Significance of C-terminal sequence elements for *Petunia* flavanone 3β-hydroxylase activity

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Abstract Flavanone 3β-hydroxylase (FHT), a 2-oxoglutarate-dependent dioxygenase (2-ODD), catalyzes the hydroxylation of (2*S*)-flavanones to (2*R*/3*R*)-dihydroflavonols in plants as a key step towards the biosynthesis of flavonols, anthocyanins and catechins. Crystallographic studies of 2-ODDs typically revealed a jelly roll in the enzyme core, and the C-terminus of the enzyme polypeptides was proposed to form a lid covering the active site cavity, thereby reducing the chances for oxidative or proteolytic damage and unfolding. Moreover, it has been proposed that in some cases the C-terminus is involved in substrate selectivity of 2-ODDs. In a systematic approach with highly active *Petunia* FHT, four C-terminally truncated enzyme forms were generated by deletion of five, 11, 24 or 29 amino acids. The recombinant FHTs preserved their substrate selectivity, but the specific activity decreased gradually with the extent of truncation. Then, an enzyme chimera was constructed by domain swapping replacing the C-terminal 52 amino acids of *Petunia* FHT by the equivalent region of flavonol synthase (FLS) from *Citrus unshiu*, an enzyme showing ambiguous FLS and FHT activity. The chimeric dioxygenase still revealed exclusively FHT activity, albeit at a moderate level only. The data predict that the selectivity of FHT is not governed by the C-terminal sequence accounting for about 13% of the enzyme polypeptide.

Key words: Flavonoid biosynthesis; Flavanone 3β-hydroxylase; Flavonol synthase; C-terminal mutation; Selectivity; *Petunia hybrida*; *Citrus unshiu*

1. Introduction

Flavonoids form a large group of secondary metabolites that serve important physiological functions during plant growth and development, but have received also attention for their pharmacological traits [1]. Investigations of the biosynthesis of flavonoids identified four 2-oxoglutarate-dependent dioxygenases (2-ODDs) catalyzing hydroxylation or desaturation reactions, which include flavanone 3β-hydroxylase (FHT) [2], flavone synthase I (FNS I) [3], flavonol synthase (FLS) [4] and anthocyanidin synthase (ANS) [5]. FHT activity converting (2*S*)-flavanones to (2*R*,3*R*)-dihydroflavonols (Fig. 1) was described initially from crude extracts of *Matthiola incana* [6] and illuminated parsley cell cultures [7]. The enzyme was cloned subsequently from *Petunia hybrida* [8] and functionally expressed as a highly active recombinant enzyme in *Escherichia coli*. Genuine FHT consists of a 41 655 Da polypeptide which, unfortunately, is prone to rapid proteolysis in crude plant extracts as well as in the extracts from recombinant expression in bacteria [8,9]. Upon proteolysis, predominantly a carboxy-terminal portion of about 3.8 kDa was removed followed by further truncations and concomitant with severe losses in enzyme activity [10]. The 3.8 kDa C-terminal portion appeared to be essential for *Petunia* FHT activity, and this might be due in part to a small far downstream sequence motif which is conserved in FHTs from widely different plants [11].

Soluble dioxygenases depend on Fe^{II} for catalysis and require a coreductant, which is often, but not always, 2-oxoglutarate. The in vitro activity of 2-ODDs is often stimulated by reducing agents such as ascorbate, which probably act in part by stabilizing the Fe^{2+} redox state of the enzyme cofactor. A number of conserved sequence motifs have been identified in true 2-ODDs and related oxygenases and oxidases, such as isopenicillin N synthase (IPNS), although the overall extent of homology may be low. These enzymes which take part in a broad spectrum of primary and secondary biosynthetic pathways in animals, plants and microorganism are often accredited with an environmental, pharmaceutical or medical impact [12–14] and have therefore been thoroughly studied. The crystallization and X-ray diffraction of 2-ODDs which include deacetoxycephalosporin C synthase (DAOCS) [15], clavaminic acid synthase (CAS) [16], carbapenem synthase (CarC) [17], proline 3-hydroxylase (P3H) [18], ANS [19], taurine/α-ketoglutarate dioxygenase [20] and the factor-inhibiting hypoxia-inducible factor [21] as well as of the related IPNS [22] have been accomplished. Irrespective of their sequence differences, these enzyme polypeptides adopt a very similar spatial configuration, in particular considering the active sites which are embedded in a double-stranded β-helix topology [23]. Furthermore, a lid function was assigned to the C-terminal portion which, in concert with conformational changes, was assumed to shield the substrate during catalysis and may assist in the coordination of the active site ligands [24]. Accordingly, removal of six amino acids from the C-terminus of *Aspergillus nidulans* IPNS [25] or *Streptomyces clavuligerus* DAOCS [15] led to considerable losses in catalytic activity, whereas deletion of 20 amino acids from the C-terminus of *Acremonium chrysogenum* DAOCS/deacetylcephalosporin C synthase

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Abbreviations: ANS, anthocyanidin synthase; DAOCS, deacetoxycephalosporin C synthase; FHT, flavanone 3β-hydroxylase; FLS, flavonol synthase; FNS I, flavone synthase I; IPNS, isopenicillin N synthase; 2-ODD, 2-oxoglutarate-dependent dioxygenase

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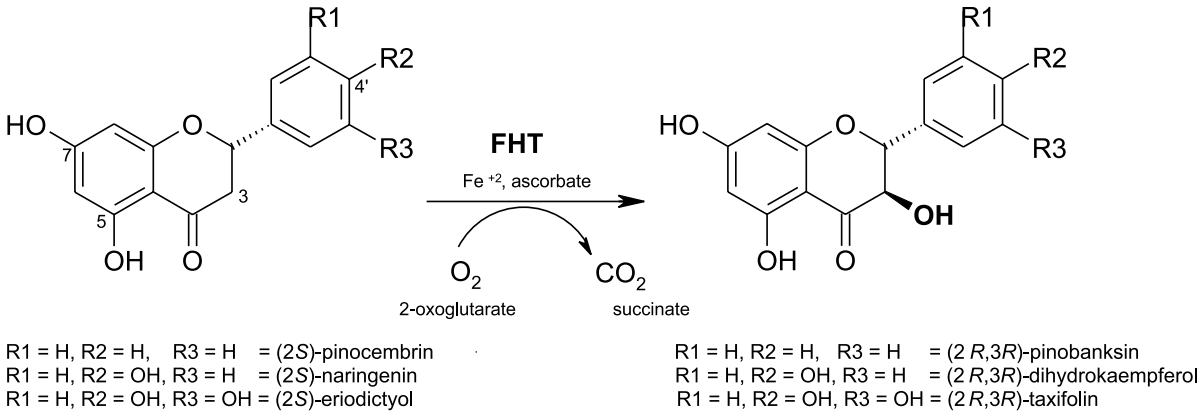


Fig. 1. FHT catalyzes the 3β-hydroxylation of (2*S*)-flavanones to (2*R*,3*R*)-dihydroflavonols, i.e. naringenin to dihydrokaempferol. The enzyme from *Petunia* accepts also eriodictyol or pinocembrin as a substrate to yield taxifolin and pinobanksin, respectively.

(DACS) did not affect penicillin ring expansion [26]. However, experiments with chimeric gibberellin C-20 oxidases clearly emphasized that the C-terminus plays an essential role for product selectivity [27]. Thus, the functional significance of the C-terminal portion must be examined individually for each 2-ODD, and the composition of conserved and variable elements in this polypeptide region might be the cause of subtle differences.

In order to decipher the relevance of conserved and variable C-terminal regions, mutation studies were done on recombinant *Petunia* FHT which had been expressed and purified in a highly active state [10]. Four C-terminal deletions of various length were introduced, and an FHT chimera was generated by replacing the *Petunia* FHT C-terminus with the corresponding sequence from *Citrus* FLS.

2. Materials and methods

2.1. Materials

Biochemicals of analytic grade were purchased from Amersham-Pharmacia (Freiburg, Germany), Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany), Roche Diagnostics (Mannheim, Germany) or Roth (Karlsruhe, Germany). Reference samples of (2*S*)-[4a,6,8-^{14}C]naringenin were collected from preparative incubations of [2-^{14}C]malonyl-coenzyme A (CoA) and 4-coumaroyl-CoA as described previously [9], employing recombinant chalcone synthase from *Pinus sylvestris* [28] and partially purified chalcone isomerase from parsley [29]. (2*S*)-[4a,6,8-^{14}C]dihydrokaempferol was prepared from (2*S*)-[4a,6,8-^{14}C]naringenin as described [10]. The purity of the labeled flavonoids was routinely verified by thin-layer radiochromatography, and the pure compounds were dissolved in ethyl acetate and stored at −20°C until use.

2.2. Mutagenesis and cDNA construction

Based on the FHT-pTZ19R plasmid [30] the FHT mutants CT1, CT2, CT3 and CT4 were generated by site-directed mutagenesis following the procedure of Kunkel et al. [31] and using custom-synthesized oligonucleotide primers (Table 1) (G. Igloi, Institut für Biologie III, Universität Freiburg). Similarly, oligonucleotide primers (FHT-*Kpn*I, FLS-*Kpn*I, Table 1) were designed for the introduction of *Kpn*I restriction sites at homologous 3′ regions of FHT and FLS cDNAs, and site-directed mutagenesis was performed as described [4,32]. The double-digested 5′-*Nco*I/*Kpn*I-3′-FHT gene fragment was isolated after purification on an 0.8% agarose gel by using the S&S BIOTRAP elektro-separation system (Schleicher&Schuell, Dassel, Germany). In order to obtain the cDNA fragment encoding the C-terminal region of FLS the FLS-*Kpn*I cDNA was spliced from the FLS-pTZ19R plasmid with *Kpn*I and *Xho*I. The 5′-*Kpn*I/*Xho*I-3′-FLS construct was isolated by agarose gel electrophoresis and assembled with the 5′-*Nco*I/*Kpn*I-3′-FHT fragment to a full-length chimeric 5′-FHT/FLS-3′ cDNA construct by ligation with the *Nco*I/*Xho*I double-digested FLS-pTZ19R vector (Fig. 2). Subsequently, the *Kpn*I restriction site of the chimeric 5′-FHT/FLS-3′ product was eliminated by point mutation in a similar manner as described above (Fig. 2). Each individual mutation was verified by dideoxy chain termination DNA sequencing [33] using the universal and reverse sequencing primers. The construction of expression vectors was performed with standard techniques [34], the mutant FHT cDNAs (CT1–CT4) and the chimeric 5′-FHT/FLS-3′ cDNA were spliced from pTZ19R vectors with *Nco*I and *Pst*I, and the inserts were subcloned into the expression vector pQE6 or in case of the chimera in pQE60. *E. coli* M15 harboring the plasmid pRep4 was transformed with the mutant FHT-pQE6 and the chimeric 5′-FHT/FLS-3′-pQE60 construct, and the cells were propagated, induced for expression and harvested as described previously [10,32].

2.3. Enzyme expression and general assays

Crude extracts from bacteria expressing mutant FHT (CT1, CT2, CT3, CT4) or the chimeric FHT/FLS polypeptide were prepared by

Table 1
Oligonucleotides for site-directed mutagenesis

Enzyme mutant	Oligonucleotide[a]	Codon exchange
FHT-CT1	5′-GAATTTC**CTA**AATGGGC-3′	GAG → TAG
FHT-CT2	5′-GGACTC**CTA**CTTAGCC-3′	TTG → TAG
FHT-CT3	5′-CTGCTG**CTA**CTTGGCC-3′	GAG → TAG
FHT-CT4	5′-CCTGCTT**CTA**GAGCCTAGC-3′	AAG → TAG
FHT-*Kpn*I	5′-CAAATGTTAT**GGTACC**ATCCATTATTG-3′	GAG → GGT, CCC → ACC
FLS-*Kpn*I	5′-CCTTGAACTTCTT**GGTACC**GTACTTTGG-3′	AAG → GGT, GCC → ACC
FHT/FLS chimera	5′-CCTTGAACTTCTT**GGCCTT**ATCCATTATTG-3′	GGT → AAG, ACC → GCC

[a]Oligonucleotides spanning the site of mutation (bold printed, base substitution underlined) in *Petunia* FHT or *Citrus* FLS were designed to introduce stop codons (FHT-CT1–CT4) or *Kpn*I restriction sites (FHT-*Kpn*I and FLS-*Kpn*I) and to reverse the *Kpn*I restriction site (FHT/FLS chimera).

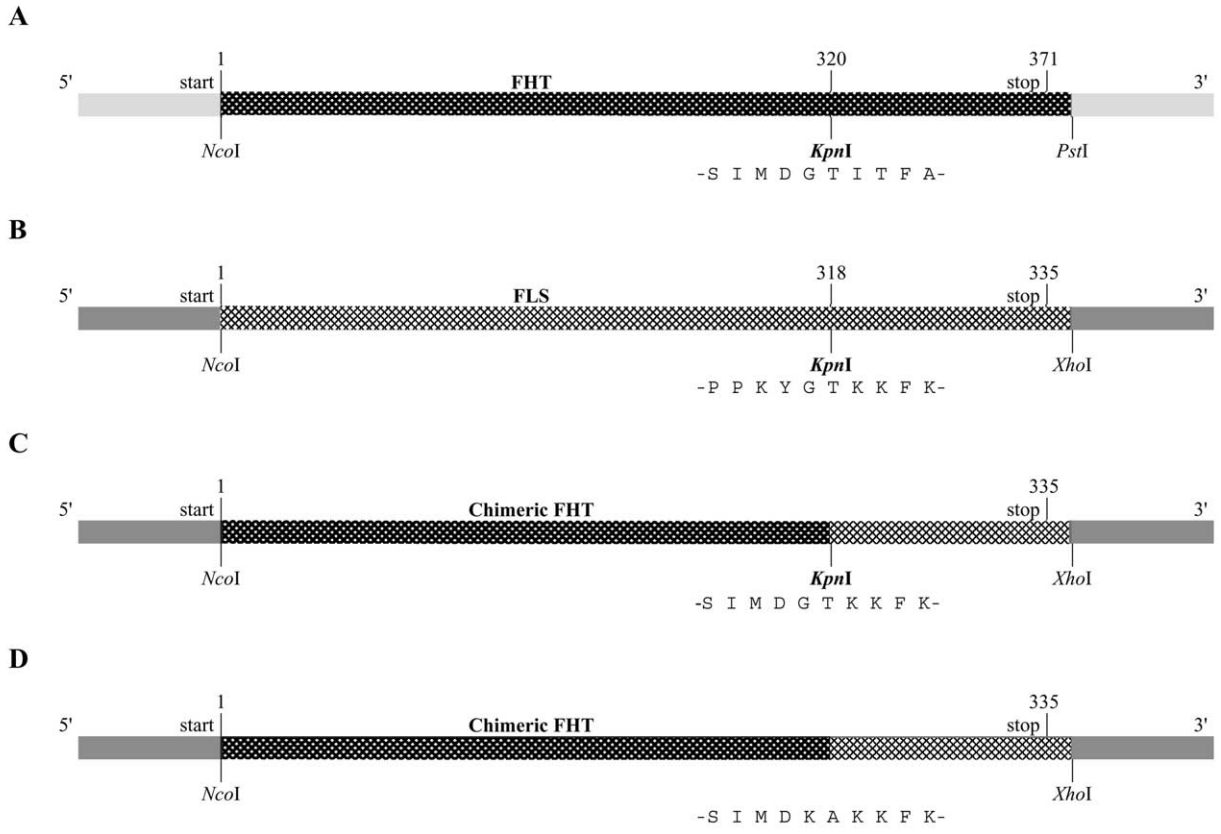


Fig. 2. Schematic mapping of plasmids for the construction of an enzyme chimera from *P. hybrida* FHT and *C. unshiu* FLS. *Kpn*I restriction sites (bold printed) were introduced into FHT-pTZ19R (A) and FLS-pTZ19R (B) by site-directed mutagenesis, and the chimeric FHT plasmid (C) was constructed by swapping of the C-terminal domain (downstream from the *Kpn*I site) of the FLS to the N-terminal FHT domain. Subsequently, the *Kpn*I restriction site of the chimera was eliminated by point mutation (D). Only the relevant restriction sites are indicated below, numbers on top refer to the amino acid residues, and sequence motifs generated at the *Kpn*I restriction sites are assigned in the bottom line.

ultrasonication followed by centrifugation (30 000×*g*, 4°C, 10 min). The clear supernatants of CT1–CT4 were precipitated with ammonium sulfate (20–55% saturation), dissolved in potassium phosphate buffer pH 5.5 (1 ml) and immediately subjected to size exclusion chromatography on a Fractogel EMD BioSEC column (Merck KgaA, Darmstadt, Germany) which had been equilibrated with Tris–HCl buffer pH 7.5 [10]. Mutant FHTs were purified to homogeneity by subsequent anion exchange chromatography on Fractogel EMD DEAE 650 (S) (Merck KgaA) in Tris–HCl buffer at pH 7.5. The protein composition of samples was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [35] and Western blotting [36], and protein was quantified after trichloroacetic acid precipitation according to Lowry [37] with bovine serum albumin as a reference. The activities of FHTs and FLS were determined as reported previously [4,32]. Labeled flavonoid products were extracted from the incubations with 150 µl ethyl acetate, separated by chromatography on cellulose thin-layer plates (Merck KgaA) in 15% aqueous acetic acid and compared quantitatively by bioimaging using a Fuji

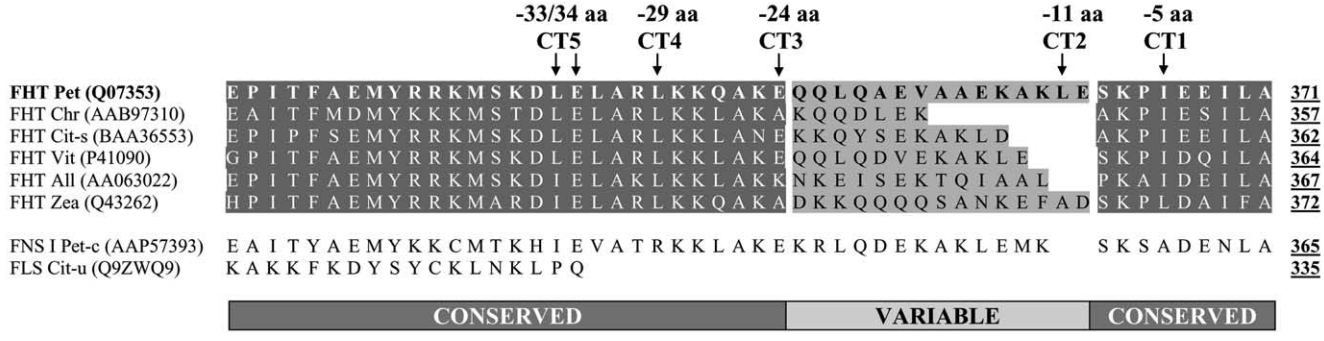


Fig. 3. Alignment of the C-terminal portions of six FHT polypeptides corresponding to the terminal 50 residues of petunia FHT. GenBank accession numbers of sequences are listed in the left margin (Pet, *P. hybrida*; Chr, *Chrysanthemum morifolium*; Cit-s, *Citrus sinensis*; Vit, *Vitis vinifera*; All, *Allium cepa*; Zea, *Zea mays*). Numbers in the right margin refer to the C-terminal amino acid of the individual sequence, and arrows mark the C-terminal end of *Petunia* FHT mutants (CT1–CT4) or of the proteolytic *Petunia* FHT fragment (CT5) isolated from crude extracts [10]. The sequence elements spanning the residues 320–348 and 363–371 in *P. hybrida* FHT are conserved and separated by a highly variable sequence (residues 349–362), as indicated in the bottom line. For comparison, the respective C-terminal portions of FNS I from *Petroselinum crispum* (Pet-c) and of FLS from *C. unshiu* (Cit-u) are also shown.

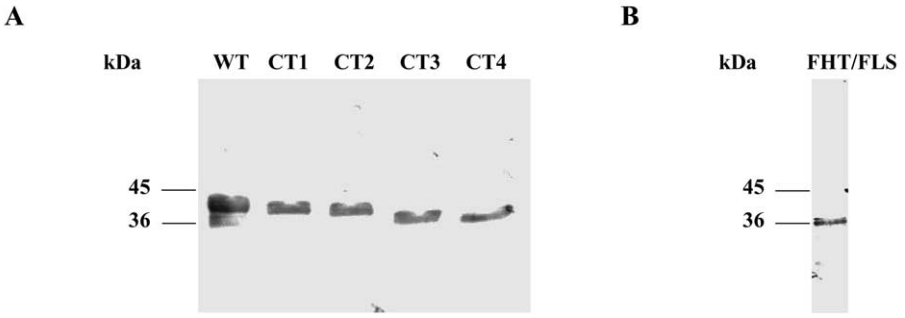


Fig. 4. Western blotting of homogeneous *P. hybrida* wild-type FHT (WT) and of FHT mutants CT1–CT4 (A; approximately 2 μg protein/lane) or of the recombinant FHT/FLS chimera in crude bacterial extract (B; 10 μg protein). The enzymes were subjected to SDS–PAGE on a 12.5% polyacrylamide gel, blotted to polyvinylidene difluoride membranes and probed with polyclonal rabbit antibodies raised against *Petunia* FHT [42]. Molecular size markers are indicated in the left margin.

BAS 1000 Bio-Imaging Analyzer with TINA software (Raytest, Straubenhardt, Germany).

3. Results and discussion

3.1. FHT deletion mutants

Previous attempts to purify *Petunia* FHT, encoded in a polypeptide of 371 amino acids, from plant tissues [9] or after expression in *E. coli* [8] revealed that the enzyme activity was almost completely lost upon deletion of a 3.8 kDa C-terminal peptide, corresponding to 33–34 amino acid residues [10]. The relevance of the C-terminal portion of five to 20 amino acids for enzyme activity had also been proposed for some other 2-ODDs and non-heme iron(II) enzymes [15,23–27], although this does not apply to all 2-ODDs. Thus a systematic approach appears necessary in each instance to correlate the extent of deletions with enzyme activity. *Petunia* FHT appeared to be particularly suited for the deletion experiments, because this hydroxylase has been expressed routinely in *E. coli* at high specific activity and purified to homogeneity [10,32]. Furthermore, FHT shows narrow specificity as compared, for example, to FLS [38–40]. A cluster alignment of 27 different FHT polypeptides revealed two strictly conserved motifs in the C-terminal region, corresponding to residues 320–348 and 363–371 in *Petunia* FHT, separated by an intervening sequence of variable length and composition (Fig. 3). Conceivably, this region contributes to the overall stability and architecture of the enzyme, but the conserved motif far downstream (ILA) might also qualify as a hydrophobic lid proposed to close the active site of 2-ODDs during catalysis [23,24]. In some FHTs, this motif is modified by a conservative exchange to IFA, whereas the mutation to NLA (Fig. 3) as in the recently cloned FNS I from parsley [40] affects the hydrophilicity. Four truncated FHT cDNAs were generated for the expression of hydroxylase deletion mutants lacking five (CT1), 11 (CT2), 24 (CT3) or 29 (CT4) amino acid residues from the C-terminus (Fig. 3). These deletions were chosen to remove the conserved downstream tripeptide only (CT1), the tripeptide including part or all of the intervening sequence (CT2, CT3) and additional amino acids from the second conserved motif (CT4).

The wild-type and mutant genes were expressed in isopropyl thiogalactose (IPTG)-induced *E. coli* cells and bacterial crude extracts were examined after SDS–PAGE separation by protein staining and Western blotting (data not shown). These experiments revealed that the wild-type and mutant FHTs were expressed at equivalent levels. The individual enzymes were purified to homogeneity from the extracts by size exclusion and anion exchange chromatographies at pH 7.5 as described elsewhere [10]. The chromatographic elution profiles of the mutants CT1–CT4 matched that of the wild-type FHT, strongly suggesting that the mutant enzymes had not undergone major conformational changes. The homogeneous FHTs were compared by SDS–PAGE and their identity was corroborated by immunoblotting (Fig. 4A).

3.2. Activities of FHT deletion mutants

The catalytic activities of the FHT mutants CT1–CT4 were assayed using (2*S*)-[4a,6,8-^{14}C]naringenin as a substrate and compared to the activity of wild-type FHT. The increase in the size of the deletions correlated with a decrease in specific activity, underlining that the C-terminal domain of *Petunia* FHT is essential for full enzymatic activity. Nevertheless, the amino acids beyond residue 346 appear to be of marginal importance, because the specific activity of mutant CT1 still reached 44% and dropped to only 27.5% of the wild-type FHT in mutant CT3 (Table 2). Most notably, the far downstream tripeptide ILA (residues 369–371) is not essentially required for catalysis, despite the fact that it is strictly conserved in FHTs. This result is reminiscent of the conclusion reached recently for DAOCS/DACS from *A. chrysogenum*, since the C-terminal deletion of 20 amino acids did not affect

Table 2
Activities of recombinantly expressed wild-type FHT and FHT mutants

Enzyme	Specific activity (mkat/kg)	Relative specific activity (%)
Wild-type	31.8	100
Mutant CT1	14.0	44
Mutant CT2	10	31.5
Mutant CT3	8.7	27.4
Mutant CT4	0.12	0.4
Mutant FHT/FLS chimera	0.09	0.3

penicillin ring expansion [26]. Further deletions of the FHT C-terminus as in mutant CT4 (29 residues) drastically diminished the FHT activity (Table 2), and, in retrospect, these results explain the activity losses which occurred in crude plant and bacterial extracts by proteolysis of the C-terminal 33/34 amino acids [10].

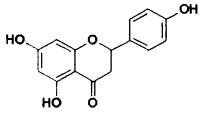
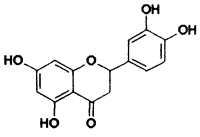
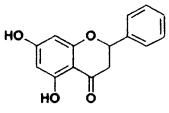
A set of potential substrates, including 4-chromanone, various flavanols (dihydrokaempferol, dihydroquercetin, dihydromyricitin), flavanones (flavanone, 4′-hydroxyflavanone, 7-hydroxyflavanone, naringenin, eriodictyol and pinocembrin) was used to examine the substrate selectivities of the wild-type and mutant enzymes. As reported for the wild-type FHT, the mutants CT1–CT4 catalyzed the 3β-hydroxylation of flavanones only (Table 3) and did not develop FLS or any other activity under the conditions employed.

3.3. FHT/FLS chimera

Several structural and kinetic investigations documented a close structural and functional relationship among the flavonoid dioxygenases, in particular between *Petunia* FHT and FLS from *Citrus unshiu* [4,38–40]. In contrast to FHT, the FLS is a bifuntional enzyme that is capable of catalyzing the FHT reaction in addition to the desaturation of dihydroflavonols. Nevertheless, the FHT and FLS differ considerably in length and in their C-terminal polypeptide composition, irrespective of the overall high degree of similarity (Fig. 3). The C-terminal differences might be responsible for the differential substrate selectivity, as exemplified very recently in case of DAOCS from *S. clavuligerus* [41]. Therefore, a domain swapping experiment was designed (FHT/FLS-pTZ19R), replacing the C-terminal 52 amino acids of FHT by the corresponding peptide region of the FLS from *C. unshiu* (Fig. 2), in order to describe more accurately the impact of the FHT C-terminus. The resulting chimeric cDNA was expressed in *E. coli*, and the expression was examined by Western blotting of the crude bacterial extract. A polyclonal antiserum raised against *Petunia* FHT detected a prominent 38 kDa polypeptide band (Fig. 4B), whereas polyclonal antibodies generated to the *C. unshiu* FLS [4] showed no cross-reaction (data not shown), suggesting that the FLS C-terminus contains inefficient epitopes.

The catalytic activity of the FHT/FLS chimera was analyzed by using labeled (2*S*)-naringenin (FHT assay) or (2*R*,3*R*)-dihydrokaempferol (FLS assay) as a substrate [11,39]. Swapping of the C-terminus greatly diminished the specific FHT activity to a level of less than 0.5% of the wild-type enzyme (Table 2), which was nevertheless sufficient for reliable enzyme assays (90 μkat/kg). However, virtually no FLS activity was detected with labeled (2*R*,3*R*)-dihydrokaempferol as substrate. In contrast to the role for substrate selectivity ascribed to the C-terminus in gibberellin C-20 oxidase [27] or DAOCS from *S. clavuligerus* [24], these results clearly demonstrated that the C-terminal portion of FHT accounting for about 13% of the polypeptide does not govern

Table 3
Substrate selectivities of recombinant wild-type and mutant FHTs[a]

Substrate	Relative activity (%) [b] WT-FHT	CT1-FHT	CT2-FHT	CT3-FHT	CT4-FHT
Naringenin 	100	100	100	100	100
Eriodictyol 	95	76	83	70	43
Pinocembrin 	38	18	14	8	n.d.

[a]Crude bacterial extracts were desalted over PD10 columns in sodium acetate buffer pH 5.5, and the enzyme activities of the eluates were determined under standard assays conditions (37°C, total volume 260 μl). The flavonoids were isolated from the incubation mixture by repeated extraction with ethyl acetate (100 and 50 μl) and reversed phase high performance liquid chromatography (HPLC) (Shimadzu, Tokyo, Japan) on a Nucleodur C18 column (250×4 mm, 5 μm; Macherey-Nagel, Düren, Germany). The elution was monitored by the absorption profile at 280, 308, 320 and 360 nm, and authentic flavonoid samples were employed as references for calibration.
[b]Values normalized with respect to the activity with naringenin; 100 corresponds to the individual specific activities listed in Table 2. The data are the average from triplicate incubations, with variations $<15\%$ between the assays.

the selectivity of this enzyme. Further investigations on the structure–activity relationship of 2-ODDs, including FHT, are necessary to assess the impact of single amino acid substitutions in the C-terminal region for substrate selectivity.

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