 25 μg of naringenin "carrier." The ethylacetate phase was dried in vacuo and residues were redissolved in 10 to 20 μL of ethylacetate and applied to 0.1-mm cellulose TLC plates (Merck, Darmstadt, Germany). Development solvent was chloroform:acetic acid:water (10:9:1, v/v/v). Reaction products were identified by comparison of the R_f of reaction products separated by various solvent systems with literature values (Swain, 1976; Doodeman et al., 1982; Stotz and Fork-

mann, 1982; Stotz et al., 1984) and with authentic flavonoids. Flavonoids separated by TLC were examined under UV light and after chromogenic treatment with $NaBH_4$/HCl to detect flavanones (Eigen et al., 1957; Horowitz, 1957). ^{3}H-labeled compounds were detected on TLC plates by autoradiography overnight after spraying plates with a 7% solution of 2,5-diphenyloxazole in diethyl ether.

For determination of the assay pH optimum, assays were conducted in 100 mM potassium phosphate buffer in the pH range 5 to 9. Assays with lowered oxygen tension were carried out essentially according to Hagmann et al. (1983). Glc oxidase (20 units), catalase (2.6 units), and Glc (0.9%) were added to the standard F3′,5′H assay. The effects on F3′,5′H activity of various compounds such as tetcyclacis and 1-aminobenzotriazole (BASF, Ludwigs Lafen, Germany), rabbit anti-Jerusalem artichoke NADPH-Cyt P450 reductase polyclonal antibody (purified on Protein A-Sepharose and a gift from Dr. I. Benveniste, Centre National de la Recherche Scientifique, Strasbourg, France), other antibodies, and various other substances (Sigma) were examined by their addition to the standard assay mixture.

CO-Reduced Minus Reduced Difference Spectra

CO-reduced minus reduced spectra (also known as the reduced, CO difference spectra) were determined by the method of Omura and Sato (1964). CO gas was introduced into detergent-solubilized samples at about 100 bubbles per min to minimize foaming. Spectrophotometric analysis of microsomal membrane suspension was carried out using a Beckman Du52 spectrophotometer, and microsomal detergent extracts were analyzed using a Hewlett-Packard HP8452A diode array spectrophotometer.

Treatment of Microsomal F3′,5′H with Detergents

Two types of experiments were conducted. In the first, 40 μL of microsomal membrane suspension (containing 200 μg of protein) was adjusted to 0 to 3% detergent and incubated at 4°C for 1 to 16 h. The entire sample was assayed for F3′,5′H activity. In a second experiment, 200-μL samples containing 200 μg of protein and 0 to 2% detergent were incubated for 10 min on ice and ultracentrifuged (Beckman Airfuge, 100,000*g*, 1 h, room temperature). Supernatant fractions containing detergent-solubilized material were assayed for F3′,5′H activity. Detergents tested were sodium deoxycholate, sodium cholate, CHAPS (Boehringer-Mannheim); Triton X-100 and Triton X-114 (Ajax Chemicals, Melbourne, Australia); Nonidet P40 (BDH); Tween 20 and Tween 80 (Sigma); Lubrol PX (Pierce, Rockford, IL); digitonin (Sigma); hexyl-, heptyl-, octyl-, nonyl-, decyl-β-D-glucopyranoside, dodecyl-maltoside (Calbiochem); Mega-8, -9, and -10 (Calbiochem); Zwittergents 3–08, 3–10, 3–12, 3–14, and 3–16 (Calbiochem); and Emulgen 911 and 913 (Kao Atlas, Tokyo, Japan). NADPH-Cyt *c* reductase was purified from *P. hybrida* flowers according to Menting (1992). Protein concentrations were determined by the method of Sedmak and Grossberg (1977).

RESULTS

F3′,5′H Activity

A typical TLC separation of the reaction products of an incubation of naringenin with cell-free extracts of flowers from *P. hybrida* mutants R51 and V23 is shown in Figure 2. Table I presents the R_f values of the compounds formed by microsomal suspensions and cell-free extracts of flowers from Old Glory Blue and V23 *P. hybrida* plants. By comparison of the R_f values of U1, U3, and U6 with published R_f values and with the migration of authentic flavonoids, these compounds were identified as the flavanones naringenin, eriodictyol, and 5,7,3′,4′,5′-pentahydroxyflavanone, respectively. Compounds U1, U3, and U6 were black under UV light. Treatment of TLC plates with the chromogenic reagent $NaBH_4$/HCl caused compounds U1, U3, and U6 to become purple-red, indicative of a reaction with flavanones. The other compounds formed from naringenin (U2, U4, U5, and U7) may be the flavones apigenin, luteolin, and tricetin. Naringenin was modified by three enzyme activities, all of which were evident in the microsomal fraction. Figure 2 shows that V23 mutant flower buds contained F3′,5′H activity, which formed eriodictyol (U3) and 5,7,3′,4′,5′-pentahydroxyflavanone (U6) from naringenin (U1). Another mutant, R51, contained a second distinct enzyme, F3′H, which formed only eriodictyol from naringenin. The third enzyme

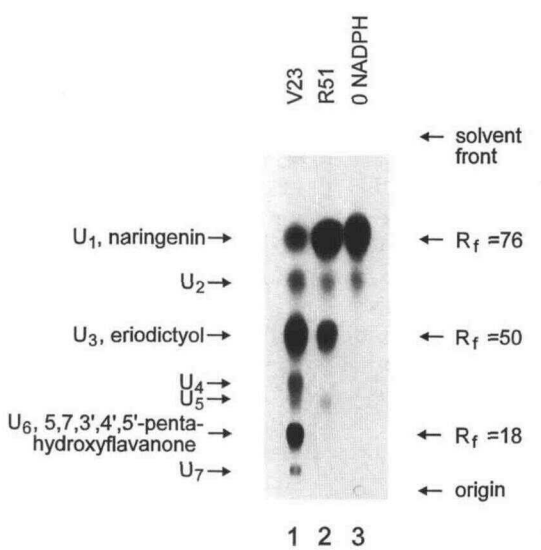

Figure 2. Typical results of a F3′,5′H assay showing an autoradiogram of compounds formed from [^{3}H]naringenin by cell-free extracts of flower buds and separated by cellulose TLC using the solvent system $CHCl_3$:CH_3COOH:H_2O (10:9:1, v/v/v). In lane 1 are products formed by flower extracts from a mutant with blue flowers and the *Hf1Hf1* genotype (V23). In lane 2 the products formed by flower extracts from a mutant with red flowers and the *Ht1Ht1* genotype are separated (R51, *hf1hf1*). Lane 3 shows compounds formed from the same assay as shown in lane 1 but without NADPH. The principal compounds formed by the action of F3′,5′H on compound U1 (naringenin) were identified as eriodictyol (U3; 3′-hydroxynaringenin) and 5,7,3′,4′,5′-pentahydroxyflavanone (U6; 3′, 5′-dihydroxynaringenin). The principal compound formed by the action of F3′H on naringenin was eriodictyol.

Table I. *R_f of flavonoids in various solvent systems*

The identities of naringenin and eriodictyol were confirmed by comparison with authentic flavanones. The identity of 5,7,3′,4′,5′-pentahydroxyflavanone was authenticated by comparison with reported R_f values (see "Materials and Methods") and from the pattern of these three flavanones when chromatographed in various solvent systems.

Flavonoid	Solvent System		
	CAW[a]	30% CH_3COOH	Forestal[b]
U1; naringenin	76	59	85
U3; eriodictyol	50	48	78
U6; 5,7,3′,4′,5′-pentahydroxyflavanone	18	37	74
Apigenin	65	22	77
Dihydroquercetin	28	64	75
Kaempferol	51	16	59
Quercetin	20	12	42
Myricetin	5	8	27
U2[c] (apigenin)	65	16	75
U4 (luteolin)	36	8	
U5 (luteolin)	28	8	53
U7 (tricetin)	8	4	33

[a] CAW represents chloroform:acetic acid:water (10:9:1, v/v/v). [b] Forestal represents acetic acid:HCl:water (30:3:10, v/v/v). [c] U2, U4, U5, and U7 represent four compounds other than the flavanones (U1, U3, and U6) produced in F3′,5′H assays with naringenin as substrate. Names in parentheses represent possible flavonoid identities.

activity appeared to form flavones from each of the three flavanones formed by the action of the F3′,5′H and F3′H. Therefore, this enzyme may be called a flavone synthase. In the absence of NADPH, V23 flower extracts failed to catalyze the conversion of naringenin into other flavonoids.

F3′,5′H activity was detected in microsomal membrane fractions isolated by two methods, differential sedimentation of membranes with $MgCl_2$ (see "Materials and Methods") and ultracentrifugation (after centrifugation of a homogenate at 20,000*g*, supernatant was recentrifuged at 105,000*g*). The membrane fraction isolated by these methods contained the ER marker enzyme NADPH-Cyt *c* (Cyt P450) reductase. Isolation of the microsomal fraction by differential sedimentation of membranes with $MgCl_2$ was less efficient than by ultracentrifugation, since the latter method yielded 4-fold more microsomal protein. Nevertheless, the pattern of hydroxyl-modified products was identical in both microsomal preparations.

The patterns of hydroxylated compounds formed from naringenin by cell-free extracts and the microsomal membrane fraction were similar whether these fractions were prepared from V23 or Old Glory Blue flowers. Apigenin, luteolin, and tricetin were always formed, providing that the respective flavanones (naringenin, eriodictyol, and 5,7,3′,4′,5′-pentahydroxyflavanone) were present (Fig. 2). When F3′,5′H or F3′H activity was low (as indicated by the production of relatively small quantities of eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone), lesser quantities of the putative flavones were formed. Although the formation of flavones occurred concurrently with flavanone synthesis, flavone production was always lower.

Substrates

Old Glory Blue flower microsomes catalyzed the conversion of naringenin to both eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone, and 5,7,3′,4′,5′-pentahydroxyflavanone was also formed from eriodictyol (Fig. 3). These reactions were dependent on NADPH. With naringenin as substrate, the formation of eriodictyol preceded that of 5,7,3′,4′,5′-pentahydroxyflavanone when the formation of reaction products was examined over time (data not presented). The same flower extracts also catalyzed the NADPH-dependent conversion of dihydroquercetin to dihydromyricetin (Fig. 3). In general, NADH supported the hydroxylation reactions very poorly. At best, a small quantity of eriodictyol was formed but 5,7,3′,4′,5′-pentahydroxyflavanone was not (Fig. 4). NADPH-dependent hydroxylation was not altered by the addition of an equimolar concentration of NADH (1.25 mM). No activity was observed in the absence of NADPH and NADH.

The dependence of hydroxylation modifications on molecular oxygen was examined by removal of oxygen with Glc oxidase and catalase (data not presented). Decreased oxygen tension resulted in a marked decrease in the formation of both eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone compared to a similar sample lacking Glc. Complete removal of O_2 from the system was unlikely to have been achieved under these experimental conditions and may explain the incomplete inhibition of the enzyme activity.

Enzyme Stability

Microsomal F3′,5′H activity was stable overnight at 4°C in the presence of 20% glycerol or Suc at pH 7.5. F3′,5′H

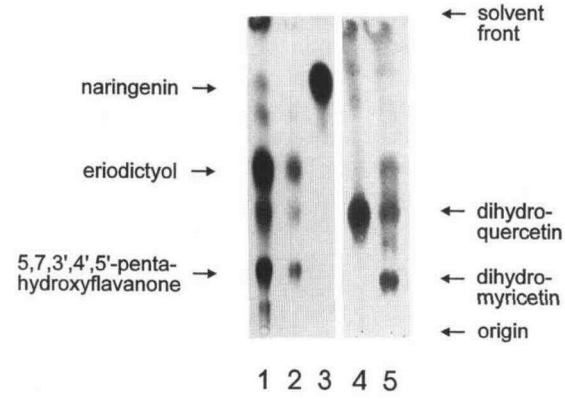

Figure 3. TLC of the compounds formed by NADPH-dependent reactions catalyzed by cell-free extracts of Old Glory Blue flower buds in the presence of various substrates. This autoradiogram shows the conversion of the substrates [^{3}H]naringenin (lane 1) and [^{3}H]eriodictyol (lane 2) to 5,7,3′,4′,5′-pentahydroxyflavanone. Lanes 3 and 4 show the migration of naringenin and dihydroquercetin marker compounds. [^{3}H]Dihydroquercetin (lane 5) was converted to dihydromyricetin.

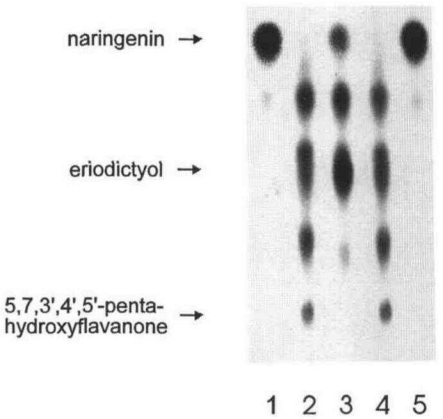

Figure 4. Autoradiogram showing the dependence of flavonoid 3′,5′-hydroxylase activity on the cofactors NADPH and NADH. Compounds formed from [^{3}H]naringenin were separated by TLC and autoradiographed. In lanes 1 to 4 are shown the products formed by microsomal membranes isolated from Old Glory Blue flower buds: lane 1, no added cofactors; lane 2, NADPH; lane 3, NADH; lane 4, both NADPH and NADH. Lane 5 shows the migration of naringenin in the absence of microsomes and cofactors.

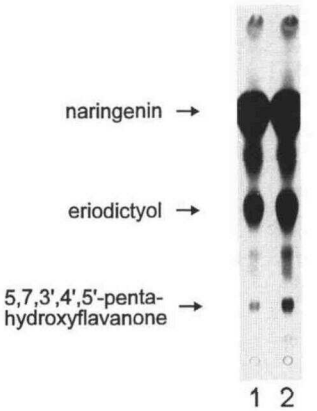

Figure 6. Autoradiogram of the effect of CO on microsomal F3′,5′H activity. Microsomal suspensions containing 100 μg of protein were gassed for 1 min with CO or air at the rate of 1 bubble per min. After incubation at 22°C for 1 h in an atmosphere of air, [^{3}H]-naringenin and NADPH were added in accordance with the standard assay procedure. Reaction products formed during a subsequent 1-h incubation were analyzed by TLC and autoradiographed. Lane 1, Sample gassed with CO; lane 2, sample gassed with air.

activity declined when microsomal suspensions were subjected to freezing and thawing. Storage at −75°C in the presence of 20% Suc for periods of greater than 1 month resulted in significant losses of enzyme activity. Highest activity was obtained with freshly harvested flower buds. The pH optimum for enzyme activity was 8.0 ± 0.3 and was determined in 100 mM potassium phosphate assay buffer.

Inhibitors

The effect of various inhibitors and antibodies on F3′,5′H activity was tested by their addition to the standard assay.

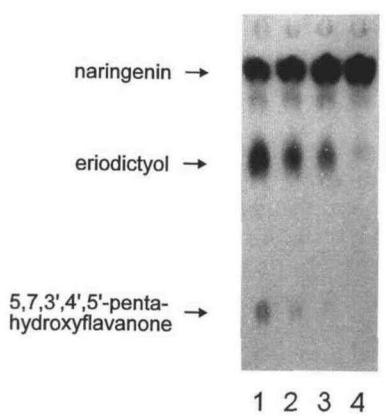

Figure 5. Autoradiogram showing the effect of an anti-NADPH-Cyt P450 reductase polyclonal antibody on F3′,5′H activity. Antibody was Protein A-Sepharose purified. Experiments were conducted by addition of buffer with or without antibody to assays of cell-free extracts of flower buds with naringenin and NADPH as substrates. The extracts were incubated for at least 30 min on ice, then assayed. Lane 1, No antibody; lane 2, plus antibody diluted 1:100; lane 3, antibody diluted 1:50; lane 4, antibody diluted 1:20.

Proteases completely destroyed the activity. Diethylpyrocarbonate (2 mM), PMSF (2 mM), and EDTA (2 mM) had no effect on activity but diethyldithiocarbamate (2 mM), *N*-ethylmaleimide (2 mM), and SDS (1%, w/v) completely inhibited activity. The plant growth regulators tetcyclacis and 1-aminobenzotriazole inhibited F3′,5′H and F3′H activities at least 20% at 100 nM and 1 mM, respectively. Inclusion of a reducing agent, 2-mercaptoethanol, was necessary to maintain maximum activity (best 20 mM). Cyt *c* (50 μM) completely inhibited activity. In addition, rabbit anti-(Jerusalem artichoke) NADPH-Cyt P450 reductase polyclonal antibody inhibited the enzyme activity (Fig. 5). This antibody was affinity purified on Protein A-Sepharose. As controls, a rabbit antiserum raised against a plant (1→3,1→4)-β-glucanase and an affinity purified rabbit antibody raised against a bacterial NADH oxidase (a flavoprotein) did not affect the activity.

The effect of CO on F3′,5′H activity was examined by bubbling CO through a microsomal suspension for 1 min. As a control, an identical sample was gassed in the same way with air. Both samples were incubated for 1 h in the presence of air, after which the samples were assayed for F3′,5′H activity for 1 h (Fig. 6). CO caused a decrease of activity compared to the control. Inhibition of microsomal F3′,5′H activity was observed to the same degree with microsomes isolated from V23 and Old Glory Blue mutants.

Without exception, the formation of flavone compounds from flavanones paralleled the formation of the flavanones; when inhibitors prevented eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone formation, the nonflavanone compounds were also not observed.

Differential Expression of F3′,5′H Activity in Developing Flowers

Flower bud extracts catalyzed the hydroxylation of naringenin at the 3′ and 5′ positions, but open flower extracts did

not. To define the relationship between F3′,5′H activity and the developmental stage, eight flower bud and open flower stages were assayed (Fig. 7). Enzyme activity was greatest in the two developmental stages just prior to flower opening (stages 5 and 6). In mature, open flower buds (stage 8) no activity was detected, but in partially open flowers (stage 7) slight activity was evident. The visual observation of anthocyanidin pigmentation coincided with the detection of F3′,5′H activity in early, developing flower bud stages.

Analysis of F3′,5′H Activity in *P. hybrida* Mutants

The relationship between the *Hf1* and *Hf2* genotypes and F3′,5′H phenotype was investigated by assaying flowers from mutant varieties for F3′,5′H activity (Table II). In all genotypes dominant for *Hf1* and *Hf2*, F3′,5′H activity was observed and F3′H activity was observed in R51 flower tubes (R51 is homozygous dominant for *Ht1* and homozygous recessive for *Hf1*). No activity was detected in flowers homozygous recessive for *Hf1*, *Hf2*, *Ht1*, and *Ht2* (Sw63), but a trace of activity (some eriodictyol was formed) was detected in the limb of Skr4. F3′,5′H activity was detected in the corolla limb but not in the tube of the *Hf2* genotype, but F3′,5′H activity was found in both limb and tube of *Hf1* genotypes. In the presence of *Ht1*, expression of F3′,5′H was intensified in the *Hf2* genotype compared to the *Hf2* genotype alone.

Table II. *Comparison of F3′,5′H phenotype with genotypes of flowers from various P. hybrida mutants*

Flower buds were chosen on the basis of their morphological similarity to stage 5 flower buds of Old Glory Blue. The limb represents the outer portion of the corolla and the tube is the narrow base of the corolla.

P. hybrida Mutant	3′,5′-Hydroxylase Activity[a]		*Hf* and *Ht* Genotypes[b]
	Limb	Tube	
V23	+++	+	*Hf1, Hf2, ht1ht1*
V30	+++	+/0	*Hf1, Hf2, Ht1, Ht2*
R51	nd[c]	0	*Ht1, hf1hf1*
Sw63	0	0	*hf1hf1, hf2hf2, ht1ht1, ht2ht2*
Sd5	+	0	*Hf2, hf1hf1, ht1ht1*
Th7	+++	+/0	*Hf1, Hf2, Ht1, Ht2*
Pa3	+++	0	*Hf2, Ht1, hf1hf1*
Dla51	++	+	*Hf1, Ht1*
Skr4	+/0	0	*hf1hf1, hf2hf2, ht1ht1*
Tb1-3	+++	+	*Hf1, Hf2, Ht1*
Tw9	+++	+	*Hf1, Ht1*

[a] Relative enzyme activities are depicted by +, ++, or +++ in order of increasing activities. The symbol +/0 represents a trace amount of enzyme activity. [b] Personal communications from E. Farcy and A. Cornu, Station de Génétique et d'Amélioration des Plantes de Dijon, Institut National de la Recherche Agronomique, France. [c] No determination was made because this mutant is *b1b1*, i.e. homozygous recessive for a mutation causing the absence of most of the limb.

Detergent Solubilization

In an attempt to solubilize active F3′,5′H, the microsomal enzyme was incubated with a variety of nonionic, anionic, and zwitterionic detergents (see "Materials and Methods" for the names of the detergents tested). Microsomal F3′,5′H activity exhibited dose-dependent inhibition by detergent. Activity significantly decreased in the range of 0.1 to 0.8% detergent and activity was generally abolished in the range 0.8 to 3%. There were threshold detergent concentrations at which the formation of eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone were concomitantly abolished, but activity could not be restored by subsequent dilution of the preparations. Following ultracentrifugation at 100,000*g* for 1 h, supernatants of detergent-treated microsomes contained solubilized proteins but no activity. Assay of supernatants and pellets following treatment of microsomes with 0 to 2% detergents showed that the enzyme was not solubilized in an active form. With increasing detergent concentrations activity was completely lost from extracted, pelleted membranes. Neither addition of leaf microsomes containing NADPH-Cyt *c* (Cyt P450) reductase, nor addition of purified NADPH-Cyt *c* reductase could restore activity. Also, there was no activity detected in the solubilized supernatant fraction of CHAPS-extracted microsomal fraction after overnight dialysis to remove the detergent.

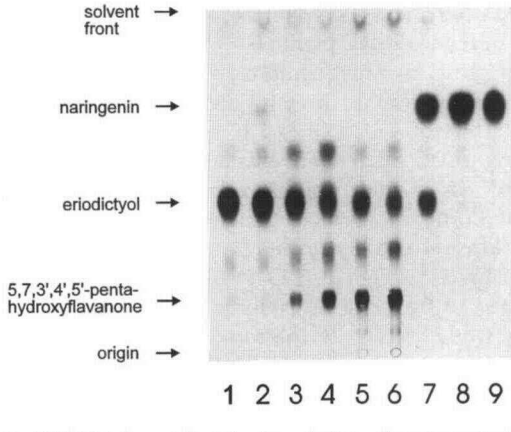

Figure 7. NADPH-dependent hydroxylation of naringenin by cell-free extracts of Old Glory Blue flowers at various stages of flower development. Flavonoid reaction products were extracted with ethylacetate, separated by cellulose TLC, and autoradiographed. The limb (outer) portion of the corolla was assayed. Selection of morphological stages was based on the definitions of Stotz et al. (1985). Lanes 1 to 8 show the compounds formed by cell-free extracts of flowers harvested at developmental stages 1 to 8, with 8 representing fully open, mature flowers. Lane 9 shows the migration of naringenin in the absence of cell-free extract and NADPH.

CO-Reduced Minus Reduced Spectra of Microsomal Fraction

Measurements of Cyt P450 content of microsomal suspensions and detergent extracts were made using the CO-reduced

minus reduced spectroscopic method (spectra not presented). Old Glory Blue microsomal suspensions exhibited a spectral maximum at 450 nm, indicating the presence of Cyts P450. Some detergents (octylglucoside, CHAPS, Emulgen 911, and, to a lesser extent, Tween 80 and Nonidet P40) successfully solubilized Cyts P450. Sodium cholate failed to give rise to a 450-nm peak. It was necessary to gas microsomal detergent extracts with CO for at least 3 min before a 450-nm shift became apparent. Peaks at 450 nm were accompanied by a trough at 468 to 470 nm. In none of the preparations were 420-nm peaks observed, which suggests the absence of Cyt P420. Although Cyts P450 were detected in this way in microsomal membranes and their detergent extracts, the spectra were severely compromised by the presence of endogenous pigments. Therefore, quantitation of Cyt P450 content in extracts of *P. hybrida* flowers was found to be unreliable.

DISCUSSION

F3′,5′H activity was detected in the microsomal membrane fraction of flower buds, whether this fraction was isolated by the $MgCl_2$-differential sedimentation method or by sedimentation at 105,000*g*. Therefore, expression of the *P. hybrida* F3′,5′H parallels other plant and mammalian monooxygenase systems (West, 1980). Microsomal preparations of F3′,5′H were generally unstable and required glycerol or Suc for preservation of activity. Properties of F3′,5′H were similar to those reported by Russell (1971) for the cinnamic acid-4-hydroxylase from etiolated pea seedlings and by Larson and Bussard (1986) for maize F3′H. Figure 4 shows that microsomal F3′,5′H converted naringenin to eriodictyol in the presence of NADH; however, the level of conversion was low in comparison to that seen in the presence of NADPH. It is possible that the slight NADH-dependent hydroxylase activity is evidence for the presence in *P. hybrida* of a NADH-Cyt b_5 reductase/Cyt b_5 system of Cyt P450 reduction (Donaldson and Luster, 1991). That NADH is a poor substrate for hydroxylation is supported by the observation that some eriodictyol is observed when F3′,5′H is assayed for short times or when inhibitors are present. When activity is low, little or no 5,7,3′4′5′-pentahydroxyflavanone is formed. In flower extracts from all *P. hybrida* mutants studied, formation of 5,7,3′4′5′-pentahydroxyflavanone occurs through the initial formation of eriodictyol (data not presented).

The effects of enzyme inhibitors on the hydroxylase activity was similar to their effects on other flavonoid B-ring hydroxylases that are believed to be Cyt P450 monooxygenases. CO partly inhibited F3′,5′H. Only a brief exposure to CO was tried because it was feared that a longer exposure to CO or indeed saturation with the gas may have resulted in the sparging of O_2, which is necessary for F3′,5′H activity. Nevertheless, the data indicate that CO inhibited the F3′,5′H even though CO treatment was followed by an aerobic incubation and assay. Some triazole fungicides, plant growth retardants, and structurally related compounds are believed to exert their effects by inhibiting the activity of particular Cyts P450. The plant growth retardant tetcyclacis inhibits Cyt P450 monooxygenases involved in GA biosynthesis (Rademacher et al., 1987), herbicide detoxification (Sterling and Balke, 1990), and sterol biosynthesis (Taton et al., 1988). The triazole compound 1-aminobenzotriazole inhibits plant Cyts P450 involved in herbicide detoxification (Cabanne et al., 1987), cinnamic acid-4-hydroxylase, and lauric acid hydroxylase (Reichardt et al., 1982) as well as mammalian (Ortiz de Montellano et al., 1984; Woodcroft and Bend, 1990) and insect Cyts P450 (Feyereisen et al., 1984). Both compounds strongly inhibited *P. hybrida* F3′,5′H activity, thereby illustrating the Cyt P450 dependence of the activity.

An antibody raised against a purified Jerusalem artichoke NADPH-Cyt P450 reductase (Benveniste et al., 1989) inhibited F3′,5′H activity. This antibody also inhibits a purified NADPH-Cyt P450 reductase from *P. hybrida* (Menting, 1992). This demonstrates that the F3′,5′H activity is dependent on a NADPH-Cyt P450 reductase. F3′,5′H activity was also inhibited by Cyt *c*, indicating that the F3′,5′H activity is dependent on an NADPH-dependent reductase capable of reducing both Cyt P450 and Cyt *c*.

Although F3′,5′H has previously been detected in microsomal fractions (Stotz et al., 1985; Britsch and Grisebach, 1986), the dependence of the activity on a Cyt P450 monooxygenase was assumed based on the properties of other B-ring hydroxylases. To our knowledge, ours is the first report of an analysis of F3′,5′H activity in flowers of *P. hybrida* mutants with a variety of *Hf* and *Ht* genotypes.

These genotypes were established on the basis of the accumulation of anthocyanin precursor compounds in mutant flowers due to mutational defects, and the phenotypic mutations were confirmed by overcoming the defects with exogenous flavonoids (Schram et al., 1984). Some of the properties of the enzymes responsible for B-ring 5′-hydroxylation have been established here, and some evidence has been provided that two such enzymes are encoded by the *Hf1* and *Hf2* genes in *P. hybrida*. Interpretation of the assay data for genotypically defined *P. hybrida* mutants indicates that both the *Hf1* and *Hf2* genotypic loci encode enzymes with F3′5′H activity. It is clear from our data that the F3′,5′H activity (phenotype) corresponds exactly to the *Hf1* and *Hf2* genotypes in *P. hybrida* mutants. Flower extracts from petunia plants with dominant *Hf* alleles but recessive for *Ht* alleles converted naringenin to both eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone. Therefore, F3′,5′H catalyzes hydroxylation at both the 3′ and 5′ positions. Since the flavonoid B-ring is free to rotate about the C_1-$C_{4'}$ axis, the 3′ and 5′ positions are geometrically equivalent. Therefore, it is interesting that the F3′H restricts its activity to the 3′-hydroxylation.

It is tempting to speculate that the active site is unable to accommodate the bulkier 3′-modified product, thereby giving rise to the observed specificity. If the *Hf2* gene (mutant Sd5, Table II) is accompanied by an *Ht1* gene (mutant Pa3), F3′,5′H activity is greater. This probably reflects an overall higher rate of eriodictyol synthesis by the two hydroxylases (F3′,5′H and F3′H) in Pa3. If more eriodictyol is produced, more is available for F3′,5′H to convert to 5,7,3′,4′,5′-pentahydroxyflavanone. This illustrates how closely the F3′,5′H phenotypic data match the genotypic data.

In comparison with other *P. hybrida* anthocyanidin biosynthetic enzymes, the pattern of F3′,5′H expression in developing flowers is unusual. Highest F3′,5′H expression was observed in the developmental stages just prior to anthesis.

It is interesting that the expression of the *P. hybrida* F3′,5′H differs from the expression of the NADPH-Cyt P450 reductase (Menting, 1992; Menting et al., 1994) because the reductase is essential for the activity of this hydroxylase. F3′,5′H is not expressed in nascent, open flower buds, but NADPH-Cyt *c* (Cyt P450) reductase is expressed at similar levels in stage 5 and stage 8 (mature) flowers. In addition to our biochemical data, the two genes corresponding to the *Hf1* and *Hf2* genes were cloned recently from *P. hybrida*, and northern analysis has shown a strong correlation among genotype, mRNA, and activity in developing flowers (Holton et al., 1993). It is probable that specific monooxygenase activities in *P. hybrida* are dependent on the expression of individual Cyts P450. In *P. hybrida* flowers, expression of the flavanone 3β-hydroxylase activity (Froemel et al., 1985) best matches the pattern of F3′,5′H expression, whereas the 3′-hydroxylase in red-flowered mutants (Stotz et al., 1985; J.G.T. Menting, unpublished data) is active in the early developmental stages. In fact, V23 stage 1 flower buds converted all of the naringenin to eriodictyol but none to 5,7,3′,4′,5′-pentahydroxyflavanone (results were identical to those of Old Glory Blue flowers in Fig. 7). This conversion to eriodictyol must be due to F3′H activity in the early developmental stages. Dihydroflavonol oxidase (Forkmann et al., 1986) is also most active in the early flower bud stages. Developmental expression of other anthocyanidin biosynthetic enzymes in flowers of other species also differ from *P. hybrida* F3′,5′H. The flavanone 3β-hydroxylase and F3′H of *Mattiola incana* are expressed in open flowers (Forkmann et al., 1980), as is the F3′,5′H of a commercial blue Dutch iris cultivar (*Iris xiphium* or *Iris tingitana*; J.G.T. Menting, unpublished data) and that of *V. hybrida* (Stotz and Forkmann, 1982). Evidently, the biosynthesis of anthocyanidins is completed prior to *P. hybrida* flower anthesis. Since anthesis is rapid, with partial opening of some stage 5 buds occurring in the interval between harvest and assay, F3′,5′H expression must be tightly regulated.

Detergent solubilization of active F3′,5′H from the microsomal fraction was unsuccessful. Evidently, the F3′,5′H was adversely affected by detergents even at levels that were too low to be useful for solubilization. Madyastha et al. (1976) discovered that microsomal membrane-associated monoterpene hydroxylase isolated from etiolated *Catharanthus roseus* (*Vinca rosea*) seedlings was inhibited at a detergent-to-protein ratio of about 0.4:1 (w/w), corresponding to the detergent-to-protein ratio at which F3′,5′H inhibition was evident in *P. hybrida* microsomes. Since the *P. hybrida* F3′,5′H could not be solubilized and remain active even though the procedure used here was similar, the F3′,5′H must be extremely unstable when removed from its native lipid environment. Hasson and West (1976) found that solubilization of microsomal kaurene oxidase with deoxycholate or Triton X-100 inhibited enzyme activity. Some activity was restored by removal of deoxycholate but not by removal of Triton X-100. Therefore, the type of detergent used for solubilization of Cyts P450 is critical for preservation of activity. Where Cyts P450 have been successfully purified, specific detergents were required for their solubilization, but recoveries of the purified enzymes were always quite poor. The best recovery of a Cyt P450 was obtained by Petersen and Seitz (1988) and was just 27%. Other recoveries were 15% for *Helianthus tuberosus* cinnamic acid 4-hydroxylase (Gabriac et al., 1985), although in a second paper it was 2% (Gabriac et al., 1991); 9% for an avocado demethylase (O'Keefe and Leto, 1989); 3.8% for soybean 3,9-dihydroxypterocarpan 6a-hydroxylase (Kochs and Grisebach, 1989), which was later reported as 0.16% (Kochs et al., 1992); and 0.2% for a *Berberis stolonifera* Cyt P450-dependent oxidase (Stadler and Zenk, 1993).

In *P. hybrida* flowers, a flavonol synthase has been shown to catalyze the oxidation of dihydroflavonols to flavonols (Forkmann et al., 1986). In *P. hybrida* mutants recessive for the 3-hydroxylase (*An3*), there is no evidence for the formation of anthocyanidins (Froemel et al., 1985), so it is reasonable to conclude that oxidation of the C-ring C_2-C_3 bond is carried out by a flavonol synthase and not by a flavone synthase. Flavonol synthases will act on dihydroflavonols and flavone synthases on flavanones. However, we have observed the apparent formation of flavones from flavanones in *P. hybrida* extracts (compounds U2, U4, U5, and U7; Table I). This enzyme activity is similar to that described by Stotz et al. (1984) in microsomal preparations from *V. hybrida* flowers. Stafford (1990b) has defined two classes of flavone synthase: type I is a soluble enzyme and type II is a microsomal enzyme. Although the genetic evidence does not support the existence of such a flavone synthase, this may be the first report of a flavone synthase type II in *P. hybrida* flowers.

The data reported here strongly suggest that F3′,5′H activity consists of a Cyt P450 and a NADPH-Cyt P450 reductase. This type of monooxygenase is distinct from the bacterial and mitochondrial systems and is the only Cyt P450-dependent monooxygenase to have been resolved in plant tissues (Donaldson and Luster, 1991). Phenolases are unlikely to be responsible for the observed flavanone hydroxylation, since these enzymes are usually soluble and they are unlikely to be sensitive to Cyt P450 inhibitors (Butt and Lamb, 1981).

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