Identification of the molecular basis for the functional difference between flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase

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Abstract Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3',5'H) are cytochrome P450 enzymes and determine the B-ring hydroxylation pattern of flavonoids by introducing hydroxyl groups at the 3'- or the 3' and 5'-position, respectively. Sequence identity between F3'H and F3',5'H is generally low since their divergence took place early in the evolution of higher plants. However, in the Asteraceae family, the family-specific low sequence identity is substantially higher. We used this phenomenon for alignment studies, in order to identify regions which could be involved in determining substrate specificity and functionality. Subsequent construction and expression of chimeric enzymes indicated that substrate specificity of F3'H and F3',5'H is determined near the N-terminal end and the functional difference between these two enzymes near the C-terminal end. The impact on function of individual amino acids located in substrate recognition site 6 (SRS6) was further tested by site-directed mutagenesis. Most interestingly, a conservative Thr to Ser exchange at position 487 conferred additional 5'-hydroxylase activity to recombinant Gerbera hybrida F3'H, whereas the reverse substitution transformed recombinant Osteospermum hybrid F3',5'H into an F3'H with low remaining 5'-hydroxylation activity. Since the physicochemical properties of Thr and Ser are highly similar, the difference in size appears to be the main factor contributing to functional difference. The results further suggest that relatively few amino acids exchanged were required for the evolutionary extension of 3'- to 3',5'-hydroxylation activity.

Keywords: Cytochrome P450; Flavonoid 3'-hydroxylase; Flavonoid 3', 5'-hydroxylase; Substrate recognition site; Chimeric gene; Site-directed mutagenesis

1. Introduction

Flavonoids are a ubiquitous group of plant secondary metabolites [1,2]. Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3',5'H) play a key role in flavonoid biosynthesis since they determine the B-ring hydroxylation pattern by introducing hydroxyl groups at the 3'- or the 3'- and 5'-position, respectively (Fig. 1). The flavanone naringenin and the dihydroflavonol dihydrokaempferol are regarded as main substrates of F3'H and F3',5'H and are converted into the 3',4'-hydroxylated eriodictyol and dihydroquercetin, respectively, by F3'H and into the 3',4',5'-hydroxylated penta-hydroxylavanone and dihydroxyflavone respectively, by F3',5'H (Fig. 1).

F3'H and F3',5'H belong to the subfamilies CYP75B and CYP75A, respectively, of the superfamily of cytochrome P450-dependent monoxygenases (P450). P450 enzymes are highly diverse and versatile and occur abundantly in all organisms, with the highest proliferation in plants [3–6]. They require molecular oxygen and NADPH as cofactors and normally are membrane-bound in eukaryotes. Though primary sequences are highly divergent, elements of secondary structure and tertiary structure are remarkably conserved [7]. Gotoh described six “substrate recognition sites” (SRSs) supposed to be involved in substrate contacting and orientation towards the catalytic centre [8].

More than 80 sequences of F3'Hs and F3',5'Hs are so far available in public databases but no information concerning the molecular basis of the functional difference. Since the divergence of F3'H and F3',5'H occurred early in the evolution of higher plants, amino acid sequences of F3'Hs and F3',5'Hs do not show more than 50% identity [9]. This high degree of difference hampers the search for putatively decisive regions. However, in members of the Asteraceae family, a type of F3',5'H was shown to have arisen independently of the other F3',5'Hs from an F3'H precursor [9]. Therefore, this Asteraceae-specific F3',5'H, represented by so far three isolated sequences, has a significantly higher similarity to F3'Hs which amounts to around 70%. In this report, this fact was used to identify regions putatively involved in the functional difference by multiple alignment studies. Subsequently, construction of chimeric cDNAs and site-directed mutagenesis were employed to assess the impact of regions and single amino acids on substrate specificity and function of F3'H and F3',5'H.

2. Material and methods

2.1. Multiple alignments

Amino acid sequences were aligned using MultAlin [10]. Each 15 sequences of F3'Hs and F3',5'Hs and 3 so far isolated F3',5'H sequences of the Asteraceae family were used and are listed in the following including GenBank Accession. F3'Hs: Arabidopsis thaliana (AF271651), Callistephus chinensis (AF313488), Gentiana triflora (AB193313), Gerbera hybrida (ABA64468), Glycine max (AF499731), 0041-537X/32.00 © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2007.06.045
Hieracium pilosella (DQ319866), Ipomoea nil (AB113264), Lobelia erinus (BAF49324), Matthiola incana (AF313491), Orzya sativa (AC021892), Osteospermum hybrid (ABB29899), Pelargonium x hortorum (AF315465), Perilla frutescens (AB045593), Petunia hybrida (AF155332), Torenia hybrid (AB057673), F3'5'Hs: Canumpana medium (D145909), Catharanthus roseus (CRO011862), Delphinium grandiflorum (AY856345), Eustoma grandiflorum (EGU72654), Gentiana triflora (D85184), Glycine max (AY117551), Gossypium hirsutum (AY275430), Nierembergia sp. (AB078514), Petunia hybrida (AY245545), Lycianthes rantonetti (AF313490), Solanum melongena (X70824), Solanum tuberosum (AY675559), Torenia hybrid (AB019295), Viola major (AB078781), Verbrana hybrid (AY064727); specific F3'5'Hs of Asteraceae: Callistephus chinensis (AAC49299), Osteospermum hybrid (ABB43031), Pericallis cruenta (ABB43030).

2.2. Construction of chimeric genes

To generate chimeric constructs consisting of cDNA fragments of Gerbera hybrid F3'5'H and Osteospermum hybrid F3'5'H, the fragments to be fused were produced in separate PCR reactions using Pfu DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). All primers used are listed in Table 1. The obtained fragments were mixed in a ratio of 1:1 (around 50 ng each) and subsequently ligated in a total volume of 20 µl for 10 min in RT using 1 U (Weiss) T4 DNA ligase (MBI Fermentas, St. Leon-Rot, Germany). The diluted ligation was used as a template for the PCR-mediated amplification of the chimeric product. Proofreading amplicons were cloned into the yeast expression vector pYES2.1/V5-His-TOPO of the chimeric product. Proofreading amplicons were cloned into the yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen, Paisley, UK). Obtained sense constructs were verified by sequencing using a commercial supplier (MWG-Biotech AG, Ebersberg, Germany).

Three cDNA fragments of Osteospermum F3'5'H (OstA, OstB, OstC) and 4 cDNA fragments of Gerbera F3'5'H (GerA, GerB, GerC, GerD) (Table 1) were generated and fused to following chimeric constructs (see also Table 4): GerOst (GerA + OstA), OstGer (OstB + GerB), GerOst6 (GerC + OstC), OstGerOst6 (OstB + GerD + OstC).

2.3. Site-directed mutagenesis

Site-directed mutagenesis was performed using the PfuUltra™ High Fidelity Polymerase (Stratagene, Amsterdam, The Netherlands) according to Stratagene's quick change II site-directed mutagenesis protocol. The mutants of Gerbera F3'5'H and Osteospermum F3'5'H to be produced are listed in Table 2 as well as the primers which were used to introduce the respective mutations into the cDNAs.

2.4. Heterologous expression in yeast

All cDNA constructs to be expressed were transformed into the yeast strain INVSc1 (Invitrogen, Paisley, UK). The yeast culture, the induction of protein synthesis by the addition of galactose and the disruption of the cell walls was performed using a modified protocol of [11] described in detail in [12]. The cell extract was centrifuged (12,000 x g, 10 min) and the supernatant was incubated on ice with 4 M NaCl (0.15 M final concentration) and PEG 4000 (100 mg/ml). The yeast microsomal fraction was pelleted by centrifugation (15,000 x g, 10 min) and resuspended in TEG buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 20% glycerol). The aliquots were shock frozen in liquid nitrogen and stored at –80 °C until further use. Protein quantities were estimated according to [13].

2.5. Enzyme assay and product identification by TLC

Enzyme assays were performed as described in detail in [14]. In brief, 10–30 µl of the isolated yeast microsomal fraction were incubated at 30 °C for 10–30 min together with 0.03 nmol of [14C]-labelled flavonoid substrates (76 Bq) from our lab collection, 10 µl of 20 mM NADPH and 0.1 M Tris–HCl, pH 7.5, in a total volume of 100 µl. The reaction mixtures were extracted with 500 µl EtOAc. The upper phases were chromatographed on precoated cellulose plates (Merck, Darmstadt, Germany) with the following solvent system: CHCl3–HOAc–H2O (10:9:1). Plates were analyzed using the FUJI BAS 1000 Bioimaging-Analyzer (Tokyo, Japan).

3. Results

3.1. Identification of regions putatively involved in the functional difference

We aligned the three so far known amino acid sequences of Asteraceae F3’5’Hs with 15 F3’H and 15 F3’5’H sequences (Table 3) and inspected the alignment for positions with...
Table 2
Primers used for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Substitution</th>
<th>Primer (5'-3')</th>
<th>Codon exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gerbera hybrida</em> F3'H</td>
<td>Tyr484Phe</td>
<td>CTATAATGGAAGGGCTTTCCGGGTTACCTTTCAAAG</td>
<td>TTA → TTC</td>
</tr>
<tr>
<td>Ho f</td>
<td>Thr487Ser</td>
<td>GCTTAAGGGCTGGCTTTGAAAGGCCC</td>
<td>ACC → ACC</td>
</tr>
<tr>
<td><em>Osteospermum hybrid</em> F3'S'H</td>
<td>Tyr484Phe + Thr487Ser</td>
<td>ATGGAAGGGCTTTCCGGGTTACCTTTCAAAG</td>
<td>TTA → TTACCC → AGC</td>
</tr>
<tr>
<td>Ho f</td>
<td>Thr487Ser</td>
<td>GCCCTTTGAAAGGCCC</td>
<td>GAC → GCC</td>
</tr>
</tbody>
</table>

Only forward primers are shown. *Gerbera hybrida* F3'H double mutant Tyr484Phe + Thr487Ser is based on the single mutant Tyr484Phe. *Osteospermum F3'S'H double mutant Phe484Tyr + Ser487Thr is based on the single mutant Phe484Tyr.

Table 3
Identification of regions bearing amino acids putatively involved in determining the functionality of F3'H and F3'S'H

<table>
<thead>
<tr>
<th>Position of the AA difference (based on the <em>Osteospermum F3'S'H</em>)</th>
<th>AA(s) at the corresponding position of F3'S'Hs of Asteraceae (n = 3) (including F3'S'Hs of Asteraceae and other families)</th>
<th>F3'S'Hs of other families (n = 15)</th>
<th>Corresponding SRS</th>
<th>Candidate region</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>A (3)</td>
<td>T (13), V (2)</td>
<td>A (15)</td>
<td>1</td>
</tr>
<tr>
<td>180</td>
<td>T (3)</td>
<td>A (14), V (1)</td>
<td>M (15)</td>
<td>1</td>
</tr>
<tr>
<td>187</td>
<td>D (3)</td>
<td>G (15)</td>
<td>S (15)</td>
<td>1</td>
</tr>
<tr>
<td>484</td>
<td>F (3)</td>
<td>Y (15)</td>
<td>F (15)</td>
<td>6</td>
</tr>
<tr>
<td>487</td>
<td>S (3)</td>
<td>T (15)</td>
<td>A (15)</td>
<td>6</td>
</tr>
</tbody>
</table>

AA, amino acid; SRS, substrate recognition site.

homogenous patterns of amino acid substitutions between the three specific Asteraceae F3'S'Hs, the F3'S'Hs of other families and the F3'Hs or between the specific F3'S'Hs of Asteraceae and of other families and the F3'Hs. In this way two candidate regions were identified, one located near the N-terminal end containing three suspicious positions, the other one located near the C-terminal end containing two suspicious positions (Table 3). The latter region corresponds to a region designated as substrate recognition site 6 (SRS6), one of the six regions which are supposed to be involved in substrate recognition and orientation towards the catalytic centre in P450s [8]. The N-terminal region is located between SRS1 and SRS2. Especially SRS4, SRS5 and SRS6 exhibit a high degree of sequence conservation with 15, 8 and 7 highly conserved amino acids out of 16, 10 and 10, respectively. Interestingly, the sequence conservation of these SRS regions extends to related P450 families such as CYP80 (N-methylcoclaurine 3'-hydroxylase) or CYP93 (flavone synthase II), too. In contrast, sequence variation is markedly higher with respect to SRS1, SRS2 and SRS3 with only 50% or less conserved amino acids.

3.2. Construction and expression of chimeric genes

To test if the above identified regions have an impact on the function, we constructed chimeric cDNAs by fusing cDNA fragments of the F3'H of *Gerbera hybrida* (*Ger F3'H*) and the F3'S'H of *Osteospermum hybrid* (*Ost F3'S'H*) and subsequently expressed them heterologously in yeast. The microsomal fractions of the yeast cultures were isolated and tested for hydroxylation activity with [14C]-naringenin (NAR) as a substrate. The first chimeric cDNA was constructed in order to obtain a hybrid protein, designated as GerOst, containing the first 174 N-terminal AS of the GerF3'H and the respective OstF3'S'H C-terminus bearing the candidate regions 1 and 2 (Table 4). The microsomal fraction showed the expected 3'-hydroxylation activity which is comparable to OstF3'S'H. To test the two candidate regions separately, two further chimeras were generated: (i) chimera OstGer containing 190 N-terminal AS of the OstF3'S'H including candidate region 1 and the respective GerF3'H C-terminus bearing candidate region 2 and (ii) chimera GerOst6 consisting of the GerF3'H N-terminus containing the candidate region 1 and the 31 C-terminal amino acids of the OstF3'S'H including candidate region 2. Microsomal fraction expressing OstGer exhibited exclusively 3'-hydroxylation activity. In contrast, chimera GerOst6 yielded in clear 3',5'-hydroxylation activity. Therefore, candidate region 2 bearing SRS6 is most likely involved in determining the function, although the overall hydroxylation activity was relatively low. Generally, overall activities of chimeras are often low, e.g. 15 out 20 chimeras between limonene 3-hydroxylase and limonene 6-hydroxylase, which exhibit a sequence identity of approx. 70%, too, did not show any or only an activity lower than 5% of the wild types [15]. We generated another chimera, OstGerOst6, by replacing the N-terminus of GerOst6 by the 190 N-terminal amino acids of OstF3'S'H, in order to test whether a higher overall activity requires the interaction of both candidate regions. Again, the microsomal fraction exhibited 3',5'-hydroxylation activity but the specific activity was not higher than that of GerOst6 (Table 5).

Moreover, we assessed substrate specificity of microsomal fractions expressing OstF3'S'H, GerF3'H and the chimeras OstGer and GerOst using NAR, eriodictyol (ERI), apigenin (Ap) and dihydrokaempferol (DHK) as substrate (Fig. 2). NAR and DHK are roughly equal substrates for OstF3'S'H whereas GerF3'H exhibits a clear substrate preference for NAR. NAR is also the best substrate for both chimeras tested. As found for OstF3'S'H, DHK is a better substrate than Ap for OstGer. In contrast, as found for GerF3'H, Ap appears to be a better substrate than DHK for GerOst. These results...
indicate an influence of the N-terminus on substrate specificity. Interestingly, the 3′,4′-hydroxylated ERI is markedly better 5′-hydroxylated by GerOst than by Ost. ERI is not at all 5′-hydroxylated by Ger and OstG.

3.3. Site-directed mutagenesis

The results of chimeric gene construction suggested that amino acids in SRS6 are involved in determining the functional difference between F3′H and F3′5′H. In SRS6, which consists of 9 amino acids, two positions are noticeable (Table 3). At position 5 of SRS6, all F3′Hs have a Tyr and all F3′5′Hs a Phe. Three positions downstream, we find a Thr for all F3′Hs, a Ser for all Asteraceae F3′5′Hs and an Ala for all F3′5′Hs of other families. To test the role of these amino acids, we performed site-directed mutagenesis experiments. The GerF3′H cDNA was mutated in order to generate a Tyr to Phe (Y484F) exchange at position 5 and a Thr to Ser (T487S) exchange at position 8 of SRS6 as well as a Y484F and T487S double mutant. The reciprocal exchanges were performed using the OstF3′5′H cDNA as a template. Furthermore, an Ala residue was established at position 8 of both GerF3′H and OstF3′5′H (T487A and S487A, respectively). The mutated cDNAs were expressed in yeast and the microsomal fractions assayed for hydroxylation activity. Since the release of 3′,4′-hydroxylated intermediates besides 3′,5′-hydroxylated products is generally observed in F3′5′H assays and depends on the assay conditions [14,16], assay conditions (140 µg and 20 min. incubation at 30°C) were used under which recombinant OstF3′5′H exhibits a 1:1 ratio of 3′,5′- to 3′-hydroxylation. Most interestingly, microsomal fraction expressing the GerF3′H T487S mutant exhibited clear 3′,5′-hydroxylation activity with a 1:10 ratio of 3′,5′- to 3′-hydroxylation. The reverse substitution reduced 5′-hydroxylation activity of OstF3′5′H 50-fold. Both mutants showed specific activities comparable to the wild types. Therefore, one conservative amino acid exchange can alter function without disturbing the overall activity and confer additional 5′-hydroxylation activity to a F3′H. Substitution of Thr to Ala enabled GerF3′H to perform 3′,5′-hydroxylation, too, but the efficiency

Table 4
Generation of chimeric F3′H/F3′5′H proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary sequence structure</th>
<th>Function</th>
<th>Spec. Activity (pkat/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OstF3′5′H</td>
<td>N</td>
<td>F3′5′H</td>
<td>2.60</td>
</tr>
<tr>
<td>GerF3′H</td>
<td>N</td>
<td>F3′H</td>
<td>3.00</td>
</tr>
<tr>
<td>GerOst</td>
<td>N</td>
<td>F3′5′H</td>
<td>1.99</td>
</tr>
<tr>
<td>OstGer</td>
<td>N</td>
<td>F3′H</td>
<td>0.58</td>
</tr>
<tr>
<td>GerOst6</td>
<td>N</td>
<td>F3′5′H</td>
<td>0.03</td>
</tr>
<tr>
<td>OstGerOst6</td>
<td>N</td>
<td>F3′5′H</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The results are based on at least three independent heterologous expression experiments. CR, candidate region; GerF3′H, Gerbera hybrida F3′H; OstF3′5′H, Osteospermum hybrida F3′5′H.

Table 5
Impact of amino acid substitutions in SRS6 on function

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Substitution</th>
<th>Ratio of 3′,5′- to 3′-hydroxylation</th>
<th>Specific activity (pkat/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbera hybrida F3′H</td>
<td>Wild type</td>
<td>0:1</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Tyr484Phe</td>
<td>0:1</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Thr487Ser</td>
<td>1:9.5</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>Tyr484Phe + Thr487Ser</td>
<td>1:11</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>Ser487Aa</td>
<td>1:44</td>
<td>1.50</td>
</tr>
<tr>
<td>Osteospermum hybrida F3′5′H</td>
<td>Wild type</td>
<td>1:1</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>Phe484Tyr</td>
<td>1:1.5</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Ser487Thr</td>
<td>1:50</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>Phe484Tyr + Ser487Thr</td>
<td>1:33</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Ser487Aa</td>
<td>1:13</td>
<td>0.94</td>
</tr>
</tbody>
</table>

The results represent mean values of at least four different incubations based on at least two independent enzyme preparations.
of 5'-hydroxylation was markedly lowered compared to the T487S mutant. The OstF3'5'H S487A mutant displayed 13-fold reduced 5'-hydroxylation activity under the chosen conditions compared to the wild type. However, using higher protein amounts, 5'-hydroxylation was markedly higher indicating that an Ala residue at position 8 of SRS6 is generally suitable for 3',5'-dihydroxylation.

The GerF3'H Y484F mutant showed only weak 3'-hydroxylation activity with NAR as a substrate. Interestingly, with ERI as a substrate, the formation of a so far unidentified product was observed. We suggest that this could be the result of 2'- or 6'-hydroxylation since the Ri value of the product is only slightly higher compared to the 3',4',5'-dihydroxylated PHF. Formation of this product was not observed with any of the OstF3'5'H mutants or wild type proteins. The GerF3'H double mutant Y484F + T487S exhibited a similar 3',5'-hydroxylation activity than the T487S single mutant with NAR as a substrate. However, using ERI as a substrate, we observed the formation of both the unidentified product and pentahydroxylavonone (PHF) in a ratio of approximately 1:1.

The F484Y mutation reduced 5'-hydroxylation activity of the OstF3'5'H to a relatively low extent. Interestingly, the F484Y + S487T double mutant exhibited a higher ratio of 3',5'-hydroxylation to 3'-hydroxylation activity than the S487T single mutant (1:33 compared to 1:50). We therefore conclude that Phe may not be indispensable for 5'-hydroxylation function but improves overall activity since spec. activities are reduced 2.8-fold for the single mutant and 4.9-fold for the double mutant compared to the wild type.

4. Discussion

Construction and expression of chimeric genes suggested that substrate specificities of F3'Hs and F3'5'Hs are defined near the N-terminal end whereas the functional difference is determined near the C-terminal end. These experimental data closely correspond to results from homology modelling of four P450s from Arabidopsis thaliana comprising F3'H and 3 other P450s involved in the phenylpropanoid pathway using crystal structure data of several bacterial and a mammalian P450 [17]. This approach resulted in the prediction that residues of SRS5, SRS6 and of the C-terminal segment of SRS4 are involved in contacting the aromatic ring of the respective substrates whereas residues located in SRS1, SRS2 and the N-terminal segment of SRS4 appear to be involved in contacting the aliphatic part. SRS3 apparently does not play a role in substrate contacting.

Our site-directed mutagenesis experiments demonstrate that single amino acid exchanges in SRS6 can alter the function of F3'5'Hs and F3'5'Hs. There are several reports about partial or total shifts in the function of P450s induced by one amino acid substitution, for example in SRS4 or SRS5 of 2-hydroxyisoflavonone synthase [18], in SRS6 of a fatty acid hydroxylase [19] or in SRS6 of limonene 6-hydroxylase [15].

As the C–C bond connecting the B and C rings of flavonoids allows free rotation, it can be proposed that 3',5'-hydroxylation occurs as a two-step process, so that rotation of the B-ring after 3'-hydroxylation allows the subsequent 5'-hydroxylation by the F3'5'H enzyme. Therefore, in contrast to the F3'Hs, the spatial catalytic site architecture of a F3'5'H should enable rotation and the appropriate adjustment of the B-ring after 3'-hydroxylation for the further hydroxylation at the 5'-position.

Substitution of the typical Thr of F3'Hs at position 8 of SRS6 with Ala or Ser which are typically present in regular F3'5'Hs and in F3'5'Hs of Asteraceae, respectively, conferred the F3'5'H of Gerbera additional 5'-hydroxylation activity. Especially Ser and Thr exhibit a high similarity with regard to their physicochemical properties which normally allows the substitution by each other [20]. Therefore, it is likely that the size of the amino acids plays the decisive role since the size of both Ala and Ser is approximately 23% smaller than that of Thr.

The substitution of Thr to Ala or to Ser, respectively, may have been key events in the ancestral F3'Hs towards the evolution of the F3'5'Hs and F3'5'Hs of Asteraceae. Since the ratios of 3',5'- to 3'-hydroxylated products achievable with the Thr to Ala and the Thr to Ser substitutions were relatively low in vitro, further amino acid substitutions appear to be necessary to improve or stabilize the 5'-hydroxylation in vitro and in vivo.

To clarify remaining questions further investigations could use a plant expression system and modelling approaches as further tools and include F3'5'Hs and F3'5'Hs of other families than the Asteraceae. To identify amino acids involved in defining substrate specificity, sequences of F3'Hs and F3'5'Hs with known substrate specificities can be used for alignment and site-directed mutagenesis studies.

Acknowledgements: We thank Bettina Deiml for valuable discussions and Niall Palfreyman for reading the manuscript.

Appendix A. Supplementary data

Amino acid alignment comprising sequences of F3'Hs, F3'5'Hs and Asteraceae F3'5'Hs. The alignment was done using the program MultAlin (Corpet, 1988). The SRS regions...
are indicated according to Rupasinghe et al. (2003). AA, amino acid; SRS, substrate recognition site. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.06.045.

References