The Roles of a Flavone-6-Hydroxylase and 7-O-Demethylation in the Flavone Biosynthetic Network of Sweet Basil*1

Anna Berim and David R. Gang

From the Institute of Biological Chemistry Washington State University, Pullman, Washington 99164-6340

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Lipophilic flavonoids found in the Lamiaceae exhibit unusual 6- and 8-hydroxylations whose enzymatic basis is unknown. We show that crude protein extracts from peltate trichomes of sweet basil (Ocimum basilicum L.) cultivars readily hydroxylate position 6 of 7-O-methylated apigenin but not apigenin itself. The responsible protein was identified as a P450 monooxygenase that prefer flavones from the CYP82 family, a family not previously reported to be involved in flavonoid metabolism. This enzyme prefers flavones but also accepts flavanones in vitro and requires a 5-hydroxy in addition to a 7-methoxyl residue on the substrate. A peppermint (Mentha × piperita L.) homolog displayed identical substrate requirements, suggesting that early 7-O-methylation of flavones might be common in the Lamiaceae. This hypothesis is further substantiated by the pioneering discovery of 2-oxoglutarate-dependent flavone demethylase activity in basil, which explains the accumulation of 7-O-demethylated flavone nevadensin.

Flavonoids are a class of physiologically and ecologically essential compounds found ubiquitously in higher plants (1). Their structural variation, reflected by more than 9,000 compounds reported to date, is achieved through “decorative” steps, such as glycosylations, acylations, prenylations, methylations, hydroxylations, and combinations of the above (2). Because chemodiversity correlates with a diversity of function, specific strategies and mechanisms of chemical structural modification are of great interest (3, 4). Numerous bioactivities have been reported for various flavonoids, turning them into attractive targets for bioengineering experiments (5, 6).

Lipophilic methylated flavonoids occur frequently in several eudicot families, such as the Lamiaceae, Asteraeaceae, and Rutaceae, but are also found in representatives of other plant families, including ferns and monocots (7, 8). Hydroxylations at positions 6 and 8 of ring A and multiple regiospecific O-methylations appear to be common modifications underlying the high lipophilicity. Notably, identical highly decorated compounds are known to be accumulated by quite distantly related species, prompting the question of whether the respective biosynthetic mechanisms in different species share any similarity.

Sweet basil (Ocimum basilicum L.) is a popular culinary herb from the mint family. The production of many characteristic flavor- and fragrance-defining compounds is restricted to peltate glandular trichomes on its aerial surfaces (9, 10). Construction of EST libraries selectively using this specialized cell type (10) facilitated fruitful studies of its metabolic and physiological processes. In addition to volatiles, basil produces specific lipophilic flavones, such as nevadensin (NEV), salvigenin (SALV), and gardenin B (GARD B) (Fig. 1) as characteristic compounds (11). We recently found that flavone biosynthesis and accumulation are also largely peltate trichome-specific (12). Within that investigation, which focused on methylation processes and pathways leading to accumulation of SALV, we identified scutellarein-7-methyl ether (SCU7Me) (Fig. 1) as a central intermediate in the metabolic network, making flavone 6-hydroxylation, a step necessary for its formation, our next object of interest. In addition, characterization of specific O-methyltransferases (OMTs) provided clues regarding biochemically favorable routes, revealing that 7-O-methylation preferentially occurred on the 6-unsubstituted substrate apigenin (API) rather than scutellarein (SCU). The combined data from the initial OMT work pointed to two candidate substrates for 6-hydroxylation in basil, API, and its 7-O-methylated derivative genkwanin (GENK) (see Fig. 1 for all structures).

Flavonoid-6-hydroxylases (F6Hs) have been insufficiently studied, in part due to their apparently restricted occurrence (13). Interestingly, the only two publications reporting molecular data for F6Hs ascribed this activity to two different enzyme

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1 This article contains supplemental Figs. 1–5.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) JKS24270, JX162212, JX162214, and JX162213.

To whom correspondence should be addressed. Tel: 509-335-0550; Fax: 509-335-7643; E-mail: gangd@wsu.edu.

2 The abbreviations used are: EST, expressed sequence tag; NEV, nevadensin; GARD B, gardenin B; SALV, salvigenin; SCU, scutellarein; SCU7Me, scutellarein-7-methyl ether; GENK, genkwanin; API, apigenin; NAR, naringenin; SAK, sakuranetin; C7Me, carthamidin-7-methyl ether; CdM, carthamidin-7,4′-dimethyl ether; AdM, apigenin-7,4′-dimethyl ether; NDM, naringenin-7,4′-dimethyl ether; LUT, luteolin; LAD, ladanein; APItriMe, apigenin-5,7,4′-trimethyl ether; ODD, 2-oxoglutarate dependent dioxygenase; OMT, O-methyltransferase; CPR, cytochrome P450 reductase; FNS, flavone synthase; F6H, flavonoid 6-hydroxylase; RACE, rapid amplification of cDNA ends; F6H-OMT, contig, group of overlapping clones.
classes. A cytochrome P450-dependent monoxygenase from soybean was most active with liquiritigenin, a 7,4′-dihydroxyflavanone, and is likely to contribute to accumulation of 6-substituted isoflavonoids (14). In contrast, a 2-oxoglutarate-dependent dioxygenase (ODD) from *Chrysosplenium americanum* preferred methylated flavonols as substrates and is involved in biosynthesis of polymethoxylated flavonols (15, 16) that are similar to methoxylated flavones in sweet basil (11). In addition, P450-dependent 6-hydroxylase activity was detected in protein extracts from *Tagetes* species, yet the molecular basis was not elucidated (17). These few examples did not allow predictions regarding the nature of this reaction in basil.

In this investigation, we found that flavonoid 6-hydroxylase in two Lamiaceae species, sweet basil and peppermint (*Mentha × piperita*), was catalyzed by monoxygenases belonging to the CYP82D family that used 7-O-methylated apigenin, but not apigenin itself, as substrate. Moreover, our results indicate that 7-O-unmethylated flavone nevadensin found in basil is produced via late 7-O-demethylation of gardenin B by an oxoglutarate-dependent dioxygenase, thereby supporting the conclusion that the pathway to 6-substituted flavones via a 7-O-methylated precursor is the only one existing in basil. These findings reveal an unprecedented loop in lipophilic flavone metabolism, further the delineation of the flavone metabolic network in basil, and add two important activities to the growing catalytic toolbox of flavonoid bioengineering.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—NADPH was obtained from EMD Biosciences, and API, naringenin (NAR), and luteolin (LUT) were from Sigma. Sakuranetin (SAK) and GENK were purchased from Extrasynthese. Non-natural flavones (apigenin-5,7,4′-dimethyl ether (APItriMe) and unnatural substrates in Tables 2 and 3) and SCU were from Indofine, and GARD B, naringenin-7,4′-dimethyl ether (NdM), and quercetin-7-methyl ether were from TransMIT (Marburg, Germany). SCU7Me, luteolin-7-methyl ether, carthamadin-7-methyl ether (C7Me), carthama-

**Plant Material**—Basil seeds were germinated in vermiculite and individually repotted into SunG ro mix. Plants were grown in growth chambers at 28 °C (24 h) under a 16-/8-h photoperiod. Light intensity of ~200 μmol m⁻² s⁻¹ was supplied by incandescent and fluorescent lamps.

**Cloning, Expression, and Yeast Microsome Preparation**—Standard molecular biological procedures were used for all experiments described. For sequences of primers used for genome walking (CYP82D33), 5′-RACE-PCR (CYP93B23), and full-length ORFs of all studied genes, see supplemental Table S1. Amplified PCR products for expression were first subcloned into pCR2.1 TOPO vector (Invitrogen), excised using appropriate restriction enzymes, and ligated into pESC-HIS (CYP93B23), pESC-URA (F6Hs), or pESC-TRP vector (cytochrome P450 reductase (CPR)) (all pESC vectors were from Stratagene). All expression plasmids were sequenced to verify sequence accuracy. INVSc1 yeast cells (Invitrogen) were sequentially transformed with vectors harboring CPR and P450. Gene expression and microsome preparations were carried out according to Pompon *et al.* (18) with the following minor changes. Disruption buffer was 100 mM Tris/HCl, pH 7.5, 1 mM DTT, and microsome storage buffer was 50 mM Tris, 0.5 mM EDTA, 10% glycerol. Protein content was determined as for trichome protein preparations. Carbon monoxide difference spectra were recorded according to Omura and Sato (19), using a Lambda 35 UV spectrophotometer (PerkinElmer Life Sciences).

**Biochemical Characterization of Recombinant CYP450s**—Linearity of reactions with respect to time and protein amount...
Flavone-6-Hydroxylase and 7-Demethylase in Basil

Analyses of Reaction Products—An LC-MS" system (LCQ Advantage system with Surveyor HPLC and photodiode array detector, Thermo) was used to evaluate F6H enzyme assays. Separation was carried out on a Discovery® HS C18 column (150 × 2.1 mm, 3 μm; Supelco) kept at 40 °C using a linear gradient of acetonitrile (B) and 5 mM ammonium formate, 0.1% formic acid buffer (A), 200 μl min⁻¹, that ran as follows: 0 min, 5% B; 2 min, 5% B; 35 min, 65% B; 40 min, 100% B; 43 min, 100% B; 47 min, 5% B; column equilibration for 7 min. For kinetic analyses, the gradient was shortened: 0 min, 5% B; 2 min, 5% B; 8 min, 43% B; 13 min, 100% B; 18 min, 100% B; 20 min, 5% B; 27 min, 5% B. Products of CYP93B23 assays were separated using methanol (B) and buffer as above (A). API and NAR were separated under isocratic conditions of 50% B and 200 μl min⁻¹ flow rate. The gradient used for assays with CdM, C7Me, SAK, and NdM as substrates was as follows: 0 min, 45% B; 12 min, 80% B; 22 min, 100% B; 27 min, 100% B; 30 min, 45% B; 37 min, 45% B. Positive mode ESI was applied for efficient ionization of (poly)methylated flavones. API and NAR were analyzed using negative ionization mode. Tuning was carried out with API for all analyses. MS settings were as follows: capillary at 275 °C and 26 V, source 5.54 kV, sheath/sweep gas flow at 33/20. For collision-induced dissociation spectra, fragmentation was achieved using 35–55% normalized collision energy, and spectra were collected using either data-dependent or data-independent mode.

Quantitative PCR—CYP82D33 and flavonoid 7-OMT transcripts were monitored using the relative quantification method (21). Assay, reagents, instrumentation, primers for the reference gene (elongation factor-1) and 7-OMT, and general considerations were as described previously (12). CYP82D33 was detected using 5'-TGATTCGTTGTTAAGGTG-3' (forward) and 5'-AGTTGTACGAACACACAGC-3' (reverse) as primers. For scaling, the relative expression of all genes in the sixth leaf pair of EMX-1 plants was set as 1. Five biological replicates of each leaf pair of both basil lines were analyzed.

RESULTS

Demonstration of 6-Hydroxylase Activity in Basil Trichomes—Based on our previous results described in the Introduction, we considered two flavones as candidate substrates for 6-hydroxylation in basil, API and GENK. Both of the most likely candidate oxygenase classes, 2-oxoglutarate-dependent dioxygenases and P450-dependent monoxygenases, are well represented in our EST databases (10), precluding preference for either of them. Therefore, crude protein extracts from isolated trichomes were incubated with each of the candidate substrates in the presence of the respective required cofactors.

When API was offered as substrate, we could detect very low 3'-hydroxylase activity and traces of 6-hydroxylase activity in a P450 assay and no conversions in ODD-based assays (Fig. 2, traces 1 and 2, and supplemental Fig. S1). The proportion of 3'-substituted flavones in basil is less than 6% (11); it is therefore not surprising that formation of LUT (3'-hydroxyl-API) from API was negligible. With GENK, no product resulted from the ODD test, but a more hydrophilic product easily identified as SCU7Me, based on its UV and mass spectra as well as retention time, was formed in the presence of NADPH (Fig. 2, traces 1 and 2), suggesting that GENK was a substrate for NADPH-dependent flavonoid 7-demethylase in basil.
The 6-hydroxylase activity with GENK was strongly reduced by cytochrome c and ketoconazole, typical P450 inhibitors, and localized to the microsomal fraction of the trichome protein extract (Table 1), further confirming that the 6-hydroxylation was catalyzed by a P450-dependent enzyme in basil.

The tests with crude trichome protein extract showed that GENK serves as a preferred and probably natural substrate for the flavonoid 6-hydroxylase in basil. From the very low yet detectable 6-hydroxylase activity with API, we were unable to absolutely rule out the possibility that some 6-hydroxylation occurs on this substrate. It appeared to be at least a much slower reaction, or it required assay conditions different from those we have offered.

Identification of Candidate P450 Genes from Basil and Biochemical Characterization of Recombinant CYP82D33—To identify candidate F6H genes, we analyzed the basil trichome EST database from four basil lines (10). One of the candidates encoded a protein belonging to the family CYP71 and was considered promising because the flavonoid 6-hydroxylase from soybean also belongs to this family (14). However, all of the encoding ESTs originated from just one of the four basil lines (MC), which contradicts flavone accumulation data in different lines (12). The recombinant protein was nevertheless tested but displayed no detectable activity with any of the offered substrates, including GENK, despite a visible peak in the CO difference spectrum (data not shown).

The second auspicious candidate contig comprised ESTs from all four basil lines, and the total EST count (54 ESTs, 0.23% of total) was highest among contigs encoding P450 proteins of unknown function. The 5’-end encoding the few missing N-terminal amino acids was retrieved using genome walking, and the encoded protein was classified as CYP82D33. Members of this CYP450 family that have been functionally characterized to date are not known to be involved in flavonoid metabolism (Fig. 3). The resulting open reading frame was cloned and expressed in yeast (supplemental Fig. S2), and the microsomal protein was analyzed for catalytic activity. To create a favorable electron transfer environment, we isolated the cDNA for CPR from basil and co-expressed it with the candidate genes. Formation of SCU7Me was detected after incubation with GENK and NADPH (supplemental Fig. S3). With API, no activity was detectable under the linear reaction conditions used to compare turnover rates. However, a very small amount of SCU, the 6-hydroxylated API, was formed when an excess of protein was incubated with an excess of this substrate. These findings fully agree with results obtained using native trichome protein as catalyst and suggest that 7-O-methylation is a prerequisite for substrate recognition by CYP82D33 and that the side activity of the same CYP82D33 protein probably accounts for the small amounts of SCU found in assays of crude trichome protein with 5 and 6). The 6-hydroxylase activity with GENK was strongly reduced by cytochrome c and ketoconazole, typical P450 inhibitors, and localized to the microsomal fraction of the trichome protein extract (Table 1), further confirming that the 6-hydroxylation was catalyzed by a P450-dependent enzyme in basil.

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**TABLE 2**

Substrate preferences of CYP82D33 and CYP82D62: Relative turnover rates with potential substrates

| Substrate   | CYP82D33<sup>a</sup> | CYP82D33<sup>b</sup> | CYP82D62<sup>c</sup> |
|-------------|----------------------|----------------------|----------------------|
| GENK        | 100.0 ± 3.1          | 100.0 ± 1.7          | 100.0 ± 2.5          |
| SAK         | 38.3 ± 0.6           | 41.6 ± 2.2           | 89.6 ± 0.8           |
| AdM         | 21.6 ± 0.7           | 39.0 ± 7.0           | 70.3 ± 0.5           |
| NdM         | 22.4 ± 0.2           | 22.4 ± 0.7           | 37.6 ± 0.4           |
| R<sub>1</sub> = H | R<sub>1</sub> = OH | 10.1 ± 0.6         | 117.7 ± 1.8          |
| R<sub>2</sub> = H | R<sub>3</sub> = OH | 6.6 ± 0.2           | 89.1 ± 1.7           |
| API         | ND                   | NT                   | ND                   |
| AdM         | ND                   | NT                   | ND                   |
| NdM         | ND                   | ND                   | ND                   |

<sup>a</sup> All substrates supplied at 5 μM, 100% = 249.9 pkat mg<sup>-1</sup> protein.

<sup>b</sup> All substrates supplied at 2.5 μM, 100% = 206.9 pkat mg<sup>-1</sup> protein.

<sup>c</sup> All substrates supplied at 5 μM, 100% = 1.56 pkat mg<sup>-1</sup> protein.

Because CYP82D33 was able to hydroxylate both flavones and flavanones, and tolerated a methylated 4'-hydroxyl group in the substrate, we tried to define minimal substrate structure requirements by offering various, in part unnatural, flavonoids. All accepted flavonoids possessed a 7-OCH<sub>3</sub> residue, in line with the earlier perception that it was required for substrate recognition (Tables 2 and 3). In addition, a free 5-hydroxyl group has to be present, because neither the 7-OCH<sub>3</sub> flavone nor the 4'-OH-7'-OCH<sub>3</sub> flavone nor APItriMe was hydroxylated. The 5-OH-7'-OCH<sub>3</sub> flavone serves as substrate; therefore, the 4'-OH is not an absolute prerequisite for activity. In addition, the 7-O-methyl ethers of quercetin and luteolin were also 6-hydroxylated, showing that the 3- and 3'-OH groups did not completely preclude CYP82D33 activity (supplemental Fig. S3).

Kinetic parameters were determined for GENK, SAK, and AdM. Saturation curves showed that all three substrates inhibited the reaction at high concentrations (supplemental Fig. S4 and Table 3). Our measurements indicate that the hydroxylation of GENK proceeds with the highest catalytic efficiency. Apparent maximal turnover and affinity constants imply that GENK is the genuine substrate for CYP82D33 and not an *in vitro* artifact. The apparent *K<sub>m</sub>* in the low micromolar range matches well with affinity constants of recently characterized basil flavonoid OMTs (12). Turnover of GENK is additionally favored by the high apparent *K<sub>c</sub>* value (Table 3).

Currently, we cannot estimate whether inhibitory substrate concentrations will accumulate at any time in the catalytically active compartment. Therefore, it remains unclear whether this *in vitro* phenomenon will translate into a physiologically relevant regulatory mechanism. Remarkably, two flavonoid 4'-OMTs were also inhibited by higher concentrations of GENK (12).

Expression of CYP82D33 in Two Basil Lines—Recent analyses of metabolite profiles in age-successive leaf pairs of two basil lines, SD and EMX-1, suggested that the rate of 6-hydroxylation was not a limiting factor, because the amounts of 6-unsubstituted flavonoids were stably low in all analyzed tissues (12). Those results also suggested that the transport processes between the secretory cells and the subcuticular storage cavity.
of the trichomes might be crucial to the accumulation of partially methylated and less decorated flavones. Although the relative abundance of AdM and GENK changed throughout leaf development, it remained under 10% of the total flavone amount (supplemental Fig. S5). Nevertheless, we chose to evaluate the transcript levels of F6H in the same tissue and used the same RNA that had been collected for the OMT analyses to ensure that the gene was indeed expressed at the times required for 6-hydroxylated flavones to be produced.

As shown in Fig. 4, the expression of CYP82D33 follows the same pattern as one of the basil flavonoid 7-OMTs, which was co-monitored for reference. The transcript levels decrease from young to old leaves, following the decreasing trichome density and progressing trichome maturation that occur as the leaves age. This characteristic expression pattern might therefore also indicate trichome-specific expression of CYP82D33, supporting its proposed involvement in a trichome-specific metabolic pathway. Although line SD was found to produce higher amounts of flavones than line EMX-1, the expression of CYP82D33 was comparable in both lines. This was not surprising, because our recent work already suggested that total flavone amounts are likely to be determined by early flavonoid pathway genes (12).

Assessment of Activities of Basil Flavone Synthase, CYP93B23—

Substrate screening revealed that flavanones served as substrates for the basil flavonoid 6-hydroxylase, CYP82D33. From the relative activities of this enzyme with different compounds, it cannot be excluded from consideration that 6-hydroxylation partially occurs at the flavanone level (Tables 2 and 3). Flavanones do not contribute significantly to the flavonoid profile found in basil. Therefore, if such early 6-hydroxylation takes place, flavone synthase (FNS), the enzyme introducing the C2=C3 double bond and converting flavanones into flavones, should display similarly broad substrate acceptance. We chose to resolve this ambiguity of reaction order by surveying the activities of the basil flavone synthase. The flavone synthase reaction is catalyzed by P450 enzymes of the subfamily CYP93B in most plant families, including the Lamiales (22). Representatives of this subfamily studied to date were mostly analyzed with a limited range of substrates that were deemed relevant, such as naringenin and eriodictyol, as well as liquiritigenin with legume proteins (23–25). Upon detecting high activity of an F6H from soybean with liquiritigenin, a flavanone, the isoflavone synthase (CYP93C1v2) was tested and found to be capable of accepting 6-substituted flavanones as substrates (14). The authors did not assess substrate preferences of the isoflavone synthase but could hypothesize that the 6-hydroxylation occurs prior to aryl ring migration in soybean.

### TABLE 3

| Substrate | \( K_m \) \( \mu M \) | \( k_{cat} \) \( s^{-1} \) | \( k_{cat}/K_m \) \( \mu M^{-1} s^{-1} \) | \( K_i \) \( \mu M \) |
|-----------|----------------|----------------|----------------|----------------|
| GENK      | 0.20 ± 0.01    | 4.97 ± 0.15   | 24.60          | 44.72 ± 3.93   |
| SAK       | 1.29 ± 0.36    | 3.25 ± 0.48   | 2.52           | 6.32 ± 1.87    |
| AdM       | 0.15 ± 0.01    | 1.64 ± 0.04   | 10.88          | 31.38 ± 6.15   |

CYP93B genes encoding putative FNSs are represented by several contigs in our EST database. The encoded proteins shared 95% identity on available sequence fragments. This high protein conservation level is likely to translate into very strong biochemical similarity, although final conclusions should await the characterization of all basil FNS isozymes. The missing 5’-end of the longest contig was obtained by RACE-PCR, and the resulting open reading frame was cloned into a yeast expression vector and co-expressed with basil CPR. The protein designated CYP93B23 shares 81% identity with an FNS from Perilla frutescens, another mint species (25) (Fig. 3). The recombinant protein converted NAR directly into API, and no 2-hydroxylated flavanone intermediates were detectable. This behavior is typical for non-legume CYP93B proteins (23). The relative turnover with higher substituted flavanones of interest was low compared with that with NAR. Under linear reaction conditions, flavone formation was detectable both with SAK and NdM (Fig. 5A, traces 1–3), but not with 6-hydroxylated derivatives C7Me and CdM (Fig. 1). Flavone formation with the latter two flavanones was not reliably detectable even after longer incubation with an excess of catalyst. Taken together with the strong preference of flavonoid 7-OMTs for apigenin as compared with naringenin (12) and assuming that none of the non-analyzed FNS isozymes is highly active with C7Me and CdM, this suggests that the major and probably only biosynthetic pathway to SCU7Me in basil appears to lead via API and GENK rather than SAK and CdM.

7-Demethylase Activity Brings Nevadensin into the Flavone Metabolic Network in Basil Trichomes—The above results indicate that 7-O-methylation is a prerequisite for the 6-hydroxylation of flavones in basil. This scenario does not offer an explanation for the accumulation of NEV, one of the major basil flavones with a free 7-OH residue. Neither previous nor current results allow us to absolutely exclude 6-hydroxylation of apigenin as a minor alternative route. However, such a route would probably not lead to the high abundance of NEV observed; nor is there convincing evidence for the existence of such a route. In addition, we have previously shown that NEV is very unlikely to serve as a precursor for GARD B (12), because it is a poor substrate for flavone 7-OMTs. As a consequence, a late removal of the 7-O-methyl group would not be reversed by subsequent remethylation and would present a viable mechanism for accumulation of NEV at high levels.
Demethylations are not known to be common in plant specialized metabolism (26). One of the few currently known instances is the N-demethylation of nicotine by CYP450-dependent monoxygenases of the family CYP82E (27). In addition, O-demethylating side activity with the alkaloid codamine was tentatively detected for CYP80G2, primarily a C-C coupling enzyme from *Coptis japonica* (28). Very recently, two *C.* *japonica*—to assess whether the 6-hydroxylation mechanism identified in basil is common in the mint family, we searched peppermint ESTs (30) for a homolog of CYP82D33 and found a 3'-terminal fragment sharing ~70% identity at the amino acid level. The corresponding 5' sequence was obtained from our transcriptome sequencing of peppermint. The full-length cDNA was amplified by RT-PCR, and the product encodes a protein with 73% identity to CYP82D33 from basil (Fig. 3). It was designated CYP82D62. The open reading frame was cloned into a yeast expression vector, and the gene was expressed analogously to CYP82D33, including co-expression with basil CPR. Microsomal protein catalyzed the 6-hydroxylation of GENK and showed the same substrate requirements (7-methoxy, 5-hydroxy residues) as its homolog from basil. A comparison of relative turnover rates with the same set of substrates as used for the basil F6H indicated that the peppermint CYP82D62 does not show the strong preference for GENK that the basil enzyme displayed (Tables 2 and 3). We did not determine kinetic parameters for CYP82D62, but preliminary experiments indicated that it is also inhibited by GENK at high concentrations. The specific activity relative to total protein content was much lower than that of basil F6H (Tables 2 and 3). It was impossible to correlate the turnover to P450 content because the CO difference spectra did not return reproducible results. It was therefore unclear whether the peppermint F6H was not well expressed or less active and whether this was due to our expression system that employed basil CPR as an electron transfer partner for the P450 enzyme from peppermint.

As for basil, we conducted enzyme assays with crude trichome protein from peppermint. These assays revealed hydroxylase activities similar to those found in basil protein extracts, with GENK being readily 6-hydroxylated by a P450-dependent enzyme and API yielding LUT and small amounts of SCU. No products were formed in dioxygenase assays with either substrate (Fig. 2, traces 3, 4, 7, and 8). Remarkably, a second product was formed from GENK (Fig. 2, trace 7). Its properties, such as the UV spectrum with a bathochromic shift of peak I maximum toward 345 nm, its mass (m/z 317), and its retention time, which was shorter than that of SCU7Me, indicated that it was likely to be the 3’-hydroxylated SCU7Me (supplemental Fig. S1). Additionally, its fragmentation pattern after loss of methyl radical was found to be identical to that of nepetin, its positional isomer (6-methoxyisoucoumarin; Fig. 2 and supplemental Fig. S1). Biochemical characterization of the responsible flavonoid 3’-hydroxylase, a CYP75B, is necessary to find out whether it has high activity with GENK or with SCU7Me, the two possible substrates. We did not detect the formation of this second product in basil, which accumulates much lower relative amounts of 3’-substituted flavonoids. At least one of the basil CYP75B isozymes found in our EST database does not seem to be active with any flavonoids except API (not shown).
In ODD assays with GARD B and peppermint trichome protein extracts, no 7-demethylation was detected. This result was expected, because peppermint does not accumulate NEV. It also suggests that the 7-\textit{O}-demethylation observed in basil is not catalyzed by a ubiquitous nonspecific enzyme. Peppermint accumulates pebrellin, a GARD B derivative possessing a hydroxyl instead of a methoxyl group at position 6. The abundance of pebrellin relative to total flavone content was shown to be low in the youngest leaves, presumably in favor of GARD B (31). Variation in relative abundances of these two compounds was proposed to be due to varying expression and activity of a flavone 6-OMT through leaf/trichome development. Our discovery of 7-\textit{O}-demethylation in basil first suggested that a regioselective 6-\textit{O}-demethylation could occur in peppermint. However, no such 6-\textit{O}-demethylation was detectable under the tested conditions, thus supporting the earlier hypothesis that an OMT is probably responsible for determining the level of pebrellin relative to GARD B.

**DISCUSSION**

Elucidation of biosynthetic pathways and mechanistic details of plant specialized metabolite accumulation is essential for further ecological, evolutionary, and metabolic engineering studies (32, 33). In the biosynthesis of polymethylated flavonoids, the late hydroxylations at positions 6 and 8 provide a basis for alternative substitution patterns and thus for the expansion of chemodiversity. This report describes cytochrome P450 monooxygenases that catalyze the 6-hydroxylation of flavones in basil and peppermint. The two proteins share high identity levels (73%), which are higher than those found for peppermint and basil flavonoid \textit{O}-methyltransferases (12), and show very similar substrate requirements. The few members of the CYP82 family (sharing 40–55% identity with CYP82D proteins; Fig. 3) that have been studied to date are not known to be involved in flavonoid metabolism but are involved in a variety of metabolic pathways. CYP82A2 from soybean yielded a type I binding spectrum with NAR and eriodictyol but did not convert either into any product (14). CYP82E4 and CYP82E5 from \textit{Nicotiana tabacum} were identified as nicotine \textit{N}-demethylases (27). CYP82N2v2 acts as protopine 6-hydroxylase in sanguinarine biosynthesis in \textit{Eschscholzia californica} (34). CYP82G1 is the homoterpene synthase in \textit{Arabidopsis} (35). An in planta biocatalysis screen in \textit{Arabidopsis} revealed that CYP82C2 and CYP82C4 are capable of 5-hydroxylating 8-hydroxypsoralen, although this might not be their natural function (36). Such functional divergence in CYP families is not unusual. For example, the family CYP71 encompasses proteins active with monoterpene (37) and sesquiterpene (38, 39) as well as indole alkaloids (40), coumarins (41), and flavonoids (14) (Fig. 3). Further functional analysis of members of the CYP82 family will reveal the degree of its divergence and the occurrence of other flavonoid-modifying enzymes within it.

The identity of the F6H in these species as a P450-dependent enzymeplaces it far apart from the F6H from \textit{C. americanum}, an oxoglutarate-dependent dioxygenase that is, however, also involved in the metabolism of polymethylated flavonoids and prefers methylated flavonols as substrates, with 7-\textit{O}-methylation recognized as a preeminently important structural element (15, 16). At the same time, the F6Hs from basil and peppermint share only low protein identity of \textless{}31% with the F6H from soybean (14), which is placed in a different P450 family (CYP71;
**Flavone-6-Hydroxylase and 7-Demethylase in Basil**

Fig. 3), indicating that their similar functions evolved independently. It is worth mentioning that the substrate preferences of CYP71D-F6H are very different from those of CYP82D-F6Hs; for example, 7-O-methylation abolishes conversion by the former enzyme, and a 5-OH group is not required for activity. Thus, the three distinct types of F6Hs studied to date at the molecular level present an interesting case of convergent evolution (42). Because the respective compounds produced by both saxifrage and soybean (43, 44) belong to different flavonoid subclasses than the basil and mint flavones, the independent origins of analogous catalytic capacities are not quite unexpected. It remains intriguing what type of catalyst mediates the 6-hydroxylation of the same flavonoid subclass, the flavones, in distantly related species and genera accumulating identical compounds, such as, for example, *Tamarix*, *Citrus*, *Ononis*, and others (7, 45, 46).

So far, our BLAST searches of *Citrus sinensis* and *Citrus clementina* genomes and EST collections, as well as *Helianthus annuus* ESTs (using Phytozome and NCBI GenBank™ online tools) only yielded protein sequences with less than 55% identity to CYP82D33, which precludes function prediction.

The finding that the P450-dependent flavone 6-hydroxylation in basil occurs via 7-O-methylated substrates matches well with our previous results (12). Based on these two extensive studies, we can complement our proposed pathway leading to NEV and expand it to reach out to NEV (Fig. 1). Pivotal roles in directing metabolite flux within this network are attributed to 7-OMT and flavone synthase. The properties of basil flavonoid 7-OMTs strongly suggest that API and not NAR is the early intermediate undergoing 7-O-methylation (12). Here, we show that although a flavone synthase (CYP93B23) is capable of converting SAK into GENK at low rates, the enzyme strongly prefers NAR as substrate (Fig. 5B), further validating the *in vivo* route via API. Because no flavone synthase activity was detected with the 6-substituted flavanones, C7Me and CdM, these compounds would not be converted into major accumulated flavones if formed by side activities of upstream enzymes. Their occurrence has not been reported for basil to date, suggesting that such parallel steps that seem possible *in vitro* are not physiologically significant. The detection of GARD B 7-O-demethylation lends critical support to the proposed biosynthetic pathway, because it allows for the accumulation of NEV in a pathway operating entirely on the level of 7-O-methylated intermediates. A detailed biochemical characterization of the underlying protein is necessary to advance our understanding of the intricate metabolic loop it facilitates and will help to determine the biosynthetic origin and position in the metabolic network of pilosin, a 7,8-dihydroxylated flavone occurring in basil (47). So far, we could not investigate the substrate preferences of the 7-demethylase due to lack of appropriate substrate (8-hydroxylated SALV). Furthermore, the identification of the molecular data for the flavonoid 7-O-demethylase in basil will be very instructive, because it will reveal whether it is more closely related to flavonoid-pathway dioxygenases or to *Papaver* alkaloid demethylases. The ODDs involved in morphine biosynthesis share ~55% identity with ODDs involved in the flavonoid pathway (flavonol synthase, flavonoid 3-hydroxylase, etc.). The basil EST database contains several highly expressed putative ODDs that also share ~50% identity with *Papaver* demethylases and are ~60% identical with some of the flavonoid-related ODDs. Remarkably, their identity with F6H from *Chrysosplenium* (16), the only ODD known to act upon the A-ring of flavonoids, is only about 30–35%. However, heterologously expressed proteins encoded by the three most promising genes did not catalyze the 7-demethylation of GARD B (not shown). We will further pursue the isolation of the responsible gene and protein in order to fill in this missing puzzle piece in the flavone biosynthetic network.

These unusual steps found in the flavone metabolic network in basil raise questions of whether similar processes occur in other species, which are both mechanistically and evolutionarily interesting. Because it appears that flavonoid 7-O-methylation is a prerequisite for 6-hydroxylation in these two mint species, the first question is whether this route is common in the Lamiaceae and whether it is shared by non-mints producing identical or very similar flavones, such as *Ablus* (48), *Rubus* (49), and *Eupatorium* (50), all of which are reported to accumulate SALV. In the Lamiaceae, phytochemical data for some groups, such as *Thymus* (51, 52), *Origanum* (53), and *Orthosiphon* (54), indicate that they only accumulate 6-substituted flavonoids possessing 7-O-methylated hydroxyl moieties and therefore could well follow the same pattern. Likewise, the discovery of the flavonoid 7-O-demethylation prompts an investigation into the frequency of demethylation in flavonoid metabolism. On the one hand, the occurrence or even prevalence of regiospecifically unmethylated compounds within the mint family (4′-OH in *Thymus*, 6-OH in *Mentha*) could result either from lack of OMT activity or from the corresponding demethylation, although we did not detect the latter in peppermint under the conditions tested and with GARD B as substrate (Fig. 6). On the other hand, it is even more interesting to consider whether a 7-O-demethylation is involved in the production of NEV in other species, such as *H. annuus* (Asteraceae) (55), *Limnophila aromatica* (Plantaginaceae) (56), *Lysionotus pauciflorus* (Gesneriaceae) (57), and *Biebersteinia orphanidis* (family unassigned) (58). To date, there are no data regarding the formation of lipophilic flavones in any of the above species. Our findings, together with previous results, provide a solid starting point for the elucidation of analogous processes in flavone metabolic networks across the plant kingdom.

Finally, the identification of a very efficient F6H adds an important catalytic activity for use in biotechnological applications. Because of their potential benefits to plants’ fitness and reported pharmacological activities, there is considerable interest in engineering production of flavonoids in both plants and microorganisms (5, 6). Although the spectrum of compounds accessible through use of F6H is limited by its substrate specificity, many of those, such as SALV, LAD, or SCU7Me, are scarce and valuable. Tests with further potential precursors and in combination with other flavonoid-modifying enzymes, including recently isolated regiospecific flavonoid OMTs from basil (12), will be necessary to fully investigate and exploit the potential of basil F6H for the production of both natural and non-natural, novel compounds.
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